

# Mass Screening Techniques for Selecting Crops Resistant to Disease



Joint FAO/IAEA Programme  
Nuclear Techniques in Food and Agriculture



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Large figure: Sporulation of cucurbit powdery mildew (*Golovinomyces cichoracearum*) on cotyledons of susceptible *Cucumis sativus* cv. Stela F1.

Circles: Detail of leaf discs with different degrees of infection 14 days after inoculation with *Golovinomyces cichoracearum*.

Photographs courtesy of A. Lebeda, Palacký University in Olomouc, Czech Republic.

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Nuclear Techniques in Food and Agriculture**



INTERNATIONAL ATOMIC ENERGY AGENCY  
VIENNA, 2010

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## FOREWORD

Diseases caused by fungal pathogens are the major causes of crop loss. Crop diseases not only reduce yield but can greatly impair the quality and stability of production year after year, undermining efforts to promote sustainable agriculture. Environmental and health hazards, resulting from the application of numerous chemical fungicides, are causing increasing concern. Well established crop breeding schemes have for a long time allowed the development of tolerant and/or resistant varieties of valuable food crops. However, these programmes are often long, fastidious and most of the time, fail to identify the positive mutant. As advances are made in mutation breeding, biotechnology and gene techniques, more rapid and cost efficient screening methods are needed to enhance the efficiency and success rate of resistance breeding programmes.

Alternative selection methods rely on scaling up the number of plants screened while reducing the space and time needed for such. Mass screening methods can be performed with well specified pathogen inocula, filtrates or purified toxins applied to plant organs or their parts, and/or in vitro produced plant material. The most important characteristic is that they mimic the infection process under natural conditions. Whereas purified toxins can be more easily quantified and replicated than inocula and crude extracts (filtrates), there are pathogens from which toxins have not yet been isolated (mostly obligate biotrophic parasites). In addition, before utilizing a toxin (mostly produced by necrotrophic pathogens) as a selection agent for disease resistance, it is important to determine whether the toxin is an essential component of the pathogenicity and disease development. Some toxins may not be needed for the full spectrum of pathogenicity and plants may be selected as toxin resistant but disease susceptible. The use of controlled inoculations and crude extracts on explants (e.g. leaf disks and detached leaves), or in vitro material, are more laborious than using toxins, but may be the best choice for plant–pathogen interactions for which reliable toxins are not known.

The source of variation for plant resistance to pathogen can be natural or induced. The use of mutagenic treatments is an effective way to induce plant variability for resistance to pathogens when this cannot be obtained by conventional selection procedures and introduced into the cultivated material through hybridization. The Joint FAO/IAEA Programme on Nuclear Techniques in Food and Agriculture promotes the use of nuclear techniques in agriculture to sustain food security and rural development. This book has been sponsored by the Joint FAO/IAEA Programme in recognition of the importance of minimizing crop losses due to fungal diseases for the effective implementation of sustainable agricultural practices, and to support mutation breeding programmes.

The impact of induced mutations on breeding of disease resistant cultivars is summarized in Chapter 1. Different aspects that must be taken into consideration when choosing the most suitable in vitro resistance screening method for a given plant–pathogen interaction are reviewed in Chapter 2. Furthermore, in vivo and in vitro mass screening methods developed for fruits, legumes, horticultural and tuber crops, with emphasis on banana (*Musa* spp.), are also compiled in this book. Banana and plantain are an important source of food security and income in many tropical areas of the world. Their vulnerability to diseases is augmented by the genetic uniformity of the commercial crops and the absence of efficient resistance sources and genes. Moreover, cultivated triploid bananas are seedless and breeding through conventional methods is challenging. Methods of resistance screening for two of the most devastating diseases of banana (Black sigatoka and Fusarium wilt) are shown. Resistance mass-screening in vivo and in vitro methods for root, vegetable, industrial, legume and fruit crops are also presented.

The IAEA would like to express its thanks to A. Lebeda (Czech Republic) for the revision of the chapters. The IAEA officers responsible for this publication are M. Miranda and M.M. Spencer of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

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## Chapter 1

### SUMMARY

#### **Improvement of crop production and disease resistance through mutation induction: The IAEA mandate**

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The post “green revolution” era of the 20th century with the numerous success stories in various parts of the world has fostered a new “gene revolution”, i.e. a more science based agronomy approach in order to warrant food security for an increasing world population in the actual context of global warming, climate variability and change, the sustainable intensification and improvement of crop production systems regarding the selection of resistance and/or tolerance to stresses should be more than ever the primary goal in plant breeding [1, 2]. This new vision of agriculture prompts the scientific community together with the policy makers to reconsider and redefine agriculture practices for the Third Millennium. Among the multiple threats to agriculture, the increase of temperature associated with the increased humidity, due to displacement of rain isohyets will have a tremendous impact on all living creatures including microbial pathogens [3].

The Joint FAO/IAEA Division, by promoting the use of nuclear techniques including mutation induction as tools for breeding new improved crop varieties, has contributed to enhance the impact of this science based approach in agriculture in developing countries [4]. The IAEA has provided several hundreds of agricultural research institutes in Member States enhanced capacity in terms of human resources as well as in providing high performance germplasm through the development of several thousands of interesting mutants in crop plants [5, 6]. These mutants with desirable characteristics have either been directly released for cultivation or used in hybridization programmes with other mutants and/or cultivars to develop new elite genotypes/cultivars [7]. The success of these mutation breeding programmes relies on the selection of “positive mutant lines” bearing the trait of interest.

On the other hand, it is clear now that many wild/original plant genetic resources vital for the present and future agricultural development are threatened by genetic erosion due to the spread of modern commercial agriculture. This, combined with the climate changes mentioned above, has increased the vulnerability of existing crops to pests, pathogens and environmental stresses. This situation led the FAO’s International Technical Conference on Plant Genetic Resources, the World Food Summit Plan of Action and the International Treaty on Plant Genetic Resources for Food and Agriculture to strongly recommend that increased attention be given to building capacities to characterize, evaluate, improve and use plant genetic resources in a sustainable manner. Therefore a project was designed under the subprogramme on Sustainable Intensification of Crop Production with the overall objective to: Develop early mass screening techniques to obtain banana plants tolerant to fungal diseases. In fact, the selection of the proper mutants has been the bottleneck in all mutation induction breeding programmes, and the difficulty is enhanced when it comes to screening for disease resistance [8]. It is therefore absolutely necessary to establish reliable, rapid and high throughput screening techniques for disease resistance in local germplasm as well as in putative mutant lines. Recent

projects represent a continuation of some previous IAEA activities which were focused on induced mutations against plant diseases [9].

Thus, papers presented in this book highlight increasing cross-cutting techniques using plant tissue culture, irradiation-induced mutation, molecular markers technology, isolation and characterization of mutants for the selection of disease resistant lines. The compilation of this volume has demanded an active participation of a number of breeders, plant physiology and phytopathology specialists. The task of correspondence with authors of the chapters in this volume and the cooperation among participants in the preparation of the manuscripts have led to very extensive and well documented chapters, which will certainly serve the purpose of facilitating the development of successful disease resistance mutation breeding programmes.

This book contains a total of 20 chapters. The first two chapters review the impact of induced mutations and *in vitro* selection on breeding of disease and pest resistant cultivars. Various aspects must be considered while choosing the most suitable *in vitro* selection technique for a given plant-pathogen interaction. The choice of selection agent is very much dependent on their origin, method of preparation, content of active substances, and effective use for screening or *in vitro* selection. Furthermore, the book covers radiation induced mutations, *in vitro* and *in vivo* mass screening methods developed for fruits, legumes, vegetables, and tuber crops, and with greater emphasis on banana (*Musa* spp.) having 6 chapters owing to it as a source of nutrition, food security and great impact on socio-economic aspects. Methods of screening against the most deadly disease of banana (Black sigatoka and Fusarium wilt), which are a great threat to sustainable banana production, are well covered. The book also includes various screening techniques (*in vitro* selection against Black sigatoka in banana by using fungal toxin juglone) for the selection against the deadly diseases of crops such as vegetables (melon and other cucurbits, onion, tomato, lettuce), industrial crops (black pepper), legumes (chickpea, peas, soybean), fruits (apple, pine apple), and tropical crops (cassava, cowpea, maize, and yam).

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## Chapter 2

### ***In vitro* screening methods for assessing plant disease resistance**

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#### **Abstract**

A combination of biotechnological and phytopathological techniques provides an alternative approach to classical resistance breeding methods. Such techniques have been increasingly used since the 1980s, in parallel with the progress in plant biotechnology. In the approach of resistance screening and selection *in vitro*, both experimental objects, i.e., the plant and the pathogen, must first be transferred to *in vitro* conditions, and finally, the plant material must be transferred back to *in vivo* conditions and adapted to the outside settings. Specific attention must be paid to the methods of pathogen preparation for use in screening and selection *in vitro*. The selection agents are classified according to their origin, the methods of preparation, nature and content of active substances, and effective utilisation for screening or selection *in vitro*. Basic principles and methodological aspects of the *in vitro* work (explant cultures, sources of *in vitro* variability, screening and selection methods, types of selection agents) as well as examples of practical applications in the breeding of different crops are critically reviewed in this chapter.

#### **INTRODUCTION**

The availability of sufficient genetic variability (i.e., biodiversity) and efficient selection procedures are two essential prerequisites in plant breeding. Genetic variability can be broadened by utilizing wild, related species in conventional crosses, whereas novel genetic characters can be obtained through mutation induction techniques or somaclonal variation. The latter two methods are particularly interesting if the desirable trait is not present in wild related species or if it cannot easily be introgressed through conventional breeding. Mutants and somaclonal variants can be selected, for instance, for disease resistance by using an appropriate selection agent (potentially derived from a pathogen). DNA alterations (i.e., mutations) that affect the gametes can be transmitted to the progeny; thus, the selected plant material is a new valuable source of genetic variability for the improvement of plant resistance to pathogens.

Selection of plants for disease resistance/tolerance is as old as agriculture itself. Plant breeders working in cooperation with plant pathologists must first decide on the priorities and methodologies in breeding for resistance to diseases. A basic knowledge about the biology of the causal agent and its relationship with the host plant is essential. This information is necessary for the development of suitable methods of screening and selecting for resistance (Russell, 1978). There is a broad range of different methodological approaches available to detect resistant genotypes and to select plants with improved resistance (e.g., Király *et al.*, 1974; Dhingra and Sinclair, 1986; Lebeda, 1986; Trigiano *et al.*, 2004; Singh and Singh, 2005), among which *in vitro* screening is one of the most high-throughput and efficient methods (Švábová and Lebeda, 2005).

Tissue culture or *in vitro* methods are being used effectively in many basic and applied areas of research in plant biology. The first studies to focus on plant-pathogen interactions *in vitro* were performed in the 1940s (Helgeson and Deverall, 1983), and several attempts to apply these methods to

plant breeding were made (Day, 1980). The possibility of selecting plants for resistance in cultures *in vitro* was probably demonstrated for the first time by Carlson (1973), who used methionine sulfoximine (MSO) as an agent for selecting cells and protoplasts of tobacco. Since that time, substantial progress has been made in this field, yielding a large number of *in vitro*-selected germplasm with the potential for developing novel disease-resistant plants (Daub, 1986). The development of this methodology is due primarily to the progress of plant tissue-culture methods. Investigation of host-pathogen interactions *in vitro* is a very useful and efficient way of gaining a better understanding of the factors that influence and are responsible for disease development (Ingram and Helgeson, 1980; Helgeson and Deverall, 1983; Huang, 2001). However, *in vitro* selections can also be difficult and time-consuming. In many articles published since 1980, many of the problems related to the theoretical and practical approaches of *in vitro* selections and their usefulness for plant breeding have been addressed (e.g., Shepard, 1981; Wenzel, 1985; Daub, 1986; Buiatti and Ingram, 1991; Graniti, 1991; for a review, see Švábová and Lebeda, 2005). This chapter provides an overview of the basic principles and methodology of *in vitro* selections for disease resistance in plants. Data, strategies and ideas related to the use of *in vitro* screening that have been published over the last two decades are summarised and discussed here. This review also includes conclusions and future prospects in this area.

## **METHODOLOGY**

### ***In vitro* cultures of plants**

*In vitro* cultures can be categorised according to: (1) purpose (micropropagation, conservation of genetic sources, reduction of pathogen transmission, induction of mutations, regeneration after genetic manipulations, production of various metabolites: e.g., biopharmaceuticals etc.); (2) type of explants (parts of roots, stems, leaves, apical tissues, floral parts; gametes; meristematic regions – lateral buds, shoot tips, immature cotyledons, zygotic embryos); and (3) type of culture (protoplasts, cell suspensions, calli, organogenic, embryogenic) (Bhojwani and Razdan, 1983).

When used in the selection of pathogen resistance, *in vitro* cultures can be grouped based on: (1) purpose (screening for stress tolerance, biochemical studies of host-pathogen interactions, selection of resistant/tolerant lines); and (2) type of selection agent (pathogen, modified pathogen, culture filtrate, phytotoxin/pathotoxin, elicitor) (Švábová and Lebeda, 2005).

*In vitro* selection methods utilise a combination of various types of plant organs or *in vitro* explants with different kinds of selection agents which may (under optimal conditions) trigger reactions similar to the responses of the plant to the pathogen. When subjected to the appropriate selection agent, plant organs or tissues that survive the selection pressure are potential sources of tolerant/resistant subclones. The difference between the selected tolerant/resistant lines and the original plant material may originate from somaclonal variation or induced mutagenesis (see below).

### **Sources of *in vitro* variability and stability**

Tissue culture- or mutation-induced variations are manifested in three ways: (1) variation within cell cultures, in which individual cells within a culture may vary in morphology and genetic make-up; (2) primary regenerants showing a non-heritable phenotypic effect, but that can be maintained as asexually propagated plants if the variation is somatically stable; and (3) heritable variation that is sexually transmitted to the offspring in a seed-derived population (Kaeppler *et al.*, 1998; cf. Jain, 2001). Depending on the crop, the last two types of variation may be efficiently used for agricultural purposes.

Somaclonal variability is a very important character in explant cultures where the step of de-differentiation (callogenesis) is included. Variability of calli subclones and cell lines occurs spontaneously (Kharabian and Darabi, 2005) or it can be induced and subsequently selected. As a result of somaclonal variation, plants regenerated from *in vitro* cultures show miscellaneous variation

in phenotype, such as in plant height (elongation/dwarfism) and changes in plant architecture (Tremblay *et al.*, 1999); flower shape, alteration in pigmentation, and number of leaves and branches (Bhatia and Ashwath, 2004); yield potential and fruit size (Tang and Tai, 2001; Ravindra *et al.*, 2004); as well as various levels of resistance to abiotic stresses, e.g., drought (Bajji *et al.*, 2004), and biotic stresses, including diseases and/or pathogens (Isaac, 1991; Evenor *et al.*, 1994; Keller *et al.*, 1994; Sebastiani *et al.*, 1994; Dan and Stephens, 1995; Jin *et al.*, 1996; Dugdale *et al.*, 2000; Mehta and Angra, 2000; Mohanraj *et al.*, 2003; Ostry and Ward, 2003). The same range of phenotype variations can be expected as the result of induced mutations.

The variation in somaclones can be either of genetic or epigenetic origin. The tissue culture system itself acts as a mutagenic system because cells experience traumatic conditions during explant isolation and culture, and may undergo a type of re-programming during plant regeneration that is different to that under natural conditions (Jain, 2001). A situation in which explants were put into stressful *in vitro* conditions was nicely illustrated in the paper of Gaspar *et al.* (2002). Unusual culture and environmental conditions, mechanical perturbation, wounding, possible air embolism due to dissection, osmotic shock caused by, for instance, a high sucrose content in the medium, abnormal mineral nutrition, unusual hormonal treatment, high relative humidity and accumulation of various gasses (ethylene) in the culture flasks, are all factors that lead to oxidative stress that may result in spontaneous mutations. Such mutations include hyper- or hypo-methylation, polyploidy/aneuploidy, chromosome strand breakage, chromosome rearrangements, transposon activity and DNA base deletions/substitutions. Similar results were also recorded by many other authors; Lopez *et al.* (2004) reported chimeric mutants in cocoa (*Theobroma cacao*) as slippage mutations or allele loss, gene amplification and gene methylation (Jain, 2001; Martin *et al.*, 2001), random changes in genomic organisation during differentiation (Sonyia *et al.*, 2001), rearrangements at the DNA level (Sanchez-Teyer *et al.*, 2003) and chromosomal aberrations (Singh, 2003; Kharabian and Darabi, 2005).

Variability in somaclones can be induced and enhanced by exposure to mutagenic substances, such as ethyl methanesulfonate (EMS), sodium azide (Hunold *et al.*, 1992), gamma- and X-rays (Saito *et al.*, 2001; Mangal and Sharma, 2002; Jain, 2005). Genetically stable mutants are considered as a valuable source of increased genetic variability for various characters, including biotic stress tolerance.

On the other hand, spontaneous somaclonal variation may be disadvantageous in cultures where the genetic identity must be preserved; that is the case in the micropropagation of commercial material where high genetic stability is an essential prerequisite. Genetic stability *in vitro* is closely dependent on organised ontogenesis in embryonic and meristematic cultures, where the period of de-differentiation and calli growth is eliminated (Novák, 1990). Genome fidelity in long-term tissue cultures was confirmed, for example, in silver birch (*Betula pendula*) (Ryyanen and Aronen, 2005) and tomato (*Lycopersicon esculentum*) (Sonyia *et al.*, 2001). Methylation of genes involved in cell differentiation and progressive elimination of cells that are capable of differentiation is proposed to be responsible for the progressive loss of organogenic potential (Jain, 2001). In long-term cultures of pea, such as a 20+ years old multiple-shoot culture of cv. 'Bohatýr', the material was thought to be genetically stable following the use of molecular markers. Repetitive microsatellite sequences did not show any differences between the cultured and original material (Griga *et al.*, 2004); neither did inter-retrotransposon amplification polymorphism (Smýkal *et al.*, 2005). Nevertheless, significant differences were found in the DNA methylation level in this long-term pea culture compared to controls. The possible consequences of increased methylation in this long-term pea culture include problematic rooting, and retarded growth and ontogenic development, which were observed after transfer *ex vitro*.

### ***In vitro* selection**

The perfect model of *in vitro* selection for disease resistance should comprise: (1) an explant culture able to generate genetic variation (or an *in vitro* mutation induction system) with high ability to regenerate resistant/tolerant, genetically stable fertile plants; (2) an easy to obtain selection agent which induces similar biochemical reactions as the pathogen *in vivo*. Backward assessment

(verification) of resistance/tolerance of lines can be proved *in vitro* via co-cultivation with the pathogen isolate (reduced growth of hyphae, reduced weight of mycelia as compared to control), and via greenhouse and field tests; and (3) molecular tools to characterise the selected resistant lines at the DNA level.

The general scheme for experiments which combines biotechnological and phytopathological approaches also has to include the following steps: (1) collection of pathogen isolates and their biological characterisation; (2) establishment of pathogen culture; (3) derivation of an effective selection agent; (4) establishment of the plant tissue culture; (5) testing of the effect (phytotoxicity) of the selection agent on the cultures *in vitro* and a statement of the selection concentrations for a particular plant-pathogen model; (6) serial selection cycles *in vitro*; (7) regeneration of explants surviving selection pressure to plants; (8) testing *in vitro*, *in vivo* and heritability analysis; and (9) assessment in field conditions under natural infection (Figure 2.1).

### ***In vitro* screening methods**

A major problem associated with the evaluation of somaclonal variation for disease resistance is the availability of efficient, reliable screening methods (Sebastiani *et al.*, 1994). The effect of the selection agent (pathogen culture, culture filtrate, phytotoxin, etc.) must be demonstrated in a preliminary experiment, where a suitable concentration range allows for a comparison of the toxic effects on susceptible and tolerant/resistant germplasm. The outcome of such preliminary experiments is to ascertain the precise dosage of selection agent that is optimal for screening resistant material, while killing or drastically reducing growth of susceptible material. These preliminary experiments must be performed for each combination of plant tissue culture and selection agent combination.

The effect of the selection agent can be assessed via various parameters, e.g., inhibition of the culture weight growth, percentage of regenerating explants, percentage of necrotic explants, colour or morphological changes evaluated with computer image analyses, evaluation of cell viability with 2,3,5 triphenyl tetrazolium chloride (TTC) vital staining (Hollmann *et al.*, 2002) or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assays (Kodama *et al.*, 1991), and staining of polysaccharides, callose, polygalacturonases and beta-glucanases (Storti *et al.*, 1991; Simoni *et al.*, 1995; Li *et al.*, 1999). The quantitative values should be further assessed via statistical analyses.

It is necessary to develop suitable techniques for *in vitro* cultivation of a particular host plant and to devise the necessary selection schemes. The principles of these techniques have been described in detail (e.g., Bhojwani and Razdan, 1983; Pierik, 1987). In Table 2.1, the basic methods for aseptic cultures of plant organ or plant tissues, which may also be used for studies of host-pathogen interactions and resistance screening are summarised. Different techniques were developed for *in vitro* plant resistance screening. In general, intact plants or various parts of plants grown in aseptic culture can be used for application of one or more selection agents in testing for resistance *in vitro*. Intact plants may be grown or co-cultivated in tubes with nutrient solution and agar inoculated with fungus (Lebeda and Buczkowski, 1986). This method has been efficiently used for resistance screening and selection of peas (*Pisum sativum*) against *Fusarium* spp. (Lebeda and Švábová, 1997), but also for physiological studies of resistance (Luhová *et al.*, 2002). Cultures of excised organs and/or isolated cotyledons, leaves, stems, shoots, fruits and roots have frequently been used for resistance screening in crop-pathogen interactions (Table 2.2, Figure 2.2; Russell, 1978; Barlass *et al.*, 1986; Lebeda, 1986; Saindrenan *et al.*, 1990; Remotti and Löffler, 1996). The most frequently used *in vitro* screening techniques are plant tissue (calli, shoots) cultures (Helgeson and Haberlach, 1980; Storti *et al.*, 1992; Koike *et al.*, 1993a; Ahmed *et al.*, 1996; Prachi-Sharma and Singh, 2002; Singh *et al.*, 2003); cell suspension (Ishida and Kumashiro, 1988; Krämer *et al.*, 1988; Koike and Nanbu, 1997; Pedras and Biesenthal, 2000) and protoplast cultures (Shepard, 1981; Sjödin and Glimelius, 1989; Koike *et al.*, 1993b; Li *et al.*, 1999); as well as anther and pollen grain culture (Bino *et al.*, 1988) (see Figures 2.3-2.7). After regeneration, the selected plants typically show a variety of phenotypes (Figure 2.8). These techniques have been applied to various crops, and the selected regenerants provided a source of germplasm with improved resistance to important diseases (Table 2.2; Švábová and Lebeda, 2005).

## Selection agents for *in vitro* screening

There are two essential prerequisites for a pathogen-derived selection agent to be considered useful for *in vitro* screening of disease resistance: (1) One or more compounds found in the selection agent should be present in infected plants; and (2) the ability of the agent to cause at least part of the disease symptoms when inoculated into healthy plants (modified from Aducci *et al.*, 1997). A comparison between the plant-pathogen interaction and the reaction of *in vitro*-cultured material to the selection agent should, ideally, result in a similar spectrum of pathogenesis-related proteins and/or enzymes commonly induced in both models (Angelini *et al.*, 1990; Lebeda *et al.*, 2001; Luhová *et al.*, 2002).

### *Natural pathogen isolates*

It is possible to utilise a live pathogen for screening disease resistance *in vitro*. However, when explants are exposed to inoculation with a natural pathogen *in vitro*, the multiple stress factors caused lead, in most cases, to death of the plant material, which is overgrown and devastated by the pathogen (Ingram and Helgeson, 1980; McComb *et al.*, 1987; Heath-Pagliuso *et al.*, 1988, 1989; Lebeda and Švábová, 1997). The conditions *in vitro* (higher humidity, reduced air velocity, media rich in nutrients) are very hospitable and favour growth of microorganisms in general. In a number of studies, different live pathogens were tested as agents for *in vitro* selection, but were found to be too devastating for the plant tissues/organs and, therefore, of limited use.

Several successful experiments have been carried out *in vitro* with live inoculums i.e., by *Clavibacter michiganensis* (Bulk *et al.*, 1991), *Xanthomonas campestris* (Hammerschlag 1990), *Plasmodiophora brassicae* (Buczacki, 1980), *Mycosphaerella musicola* (Trujillo and De Garcia, 1996), *Alternaria alternata* (Takahashi *et al.*, 1991), *Fusarium solani* (Huang and Hartman, 1998), *Colletotrichum trifolii* (Cucuzza and Kao, 1986), *Peronospora tabacina* (Tuzun and Kuć, 1987), *Phytophthora cinnamoni* (McComb *et al.*, 1987; Cahill *et al.*, 1992) and powdery mildews (*Blumeria graminis*, *Erysiphe pisi*, *Sphaerotheca pannosa*, *Uncinula aceris*) (Webb and Gay, 1980). Therefore, the pathogen is mostly used more or less modified to various selection agents.

### *Modified fungal cultures*

Inactivation of the pathogen is often applied to circumvent the problems associated with excessive growth of the pathogen during *in vitro* selection, which leads to the unselective death of the plant material. After some time in flasks or Petri dishes, the culture containing the pathogen isolate is inactivated by autoclaving, and then overlaid with an additional layer of medium; together, these layers form a so called “double layer” (Ahmed *et al.*, 1991; Švábová and Griga, 1997).

Inactivated cultures represent a complex mixture including small portions of the pathogen isolated. Crude culture filtrates can be obtained after cultivating agar disks with fungal mycelia on the surface of the liquid nutrient solution and filtering through a layer of cellulose wadding. Culture filtrates contain a mixture of fungal metabolites and are very often used for selection, with many examples of positive results (Cvikrová *et al.*, 1992; Mezzetti *et al.*, 1992; Jayasankar and Litz, 1998; Prachi-Sharma and Singh, 2002; Thakur *et al.*, 2002; Singh *et al.*, 2003).

Fungal culture filtrates may contain a spectrum of secondary metabolites, such as polysaccharides, oligosaccharides (Peros and Chagvardieff, 1987), proteins, glycoproteins, unsaturated fatty acids, stem from the cell walls, cytoplasm of the bacteria or fungi, growth regulators such as auxin, kinetin and gibberellic acid (Gentile *et al.*, 1992), along with toxins that may play a role as co-determinants of pathogenicity during disease development (Buiatti and Ingram, 1991; Crino, 1997; Švábová and Lebeda, 2005). Toxins were found in various filtrates of the two main types of plant-pathogen interactions, i.e., host-selective and non-host-selective. The application of filtrates to cultures *in vitro* can trigger the elicitation of various defence responses, e.g., phytoalexins; activity of certain enzymes (Saindrenan *et al.*, 1990; Crino, 1997; Lebeda *et al.*, 2001); accumulation of phenolic acids (Cvikrová

*et al.*, 1992), total phenols, peroxidases and beta 1,3-glucanase (Lebeda *et al.*, 2001; Singh *et al.*, 2003); and chitinase (Jayasankar and Litz, 1998).

It is well known that inoculation with culture filtrates of some plant pathogenic fungi can produce disease-like symptoms and may also be used to select for resistance (Wenzel, 1985; Daub, 1986; Buiatti and Ingram, 1991; Crino, 1997; Švábová and Lebeda, 2005). Culture filtrates are mostly produced by fungal cultivation in liquid media and subsequent separation of the solid and liquid parts of the culture. The liquid part of the culture is used as the selection agent. This approach yields a variety of selection agents whose composition ranges from the absence of any toxin in the filtrate (in which cases the symptoms are caused by other fungal metabolites) to filtrates that contain uncharacterised active toxins (Daub, 1986). Crude culture filtrates allow easy bioassay and screening for toxic effects on plants, cuttings, leaf disks or even cell suspension cultures of the host species (Isaac, 1991). Crude culture filtrates have been used as selective agents in numerous disease resistance studies in which they exhibit phytotoxic activity (Chen and Swart, 2002; Švábová and Lebeda, 2005). Phytotoxicity of cell-free culture filtrates of *F. solani* f. sp. *glycines* has been proved, for instance, in cuttings of soybean (*Glycine max*) seedlings (Hartman *et al.*, 2004).

The application of metabolites with various levels of toxicity in *in vitro* selection for resistance has some advantages when compared with natural conditions (Nedělník and Řepková, 1998): (1) unfavourable weather and climate conditions are avoided, thus enabling easier and more precise the assessment of quantitative differences in polygenic traits; (2) a large number of individuals can be tested in a small space; (3) it is easier to manipulate with large populations of mutants, haploids, and somaclones with higher variability in the genome; and (4) mass screening of mutants for resistance is facilitated.

#### *Phytotoxins and pathotoxins*

An alternative approach to crude extracts is to purify substances from fungal or bacterial cultures that have a toxic effect on the plants. The toxic substances, known as phytotoxins, first need to be identified, then separated from crude extract and purified. Phytotoxins that are known to play a role in pathogenesis are called pathotoxins (Graniti, 1991; Švábová and Lebeda, 2005). Particularly useful are the non-host-selective toxins that act primarily by inhibiting active plant defence processes (Buiatti and Ingram, 1991). A number of different phytotoxic substances have been used as selection agents in resistance screening, e.g., a toxin from *Drechslera teres* for selection of barley (*Hordeum vulgare*) calli cultures (Hunold *et al.*, 1992), malseccin from *Phoma tracheiphila* for selection of lemon tree (*Citrus limon*) calli and protoplast cultures (Gentile *et al.*, 1992), 2,4,8-trihydroxytetralone from *Mycosphaerella fijiensis* in micro-cross section cultures of banana (*Musa* sp.) (Okole and Schultz, 1997), colletotrichin from *Colletotrichum gloeosporioides* for the selection of embryogenic mango cultures (*Mangifera indica*) (Jayasankar *et al.*, 1999), solanapyrone A,B,C from *Ascochyta rabiei* for the selection of shoot cultures of chickpea (*Cicer arietinum*) (Hamid and Strange, 2000), phomalide from *Phoma lingam* in cell suspensions of rape (*Brassica rapa*) (Pedras and Biesenthal, 2000), AM-toxin from *Alternaria alternata* for selection of apple (*Malus domestica*) shoot cultures (Saito *et al.*, 2001; Chapter 9), and many others (Švábová and Lebeda, 2005) (Tables 2.3 and 2.4).

Phytotoxins are considered microbial metabolites that may damage or harm plants at very low concentrations (Graniti, 1991). The idea that metabolites produced by plant pathogens could be detrimental to plants originated from the experiments of de Bary (1886) and the concept was advanced by Gäumann (1954) who stated that microorganisms are pathogenic only if they are toxigenic. Recently, more than 250 phytotoxic metabolites produced by plant pathogenic bacteria and fungi have been isolated, purified and structurally characterised. Relatively little is known about their biosynthesis and metabolism, and much less about the genetics underlying their production (Huang, 2001).

Toxin-producers are found among some bacteria (e.g., *Pseudomonas*, *Burkholderia*, *Clavibacter*, *Streptomyces*, *Xanthomonas*) and a number of fungi (e.g., *Alternaria*, *Ascochyta*, *Bipolaris*, *Botrytis*,

*Ceratocystis*, *Cercospora*, *Cochliobolus*, *Colletotrichum*, *Drechslera*, *Fusarium*, *Phoma*, *Pyrenophora*, *Sclerotium*, *Septoria*, *Stemphylium*, *Verticillium*) (Huang, 2001; Švábová and Lebeda, 2005) (Tables 2.3 and 2.4).

There are two main known roles of pathogen-produced toxins: (1) initiation of disease; and (2) disease development. Toxins are classified based on their mode of action; those that are responsible for the onset of disease and are essential for causing the disease are known as “pathogenicity factors”, and those that are required for the development and increase in the extent of the disease are known as “virulence factors” (Yoder, 1980). The pathological significance of phytotoxins can be assessed by evaluating the correlation between: (1) the production of toxin and disease occurrence of the pathogen; and (2) sensitivity to the toxin and susceptibility of the plant to the disease (Yoder, 1981).

The toxins/phytotoxins are either host-selective (HST, or host-specific) or non-host-selective (NHST, or non-host-specific) according to their specificity in a given plant-pathogen interaction (Mitchell, 1984; Walton and Panaccione, 1993). The HST group comprises a limited number of phytotoxins that meet the following criteria: (1) the toxin and its producer have similar host specificity; (2) the virulence of the pathogenic strains is positively correlated to their capacity to produce the toxin; and (3) the toxin is able to produce, in susceptible plants, symptoms characteristic of the disease caused by the pathogen (Huang, 2001). HSTs are toxic to plant species or cultivars susceptible to the pathogens producing these toxins, and there is a correlation between sensitivity to the toxin and susceptibility of the plant to the pathogen (Knogge, 1996).

NHSTs form a larger group of toxins and include compounds that do not reproduce the patterns of resistance and/or susceptibility responses of the host to the pathogen infection. They are thought to be broad-spectrum toxins that affect a large number of crops (Graniti *et al.*, 1991). These toxins act primarily by inhibiting active defence processes such as detoxification or stimulation of membrane-localised H<sup>+</sup>-ATPase activity (Knogge, 1996). However, there are also known toxins with a specific mode of action, e.g., toxins responsible for plant wilting (Van Alfen, 1989).

Plant pathologists have concerned themselves mainly with the chemistry and biochemistry of phytotoxins, and their roles in plant pathogenesis (Huang, 2001). However, in the past two decades, research has been extended to the use of phytotoxins as tools in screening and breeding for disease resistance (Švábová and Lebeda, 2005). The general strategy is based on considerable evidence that there is a correlation between tolerance/resistance to phytotoxins and resistance to the pathogen. Phytotoxins and culture filtrates of pathogens have been used in root growth, leaf chlorosis and lesion development assays in screening early segregating populations. They have also been used for *in vitro* selection of tolerant protoplasts, cells and calli, which are subsequently regenerated into putative resistant plants (Huang, 2001; Švábová and Lebeda, 2005).

### *Bacterial phytotoxins*

Generally, the bacterial phytotoxins are either glycosides (glycopeptides) or low molecular weight compounds derived from amino acids and peptides (Strobel, 1976). There are at least six groups of bacterial phytotoxins: tabtoxin, coronatine, thaxtomins, phaseolotoxin, syringomycin and rhizobitoxine (Huang, 2001; for examples, see Table 2.3).

Tabtoxin is a dipeptide produced by certain strains and pathovars of *Pseudomonas syringae* that cause wildfire or halo blight diseases. Tabtoxin was detected in *P. syringae* pv. *tabaci*, causing wildfire of tobacco (*Nicotiana tabacum*) plants. At first, crude toxin preparations from *P. syringae* pv. *tabaci* called “wildfire toxin” were produced, and were later characterised as tabtoxins, represented by tabtoxine and threonine. According to Strobel (1976), tabtoxins are capable of inducing chlorosis in plants. However, Kinscherf *et al.* (1991) found that tabtoxin production alone is not sufficient to cause disease. The possibility that plasmids may be involved in tabtoxin production has been investigated (Huang, 2001).

Coronatine is produced by several *P. syringae* pathovars and was first detected in liquid cultures of *P. syringae* pv. *atropurpurea*. When applied to leaves of Italian ryegrass (*Lolium multiflorum*), chlorosis and browning occurred, similar symptoms as after inoculation with live *P. syringae* pv. *atropurpurea* (Mitchell, 1984).

Thaxtomins are a group of 2,5-dioxopiperazines produced by *Streptomyces scabies*, *S. acidiscabies* and *S. ipomoeae*, the causal organisms of potato common scab, acid scab and sweet potato pox (Toth *et al.*, 1998). Thaxtomins induce the formation of scab-like lesions on potato (*Solanum tuberosum*) tuber. A positive correlation between pathogenicity and thaxtomin production was recorded on potato slices (King *et al.*, 1991).

Phaseolotoxin is a non-host-specific and extracellular toxin produced by *P. syringae* pv. *phaseolicola* and the causal agent of halo blight of bean, which induces a chlorotic halo on treated leaves. Structurally, phaseolotoxin is a tripeptide (Huang, 2001). The primary function of phaseolotoxin is inhibition of carbamoyltransferase, which is an enzyme involved in chlorophyll synthesis (Patil, 1974). The production of this toxin contributes significantly to the virulence of *P. syringae* pv. *phaseolicola*, whereas avirulent strains are not able to produce toxins in culture (Mitchell, 1984).

Another group of bacterial toxins is formed by syringopeptin, syringomycin and syringotoxin. Biochemically, these toxins are peptides (Huang, 2001). Syringomycin was characterised in isolates of *P. syringae* pv. *syringae*, a pathogen of stone fruit trees. It has been suggested that syringopeptin primarily affects cellular membranes (Backman and DeVay, 1971). Syringotoxin, like syringomycin, is also produced by *P. syringae* pv. *syringae*; however, only by strains pathogenic to *Citrus* spp., which do not produce syringomycin. Syringotoxin differs from syringomycin by slower migration on polyacrylamide gel electrophoresis and by different biocidal activity against bacteria and fungi (Gonzalez *et al.*, 1981).

Tagetitoxin was identified by *P. syringae* pv. *tagetis*, causing a leaf spot of marigolds. Isolated and purified tagetitoxins cause chlorosis of the apical stem part of zinnia plants two to three days after application (Mitchell and Durbin, 1981).

Rhizobitoxine is produced by certain strains of the *Bradyrhizobium* (*Rhizobium*) *japonicum* bacterium, which fixes nitrogen in soybean plants, and by *Pseudomonas andropogonis*, which is the causal agent of bacterial stripe and leaf spot in corn, sorghum and legumes (Huang, 2001). The toxin was isolated from chlorotic leaves, nodules and bacterial cultures; and is non-host-specific because it produces chlorosis in many plant species (Owens *et al.*, 1965). Rhizobitoxines were found to be an antimetabolic enol-ether amino acid (Owens *et al.*, 1972).

#### *Fungal host-selective toxins (HSTs)*

Fungal HSTs comprise a group of structurally complex and chemically diverse metabolites produced by plant pathogenic strains of certain fungi (Wolpert *et al.*, 2002). HSTs have been mostly described in necrotrophic pathogens. This group includes fungi such as *Alternaria*, *Bipolaris* (*Helminthosporium*), *Corynespora*, *Fusarium*, *Phyllosticta*, and others. Most of the known HSTs are low molecular weight secondary metabolites (e.g., cyclic peptides, terpenoids, oligosaccharides, polyketides and sesquiterpene glycosides), showing great diversity in their chemical, biological and physiological effects. The same compound is often produced by several taxonomically different species and/or single species can produce various toxins (Goodman *et al.*, 1986; Kohmoto and Otani, 1991). HSTs function as essential determinants of pathogenicity or virulence. Investigations into the molecular and biochemical responses to these disease determinants reveal responses typically associated with host defence and incompatibility induced by avirulence determinants (Wolpert *et al.*, 2002).

However, from the point of view of the host plant, there is no common genetic pattern of response to HSTs and the fungi producing them. Genetic analyses have shown various genetic patterns of sensitivity to HST, e.g., nuclear inheritance – monogenic (dominant, semi-dominant, recessive) or cytoplasmic inheritance (Walton and Panaccione, 1993). The most important and well known HSTs are produced by *Alternaria* (Nishimura and Kohmoto, 1983; Huang, 2001), *Bipolaris* (*Helminthosporium*), *Drechslera* (Huang, 2001) and *Phyllosticta maydis* (Desjardins and Hohn, 1997; Huang, 2001). A survey of the most important HSTs used for experimental purposes and resistance selection is summarised in Table 2.3.

Several host-specific *Alternaria* toxins are known (Nishimura and Kohmoto, 1983). AAL-toxin, an amino acid-derived phytotoxin, is produced by *A. alternata* f. sp. *lycopersici*, the causal fungus of tomato stem canker. A detached leaf bioassay showed that AAL-toxin causes necrosis on susceptible tomato plants. However, electrolyte leakage was not detected prior to the onset of necrosis, indicating that the plasma membrane is not the primary target site of AAL-toxin (Huang, 2001). Tomato cell suspension cultures are sensitive to AAL-toxin (Fuson and Pratt, 1988).

Helminthosporoside (HS-toxin) is produced by *Drechslera sacchari*, the causal organism of eye spot disease of sugarcane. It is a sesquiterpenoid toxin (Huang, 2001). HS-toxin produces reddish-brown streaks only on those clones of sugarcane that are susceptible to the fungus. Sensitive clones possess a membrane protein that recognises and binds the toxin. The disintegration of the outer chloroplast membrane is the earliest cytological disturbance in susceptible plants, and this is accompanied by a strong decrease in CO<sub>2</sub> fixation (Brown *et al.*, 1982).

Victorin (HV-toxin) is produced by *Drechslera victoriae*, which causes foot and root rot, and leaf blight of oat, and only affects susceptible oat cultivars. Chemically, this toxin is a polypeptide (Huang, 2001). The earliest detectable effect of victorin in susceptible plants is an increase in the permeability of the plasma membrane to electrolytes (Wheeler and Black, 1963). Protoplasts from susceptible cultivars begin to burst almost immediately after exposure to victorin, whereas protoplasts from resistant cultivars are not affected (Sammadar and Scheffer, 1968).

HC-toxins are produced by race 1 of *Helminthosporium carbonum* (*Cochliobolus carbonum*, *Drechslera zeicola*), which is the causal agent of one of the most serious diseases in the recent history of plant pathology called corn leaf blight (Goodman *et al.*, 1986). HC-toxin is a cyclic tetrapeptide phytotoxin (Huang, 2001) that causes chlorotic symptoms by inhibiting chlorophyll biosynthesis in etiolated leaves of susceptible cultivars of maize (Rasmussen and Scheffer, 1988). There are indications that the HC-toxin inhibits histone deacetylases in maize (Ransom and Walton, 1997).

HMT-toxins (T-toxins) are produced by *Helminthosporium maydis* (*Cochliobolus heterostrophus*, *Bipolaris maydis*) race T and are highly toxic to cells of corn possessing Texas male sterile (Tms) cytoplasm. Chemically, HMT-toxins are linear polyketols (Huang, 2001). It has been shown that HMT-toxins preferentially inhibit root growth of T-cytoplasm but have little effect on resistant corn with N-cytoplasm (Smedegaard-Petersen and Nelson, 1969).

The PC-toxin is a polypeptide produced by *Periconia circinata*, which is a pathogen of grain sorghum. The symptoms produced by this toxin are similar to those seen in plants inoculated with the fungus (Goodman *et al.*, 1986). The mode of action of this toxin is similar to that of victorin, i.e., it causes an increased respiration rate, decreased growth, decreased protein synthesis and disturbance of membrane function (Pringle and Scheffer, 1967).

PM-toxins are produced by *P. maydis*, which is the causal agent of corn yellow leaf blight. The pathogen attacks only Tms corn (T-cytoplasm) and produces host-specific, linear polyketol phytotoxins (Huang, 2001). PM-toxins selectively inhibit seedling root growth, induce leaf chlorosis and increase leakage of electrolytes in corn leaves with T-cytoplasm (Goodman *et al.*, 1986).

### *Fungal non-host-selective toxins (NHSTs)*

NHSTs have been identified in various groups of fungi, and are mostly necrotrophic or cause vascular diseases (e.g., *Alternaria tenuis*, *A. mali*, *Ceratocystis ulmi*, *Cochliobolus miyabeanus*, *Colletotrichum* spp., *Fusarium* spp., *Fusicoccum amygdali*, *Leptosphaeria maculans*, *Pyricularia oryzae*, etc.). NHSTs consist of a broad spectrum of toxins affecting various plant species. As for their chemistry, they comprise mostly macromolecular compounds such as amino acid derivatives, peptides, cyclic tetrapeptides and diterpenoids (Mitchell, 1984; Ballio, 1991; Huang, 2001). The genetic pattern of plant responses to NHSTs is not well known. A survey of the most important NHSTs used for experimental purposes and resistance selection is summarised in Table 2.4.

Many types of NHSTs are produced by *Fusarium* species. One such example is fusicoccin, which is produced by *F. amygdali*, and is involved in wilt disease of almond (*Prunus dulcis*) and peach (*Prunus persica*). Fusicoccin is a diterpenoid glycoside and affects cellular transport processes (Ballio, 1978).

There are also numerous other toxins produced by *Fusarium* species that cause diseases that are collectively known as Fusarium wilt; such toxins are fusaric acid (FA), lycomarasin, moniliformin, naphthazarin, sambucin and beauvericin (Luz *et al.*, 1990; Řepková and Nedělník, 1998; Desjardins and Proctor, 2001; Kuzniak, 2001; Zemánková and Lebeda, 2001; Švábová and Lebeda, 2005). *Fusarium* species producing FA are represented by *F. moniliforme*, *F. napiliforme*, *F. thapsinum*, *F. nygamai*, *F. sachari*, *F. fujikuroi*, *F. proliferatum*, *F. subglutinans*, *F. sambucinum*, *F. crookwellense*, *F. heterosporum*, *F. oxysporum* complex and *F. solani* complex (Desjardins and Proctor, 2001). The primary effect of FA is an increase in cell permeability. Lines of banana (*Musa* sp.), gladiolus (*Gladiolus* sp.) and pineapple (*Ananas comosus*) that were selected with FA also show increased tolerance to *F. oxysporum* (summarised by Švábová and Lebeda, 2005).

Generally, species in the *F. oxysporum* complex are known to produce enniatins, FA, moniliformin, naphthazarins and sambutoxins, but are not known to produce fusarins, fusarochromanone, fusaproliferin, fumonisins, trichothecenes or fusaproliferin (Desjardins and Proctor, 2001). Lycomarasin is a tripeptide produced by *F. oxysporum* f. sp. *lycopersici* and a few other species. Naphthazarin is produced by *F. oxysporum* f. sp. *pisi* and *F. solani*, and acts by inhibiting the anaerobic decarboxylation of pyruvate (Isaac, 1991; Baker and Nemeč, 1994, 1997). Beauvericin is a cyclodepsipeptide metabolite, closely related to the enniatins (Moretti *et al.*, 1995) and an important secondary metabolite of various phytopathogenic *Fusarium* species (Abdalla *et al.*, 2000). Although beauvericin did not cause any symptoms in the roots of the tested plants (melon, tomato, wheat and barley), it showed the highest toxicity towards the protoplasts of all plants tested (Sagakuchi *et al.*, 2000). Recent data suggest that the production of beauvericin and enniatin is not related to the pathogenicity of *F. oxysporum* f. sp. *melonis* or to the differential specificity of the races (Moretti *et al.*, 2002). It has been generally concluded that the non-selective toxic activity of beauvericin is due to its ability to induce pores in biological membranes, resulting in a disturbance of the normal gradients of physiologically important monovalent cations across membranes (Lemmens *et al.*, 2000).

Species in the *F. solani* complex produce FA, naphthazarins and trichothecenes. Those species with *Giberella* sexual state produce fusarins and moniliformin that are not found in species with *Nectria* sexual state (Desjardins and Proctor, 2001).

Trichothecenes are another very important group of *Fusarium* mycotoxins. The possible role of trichothecenes in plant pathogenesis was initially assessed with UV-blocked mutant strains of *F. sporotrichoides*. The acute phytotoxicity of trichothecenes and their occurrence in infected plant tissues suggest that this mycotoxin plays a role in the onset of pathogenesis by *Fusarium* (Desjardins and Proctor, 2001; Desjardins, 2003). Among the *Fusarium* toxins identified to date, however, only trichothecenes and enniatins have been confirmed as virulence factors in plant pathogenesis. The reduced virulence of *F. avenaceum* with a disrupted enniatin synthetase gene was proven in experiments with potato tubers (Herrmann *et al.*, 1996). Localisation studies of trichothecenes indicated that these toxins could be detected in host tissues at an early stage of infection (Kang and

Buchenauer, 2002). It was proven that trichothecene production contributes to the virulence of *F. graminearum* (Miller *et al.*, 2001; Proctor *et al.*, 2002). Similar results were demonstrated by *F. culmorum* where correlations were found between the aggressiveness and deoxynivalenol content (Hestbjerg *et al.*, 2002; Mesterházy, 2002). Coleoptile tissues of wheat (*Triticum aestivum*) cultivars that were resistant to Fusarium head blight were 10 times more resistant to deoxynivalenol and some other metabolites (including 3 acetyl deoxynivalenol and dihydroxycalonectrin) than disease-susceptible cultivars (Miller *et al.*, 2001).

There are several NHSTs such as tentoxin, a peptide phytotoxin, that have been found in cultures of *Alternaria tenuis* and *A. mali* (Okuno *et al.*, 1975). These toxins cause chlorosis as the result of interference with plastid development and a reduction of chlorophyll synthesis. These symptoms were observed on various plants such as cotton (*Gossypium hirsutum*), citrus (*Citrus limon*), cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*) (Mitchell, 1984).

The causal agent of Dutch elm disease (*Ceratocystis ulmi*) produces a high macromolecular weight NHST called cerato-ulmin (Takai *et al.*, 1983). Ophiobolin (cochliobolin) is a toxin produced by a number of fungi, in particular by *C. miyabeanus* (*Helminthosporium oryzae*) which causes rice leaf spot disease. Ophiobolin is a sesquiterpene that alters the plasma membrane structure and phenolic metabolism. The polymerisation of phenolics gives rise to the brown pigments that appear in the necrotic areas of host tissues (Isaac, 1991).

#### *Fungal cell wall components (elicitors)*

Some components isolated from the cell walls of pathogenic fungi are called elicitors and have the ability to trigger plant defence responses. Fungal elicitors were first used by Buiatti *et al.* (1985) to select carnations resistant to *F. oxysporum* and by Scala *et al.* (1985) to select resistant tomatoes. Similar experiments were performed later, also with carnation, by Trillas and Azcón-Bieto (1995). Other experiments with cell wall components of *F. oxysporum* were carried out for selection of tomato (Storti *et al.*, 1992). Alfalfa protoplast and cell cultures were selected with fungal cell-wall components and heat-released elicitors for resistance to *Verticillium albo-atrum* (Koike *et al.*, 1993b; Koike and Nanbu, 1997).

## **APPLICATIONS**

Table 2.2 summarises the practical applications of *in vitro* selection of disease resistance in order to obtain breeding lines that are tolerant of or resistant to certain pathogens. The similarities between the *in vitro* screening systems and whole plant-pathogen interactions were recently compiled in a comprehensive review (Švábová and Lebeda, 2005).

The possibility of using pathogen isolates, extracts or toxins as selection agents to screen *in vitro* cultures of host plants is now well established. Also, the application of selection agents during plant *in vitro* regeneration results in plant lines showing an increased degree of resistance to diseases. In the past two decades, more than 100 research articles that used *in vitro* selection methods for the improvement of resistance to phytopathogens were published. Over 30 plant species and their different types of *in vitro* cultures were examined, and various selection agents were obtained from about 40 plant pathogens. Such methodology often utilises some type of explant cultures (excised organ or part of organ, meristem tip culture, anther or pollen, callus, cell or tissue suspension, protoplast culture) of the host plants that are treated with various selective agents (natural isolate of pathogen, modified pathogen culture, culture filtrates, toxins, elicitors), which in turn elicit reactions that parallel those by the pathogens. Their application successfully resulted in resistant lines in various important crops including banana (*Musa* sp.), carnation (*Dianthus caryophyllus*), grapevine (*Vitis vinifera*), strawberry (*Fragaria ananassa*) and wheat (*Triticum aestivum*). Nowadays, these techniques are an important complement to classical breeding methods. Some examples of the application of these techniques in the improvement of crop resistance are shown in Table 2.2.

## CONCLUSIONS

Molecular studies reveal that stable genetic changes have occurred in plant materials that were selected *in vitro* for their increased tolerance/resistance to the selection agents (Kodama *et al.*, 1991; Knogge, 1996; Loon, 1997; Wojtaszek *et al.*, 1997; Bettini *et al.*, 1998; Jayasankar *et al.*, 2003). The resistant material often exhibits changes in the expression of pathogenesis-related and/or anti-fungal proteins. Fundamental knowledge about the role of particular selective components in pathogenesis and their mode of action are now crucial points for further development in the area of *in vitro* selection studies.

This chapter shows that significant progress has been made in phytotoxin research during the last two decades, particularly in: (1) elucidating the chemical structures of phytotoxins and their relationship to biological function; (2) the genetics of phytotoxin production; (3) the mode of action of phytotoxins at the molecular level; and (4) in proving that phytotoxins are efficient tools for screening plants for disease resistance.

In the case of *in vitro* selection techniques, which use some of the selective agents mentioned above, the resulting changes in phenotypic characters (e.g., resistance/tolerance to diseases) can be interpreted as potential mutations in the plant DNA sequence. Nevertheless, caution is advised when interpreting the results because similar phenotypes can be caused by epigenetics. Such epigenetic interactions frequently occur, but are not transmitted to the progeny (Bulk, 1991). Therefore, genetic analyses of inheritance in selected lines must be performed and the results carefully interpreted.

*In vitro* selection can considerably shorten the time needed for the selection of desirable traits. *In vitro* selection pressure suffers minimal influence from exterior environmental conditions, and can precede and complement field selections. *In vitro*-selected putative variants should be tested in the field to confirm the genetic stability of the selected trait (Jain, 2001), and genetically stable somaclones or mutants can then be used directly as elite varieties or introduced into plant breeding programmes.

Last but not least, *in vitro* selection of somaclonal variants and induced mutants is not protected under intellectual property regulations, nor is it subject to public safety concerns that currently hamper transgenic (i.e., GMO) approaches for the development of new crop cultivars. Thus, *in vitro* selection is a promising, non-transgenic approach, which offers an attractive alternative method for producing improved cultivars (Jayasankar *et al.*, 2003).

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**Table 2.1.** Methods of aseptic and *in vitro* culture of plant organs and plant tissues which may be used for resistance screening\*

Culture type	Description
Excised organ or part of organ culture	The culture of whole or parts of excised roots, leaves, stems, flowers, fruits etc.
Meristem (tip) culture	The regeneration of whole plants from excised stem apical or axillary meristems, via organogenesis or embryogenesis
Anther or pollen culture	The regeneration of haploid or homozygous diploid callus or plants from pollen or anther cells
Callus (tissue) culture	The culture of disorganised masses of tissue on solid media. Callus of some species may be induced to differentiate tissues or organs by changing the hormone and nutrient balance of the culture medium
Cell or tissue suspension culture	The culture of suspensions of single cells and/or cell aggregates in liquid media. Cells of some species may be induced to give rise to embryoids and then plants in culture by changing the hormone and nutrient balance of the medium
Protoplast culture	The enzymatic or mechanical isolation and culture of cell protoplasts. Protoplast fusion, wall regeneration and normal or hybrid plant regeneration are all possible with certain species

\*Modified according to Ingram (1976).

**Table 2.2.** Examples of successful application of *in vitro* resistance screening in selection and plant breeding\*

Crop (plant species)	Type of <i>in vitro</i> culture	Pathogen (selective agent)	Effect on resistance or other results of the study	References
<b>Excised organ or part of organ culture</b>				
alfalfa ( <i>Medicago sativa</i> )	separated cotyledons	<i>Colletotrichum trifolii</i> (spore suspension)	useful screening method which correlates with other greenhouse tests	Cucuzza and Kao (1986)
alfalfa ( <i>M. sativa</i> )	stem cuttings and leaf infiltration assay	<i>Verticillium albo-atrum</i> (filtrate)	symptoms on stems and leaves after filtrate treatment were typical for field-infected plants; the filtrate has a potential for use in screening for resistance	Ireland and Leath (1987)
cowpea ( <i>Vigna unguiculata</i> )	leaf disks	<i>Phytophthora cryptogea</i> (filtrate)	induction of resistant response and elicitation of phytoalexins	Saindrenan <i>et al.</i> (1990)
grape vine ( <i>Vitis vinifera</i> )	culture of sterile rooted shoots	<i>Plasmopara viticola</i> (co-cultivation with isolate-dual culture)	resistance to downy mildew was expressed <i>in vitro</i> ; technique can be used for screening and selection for host resistance	Barlass <i>et al.</i> (1986)
onion ( <i>Allium cepa</i> )	germinating seeds	<i>Alternaria porri</i> (filtrate)	reduction in seed germination and seedling vigour after filtrate treatment	Gupta <i>et al.</i> (1986)
pea ( <i>Pisum sativum</i> and wild species)	roots tube test	<i>Fusarium solani</i> , <i>F. oxysporum</i> (inoculation)	broad variation in sensitivity and/or resistance among wild species collection; several potential sources of resistance were selected	Lebeda and Švábová (1997)
pineapple ( <i>Ananas comosus</i> )	leaf segments and wounded plantlets from tissue cultures	<i>Fusarium subglutinans</i> (filtrate, fusaric acid - FA)	susceptible cvs. were sensitive to culture filtrate whereas resistant cvs. showed tolerance; using filtrate allowed the selection of resistant plants to fungus itself	Borrás <i>et al.</i> (2001)
soybean ( <i>Glycine max</i> )	intact plants	<i>Fusarium solani</i> f. sp. <i>glycines</i> (inoculation, filtrate)	positive correlation between AUDPC of inoculated plants and cut seedling test using culture filtrate	Huang and Hartman (1998)
sugar cane ( <i>Saccharum officinarum</i> )	<i>in vitro</i> micropropagated culture of intact plants	<i>Ustilago scitaminea</i> (inoculation)	correlation between <i>in vitro</i> response and resistance of plants was proven	Fereol (1984)

tobacco ( <i>Nicotiana tabacum</i> )	separated leaves	<i>Phytophthora cryptogea</i> (filtrate)	study of phytotoxicity of individual filtrate fractions	Bonnet <i>et al.</i> (1985)
tobacco ( <i>N. tabacum</i> )	intact leaves; cell and protoplast culture	<i>Alternaria alternata</i> (AT toxin)	the toxin induced typical symptoms on leaves of susceptible cultivars; cells of resistant cvs. survived toxin treatment better than those of susceptible cvs; field tolerance among cvs. is expressed at the cultured cell level	Ishida and Kumashiro (1988)
tomato ( <i>Lycopersicon esculentum</i> )	somaclones derived from leaves, cotyledons and hypocotyls	<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> (suspension)	limited potential of somaclonal variation as a source of resistance to bacterial cancer was shown	Bulk <i>et al.</i> (1991)
<b>Meristem, shoot and embryogenic culture</b>				
apple tree ( <i>Malus domestica</i> )	shoot culture	<i>Gymnosporangium juniperi-virginianae</i> (inoculation)	shoots from resistant variety were not affected by the fungus; differences in resistance between cotyledonary or embryo axis shoots were observed	Joung <i>et al.</i> (1987)
banana ( <i>Musa</i> )	shoot-tip culture	<i>Mycosphaerella fijiensis</i> (secondary metabolites)	2,4,8-THT(trihydroxytetralone) activated phenylalanine-ammonia lyase; necrotic microlesions and activation of defence mechanism in resistant cv. led to incompatible reaction	Hoss <i>et al.</i> (2000)
banana ( <i>Musa</i> )	multiple-shoot culture	fusaric acid	tolerant variant selected with FA after chemical mutagenesis	Matsumoto <i>et al.</i> (1995)
chickpea ( <i>Cicer arietinum</i> )	shoots	<i>Ascochyta rabiei</i> , <i>Fusarium oxysporum</i> (filtrates, toxins solanapyrone A, B, C)	sensitivity to the toxins was correlated with susceptibility to the disease; selection of plants with well expressed glutathion/glutathion-S-transferase may be one means of improving resistance	Hamid and Strange (2000)
eucalyptus ( <i>Eucalyptus marginata</i> )	micropropagated clones	<i>Phytophthora cinnamomi</i> (suspension)	primary roots of resistant micropropagated lines were able to restrict and confine colonisation by <i>P. cinnamomi</i>	Cahill <i>et al.</i> (1992)
pea ( <i>Pisum sativum</i> )	tube test, multiple-shoot culture, dual culture	<i>Fusarium solani</i> , <i>F. oxysporum</i> (inoculation, culture filtrate, autoclaving inactivated fungus)	comparison of the response using three <i>in vitro</i> methods	Švábová <i>et al.</i> (1998)

pea ( <i>P. sativum</i> )	tube test, multiple-shoot culture, dual culture	<i>Fusarium</i> spp. filtrates	regenerants were tested in field conditions and artificially inoculated substrates; improved resistance evaluated with infection degree was observed	Švábová and Odstřčilová (2001)
strawberry ( <i>Fragaria vesca</i> )	clones derived from shoot apex calli	<i>Alternaria alternata</i> (inoculation)	resistant lines revealed from surviving inoculated calli clones were obtained	Takahashi <i>et al.</i> (1992)
wheat ( <i>Triticum aestivum</i> )	embryogenic culture	<i>Septoria nodorum</i> (toxic extracts)	good correlation between field resistance and embryo resistance <i>in vitro</i>	Keller <i>et al.</i> (1994)
<b>Callus culture</b>				
apple tree ( <i>Malus domestica</i> )	callus culture	<i>Venturia inaequalis</i> (co-cultivation)	study of ultrastructural interactions of resistant and susceptible varieties (similar relationships)	Beech and Gessler (1986)
banana ( <i>Musa</i> )	callus, intact plants	<i>Cercospora musae</i> , <i>Mycosphaerella musicola</i> (inoculation)	both calli and plants showed similar levels of survival after inoculation	Trujillo and Garcia (1996)
barley ( <i>Hordeum sativum</i> )	callus culture derived from immature embryos	fusaric acid	plants regenerated from calli selected on FA were resistant to the toxin (75-100% concentration)	Chawla and Wenzel (1987)
barley ( <i>H. vulgare</i> )	callus culture derived from immature embryos	<i>Drechslera teres</i> (toxin)	nine progeny of S2 showed a correlation between toxin tolerance and resistance against the pathogen	Hunold <i>et al.</i> (1992)
camrathion ( <i>Dianthus caryophyllus</i> )	callus culture	<i>Fusarium oxysporum</i> (filtrate)	resistant calli were used for plant regeneration – 32% plants had considerable resistance against pathogen in field conditions	Thakur <i>et al.</i> (2002)
cauliflower ( <i>Brassica oleracea</i> var. <i>botrytis</i> )	callus culture	<i>Xanthomonas campestris</i> pv. <i>campestris</i>	mutagenised calli were selected on 30% culture filtrate; high level of correlation between resistance of calli to the filtrate and resistance of regenerated plants to pathogen was observed	Mangal and Sharma (2002)
celery ( <i>Apium graveolens</i> )	callus and suspension cultures	<i>Septoria apiicola</i> (filtrate)	positive response of continual culturing on the percentage of cells surviving in selections	Evenor <i>et al.</i> (1994)
chickpea ( <i>Cicer arietinum</i> )	callus culture	<i>Fusarium oxysporum</i> (filtrate)	inhibition of callus growth; analysis of total phenols, peroxidase and $\beta$ -1,3-glucanase	Singh <i>et al.</i> (2003)

common bean ( <i>Phaseolus vulgaris</i> )	calli	<i>Colletotrichum lindemuthianum</i> (culture filtrate)	activities differential response of bean callus from sensitive and/or resistant lines was observed; toxic compounds of the filtrate were analysed; pathogenicity could be related with common filtrate substances	Fernandez <i>et al.</i> (2000)
corn ( <i>Zea mays</i> )	calli	<i>Helminthosporium carbonum</i> (toxin HC)	an attempt to regenerate calli resistant to HC toxin failed (due to inability to identify resistant cells among slow growing callus cells)	Wolf and Earle (1990)
eggplant ( <i>Solanum melongena</i> )	callus culture	<i>Verticillium dahliae</i> (filtrate)	reliable screening system <i>in vitro</i> was developed; selection for tolerant lines on cellular level	Koike <i>et al.</i> (1993a)
elm ( <i>Ulmus americana</i> )	callus culture, stem cuttings	<i>Ceratocystis ulmi</i> (filtrate)	reduction in callus growth of susceptible variety on media with filtrate; correlation between callus reaction and cut stem assay	Pijut <i>et al.</i> (1990)
eucalyptus ( <i>Eucalyptus marginata</i> )	callus culture	<i>Phytophthora cinnamomi</i> (agar blocks of fungus culture)	hyphal growth on callus correlated with susceptibility of the plant	McComb <i>et al.</i> (1987)
gladiolus ( <i>Gladiolus</i> sp.)	shoots and callus cultures; intact corms	<i>Fusarium oxysporum</i> f. sp. <i>gladioli</i> , fusaric acid	shoot assay and ion-release with intact cormels gave significantly coinciding results; part of the Fusarium resistance is based on insensitivity to fusaric acid	Löffler and Mouris (1992), Remotti and Löffler (1996)
oat ( <i>Avena sativa</i> )	calli	<i>Helminthosporium victoriae</i> (toxin – victorin)	insensitivity to toxin was heritable; specific resistance can be selected in tissue cultures of oats	Rines and Luke (1985)
onion ( <i>Allium cepa</i> )	callus and shoot cultures	<i>Pyrenochaeta terrestris</i> (filtrate)	calli exposed to the filtrate reflected the degree of whole plant susceptibility	Gourd <i>et al.</i> (1988)
potato ( <i>Solanum tuberosum</i> )	calli and bulbs	<i>Verticillium albo-atrum</i> (inoculation and filtrate)	hyphal colonisation on tuber disks and calli; calli development on media containing culture filtrate are good markers for <i>in vivo</i> resistance	Koike <i>et al.</i> (1996)
potato ( <i>S. tuberosum</i> )	callus culture	<i>Erwinia carotovora</i> subsp. <i>carotovora</i> (inoculation and bilayer culture)	calli resistant to <i>E. carotovora</i> were identified, number of regenerants were too low to determine correlation between <i>in vitro</i> response and tuber resistance	Taylor and Secor (1990)

tomato ( <i>Lycopersicon esculentum</i> )	calli	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> (filtrate and mycelial cell wall components-elicitors)	correlation between <i>in vivo</i> resistance and <i>in vitro</i> hypersensitive response and phytoalexin induction, but tolerance to toxic filtrate <i>in vitro</i> is not indicator of <i>in vivo</i> resistance	Scala <i>et al.</i> (1985)
tomato ( <i>L. esculentum</i> )	callus and cell suspension culture	<i>Clavibacter michiganensis</i> (toxin)	toxin-tolerant cell lines were developed	Krämer <i>et al.</i> (1988)
wheat ( <i>Triticum aestivum</i> )	calli on double layer	<i>Fusarium graminearum</i> and <i>F. culmorum</i> (culture inactivated by autoclaving)	3% of R <sub>2</sub> plants were found to be more resistant than the original cultivars	Ahmed <i>et al.</i> (1991)
wheat ( <i>T. aestivum</i> )	calli	<i>Fusarium culmorum</i> and <i>F. graminearum</i> (filtrate)	35.7% of lines were more resistant than the original cultivars	Ahmed <i>et al.</i> (1996)
<b>Cell or tissue suspension culture</b>				
alfalfa ( <i>Medicago sativa</i> )	cell suspension culture	<i>Verticillium albo-atrum</i> (inoculation and heat-released elicitors)	treatment with conidia and heat-released elicitors induced a large increase in PAL activity in resistant cell lines	Koike and Nanbu (1997)
alfalfa ( <i>M. sativa</i> )	cell culture	<i>Fusarium oxysporum</i> (filtrate)	cell cultures derived from genotype susceptible to <i>F. oxysporum</i> accumulated double the amount of phenolic acids than the resistant ones	Cvikrová <i>et al.</i> (1992)
apple tree ( <i>Malus domestica</i> )	mesophyll cells	<i>Phytophthora cactorum</i> (culture filtrate)	virulent strain showed an increase in fluorescence; possibility of using fluorescence measurement as a screening system and filtrate as selective agent	Mezzetti <i>et al.</i> (1992)
celery ( <i>Apium graveolens</i> )	somaclones revealed from cell suspension cultures	<i>F. oxysporum</i> (inoculation by soaking of roots in suspension)	resistant phenotypes were identified; resistance conditioned by more than one locus	Heath-Pagliuso <i>et al.</i> (1988)
coffee tree ( <i>Coffea arabica</i> )	cell and protoplast culture	<i>Colletotrichum kahawae</i> (filtrate)	selective effect of filtrate on protoplast was proven; system can be used for selection and screening	Nyange <i>et al.</i> (1997)
rape ( <i>Brassica juncea</i> , <i>B. napus</i> ), mustard ( <i>Sinapis alba</i> )	cell suspension cultures	<i>Phoma lingam</i> (phomalide), <i>Alternaria brassicae</i> (destruxin)	differential phytotoxicity of phomalide and direct correlation with plant disease resistance; destruxin – less clear relationship to disease resistance	Pedras and Biesenthal (2000)

soybean ( <i>Glycine max</i> )	suspension cell culture; stem cuttings	<i>Fusarium solani</i> f. sp. <i>glycines</i> (filtrate)	positive correlation between foliar symptom severity and viability staining of cell cultures	Li <i>et al.</i> (1999)
tomato ( <i>L. esculentum</i> )	cell cultures	<i>Alternaria alternata</i> (AL-toxins)	cells revealed from resistant cultivar were not affected significantly; host-selective toxicity was expressed on the cell level as well as plant tissue	Kodama <i>et al.</i> (1991)
<b>Protoplast culture</b>				
alfalfa ( <i>M. sativa</i> )	protoplast culture	<i>Verticillium albo-atrum</i> (filtrate and cell-wall components)	protoplasts of susceptible variety were sensitive to the low molecular weight fractions of the filtrate	Koike <i>et al.</i> (1993b)
grape vine ( <i>V. vinifera</i> )	protoplasts	<i>Botrytis cinerea</i> (filtrate)	the plating efficacy and embryogenic competence of regenerated calli decreased with increasing filtrate concentration	Reustle and Matt (2000)
hop ( <i>Humulus lupulus</i> )	protoplast and cell suspension culture	<i>Verticillium albo-atrum</i> (filtrate)	cytotoxicity of the culture filtrates correlated both to the virulence of the isolates and the resistance of the cultivars	Connel <i>et al.</i> (1990)
rape ( <i>Brassica oleracea</i> ), tobacco ( <i>N. tabacum</i> ), potato ( <i>S. tuberosum</i> )	protoplasts, calli; intact plants	<i>Phoma lingam</i> (inoculation, toxic metabolites, sirodesmin PL)	cells of the host plant rape were sensitive to the toxin of <i>Phoma</i> , but the non-host tobacco and potato cells remained resistant	Sjödín <i>et al.</i> (1988)
rape ( <i>B. napus</i> , <i>B. juncea</i> , <i>B. carinata</i> , <i>B. nigra</i> )	protoplasts; cell aggregates	<i>Phoma lingam</i> (toxin – sirodesmin PL)	– toxin has host-selective properties clear correlation between resistance to <i>P. lingam</i> and insensitivity to sirodesmin PL is present; toxin can be used to distinguish resistant and susceptible material both <i>in vitro</i> and <i>in vivo</i>	Sjödín and Glimelius (1989)
tobacco ( <i>N. tabacum</i> )	protoplast-derived calli	<i>Pseudomonas syringae</i> pv. <i>tabaci</i> and <i>Alternaria alternata</i> (toxins)	assay of R <sub>1</sub> generation derived from toxin-resistant calli indicated the inheritance of resistance	Thanutong <i>et al.</i> (1983)
tomato ( <i>L. esculentum</i> )	protoplasts, calli; intact plants	<i>Fusarium oxysporum</i> (fusaric acid, inoculation)	single dominant gene type of resistance to <i>Fusarium wilt</i> was obtained after <i>in vitro</i> selection on FA	Shahin and Spivey (1986)

\*Modified according to Šváblová and Lebeda (2005).

**Table 2.3.** Host-selective toxins (HSTs) produced by plant pathogenic fungi and bacteria

Pathogen	Host plant	Toxin	References
<b>Fungal pathogens</b>			
<i>Alternaria mali</i>	apple, pear	AM-toxin	Nishimura and Kohmoto (1983), Kohmoto and Otani (1991), Saito <i>et al.</i> (2001)
<i>A. citri</i>	citrus	AC-toxin, ACRL-toxin	Gardner <i>et al.</i> (1985), Kohmoto and Otani (1991) Nishimura and Kohmoto (1983)
<i>A. kikuchiana</i>	japanese pear knapweed	AK-toxin maculosin	Nishimura and Kohmoto (1983), Kohmoto and Otani (1991) Upadhyay and Mukerji (1997)
<i>A. alternata</i>	strawberry	AF-toxin	Nishimura and Kohmoto (1983), Kohmoto and Otani (1991)
<i>A. fragariae</i>	tangerine	ACT-toxin I, II, ACTG-toxin A,B	Kohmoto and Otani (1991)
<i>A. citri</i>	tobacco	AT-toxin	Nishimura and Kohmoto (1983), Ishida and Kumashiro (1988), Kodama <i>et al.</i> (1990), Kohmoto and Otani (1991)
<i>A. longipes</i>	tomato	AL-toxin, AAL-toxin	Kodama <i>et al.</i> (1991), Kohmoto and Otani (1991), Chen <i>et al.</i> (1992), Nishimura and Kohmoto (1983)
<i>A. alternata</i> f. sp. <i>lycopersici</i>	rape	destruxin B, homodestruxin B, destruxin B2	Tewari and Bains (1997), Upadhyay and Mukerji (1997), Pedras and Biesenthal (2000)
<i>A. brassicae</i>			
<i>A. helianthi</i>	sunflower	radicin, deoxyradicin	Nishimura and Kohmoto (1983)
<i>A. temis</i>	pigeonpea	tentoxin	Nishimura and Kohmoto (1983), Kohmoto and Otani (1991), Upadhyay and Mukerji (1997)
<i>Corynespora cassiicola</i>	tomato	CC-toxin	Kohmoto and Otani (1991), Upadhyay and Mukerji (1997)
<i>Drechslera zeicola</i>	maize	HC-toxin, HC-toxin I, II, III, HMT-toxin	Buiatti and Ingram (1991), Kohmoto and Otani (1991), Wolf and Earle (1990), Walton and Panaccione (1993)
<i>Helminthosporium carbonum</i>			Yoder (1980)
<i>D. maydis</i>	maize	HC-toxin	Walton and Panaccione (1993)
<i>(C. heterostrophus)</i>			
<i>D. sacchari</i>	sugarcane	helminthosporoside, HS-toxin A,B,C	Yoder (1980), Kohmoto and Otani (1991), Upadhyay and Mukerji (1997)
<i>(Bipolaris sacchari,</i> <i>Helminthosporium sacchari)</i>			

<i>D. victorinae</i>	oat	HV-toxin (victorin C); victorin	Rines and Luke (1985), Kohmoto and Otani (1991), Walton and Panaccione (1993)
( <i>Cochliobolus victorinae</i> )			
<i>Periconia circinata</i>	sorghum	PC-toxin, peritoxin A,B	Kohmoto and Otani (1991), Walton and Panaccione (1993), Upadhyay and Mukerji (1997)
<i>Phyllostica maydis</i>	maize	PM-toxin A,B,C,D	Kohmoto and Otani (1991), Upadhyay and Mukerji (1997)
<i>Phoma lingam</i>	rape	phomalide	Pedras and Biesenthal (2000)
<i>Phoma tracheiphila</i>	lemon	malseccin, mellein	Gentile et al. (1992), Parisi et al. (1993)
<b>Bacterial pathogens</b>			
<i>Pseudomonas syringae</i> pv. <i>lycopersici</i>	tomato	syringolide	Walton and Panaccione (1993)
<i>P. syringae</i> pv. <i>phaseolicola</i>	beans	phaseolotoxin	Yoder (1980), Moore et al. (1984)
<i>P. syringae</i> pv. <i>tabaci</i>	tobacco	tabtoxin	Yoder (1980), Shaw (1997)

**Table 2.4.** Non-host-selective toxins (NHSTs) produced by plant pathogenic fungi

<b>Pathogen</b>	<b>Host plant</b>	<b>Toxin</b>	<b>Reference</b>
<i>Cercospora kikuchii</i>	soybean	cercosporin	Daub and Ehrenshaft (1997)
<i>C. beticola</i>	sugar beet	CB-toxin, beticolins, cebetins	Jalal <i>et al.</i> (1992)
<i>C. arachidicola</i>	peanut	dothistormin	Jalal <i>et al.</i> (1992)
<i>Fusarium</i> spp.	barley	fusaric acid	Chawla and Wenzel (1987)
	banana	fusaric acid	Matsumoto <i>et al.</i> (1995)
	alfalfa	fusaric acid	Řepková and Nedělník (1998)
<i>F. culmorum</i>	cereals	trichothecenes (DON)	Bruins <i>et al.</i> (1993), Hestbjerg <i>et al.</i> (2002), Kang and Buchenauer (2002), Mesterházy (2002)
<i>F. graminearum</i>	wheat	DON	Mesterházy (2002)
	maize	DON, Ac-DON	Proctor <i>et al.</i> (2002)
<i>F. moniliforme</i>	gladiolus, rice, maize	fumonisin B	Abbas <i>et al.</i> (1995)
<i>F. solani</i>	lemon-tree	dihydrofusarubin, isomarticin, naphthazarins	Nemec <i>et al.</i> (1989)
	pea	marticins, fusarubin,	Baker and Nemeč (1997)
	soybean	monorden	Baker and Nemeč (1994)
<i>F. subglutinans</i>	pineapple	fusaric acid	Borrás <i>et al.</i> (2001)
<i>F. oxysporum</i>	pea	isomarticin, fusaric acid, pyridine derivative toxin,	Baker and Nemeč (1997)
	jimsonweed	moniliformin	Abbas <i>et al.</i> (1995)
	tomato	fusaric acid	Kuzniak (2001)
f. sp. <i>gladioli</i>	gladiolus	fusaric acid	Remotti and Löffler (1996)
f. sp. <i>lilii</i>	lily	fusaric acid	Löffler and Mouris (1992)
f. sp. <i>melonis</i>	muskmelon	beauvericin	Moretti <i>et al.</i> (2002)
f. sp. <i>vasinfectum</i>	(particular host plants)	fusaric acid	Luz <i>et al.</i> (1990)
<i>F. verticillioides</i>	maize	fumonisin	Proctor <i>et al.</i> (2002)
<i>Ascochyta pisi</i>	pea	ascochitine, ascosalitoxin, pinolidoxin	Strange (1997)
<i>A. pinodes</i>	pea	ascochitine,	Evidente <i>et al.</i> (1993)
<i>A. fabae</i>	fababean	pyrenolide A,	Beed <i>et al.</i> (1994)
<i>A. hyalospora</i>	lambquarters	hyalopyrone,	Venkatasubbaiah and Chilton (1992)
	chrysanthemum	chrysanthone A,B,C	
<i>A. chrysanthemi</i>		ascotoxin,	
<i>A. imperfecta</i>		ascochlorin	Strange (1997)

<i>A. viciae</i>	lucerne, clover vetch	ascofuranone, ascofuranol cytochalasins A, B, U, V, T, F, 7-0- acetylalachalasin B, cytochalasin A, B solanapyrone A, C solanapyrone sirodesmin PL, phomalide	Strange (1997) Strange (1997) Strange (1997) Vurro <i>et al.</i> (1992) Hamid and Strange (2000) Strange (1997) Buiatti and Ingram (1991), Pedras <i>et al.</i> (1999) Hoss <i>et al.</i> (2000), Okole and Schulz (1997) Keller <i>et al.</i> (1994)
<i>A. heteromorpha</i>	oleander		
<i>A. lathyri</i>	vetchling		
<i>A. rabiei</i>	chickpea		
<i>Alternaria solani</i>	potato, tomato		
<i>Leptosphaeria maculans</i> ( <i>Phoma lingam</i> )	oilseed rape		
<i>Mycosphaerella fijiensis</i>	banana	2,4,8- trihydroxytetralone	
<i>Septoria nodorum</i>	wheat	mellein	

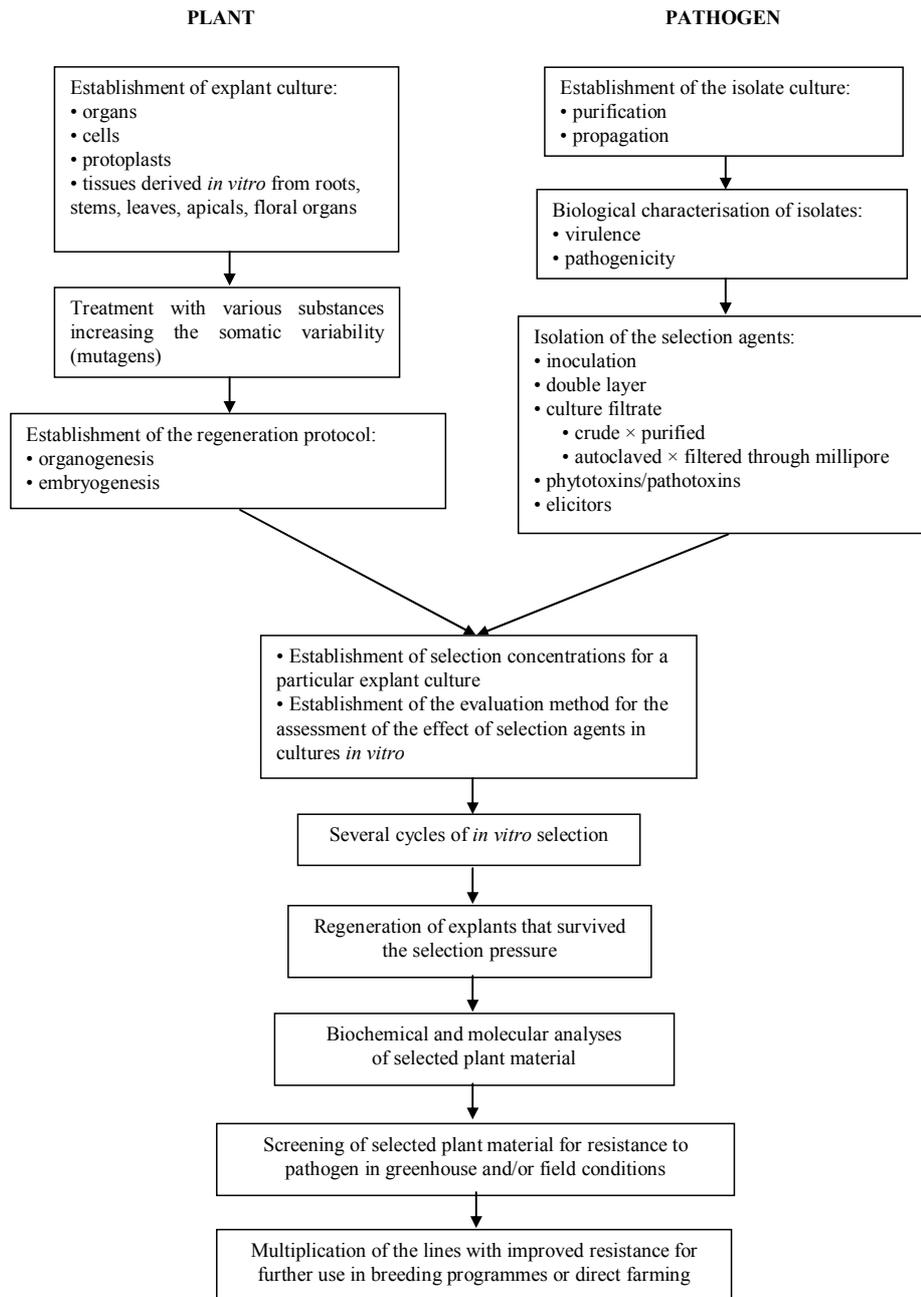
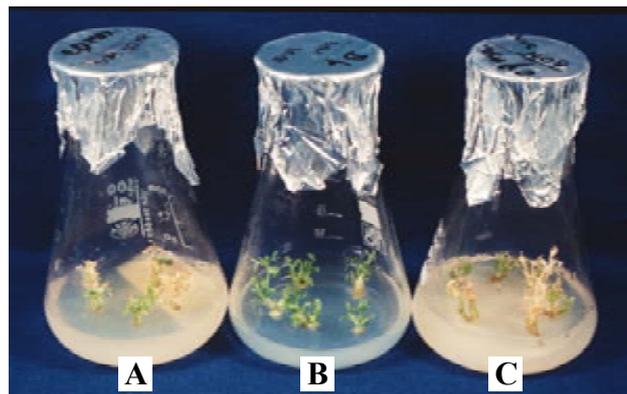


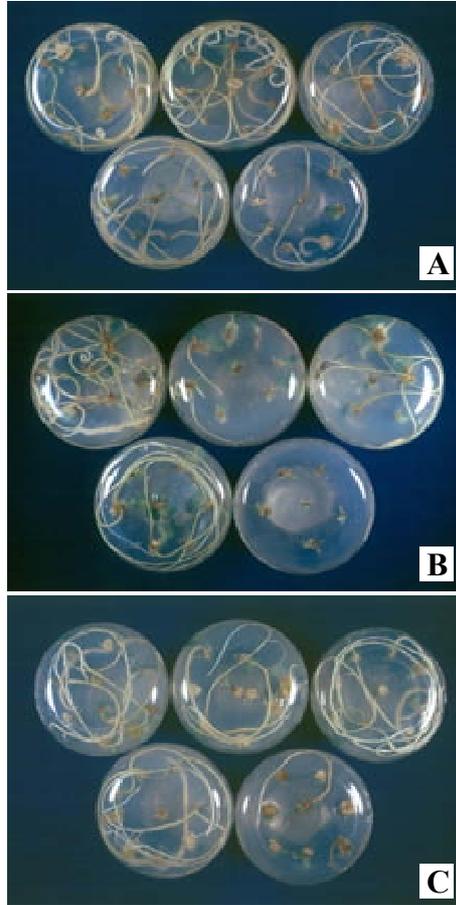
Figure 2.1. General scheme of *in vitro* screening for disease resistance and selection.



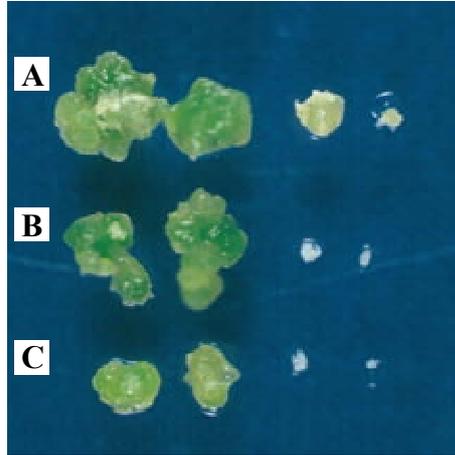
**Figure 2.2.** Culture of pea (*Pisum sativum*, line HM6) root segments and their response to *Fusarium oxysporum* filtrate. Concentration of the filtrate from left to right: 0 (control), 0.1, 1, 2, 5 and 10%. Bar: 10 mm. Photograph: M. Griga.



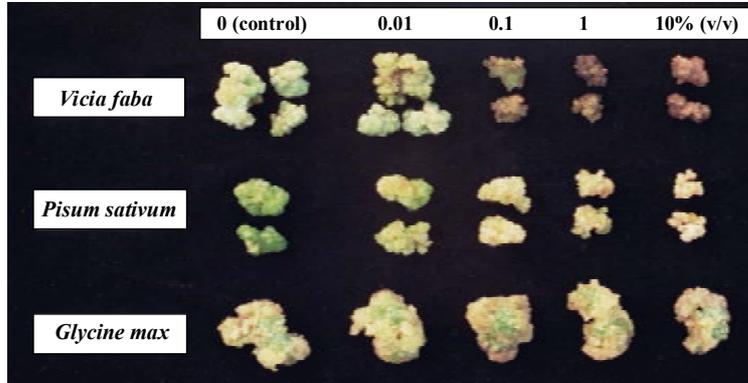
**Figure 2.3.** Culture of pea (*Pisum sativum*) shoot clusters grown on a “double-layer” medium. (A) *Fusarium oxysporum*; (B) Control; (C) *F. solani*. The first layer was inoculated with the fungus and grown for four weeks, then inactivated by autoclaving and overlaid by a second layer of medium suitable for the explant culture. In this particular case, a CDA medium was used as the first layer for growing the fungus, and an MS medium with macro- and micro-elements (B5 vitamins, FeEDTA, inozitol, sucrose, 0.1 mM NAA and 20 mM BAP) was used for growing the shoots. Note the presence of green resistant/tolerant plantlets in (A) and (C). The photograph was taken four weeks after cultivation. Photograph: M. Griga.



**Figure 2.4.** Differences in rooting of pea (*Pisum sativum*) shoots on media supplemented with different *Fusarium* filtrates (10% [v/v]). From left to right: control, *F. oxysporum*, *F. poae*, *F. solani*, *F. semitectum*; (A) cv. 'Komet'; (B) cv. 'Colt'; (C) line DP 1059. Photograph: M. Griga.



**Figure 2.5.** Effect of different concentrations of fusaric acid (FA) on callus culture of *Pisum sativum* (line 19/1). Concentration of FA in (A) 0 (control): simultaneous incidence of green and yellowish calli; (B) 2.5  $\mu\text{M}$ : incidence of explants growing into normal calli and without any development; and (C) 5  $\mu\text{M}$ : incidence of explants growing into calli with retarded growth and explants without any development. Photograph: M. Griga.



**Figure 2.6.** Effect of *Fusarium semitectum* filtrate on callus culture of *Vicia faba*, *Pisum sativum* and *Glycine max*. The concentration of *F. semitectum* filtrate is shown. Photograph: M. Griga.



**Figure 2.7.** Multiple-shoot culture of pea (*Pisum sativum*, line HM6) after application of *Fusarium oxysporum* filtrate (10% [v/v]). (A) Variation in shoot formation; (B) Variation in rooting intensity of plantlets. Photograph: M. Griga.



**Figure 2.8.** Growing of pea (*Pisum sativum*) plantlets in non-sterile conditions in the greenhouse after selection for resistance to *Fusarium* spp. Photograph: M. Griga.



## Chapter 3

### Mass-screening techniques for early selection of banana and plantain crops resistant to major diseases and pests

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#### Abstract

Different *in vitro* selection techniques that are used for selecting banana and plantain plants resistant to diseases and pests are discussed in this chapter with a focus on Black sigatoka disease. Explants that are suitable for *in vitro* selection include shoot meristems, micro-cross sections, callus, cell suspension and protoplasts. A double selection system using the crude filtrate and the purified host-specific toxin 2,4,8-trihydroxytetralone (2,4,8-THT) is described. The optimum concentration of the host-specific toxin 2,4,8-THT and the non-host-specific toxin juglone used for selection and the mode of action of the purified toxins are also shown. A combination of mutation induction and effective selection methods appears to be the most effective strategy to improve tolerance to diseases in banana and plantains.

#### INTRODUCTION

Bananas and plantains (*Musa* spp.) are among the most important staple food crops for several million people around the world. They rank fourth among food crops after rice, wheat and maize (CGIAR, 1993). Bananas and plantains play a major role in nutrition and well being, as well as in the cultural life of millions of people living in the tropics and sub-tropics. In many developing countries, bananas and plantains are widely used as a food supplement or as a staple food, while dessert bananas are a common food in Europe, the USA and Canada. Total world production of bananas and plantains is estimated at 75 million and 33 million metric tons, respectively (FAO, 2005).

The performance of banana and plantain crops, like that of many other crops, is limited by major diseases and pests. The major diseases and pests that affect banana and plantain production are Black sigatoka (black leaf streak) caused by *Mycosphaerella fijiensis*, Panama disease caused by *Fusarium oxysporum* f. sp. *cubense*, moko disease caused by *Ralstonia solanacearum*, bunchy top disease caused by the bunchy top virus, nematode diseases caused by *Radopholus similis*, *Pratylenchus* spp., *Meloidogyne* spp. etc., and corm borer caused by *Cosmopolites sordidus*. These diseases and pests usually lead to significant losses in food production and income. Consequently, the largest single component in the cost of production of bananas and plantains is disease and pest control. Current control measures, which comprise approximately 40% of total production costs in commercial plantations and small holder farms, vary from cultural and biological measures to chemical control (Ganry, 1993).

Breeding banana and plantain for resistance to diseases and pests is urgently needed by small-scale and commercial producers. However, these crops have very low clonal multiplication rates and most of the commercial varieties are polyploid with extremely poor seed production. These barriers impede sexual hybridisation and consequently slow down genetic improvement of the crop. The ability to culture bananas and plantains *in vitro*, is allowing researchers to develop different biotechnological methods, which they hope to deploy in producing new improved clones that are resistant to diseases

and pests. The different non-conventional breeding approaches which may be used to improve these crops to be disease and pest-resistant include genetic engineering, mutation breeding and *in vitro* selection using pathogen culture filtrates or purified toxins from the pathogens.

Genetic engineering would involve the introduction of a well characterised disease- or pest-resistant gene into banana or plantain by means of *Agrobacterium*-mediated transformation, microprojectile particle bombardment or electroporation. The plants would have to be tested, both in the laboratory and the greenhouse, for gene stability and integration before screening for the major diseases and pests.

## MATERIAL AND METHODS

In the case of mutation breeding, genetic variation is essential for the creation of plants with superior agronomic traits. However, spontaneous mutations occur at an extremely low frequency. Spontaneous somatic mutations have played an essential role in the speciation and domestication of plantains and bananas. Some authors even claim that all the bananas and plantains that we grow and eat were selected in prehistory from spontaneous mutations (Buddenhagen, 1986). Some of these spontaneous mutants in *Musa* (members of the 'Cavendish' subgroup and plantains) now play a very important role in banana and plantain production. Mutagenic agents such as radiation and certain chemicals can be used to induce mutations at a higher frequency and generate genetic variation from which desired mutants may be selected (Roux, 2004). Mutation breeding should be encouraged because the acceptance rate of this technology is higher than that for genetic transformation.

*In vitro* selection techniques used for banana and plantain are analogous to those used in prokaryotic systems, where mutations can be efficiently induced and variants selected and isolated at the cellular level. *In vitro* selection represents an immediate and inexpensive way of generating and selecting banana and plantain variants with tolerance to either the pathogen or its toxin from susceptible varieties, as compared to classical breeding methods (Evans and Sharp, 1986). Pathogens or their metabolites could be used as selection agents to enhance resistance within tissue cultures and to select resistant individuals.

The purified forms of these metabolites are known as toxins. Toxins produced by pathogens have been categorised based on several criteria, such as their chemical nature, mode of action, identity of the microorganisms producing them, and most definitions take into consideration their involvement as primary or secondary determinants of pathogenesis (Yoder, 1980). Toxins are primary determinants of pathogenesis when they act as the key element in infection initiation and symptom development. They are secondary determinants when they only modify the symptoms' intensity (El Hadrami *et al.*, 2005b). In order to assess the involvement of toxins in pathogenesis, commonly used criteria include: (1) host specificity, (2) presence in infected plants, (3) toxin production at a key step of disease development, (4) induction of typical disease symptoms and (5) degree of correlation between the quantity of toxin produced *in vitro* and the pathogenicity level (Scheffer, 1976; Yoder, 1980; Markham and Hille, 2001; El Hadrami *et al.*, 2005b; Švábová and Lebeda, 2005). Several experimental approaches have been developed in order to evaluate the role of toxins in host-pathogen interactions (Wolpert *et al.*, 2002). The most convenient seems to be the inactivation or specific elimination of the toxin from the system, followed by the observation of the modifications that occur during the initiation, establishment or expression of the host-pathogen interaction. For toxins or culture filtrates to be used as the selection agent *in vitro*, they will need a competent explant source that will be used for selection.

In any non-conventional breeding programme of banana and plantain, a suitable, highly regenerative explant source is needed. Shoot meristem, protoplasts, cell suspensions, callus and micro-cross sections have been used. Shoot meristems are usually generated from field-grown materials. The process involves sterilising the shoot tips in 30% commercial bleach solution (Domestos) containing 5% of sodium hypochlorite for 30 minutes. After sterilisation, the shoot tips are rinsed with sterile

distilled water or placed directly into Murashige and Skoog (MS) basal salt medium with vitamins (Murashige and Skoog, 1962). The MS basal salt medium is usually supplemented with ascorbic acid (80 mg/l), sucrose (30 g/l) and phytohormones: benzylaminopurine (BAP) (2-4 mg/l) and indole acetic acid (IAA) (0.2 mg/l). The pH is usually brought to 5.8 before 3 g/l of gelrite is added for autoclaving at 121°C for 25 minutes. After three subcultures, the shoot meristems can be used for transformation, mutation or *in vitro* screening.

A second explant source that is used by many laboratories involves the use of micro-cross sections (Okole and Schulz, 1996). Micro-cross sections are prepared either with a microtome or a scalpel by cutting a thin section of 300-400 µm from the corm of an *in vitro* tissue culture plant, which has been growing on a multiplication medium. The section normally produces several shoot buds with the addition of the appropriate phytohormones in the medium. These buds will eventually regenerate into plantlets. With this technique, it is possible to regenerate up to 15 plantlets from one explant without any morphological changes when compared to the controls. The tissues are also small and flat, and will have better contact with the pressure source, be it toxins, culture filtrate, or chemical or radiation mutants. Micro-cross sections can also be used to generate callus and cell suspension cultures with modifications of the phytohormones added.

The third type of explants used are male flower buds. It has been reported by several researchers (Escalant *et al.*, 1994; Sági *et al.*, 1995; Becker *et al.*, 2000; Ganapathi *et al.*, 2001) that male flowers are the most responsive starting material for initiating embryogenic cultures, especially for the 'Cavendish' group. The process described by these authors involves culturing the male flower buds on MS medium supplemented with 18.10 mM 2,4-dichlorophenoxyacetic acid (2,4-D), 5.37 mM naphthalene acetic acid (NAA), 5.71 mM IAA and 4.09 mM *d*-biotin with 30 g/l sucrose. After 2-3 months, the floral primordial shows enlargement and the development of whitish embryogenic callus. Embryogenic callus will continue to proliferate when subcultured on MS medium supplemented with 0.22 mM BAP and 1.14 mM IAA. If the cultures stay on this medium, somatic embryos will start developing during subcultures on the same medium. Green plumule and subsequently complete plantlets will emerge from the embryos followed by the development of roots within a span of 6 to 8 weeks upon transfer to half strength MS medium supplemented with 0.5 g/l malt extract (ME) and 0.1% activated charcoal (Ganapathi *et al.*, 2001).

Techniques of genetic transformation include: (1) electroporation of protoplasts, which have been isolated from an embryogenic cell suspension of the cooking banana cultivar 'Bluggoe' (Sági *et al.*, 1995); (2) the procedure of micro-projectile bombardment with DNA-coated particles which has been applied to embryogenic cell suspensions of 'Bluggoe', dessert bananas 'Williams' and 'Grand Naine' and the plantain 'Three Hand Planty' (Sági *et al.*, 1995; Becker and Dale, 2004); and (3) a combined *Agrobacterium* and microprojectile bombardment approach recently reported by May *et al.* (1995) for the dessert banana 'Grand Naine'. May *et al.* (1995) used corm slices as explants and *Agrobacterium* infection after wounding of meristematic tissue by using particle bombardment. Transformation by *Agrobacterium* was unexpected, because infection with *Agrobacterium* was formerly considered to be unsuitable for a monocotyledonous plant like banana.

Application of genetic transformation techniques has already resulted in regenerated transformed plants from cvs. 'Bluggoe' and 'Grand Naine' expressing marker genes such as the *uidA* gene coding for bacterial β-glucuronidase or an antibiotic resistance gene. However, there are currently no published reports about the expression of genes in banana or plantain, which might offer protection against a fungal disease. Sági *et al.* (1995) recently indicated that they intend to transfer genes into banana and plantain cultivars coding for cysteine-rich peptides isolated from seeds of different plant species (Cammue *et al.*, 1993). These peptides have shown high anti-fungal activity to *M. fijiensis* and *F. oxysporum* f. sp. *cubense in vitro*, while at the same time they are not toxic to human and plant cells.

### ***In vitro* selection using culture filtrates and toxins**

The two most important diseases studied in this regard are Black sigatoka disease and Panama disease. For this chapter the focus will be more on Black sigatoka disease.

The strategy is based on the use of more or less purified culture filtrates of *M. fijiensis* for Black sigatoka or *F. oxysporum* f. sp. *cubense* for Panama disease as a selection agent. Essential conditions for attributing the function of a disease determinant to a toxin or culture filtrate are: (1) the demonstration that the toxin or culture filtrate occurs in infected plants, and (2) the ability of the toxin to cause at least a portion of the syndrome when placed in healthy plants (Aducci *et al.*, 1997). Although toxins and culture filtrates may play important roles in some plant diseases, their possible use for selecting resistant genotypes of the host *in vitro* depends on two observations: (1) all reliably identified isolates of the fungus produce one or more toxins in culture; and (2) the symptoms of the disease (chlorosis, necrosis, breakage of the stem, etc.) are also elicited by the toxins alone (Hamid and Strange, 2000).

The phytotoxicity of *M. fijiensis* and *F. oxysporum* f. sp. *cubense* culture filtrates and toxins has been demonstrated in bioassays carried out on leaves of banana and plantain (Okole, 1995; Hoss *et al.*, 2000; Lepoivre 2003; Busogoro *et al.*, 2004; Gimenez and Colmenares, 2004; El Hadrami *et al.*, 2005a). Most of these compounds are non-host-selective toxins. The symptoms caused by toxins depends on the chemical nature of the compound and the properties of the plant. The most common symptoms that can be seen with the naked eye are wilting, necrosis, water-soaking and chlorosis. *In vitro* symptoms include slow or rapid cellular collapse, membrane leakages, accumulation of toxic metabolic intermediates, or ultrastructural alterations in organelles (Walton and Panaccione, 1993; Walton 1996).

Purification of crude extracts of *M. fijiensis* has allowed identification of a set of phytotoxic metabolites found in the pathogen culture filtrates. These compounds are 2,4,8-trihydroxytetralone (2,4,8-THT), 5-hydroxy-1,4-naphthalenedione (commonly called juglone), 2-carboxy-3-hydroxycinnamic acid, a dimethyl ester of 2-carboxy-3-methoxycinnamic acid, isochrocinic acid and 4-hydroxycytalone (Stierle *et al.*, 1991). Fijiensin, another metabolite of *M. fijiensis* that is characterised by a more complex structure than the previous molecules, exhibited no differential reaction to various cultivars despite the specificity of this molecule on banana (Upadhyay *et al.*, 1990). Out of the list of compounds produced from the culture filtrate, only 2,4,8-THT has been shown to be host-specific (Okole and Schulz, 1996; Hoss *et al.*, 2000).

The process normally used for *in vitro* selection is outlined in Figure 3.1. A double selection system was applied for selecting banana and plantain resistant plants. This involved selection first in the crude filtrate of *M. fijiensis* followed by a second round of selection using the host-specific purified toxin 2,4,8-THT. For that, micro-cross sections (400  $\mu\text{m}$ ), from which shoot buds can be regenerated, were placed in a 9 cm Petri dish. The best concentration when using the crude filtrate was adding 25% of the crude filtrate into MS multiplication medium described above. The dish containing the micro-cross sections and the crude filtrate was kept under light conditions of 90  $\mu\text{E}/\text{m}^2/\text{s}$  for four days. After treatment with the crude fungal filtrate, the sections were transferred to a multiplication medium without the addition of the crude fungal filtrate. Shoot buds that developed after four weeks on the brown tissues were regenerated to plantlets.

For double selection, micro-cross sections were again prepared from the plantlets resistant to the crude filtrate. They were placed for 48 hours under light conditions of 90  $\mu\text{E}/\text{m}^2/\text{s}$  on a banana multiplication medium containing 20  $\mu\text{g}/\text{ml}$  2,4,8-THT in the medium. A stock solution of the synthetic melanin inhibitor tricyclazole (5% ethanol [v/v]) (5-methyl-1,2,4-triazolo[3,4-b]-[1,3]benzothiazole, Dow AgroSciences LLC, Indianapolis, IN, USA) was added to the medium before autoclaving. After transfer of micro-cross sections onto toxin-free medium, shoot buds produced after four weeks were regenerated to plantlets and established in soil for *in vivo* screening using the fungus *M. fijiensis*.

### ***In vivo* screening**

Banana and plantain plantlets, ~20 cm in height, which were regenerated from micro-cross sections after the double selection protocol, were screened for fungal resistance with conidial suspension ( $4 \times 10^4$  conidia/ml) of different isolates collected from four different countries (Cameroon - isolate 200, Honduras - isolate 400, Papua New Guinea - isolate 309 and Nigeria - isolate 100). Some of these isolates were kindly supplied by Dr. X. Mourichon CIRAD/IRFA, Montpellier, France and Dr. B. Fullerton, Mt. Albert Research Centre, New Zealand. Inoculation on the leaves was performed following the method described in Figure 3.1. Symptoms on the leaves, such as chlorosis and brown to black lesions resulting from *M. fijiensis* infection were observed on the plants 40-60 days after inoculation. Each treatment had a control plant. Resistant plants had no symptoms or chlorosis; tolerant had 2-3 dark brown spots/flecks, while susceptible plants showed typical Black sigatoka symptoms.

### **RESULTS AND DISCUSSION**

There were 150 regenerated plants from the micro-cross sections of the three different banana and plantain cultivars (Table 3.1). The plants that were regenerated from the double selection procedure were later screened against fungal isolate 100 from Nigeria in the growth room. From Table 3.1, it could be seen that the different cultivars responded differently to the fungus in the growth room. Of the three cultivars tested, 'Petite Naine' had the highest percentage of resistant plants (19%), while 'Horn' plantain had the lowest (10.7%). All the control non-treated plants that were regenerated from micro-cross sections not treated with the crude filtrate or purified toxin were severely infected and showed typical Black sigatoka symptoms.

Since the cultivar 'Petite Naine' had the highest percentage of resistant plants, it was further multiplied and tested against four isolates of the fungus from different countries. The results in Table 3.2 show that the level of resistance of 'Petite Naine' plantlets varied with the different isolates used. The highest percentage of resistant plants came from plants treated with the fungal isolate from Nigeria (48%), while the lowest percentage of resistant plants (29%) was for plants inoculated with the isolate from Papua New Guinea.

The resistant plants from both Tables 3.1 and 3.2 were further multiplied (Figure 3.2) for a field experiment that was carried out in a big commercial plantation in Cameroon (SP&P). Unfortunately, the results were not conclusive, because of the indiscriminate spraying method used to control disease in big plantations. Spraying is normally done with small aeroplanes and it was difficult to isolate the experimental plot from the plantation bananas. There was always a carry over of the chemical spray to the experimental plot. Although the results looked good, it was not possible to draw conclusions from the data obtained.

Generation of disease- or pest-resistant plants can be carried out through identification of natural variants, chemically or by induced mutants or by using a genetic engineering approach. This was the first report in which *Musa* micro-cross sections were used for selection to a host-specific fungal toxin and subsequent regeration of resistant cell lines after challenging with the toxins and the fungus (Okole and Schulz, 1997). In general, the technique of using micro-cross sections in selection studies is comparable to other techniques where shoot tips, callus or cell suspension culture have been used in banana and plantain as an explant source (Matsumoto *et al.*, 1995; Trujillo and De Garcia, 1996; Hoss *et al.*, 2000; Gimenez and Colmenares, 2004; Reyes-Borja, 2005). Early reports of the ability of *M. fijiensis* to produce toxic metabolites *in vitro* were published by, Molina and Krausz (1989), Natural (1989, Upadhyay *et al.* (1989), and Lepoivre and Acuna (1990). In these reports, they did bioassay studies on leaves of tissue culture banana plants using crude filtrate metabolites and, in some cases, the purified compounds. In all of their results, they reported biological activity on the leaves with activity being correlated with the different levels of resistance of the banana or plantain plants.

Breeding for resistance is the most promising strategy for fighting Black sigatoka disease, especially in small farmer plantations. *M. fijiensis* produces many phytotoxins. The most studied of these phytotoxins are the pentaketide metabolites, 2-hydroxyjuglone, juglone (a non-host-specific toxin) and 2,4,8-THT (host-specific). Both toxins can be used, jointly with the fungus in field and growth room controlled conditions, for screening banana and plantain cultivars for Black sigatoka resistance. The non-host-specific phytotoxin has been shown to act on chloroplasts and disturbs the proton electrochemical gradient across the plasmalemma membrane. The purified toxin has a direct inhibitory effect on the electron transfer properties of purified banana chloroplasts. Furthermore, an involvement of the oxidative burst during the interaction has been suggested (Busogoro *et al.*, 2004; El Hadrami *et al.*, 2005a). Mora *et al.* (2002) carried out a study to determine the best concentration to differentiate two *Musa* cultivars 'Fougamou' (resistant) and 'Grand Naine' (susceptible). They reported that the concentration of 100 mg/l was able to produce necrotic symptoms in both cultivars; nevertheless, with the 50 mg/l concentration, it was possible to obtain differences between the two cultivars.

The second pentaketide 2,4,8-THT has been summarised by Hoss *et al.* (2000) as follows: Following recognition and enhancement of fungal metabolism in a resistant cultivar such as 'Yangambi Km 5', an increase of phenylalanine-ammonia lyase (PAL) activity is considered to be the first detectable specific reaction of host tissues leading to the biosynthesis of secondary plant compounds. The elicitation of this process is accompanied by necrotic micro-lesions which are often categorised phenotypically as a hypersensitive reaction. Other responses, such as phytoalexin accumulation, as observed in the resistant or tolerant cultivars, are the result of the effects that cause incompatibility between host and pathogen. Therefore, they concluded that the rapidity and spatial extension of high 2,4,8-THT concentrations at the sites of close contact between host tissue and fungal hyphae are determinants of the resulting interaction, ranging from susceptibility to resistance inside a host reaction continuum. The *Musa* spp.-*M. fijiensis* pathosystem, therefore, differs from other known plant-pathogen relationships by the fact that host specificity is determined at the cultivar level due to an activation of fungal metabolism, which leads on its part to an elicitation of a defence reaction and results in an incompatible interaction. Stierle *et al.* (1991) further reported that the most abundant of the host-specific phytotoxic compounds produced by *M. fijiensis* is 2,4,8-THT, which induces necrotic lesions at 5 µg/5 µl in less than 12 hours on sensitive cultivars of bananas.

The application of tricyclazole to healthy leaf tissue did not cause any visible phytotoxic symptoms on the leaves, but the leaves of the plants treated with tricyclazole and the toxin showed pronounced necrosis within a few days of incubation. The synthetic compound tricyclazole has been used as an inhibitor of two reduction systems of melanin biosynthesis, causing accumulation of important intermediate compounds of pentaketide metabolism. This could hypothetically enhance 2,4,8-THT biosynthesis (Stierle *et al.*, 1991; Hoss *et al.*, 2000).

## CONCLUSIONS

Bananas and plantains (*Musa* spp.) belongs to the most important staple food crops worldwide. Production of these crops is limited by many diseases and pests. Resistance breeding is urgently needed because diseases and pest control measures comprise ca 40% of total production costs. One of the most efficient banana and plantain breeding approaches is *in vitro* selection for resistance by using culture filtrates and toxins produced by most dangerous fungi, i.e. *Mycosphaerella fijiensis* (causing Black sigatoka) and *Fusarium oxysporum* f. sp. *cubense* (causing Panama disease). Literature data and our own experiments clearly demonstrated that both fungi produce phytotoxic compounds which are mostly non-host-selective toxins (e.g. juglone), however, also host-selective toxins (e.g. 2,4, 8-THT). In this paper we focused on *in vitro* selection to *M. fijiensis*. Shoot meristems, micro-cross sections, callus, cell suspension and protoplasts are suitable for *in vitro* selection for resistance. A double selection system showed high efficiency with using: a) the crude filtrate, b) the purified host-specific toxin 2,4,8-THT, as well as c) the non-host-specific toxin juglone. The most suitable concentrations of both toxins were suggested for *in vitro* selection. It is evident that induction of mutations and

following selection *in vitro* by using of toxin technology could be very efficient mass-screening approach in early stages of banana and plantains breeding for resistance/tolerance to fungal diseases.

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**Table 3.1.** *In vivo* response of the toxin-treated banana and plantain plants to *Mycosphaerella fijiensis* (isolate 100)

<i>Musa</i> cultivar	No. of toxin-resistant plants regenerated	Disease reaction <sup>1</sup>			% Resistance <sup>2</sup>
		R	T	S	
‘Williams’	150	22	61	67	14.7
‘Petite Naine’	150	29	78	43	19.3
‘Horn Plantain’	150	16	75	59	10.7

<sup>1</sup>R = resistant; T = tolerant; S = susceptible; <sup>2</sup>% Resistance represents total number of resistant plants/total number of toxin-resistant regenerated plants × 100. Source: Okole and Schulz (1997).

**Table 3.2.** *In vivo* response of plantlets regenerated from toxin-resistant ‘Petite Naine’ plants to different *Mycosphaerella fijiensis* isolates

<i>M. fijiensis</i> isolate <sup>1</sup>	No. of fungal toxin-resistant plants	Disease reaction <sup>2</sup>		
		R	T	S
100	50	48	2	0
200	50	40	9	1
309	50	29	18	3
400	50	38	10	2

<sup>1</sup>Origin of isolates: isolate 100 - Nigeria; isolate 200 - Cameroon; isolate 309 - Papua New Guinea; isolate 400 - Honduras. <sup>2</sup>R = resistant; T = tolerant; S = susceptible. Source: Okole and Schulz (1997).



**Figure 3.1.** Selection procedure for production of Black sigatoka disease-resistant plants. After brushing off the waxy layers on the lower surface of the leaves, the conidia and mycelium are sprayed evenly on both sides of the leaves with a spray gun to allow uniform spread on the leaves. Inoculated plants are kept in a growth room with the temperature adjusted to 26°C with a 16:8 hour light: dark photoperiod (light intensity: 45-50  $\mu\text{E}/\text{m}^2/\text{s}$ ) and high humidity. Visible disease symptoms of Black sigatoka usually appear from 40-60 days in control plants (Okole *et al.*, 2000).

## Chapter 4

### **Mutation induction by gamma-rays and carbon ion beam irradiation in banana (*Musa spp.*): a study with an emphasis on the response to Black sigatoka disease**

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#### **Abstract**

Gamma-rays and carbon ion beam irradiation methods were applied to study critical doses, genetic variability and the response to Black sigatoka disease. ‘Cavendish Enano’, ‘Williams’, ‘Orito’ and ‘FHIA-01’ cultivars of banana were studied. Both gamma-rays and carbon ion beam irradiation methods had different biological effects when banana explants were exposed to them. In both methods, increased dose caused increased mortality. ‘FHIA-01’ tolerated high doses of gamma-rays but was susceptible to high doses of carbon ion beam irradiation. The results suggest that the response in ‘FHIA-01’ can be explored using other dose intervals between 150 and 300 Gy. Weight and height were also reduced drastically when high doses of gamma-rays and carbon ion beams were applied. The LD<sub>50</sub> of cultivars ‘FHIA-01’ and ‘Orito’ revealed high sensitivity to both gamma-rays and carbon ion beams. DNA deletion in ‘FHIA-01’ occurred by using gamma-rays at doses of 200 and 300 Gy, suggesting that ‘FHIA-01’ is definitely a promising cultivar with a high sensitivity response to gamma-ray exposure, and that there is a high chance of improving its fruit quality by mutation induction. Sigmoid drooping leaf, a putative mutation of ‘FHIA-01’, was generated. This mutation is heritable as mother plant and sucker showed the same characteristics. Future research could be conducted on the relationship of leaf shape to fruit quality and production. Hexaploid cells were detected by flow cytometry (five plants in ‘Cavendish Enano’ and one in ‘Williams’), signifying that chromosome duplication can be induced by carbon ion beams. Variation in the leaves such as being abnormal, double, long, rudimentary, spindled and yellow spotted leaf was visible, suggesting that long-term chronic irradiation (gamma-rays) directly affects active cell division at the meristem level, resulting in severe damage or even death of the meristems. During the juglone toxin experiment on gamma-ray-irradiated plants, 20 plants were selected from the ‘Orito’ lot, eight in ‘Williams’ and five in ‘Cavendish Enano’. In the carbon ion beam experiments, six plants of ‘Williams’ and two of ‘Cavendish Enano’ were selected as possible candidates with a better response to Black sigatoka disease. In addition, following irradiation with a carbon ion beam, a fast growing plant was observed and selected as earliness is an important characteristic for shortening the crop life cycle. Finally, field

experiments throughout the whole plant cycle are needed to evaluate mutated traits for fruit quality, yield and post-harvest characteristics for a final selection.

## INTRODUCTION

Due to low reproductive fertility and high polyploid levels in banana, traditional hybridisation breeding techniques remain difficult (Rowe, 1984). Diseases are among the most important factors seriously affecting banana production worldwide. Among them, Black sigatoka disease caused by *Mycosphaerella fijiensis* Morelet, being the most destructive disease to attack the leaves, is one of the most serious constraints of banana cultivation (Craenen and Ortiz, 1996). It causes yield losses of 50% or more. Most research focuses on generating cultivars tolerant/resistant to this disease. Thus, radiation breeding could prove to be a viable method in banana breeding work. The use of mutagens has been acknowledged to be a reliable method for breeding plants with improved characteristics in many crops. In this study, nuclear techniques, especially the use of  $^{60}\text{Co}$  and  $^{137}\text{Cs}$  as sources of gamma-rays and carbon ion beams, have been coupled with *in vitro* techniques, to induce mutation, as a source of genetic variability for potentially improved and more desirable traits. At the same time, genetic variation has been noted when tissue culture techniques are used and to a greater extent when these variations are induced by the use of mutagens (Bermúdez *et al.*, 2002; García *et al.*, 2002).

Using gamma-rays, Roux (2004) reported different banana mutants with improved morphological characteristics of bunch size and cylindrical shape (mutant line name: ‘Klue Hom Thong KU1’) in Thailand, and plant height (dwarfness) (mutant line names: ‘SH-3436-L9’ and ‘6.44’) in Cuba. Mutant lines with increased tolerance to *Fusarium oxysporum* (‘Mutiara’ and ‘Novaria’) developed in Malaysia, and to the toxin of *M. fijiensis* (‘GN35-I to GN35-VIII’) developed by the IAEA were also reported.

The ion beam technique has recently been used rather than gamma-rays to produce a wide range of mutants. Fukuda *et al.* (2004) mentioned that ion beams can frequently produce large DNA alterations such as inversions, translocations and large deletions rather than point mutations, resulting in the production of desirable characteristics. Yu (2006) stated that the most important application of artificially-induced mutations is in mutation breeding, using sexual or asexual offspring to induce ideal genotypes. Since the biological effects of ion beams, as a new mutagen, were discovered, this technique has progressively received increasing attention. Ion beams integrate the factors of mass, energy and charge, inducing damage to the biological materials, thereby displacing, recombining and compounding the biological molecules and atoms.

The objectives of this research, conducted to find mutations, especially in response to Black sigatoka disease, are listed below:

- (1) To apply both tissue culture and irradiation techniques to induce genetic variability for mutant selection, and to determine irradiation sensitivity ( $^{60}\text{Co}$ ,  $^{137}\text{Cs}$  and carbon ions) in both explant and plantlet stages of banana.
- (2) To conduct studies on irradiated materials with the aim of finding a Black sigatoka disease-tolerant or resistant banana mutant, by using juglone toxin and conidial cultures of *M. fijiensis* as inoculation techniques to evaluate disease resistance at early stages of banana *in vitro*.

## MATERIAL AND METHODS

### Plant material

Four cultivars of banana were irradiated using gamma-rays and carbon ion beams. ‘Cavendish Enano’ (*M. acuminata* AAA) belongs to the ‘Cavendish’ subgroup, has an intermediate plant height, a normal bunch and an acceptable commercial fruit quality. This triploid cultivar is susceptible to Black

sigatoka disease and is one of the most important ‘Cavendish’ subgroup cultivars cultivated worldwide. It is also well known as ‘Grand Naine’. ‘Williams’ (*M. acuminata* AAA) is also a triploid belonging to the ‘Cavendish’ subgroup. It has a normal plant, a good bunch and good fruit quality, but is highly susceptible to Black sigatoka disease. ‘Orito’ (*M. acuminata* AA) is a favourite cultivar in Ecuador. It grows in humid areas and most of the plantations are organic, since the farmers cultivate with a minimum of cultural practices. This cultivar possesses small finger, sweet and delicious and has a high carotenoid content compared to ‘Cavendish’. It is susceptible to Black sigatoka disease but less so than the ‘Cavendish’ type. This cultivar is also called ‘Baby banana’ on the organic banana market. ‘FHIA-01’ (*M. acuminata* AAAB) is a tetraploid hybrid, and very resistant to Black sigatoka disease. Bunch weight ranges from 39 to 56 kg, with the number of functional leaves at the harvest stage ranging from 9 to 11. Plant height is ~4 m, with finger number ranging from 170 to 229. The fruit possess a very different taste to that of the ‘Cavendish’ type. This cultivar was included in this experiment as a control to compare cultivars resistant or susceptible to Black sigatoka disease.

### **Gamma-ray and carbon ion beam characteristics and irradiation doses**

Gamma irradiation was carried out in collaboration with the National Institute of Radiation Breeding, National Institute of Agrobiological Science, located in Hitachiohmiya, Ibaraki Prefecture, Japan. Regenerated plants and explants were treated using gamma-rays provided in facilities such as the “Gamma room” ( $^{60}\text{Co}$ ), “Gamma field” ( $^{60}\text{Co}$ ) and “Gamma greenhouse” ( $^{137}\text{Cs}$ ). In the “Gamma room” facility, the doses were: 0 (control), 50, 100, 150, 200, 300 and 500 Gy. In the “Gamma field” facility, plantlets were treated with irradiation doses of 0.5, 1 and 2 Gy, and in the “Gamma greenhouse” facility, plantlets were irradiated with three doses: 0.25, 0.50 and 0.75 Gy (20 hours/day).

Regarding the research on carbon ion beams, irradiation was conducted at the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), Japan Atomic Energy Agency (JAEA), Japan. Carbon ions with a total energy of 320 million electron volts (MeV) were generated by an Azimuthally Varying Field (AVF) cyclotron. The physical properties of the carbon ions were as follows: the incident energy at the target surface was 311 MeV (25.9 MeV/u), the range of the ions in a target was 2.2 mm, and the mean linear energy transfer (LET) in a target was estimated to be 137.6 keV/ $\mu\text{m}$ . ‘Cavendish Enano’ and ‘Williams’ samples were irradiated with doses of 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 Gy as a first trial to scan the best dose, and later on a second irradiation was conducted using doses of 0, 0.5, 1, 2, 4, 8 and 16 Gy on ‘Williams’, ‘Orito’ and ‘FHIA-01’.

### **Plant and explant conditioning pre- and post-irradiation**

The initial material consisted of *in vitro* micro-propagated plants prior to the establishment of the experiments. Four week old *in vitro* shoot tips from the four banana cultivars were used as a source of explants for both “Gamma room” and carbon ion beam irradiations (Figure 4.1A and D, respectively). These shoot tips were grown in a multiplication solid medium that consisted of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 6-benzylaminopurine (BAP; 2.25 mg/l), indole-3-acetic acid (IAA; 0.05 mg/l), sucrose (20 g/l) and agar (9 g/l) at pH5.6. For the “Gamma room” experiment, ‘Cavendish Enano’, ‘Williams’, ‘Orito’ and ‘FHIA-01’ varieties were used. The irradiation doses were applied six days after planting the explants in the solid medium. The survival rate (%), height (cm) and weight (g) of the shoots were determined one month after irradiation. For fast propagation, during pre- and post-irradiation, the explants were transferred to multiplication liquid medium (same as above without agar) by dividing the corm of each.

For the carbon ion beam irradiation, banana explants as thin as 2 mm were requested to allow total penetration of the ion beam. Prior to irradiation, an experiment was conducted to clarify the regeneration rate of the thinner banana explants. Cultivars ‘FHIA-01’ and ‘Williams’ were used for that experiment. Two types of slicing or cutting methods (vertical and horizontal slices) were applied to the corms (i.e., banana rhizome). The slices were placed in a regeneration liquid medium, containing MS medium supplemented with BAP (5 mg/l) and sucrose (20 g/l) at pH 5.6. 25 slices

were placed in a 300 ml Erlenmeyer flask containing 100 ml of medium. Five flasks (replications), containing the same number of explants, were prepared. The explants were stirred in a shaker at 100 rpm. The regeneration rate (%) and weight (g) of explants were recorded. From the results, the slicing method allowed the selection of the highest number of regenerated plants which were applied in the establishment of the experiment for carbon ion beam irradiation.

For the first irradiation with the carbon ion beam, vertical slices from four week old shoot tips of ‘Cavendish Enano’ and ‘Williams’ were placed in 6 cm diameter plastic dishes containing multiplication solid medium and were covered with sterilised Kapton films (thickness: 8  $\mu\text{m}$ , Toray-Dupont, Japan) in order to prevent the loss of energy of the carbon ions. The explants were planted on a Petri dish two days before irradiation. For this purpose, 20 explants/dish  $\times$  2 dishes (40 explants per dose) were used, giving a total of 400 explants per cultivar (800 explants considering both cultivars). Two days after irradiation, the explants were transferred to a 100 ml Erlenmeyer flask containing 50 ml of MS liquid medium. 19 days later, growth of the explants was evaluated using the following parameters: weight (g), height (cm), survival rate (%) and LD<sub>50</sub> (lethal dose 50%; i.e., irradiation dose that causes 50% of mortality in test explants).

In the case of the chronic irradiation, both “Gamma field” and “Gamma greenhouse” facilities were used (Figure 4.1B, and C). The experiments were carried out using acclimated plantlets ~20 cm in height growing in 40 cm diameter pots. Plants at the “Gamma field” facility were irradiated for a period of 34 days. One plant per treatment and per cultivar was used, except for the cultivar ‘Orito’ for which the dose of 1 Gy was omitted. At the “Gamma greenhouse” facility, the banana plants were irradiated for a period of nine months. One plant per dose was irradiated in ‘Williams’. Two plants per treatment at doses of 0.25 and 0.5 Gy, and only one plant at a dose of 0.75 Gy were irradiated for the cultivars ‘Orito’, ‘Cavendish Enano’ and ‘FHIA-01’. To propagate the irradiated plants, the meristems were excised in aseptic conditions and cut into several explants. The meristems were placed into a multiplication solid medium for *in vitro* culture. Explants from this experiment were sub-cultured three times to increase plant numbers for post-irradiation studies.

In general, after irradiation either by gamma-ray or by carbon ion beam methods, the explants were immersed in the multiplication liquid medium and stirred in a shaker at 100 rpm. During all the maintenance stages in the tissue culture room, the temperature was kept at 27°C with a 16:8 hour light: dark photoperiod (light intensity: 65  $\mu\text{mol}/\text{m}^2/\text{s}$ ). After irradiation, initiation solid medium, multiplication liquid medium and regeneration solid medium were used to propagate the explants into a large population to study the mutations. For a better understanding of the sequence of this experiment, a schematic drawing is shown in Figure 4.2.

### **Relative DNA content measured using flow cytometry**

To analyse the relative DNA content of banana leaf samples, a PAS Flow Cytometer (Partec) equipped with a mercury arc lamp that was suitable for analysis of samples stained with a CyStain UV kit was used. The samples were prepared using 400  $\mu\text{l}$  of nuclei extraction buffer and approximately 0.5  $\text{cm}^2$  of banana leaf tissue on a plastic 55 mm Petri dish. Then, using a sharp razor blade, the samples were chopped for 30 to 60 s and filtered through a Partec 50  $\mu\text{m}$  cell-trics disposable filter using a test tube to collect the filtrate. Samples were incubated in ice for 30 s to 5 min. After incubation with the extraction buffer, 1.6 ml of staining solution was added to the test tube and the sample was immediately analysed in the flow cytometer to obtain the fluorescent index (FI).

Non-irradiated ‘Cavendish Enano’, ‘Williams’, ‘FHIA-01’ and ‘Orito’ were used as controls to compare with the irradiated material. In order to assess FI values, an internal control must be used that has a ploidy level different than that of the test sample whose relative DNA content is being calculated. In this case, non-irradiated ‘Orito’ (diploid) was used as an internal control for the irradiated triploids ‘Williams’ and ‘Cavendish Enano’, and tetraploid ‘FHIA-01’. To compare irradiated samples of ‘Orito’, non-irradiated ‘Cavendish Enano’ was used as the internal control.

The Relative Nuclear DNA Content (RDC) was calculated using the FI peak mean value of the test sample divided by the FI peak mean of the internal control. The resulting values of RDC were analysed by frequency distribution. Flow cytometry analysis of gamma-ray-irradiated plants was carried out using 20 plants for each dose and cultivar, plus 20 non-irradiated and 'Orito' as control plants. Only the plants resulting from the "Gamma room" experiment were analysed by frequency distributions. Regarding the carbon ion beam-irradiated samples, 115 samples were analysed using 'FHIA-01' as the control.

After analysis of the relative DNA content, 'FHIA-01' was found to be the most affected cultivar among the irradiated plants at the higher doses (200-300 Gy). Plants with a reduced DNA content were selected. The plants were kept in big pots with soil and later on were transferred to the greenhouse and maintained under controlled temperature conditions.

### **Juglone toxin screening**

Prior to screening the irradiated banana plants obtained from gamma-ray and carbon ion beam irradiation, trials using juglone toxin were conducted on non-irradiated plants of 'Cavendish Enano', 'Williams' and 'Orito', which are susceptible to Black sigatoka disease. 'FHIA-01', which is resistant, was included in this experiment as a resistance indicator control. Various concentrations of juglone were applied, in order to select the most suitable concentration for producing differentiable necrotic area between non-irradiated susceptible cultivars and the resistant control. Juglone (5-hydroxy-1,4-naphthoquinone) is one of the most active toxins of the seven compounds produced by *M. fijiensis* Morelet, that induce the formation of necrotic lesions on plants leaf cells (Stierle *et al.*, 1991; Strobel *et al.*, 1993). This toxin was used in this experiment to screen young plants as an indicator of resistance/tolerance to Black sigatoka disease.

Non-irradiated *in vitro* derived plants, bearing 4-6 leaves and with a height of 15-20 cm, were used for this experiment. Four plants per cultivar and four leaf disks for each concentration were used to evaluate extended necrosis induced by juglone on the leaf disks of banana. Low concentrations of juglone (10, 15, 20, 25, 30, 35, 40, 50, 55 and 60 ppm) were initially tested; however, these concentrations did not induce any necrosis on the disks 24 and 48 hours after inoculation. A second trial using concentrations of 100, 150, 200, 250, 300 and 350 ppm was conducted. Leaf disks were immersed in 2 ml of juglone solution contained in a small Petri dish (diameter: 40 mm) and kept in an illuminated incubator at 26°C for 24 and 48 hours. The disks were obtained from the second expanded leaf with a cork borer of radius 6 mm (113.09 mm<sup>2</sup>). Leaf disks from 'FHIA-01' (Black sigatoka disease-resistant) were included as a resistance indicator to compare with the irradiated susceptible cultivars ('Cavendish Enano', 'Williams' and 'Orito').

After inoculation using the toxin, leaf disk photos were taken with an Olympus Camedia Digital Camera C-5050 Zoom. The Leaf disk necrotic area = LDNA (%) was measured using the GIMP 1.2 software by counting the number of pixels of the full disk and the number of pixels of the green area by selecting hand-drawn regions and obtaining the value for the necrotic area by subtraction (Figure 4.3) as is expressed in the following formula:

$$\text{LDNA (\%)} = \frac{\text{Pixels of the full disk} - \text{Pixels of the green area}}{\text{Pixels of the full disk}} \times 100$$

Based on the findings of the preliminary experiments, juglone at concentrations of 100 or 150 ppm was sufficient to obtain clear differences between the resistant and susceptible cultivars. This served as an indicator to differentiate the juglone-tolerant response among irradiated plants. Thus, using this criterion, irradiated plants were screened using 150 ppm of juglone. In the present experiment, we confirmed that juglone at different concentrations is able to induce marginal necrosis in banana leaf disks, based on the application of the method to black spot disease of Japanese pear (Sanata, 1988).

For the gamma-irradiated population, the disks of a 6 mm in diameter were taken from the second expanded leaf of each cultivar and immersed in a Petri dish (diameter: 30 mm) containing 10 ml of juglone solution. Samples were kept in an illuminated incubator at 26°C for 24 hours. 'FHIA-01' was also included as the indicator of resistance. Photographs of the leaf disks were processed as mentioned above. A total of 208, 179 and 307 plants were screened in 'Cavendish Enano', 'Williams' and 'Orito', respectively. Data were analysed by frequency distributions, and values divided into nine classes. The lowest LDNA (%) class values (necrosis produced by using 150 ppm of juglone on the leaf disks) were selected, considering that those values were around 9% of LDNA (%) which was the value obtained for the resistance indicator 'FHIA-01'.

In the case of the plants irradiated with carbon ion beams, the leaf disks (five disks per plant) were taken from the second expanded leaf of cultivars 'Williams' and 'Cavendish Enano' from the field-planted experiment. Leaf disk samples of 'FHIA-01' (indicator of resistance) were obtained from sucker plants available in the banana collection of the Estación Experimental Tropical Pichilingue, Ecuador. The value of the resistance indicator 'FHIA-01' was calculated at ca. 42.2%. A solution containing 150 ppm of juglone was prepared using distilled water. 10 ml were dispensed into a Petri dish (90 mm) and the leaf disks were immersed in the solution. The leaves were rinsed with distilled water and disks with a radius of 7.5 mm (176.71 mm<sup>2</sup>) were removed using a cork borer. Petri dishes with leaf disk samples were kept in the light and at room temperature for 24 hours. A total of 435 leaf disks were analysed (87 plants × 5 leaf disks/plant). After 24 hours, photographs of the leaf disks were taken and processed in the same way as described above.

The lowest values of LDNA (%) of the gamma-ray population were selected; however, in the case of the population irradiated with carbon ion beams, the LDNA (%) data were combined with the values for the Disease Development Period (DDP; measured in days) and Infection index (II; measured in %) in order to select candidates using a regression analysis.

### **Plant regeneration and acclimatisation for Black sigatoka disease assessment**

Most of the plants evaluated for Black sigatoka disease were from the carbon ion beam-irradiated plants due to the high mortality of the plant material. After the irradiated explants were sub-cultured three times to regenerate shoots, they were planted individually into a test tube containing 10 ml of solid MS medium, and 0.5 mg of activated charcoal for rooting. A total of 1707 rooted plantlets were transferred to sterilise plastic bags and transported to Ecuador (Station Experimental Tropical Pichilingue; Instituto Nacional de Investigaciones Agropecuarias, INIAP) for field experiments.

Immediately after arrival in Ecuador, plantlets were kept for two days in a tissue culture room at 26°C with a 16:8 hour light: dark photoperiod (82 μmol/m<sup>2</sup>/s) to recover photosynthesis. They were then transferred to a soil substrate bed (with a 1:1 mixture of soil and decomposed rice husk). The plantlets were covered with a plastic sheet to avoid dehydration under greenhouse conditions. Only 87 plants survived the nursery acclimatisation. The high mortality might have been caused by unfavourable transportation conditions and pathogen attacks. These materials were used for the experiments for Black sigatoka inoculation at the nursery and, later on, in field conditions.

On plants of ~30 cm in height, three leaves per plant were inoculated, and the younger expanded leaf was marked as the first leaf for inoculation by a conidial solution (Figure 4.4). The second and third successive young emitted leaves were inoculated by fragments of the diseased banana leaves (Figure 4.5). Conidial cultures of *M. fijiensis* at a concentration of  $1.5 \times 10^6$ /ml were produced. After inoculation, the plants were kept at 26°C with a high relative humidity (~85%) in a dark incubation room for 48 hours. For second and third leaf inoculation, leaf fragments from plants severely infected by Black sigatoka were placed at the base and inside the canopy of each plantlet as a potential natural inoculum. A fickle cotton sheet moisturised three times a day was provided to cover all the plantlets to ensure sporulation and to enhance the inoculation.

Tolerance to Black sigatoka disease on inoculated banana plants was evaluated by two indices: DDP (days) and II (%). DDP (days) was expressed as days until the full development of a spot with a dry grey centre, using the stages of symptoms described by Fouré's scale from the time of inoculation (Orjeda, 1998). The disease severity determined by II (%) was calculated using the values obtained from the Stover's scale modified by Gauhl (Orjeda, 1998).

### **Putative mutants and factor of effectiveness-FE (%)**

Several putative mutants were produced by gamma-ray and carbon ion beam irradiation. Mutants showing traits such as juglone tolerance, low relative DNA content, dwarfism, sigmoid drooping leaf, fast growth of sucker, good response to Black sigatoka disease, fast growth of plantlet, hexaploid plants and several kinds of leaves were observed.

A factor of effectiveness-FE (%) was used to measure the efficiency of the mutagens based on data obtained from phenologic and phenotypic variations. FE (%) was calculated with a modified formula by Walther (1969) cited by Bhagwat and Duncan (1998) and described as follows:

$$FE (\%) = \frac{\text{Total number of variations} \times 100}{\text{Total number of plants treated}}$$

### **Statistical analysis**

The statistical one-way analysis of variance by Tukey-Kramer ([www.jmp.com](http://www.jmp.com), JMP, Version 5) was used to analyse data from the gamma-ray population. For carbon ion beam irradiation, the data were processed using analysis of variance (General AOV/AOCV, analytical software Statistics for Windows version 2.0) followed by Tukey analysis ( $p \leq 0.05$ ). The radiosensitivity was evaluated as the survival rate-lethal dose ( $LD_{50}$ ), and analysed by exponential regression. The  $LD_{50}$ , which determines the dose (Gy) necessary to kill half of the irradiated population, is the value used to assess acute toxicity.

## **RESULTS AND DISCUSSION**

### **Explant slicing conditioning prior to ion beam irradiation**

The analysis of variance for regeneration rate (%) reported high significance for the two slicing methods; however, there were not significant differences between cultivars. In contrast, the weights of the explants were different between cultivars; however, no significant differences were found between the two slicing methods. These results suggest that the regeneration rates (%) of 'Williams' and 'FHIA-01' were affected by the type of slicing method to the same extent, but that the weight of the explants was not affected by this factor.

Figure 4.6 shows the relationships of the explants' weight (A) and regeneration rate (B) with the slicing methods (vertical and horizontal) in 'Williams' and 'FHIA-01'. Vertically-sliced cuttings showed the highest regeneration rate (%) in both cultivars as is shown in Figure 4.7. Regeneration rates of 60-70% were observed in 'Williams' and 'FHIA-01' using the vertical cutting method, in contrast to only 37-43% in horizontally-sliced cuttings. The weights of the explants were similar in both cultivars when the corm shoot tips were cut vertically. Horizontal slicing slightly affected 'Williams' although there were no significant differences between the two slicing methods. Explants from this cultivar weighed, on average, 0.6 g when using the horizontal slicing method, compared to 0.9 g when using the vertical slicing method. Finally, the vertical slicing method was selected as the best method for regenerating banana explants as thin as 2 mm and this type of slice was obtained from shoot tips as a material for carbon ion beam irradiation.

## Gamma-ray and carbon ion beam biological effects on banana shoots

The application of radiation in the “Gamma room” facility showed that all the cultivars were affected by the irradiation doses as reported by Reyes-Borja *et al.* (2005) (Figure 4.8A-C). The survival rate decreased with increasing doses, and is cultivar-dependant. Differences in the sensitivity to the doses of gamma-rays between ‘Cavendish Enano’ and ‘FHIA-01’ were significant at the 5% level. ‘Williams’ and ‘Orito’ showed a higher significance at the 1% level. ‘Williams’ and ‘FHIA-01’ showed a superior height, weight and survival rate when the dose reached 150 Gy, while ‘FHIA-01’ tolerated doses of up to 500 Gy, but with a very low survival rate (Figure 4.8A). ‘Cavendish Enano’ and ‘Orito’ appeared to be more sensitive to gamma-ray exposure, as evidenced by the low values of plant height and weight recorded in these cultivars. Figure 4.9 shows the biological effect of gamma-rays significantly reducing the growth of the explants as the doses increased.

Irradiation had a similar effect on plantlet height and weight across all cultivars with increasing radiation doses. According to the Tukey-Kramer analysis, the four cultivars showed significant differences ( $p \leq 0.01$ ). Doses  $>150$  Gy strongly affected the weight of the shoots. Plantlet height decreased in all cultivars with increasing dose rate. The difference between ‘Cavendish Enano’ and ‘FHIA-01’ was significant at the 5% level ( $p \leq 0.05$ ), and that between ‘Williams’ and ‘Orito’ was significant at the 1% level ( $p \leq 0.01$ ). ‘Cavendish Enano’ was significantly different ( $p \leq 0.05$ ) across dosages. The effect was more pronounced in ‘Cavendish Enano’, even at the lowest dosage, resulting in low growth when  $>150$  Gy was applied. ‘FHIA-01’ at a dose of 200 Gy still showed vigorous growth, but this was reduced strongly when dosage was increased to 500 Gy. ‘Williams’ and ‘Orito’ could not tolerate radiation dosages  $>200$  Gy. However, the height in ‘Williams’ remained similar even when doses were increased to 50, 100 and 150 Gy (Figure 4.8C).

Figure 4.10A shows the optimum range dose of gamma-rays obtained in ‘Cavendish Enano’, ‘Williams’, ‘Orito’ and ‘FHIA-01’ through the LD<sub>50</sub>. Survival rate (%) values were used to calculate the LD<sub>50</sub> by exponential regression as an analysis to assess acute sensitivity in all four cultivars. The LD<sub>50</sub> was highest in ‘Williams’ (83.94%) and lowest in ‘Orito’ (65.0%);  $\pm 5\%$  was aggregated to the LD<sub>50</sub> value of each cultivar to select the optimum irradiation doses of gamma-rays. Consequently, the optimum doses for ‘Cavendish Enano’ ranged from 74.0 to 81.8 Gy, for ‘Williams’ from 79.7 to 88.1 Gy, for ‘Orito’ from 61.8 to 68.3 Gy, and for ‘FHIA-01’ from 73.2 to 80.9 Gy.

These results are in agreement with those obtained by Novak *et al.* (1990), who reported that *Musa* cultivars exhibit significant differences in radiosensitivity and post-irradiation recovery. These differences depend on the ploidy level and the hybrid constitution (genomes A and B). In their study, the tetraploid cultivar ‘SH-3436’ (AAAA) exhibited the lowest level of radiation damage, whereas the diploid cultivar ‘SH-3142’ (AA) was the most sensitive to gamma irradiation, which is in agreement with the results obtained in the present study because ‘Orito’ (diploid AA) also showed high sensitivity. However, triploids seemed to be unstable, since ‘Williams’ was less affected by irradiation than ‘Cavendish Enano’. A similar trend was observed by Novak *et al.* (1990), who showed that the highest dose (35-40 Gy) of gamma-rays was suitable for mutation induction in the triploid cultivar ‘Highgate’, but also in the tetraploid cultivar ‘SH-3436’.

Regarding carbon ion beam irradiation, the biological effects in both ‘Cavendish Enano’ and ‘Williams’ (first irradiation) were as follows. The analysis of variance for the height of explant showed significant differences between cultivars and among ion beam doses ( $p \leq 0.05$ ). As shown in Figure 4.8E,F, the weight and height of plantlets were affected when the Gy doses were increased irrespective of cultivar. The highest growth behaviour in terms of weight and height was observed when doses  $\leq 2$  Gy were applied. These parameters decreased when 4 Gy or higher were used.

The survival rate (%) of the explants is presented in Figure 4.8D. In general terms, the higher the irradiation dose used the higher was the mortality rate, but doses of 8 Gy and lower did not seem to

have any effect on survival rates (Reyes-Borja *et al.*, 2007). Figure 4.11 shows banana plantlets of ‘Cavendish Enano’ and ‘Williams’ affected by different doses of ion beam (Gy), 19 days after irradiation. Hase *et al.* (2002) reported that high-LET radiation such as heavy-ion beams have greater biological effects than low-LET radiation such as gamma-rays and X-rays. High-LET causes a reduction in survival and a linear increase in the frequency of aberrant cells. Thus, it is possible that the induction of chromosome aberrations depended on the LET.

The LD<sub>50</sub> values for both ‘Cavendish Enano’ and ‘Williams’ (carbon ion beam first irradiation) were obtained by exponential regressions. The LD<sub>50</sub> for ‘Williams’ was established at 13.5 Gy and for ‘Cavendish Enano’ at 15.0 Gy indicating that the former would be more sensitive to the ion beams than the latter. We only discuss the survival rate and LD<sub>50</sub> for the batch of explants irradiated second with carbon ion beams in ‘Williams’, ‘Orito’ and ‘FHIA-01’. The analysis of variance for survival rate showed significant differences between the ion beam doses, but there was no difference between cultivars. The survival rate of the explants was similar when 0-8 Gy were applied, but with 16 Gy this variable was affected. The LD<sub>50</sub> values for ‘Williams’, ‘Orito’ and ‘FHIA-01’ were estimated at 9.0, 3.8 and 4.6 Gy, respectively, indicating that ‘Orito’ and ‘FHIA-01’ were more sensitive to the ion beams than ‘Williams’. Even though ‘Williams’ was irradiated twice, with LD<sub>50</sub> values equal to 13.5 Gy and 9 Gy for the first and second irradiations, respectively, it was still more resistant to ion beams than ‘Orito’ and ‘FHIA-01’.

As a consequence, when considering the data from the first and second irradiations, 5% were added and subtracted from each LD<sub>50</sub> value of each cultivar to designate the optimum irradiation dose range of carbon ion beams. When this was done, the optimum doses for ‘Williams’ ranged from 12.8 to 14.2 Gy, for ‘Cavendish Enano’ (average of the two irradiations) from 14.3 to 15.8 Gy, for ‘FHIA-01’ from 4.4 to 4.8 Gy, and for ‘Orito’ from 3.6 to 4.0 Gy. Figure 4.10B shows the optimum dose range for carbon ion beam irradiation in each cultivar.

Plants irradiated over a period of 34 days in the “Gamma field” facility did not show any phenotypic variation; however, plants kept at the “Gamma greenhouse” facility for a period of nine months showed unique characteristics as reported by Reyes-Borja *et al.* (2005) (Figure 4.12). Novak *et al.* (1990) reported the presence of considerable phenotypic variation among the plants regenerated from shoot-tips after mutagenic treatment. At the early stages of plant development, the irradiation affected the emergence and expansion of the younger leaves, and several plants formed compact leaf rosettes. Aberrant morphology of the laminae was observed mainly in younger leaves due to damage of the apical meristem. The results from the present study in the “Gamma greenhouse” facility are in agreement with the findings reported by Novak *et al.* (1990). We also found damage to meristematic cells as shown in Figure 4.13. Double and spindled leaves were the most relevant characteristics (Figure 4.12B,D), while yellow spot leaf was the most frequently observed characteristic in ‘Cavendish Enano’, ‘FHIA-01’ and ‘Orito’ (Figure 4.12E), except for ‘Williams’.

### **Measuring relative DNA content variation with flow cytometry**

A flow cytometer was used to measure the relative DNA content of plants irradiated with gamma-rays. The relationships between the irradiation doses and the relative DNA content showed that the values from the three cultivars (‘Cavendish Enano’, ‘Williams’ and ‘Orito’) were almost the same, with an imperceptible variation for all the applied doses. However, a perceptible variation was observed in cv. ‘FHIA-01’ when the dose was increased. The variation in ‘FHIA-01’ was better observed when a frequency distribution of the relative DNA contents was analysed (Figure 4.14). The analysis detected a reduction in relative DNA content with increasing irradiation dose (Gy). The lowest value was 1.947 (96% of donor), observed at 300 Gy. These results suggest that deletion of DNA occurred when high doses were applied in ‘FHIA-01’, exhibiting a shift to the left, indicating a loss of chromosomes or chromosomal parts.

A report by the FAO/IAEA (2002) states that flow cytometer screening was started to identify aneuploid mutants in irradiated banana plants. Aneuploid mutants can be detected through chromosome counts, but this is a rather time-consuming process. Results obtained by flow cytometry were compared to chromosome counting in meristem shoot-tip cells. It was shown that flow cytometry is sensitive enough to detect aneuploidy in *Musa*. Dolezel *et al.* (2002) reported that flow cytometry was used to determine ploidy levels of *Musa* accessions. Among 890 accessions, 2% of mixoploid plants were detected. In the annual report published by the National Institute of Agro-biological Sciences (2002), it was reported that the rates of the hairless mutation in sugar cane obviously increased with increasing irradiation dose. A very high mutation rate was observed at 200 Gy and all the hairless mutants had 1.4% less DNA than the donor. DNA deletions frequently occurred at doses >100 Gy.

The relative DNA content was measured in 115 samples from the population subjected to carbon ion beam irradiation. The flow cytometer reported hexaploid cells among the analysed samples (Figure 4.15). In 'Cavendish Enano', five hexaploid plants were found when the applied ion beam dose was 4 Gy. In 'Williams' variety just one hexaploid plant was observed. These results suggested that carbon ion beam irradiation at 4 Gy can produce a duplication of the chromosomes. According to Yu (2006), if a normal chromosome is increased by a segment with the same sequence, it is said that duplication occurred. If the duplicated segment follows the original sequence, it is called tandem duplication; if the segment is linked to the reverse sequence, it is called a reverse duplication. The same author also reported that a large amount of chromosomal lagging can definitely induce changes in chromosome number and can, thus, possibly result in aneuploidy. Using N-ion implantation doses of D3 ( $3 \times 10^{16}$  ions/cm<sup>2</sup>) and D4 ( $4 \times 10^{16}$  ions/cm<sup>2</sup>), monomers with a chromosome number of 41 appeared in wheat 'Premebi'. In 'Rye AR1' with dose D4, monomers with chromosome numbers of 19 appeared, 15 of them being normal chromosomes and 4 being trisomic B chromosomes.

### **Screening of sensitivity to juglone toxin**

In the experiment using juglone at concentrations of 100, 150, 200, 250, 300 and 350 ppm, and evaluating 24 and 48 hours after inoculation, no significant differences with respect to time or dose were noted among susceptible and resistant ('FHIA-01') cultivars as the toxin continued to induce necrosis even 48 hours after treatment (Figure 4.16). The results suggest that all the damage caused to cells by the juglone occurred just after 24 hours in the susceptible cultivars. The figures show that an increase in concentration of juglone was concomitant with an increase in the extent of necrosis in the leaf disks. The mechanisms of the stopped necrosis after 24 hours in susceptible cultivars and the increased necrosis after 48 hours in the resistant cultivar 'FHIA-01' are still unknown. Thus, an in depth study of the cellular interaction with juglone at the morphological, molecular and biochemical levels should be carried out.

As a consequence, embedding leaf disks for 24 hours was the selected time to inoculate the irradiated materials. In relation to this, significant differences across various concentrations of juglone using the Tukey-Kramer ( $p < 0.05$ ) test (Table 4.1) were detected. Differences were also observed among the cultivars tested. 'FHIA-01' was the least affected across the concentrations tested in contrast to the susceptible cultivars 'Cavendish Enano', 'Williams' and 'Orito' (Figure 4.16 E, F). The effectiveness of the juglone concentrations is shown in Figure 4.17.

This experiment was undertaken to determine a concentration of juglone sufficient to induce necrosis on banana leaf disks and allow discrimination among the irradiated plants. As previously mentioned, juglone is one of the seven toxins produced by *M. fijiensis* Morelet as it attacks the leaf cells of the plant, resulting in necrotic tissues. In this experiment, we confirmed that various concentrations of juglone can induce necrosis in banana leaf disks. The results showed that a concentration of 150 ppm of juglone was sufficient to induce differentiable necrotic areas among susceptible and resistant cultivars. Thus, 150 ppm of juglone solution was used for screening the irradiated population. At a concentration of 350 ppm, juglone induced necrosis among susceptible and resistant cultivars at a

similar intensity. Thus, the use of high concentrations of juglone is not ideal for conducting screening trials since even the highly resistant cultivar 'FHIA-01' was affected. Molina and Krausz (1988) observed a clear variation between the Black sigatoka disease-susceptible cultivar 'Grand Naine' and the highly Black sigatoka disease-resistant line 'IV-9'. This variation was observed with the needle-piercing method using phytotoxic extract from the pathogen, and measuring the diameter of the area of necrotic tissue. The toxic activity of the extracts can, apparently, be used to screen banana and plantain breeding materials rapidly even at a very early stage of plant growth.

In the present study, regarding the plants irradiated with gamma-rays, a total of 208, 179 and 307 plants were screened in 'Cavendish Enano', 'Williams' and 'Orito', respectively. Plants showing low levels of necrosis (~4-9% produced by using 150 ppm of juglone on the leaf disks) were selected. Out of these, 5, 8 and 20 plants were selected in 'Cavendish Enano', 'Williams' and 'Orito', respectively. Figure 4.18 shows the frequency distribution as expressed by class limits of LDNA (%) in the three cultivars. The values in these plants were lower than the resistance indicator value (9.5%) of 'FHIA-01' that was previously calculated.

In studies reported by the FAO/IAEA (2002), it was found that of ~4000 irradiated 'Grande Naine' plants screened, 19 putative mutants were selected for their tolerance to 25 ppm of juglone; however, new screening for resistance to *M. fijiensis* in these plants still has to be confirmed through inoculation with the fungus, which is a very slow process. On the other hand, the results obtained by Lepoivre *et al.* (2002) confirm the possibility of selecting banana plants resistant to *M. fijiensis* metabolites; however, in their research this approach did not result in a higher level of resistance to black leaf streak disease.

Lepoivre *et al.* (2002) reported that chloroplasts are a target site of juglone. When juglone was used, swelling chloroplasts were observed by electron microscopy in ethyl acetate crude extract (EaCE)-treated leaves. Upon observation, 'Fougamou' (a partially resistant cultivar) chloroplasts appeared to be less affected by juglone than those in 'Grand Naine'. These results suggest that the chloroplasts are one of the primary action sites of juglone.

Different screening methods have been mentioned by different authors (Molina and Krausz, 1988; Harelimana *et al.*, 1997; Lepoivre *et al.*, 2002). The leaf puncture bioassay has been widely used to assess host tolerance/resistance to Black sigatoka disease or juglone; however, although detached banana leaves or the injection of crude toxic extract into the leaves is easy, neither method is sensitive or quantitative. In this study, since the leaf disks of the resistant cultivar (in this case 'FHIA-01') were less affected by 150 ppm juglone compared to the susceptible cultivars, the leaf disk immersion method was a simple and rapid method to measure tolerance/resistance to juglone. Limited necrosis was observed in susceptible irradiated cultivars (selected ones); however, new studies to reconfirm the tolerance/resistance to juglone on the selected material must be undertaken. On the other hand, Van Harten (1998) has mentioned that selection using phytotoxins and culture filtrate are more effective than the use of the pathogen itself. Nevertheless, the selected materials in the present study must be assayed under field conditions, applying natural inoculums of Black sigatoka to confirm that the use of juglone is an adequate means of evaluating resistance/tolerance or susceptibility at early stages of plant growth.

The results of the LDNA (%) obtained from carbon ion beam-irradiated plants are shown in Figure 4.19C, F in both 'Cavendish Enano' and 'Williams', respectively (Reyes-Borja *et al.* 2007). In this case, the results of LDNA (%) were combined with DDP (days) and II (%) for candidate selection. As shown in Figure 4.19C,F, lower LDNA (%) values varied from 38.0 to 44.9% in irradiated 'Cavendish Enano' and from 33.0 to 39.9% in 'Williams' (indicated between dotted vertical lines). Conversely, LDNA (%) values from non-irradiated plants did not show such low values. Therefore, the low LDNA (%) values in irradiated plants might be attributed to tolerance to Black sigatoka disease, possibly due to slower penetration of the toxin into the cells as the result of mutations. Leaf-disk bioassays have been reported to be effective for evaluating resistance in several crops. Ostry *et al.*

(1988) working with *Septoria musiva*, a disease of *Populus* spp., described that this method was sufficiently sensitive to distinguish between clones with high, moderate or low resistance. Etame (2003) inoculated banana genotypes showing different reactions to Black sigatoka with juglone toxin and the pathogen for selecting resistant plants. The genotypes resistant to juglone toxin ('Fougamou', 'Pisang madu', 'M53' and 'Klutuk') also showed resistance to the pathogen, although some cultivars resistant to *M. fijiensis* were susceptible to juglone. In conclusion, this method should only serve as a preliminary screening technique before field tests.

### **Combining DDP (days) and II (%) with LDNA (%) to select plants tolerant/resistant to Black sigatoka disease**

As shown in Figure 4.19A, D, the frequency distribution by class limits of the DDP (days) in both cultivars, the higher class limit values were clearly separated from the lower ones, as indicated between dotted vertical lines, ranging from 53.0 to 59.9 days and from 50.0 to 54.9 days in 'Cavendish Enano' and 'Williams', respectively. The lowest II (%) values in 'Cavendish Enano' corresponded to 25.0-34.9 days and, in 'Williams', to 27.0-36.9 days, as marked between dotted vertical lines, showing a slight variation contrasting greatly with the higher values (Figure 4.19B,E).

Using the II (%) to evaluate a hybrid population of plantain, Cohan *et al.* (2003) demonstrated that 'CRBP-39' (AAAB) was extremely resistant to Black sigatoka disease in three developmental phases: the vegetative phase at 6 months, the flowering phase and the harvest phase. II (%) is, therefore, a very useful parameter for evaluating the resistance of plants to this disease. In this research, the II (%) allowed us to observe inter-individual variation in response to Black sigatoka disease, which varies from very susceptible to tolerant among the plants regenerated from irradiated explants, when subjected to the inoculum. The effect of the irradiation probably causes DNA alteration as mentioned by Fukuda *et al.* (2004), and results in expanding the variation in relation to this pathogen.

The appearance of fewer symptoms following infection is closely related to a response by the plant defence mechanism. By crossing two susceptible triploid plantain cultivars ('Bobby Tannap' and 'Obino 1 Ewai') as female parents with the resistant wild, diploid banana 'Calcutta 4', Ortiz and Vuylsteke (1994) obtained segregated progeny with a durable horizontal resistance. In the case of the progeny with a partially resistant response, slow lesion development and, ultimately, reduced sporulation were observed. Ortiz and Vuylsteke (1994) also discussed that the possible mechanisms of Black sigatoka disease resistance were expressed by different pathways such as the synthesis of phytoalexins, the production of lignin or suberin, polyphenolic content (higher in resistant cultivar), low stomata density and increased epicuticular wax. However, the mechanism still remains obscure.

Taking the results of the three parameters DDP (days), II (%) and LDNA (%) together, variations in relation to tolerance to Black sigatoka disease have been expanded in the banana population established from *in vitro* plantlets irradiated with carbon ion beams, suggesting that ion beam irradiation is a useful tool for mutation breeding in banana.

For selecting tolerant/resistant plants, the variables DDP (days), II (%) and LDNA (%) were combined by linear regression to assess the response to Black sigatoka disease in the irradiated materials. The linear regression permitted us to categorise the plants that showed a better response to this disease. LDNA (%) regression versus II (%) showed high significance ( $p \leq 0.01$ ) compared to LDNA (%) versus DDP (days), and DDP (days) versus II (%) that were significant at the 5% level ( $p \leq 0.05$ ) in 'Williams' (Figure 4.20). The regression among the three combined variables permitted us to select six plants in 'Williams' (with code numbers 'W 16 II 74' (1), 'W 128 I 67' (2), 'W 1 II 148' (3), 'W 8 II 13' (4), 'W 1 II 19' (5) and 'W 1 II 31' (6) showing high relationships) and two plants in 'Cavendish

Enano' (with code numbers 'CE 4 II 30' and 'CE 64 I 5' - data not shown) as candidates with increased tolerance to Black sigatoka disease.

Regarding the complete assessment of the candidate plants, field experiments based on the whole plant cycle are necessary to evaluate not only the response to Black sigatoka disease but also the fruit quality, potential production and post-harvest parameters, which are valuable components for final selections.

### **Putative mutants and factor of effectiveness-FE (%)**

Summarising the effectiveness of both gamma-rays and carbon ion beams, putative mutants such as juglone tolerance, low relative DNA content, dwarfism, sigmoid drooping leaf, fast growth of sucker, good response to Black sigatoka disease, fast growth of plantlet, hexaploid plants and several kinds of leaves were obtained (Table 4.2). An interesting sigmoid drooping leaf mutant was produced in cultivar 'FHIA-01' by applying high doses of gamma-rays. The characteristics were observed in the first and second generations (mother plant and sucker), suggesting that this characteristic is heritable. Sigmoid drooping leaf is a peculiar characteristic in certain *Musa* cultivars such as 'FHIA-03', which is a cooking banana (AABB) with high production and good vigour (Reyes-Borja, 1995). Figure 4.21 shows a comparison of the putative mutant sigmoid drooping leaf with the normal growth of 'FHIA-01'. A fast growing plantlet (Figure 4.22) was also observed among the irradiated cultivars. This plant, selected as fast growing, has a very useful characteristic as it may result in earliness of fruit harvesting. Regarding the hexaploid cells that we found by measuring the relative DNA content, this could be a way of causing chromosome duplication, efficiently and fast, especially on improved diploids possessing resistance that could be used as parental lines for banana breeding. Gamma-ray irradiation also produced a putative dwarf of 'FHIA-01' but, unfortunately, resulted in death during development. The low relative DNA content that resulted in 'FHIA-01' is evidence for the effectiveness of the irradiation. This directly affects phenologic and phenotypic characteristics as confirmed by the variations obtained in 'FHIA-01'.

## **CONCLUSIONS**

The methodologies applied in the course of this research were highly efficient in developing the main process related to mutation induction in banana. The technique used to measure relative DNA content was very useful for detecting alterations at the DNA level caused by irradiation, and allowed us to select plants that during cropping expressed good putative mutations as shown for cultivar 'FHIA-01' irradiated with gamma-rays. Still more useful were the results, by which this technique detected hexaploid cells, suggesting that chromosome duplication had occurred. This kind of material can be interesting as parental lines for breeding by crossing, especially when developed from diploids resistant to Black sigatoka disease. The technique using leaf disks exposed to juglone toxin permitted to discriminate between cultivars that are resistant or susceptible to Black sigatoka disease. Additionally, it also allowed selecting candidates from plants irradiated at earlier stages. Combining DDP (days) and II (%) with LDNA (%) produced more accurate results. Nevertheless, an additional selection method must involve subjecting the selected material to natural inoculum to study the disease indices, especially at the flowering and the harvest stages for at least 3 or 4 cycles for final selection.

Gamma-ray and carbon ion beam irradiation produced the same trends in survival rate when banana explants were subjected to irradiation. However, the methods differ in terms of LET, with carbon ion beam irradiation being effective at low doses. The cultivars most sensitive to the irradiation among those used in this experiment were 'Orito' (gamma-rays and carbon ion beam) and 'FHIA-01' (carbon ion beam). A sigmoid drooping leaf plant of 'FHIA-01' and plants weakly affected by Black sigatoka disease and juglone toxin were obtained. In addition, a single plant of 'Cavendish Enano' irradiated with a carbon ion beam under greenhouse conditions showed fast growth among a group of plants. Regarding the selected plants only weakly affected by Black sigatoka disease, field experiments considering the whole plant cycle must be undertaken to confirm not only the Black sigatoka response

but also fruit quality and potential production and post-harvest evaluations as necessary components for final selections. Additionally, field studies of mutant plants such as sigmoid drooping leaf, fast growth, hexaploids and diminished DNA content plants must also be carried out to confirm whether the new traits involve single or linked genes.

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**Table 4.1.** LDNA (%) of banana leaf disks in different concentrations of juglone

Juglone (ppm)	LDNA (%) of banana leaf disks affected by juglone			
	‘Cavendish Enano’	‘Williams’	‘FHIA-01’	‘Orito’
0	0.0	0.0	0.0	0.0
100	11.0 ± 1.8 cA	11.1 ± 1.9 cA	6.0 ± 2.6 dB	14.5 ± 1.5 dA
150	14.9 ± 1.2 bcA	13.6 ± 1.8 cA	9.5 ± 1.9 cdB	17.1 ± 1.9 cdA
200	19.6 ± 1.7 abA	14.7 ± 0.9 bcB	11.5 ± 3.8 bcdB	19.4 ± 2.0 bcdA
250	22.1 ± 5.8 abA	18.1 ± 1.9 abAB	12.3 ± 1.1 bcB	22.4 ± 1.8 abcA
300	24.7 ± 4.5 aA	20.2 ± 1.5 aAB	15.5 ± 2.0 abB	23.3 ± 3.1 abA
350	24.4 ± 3.9 aA	21.7 ± 1.7 aA	19.3 ± 3.2 aA	25.7 ± 4.7 aA

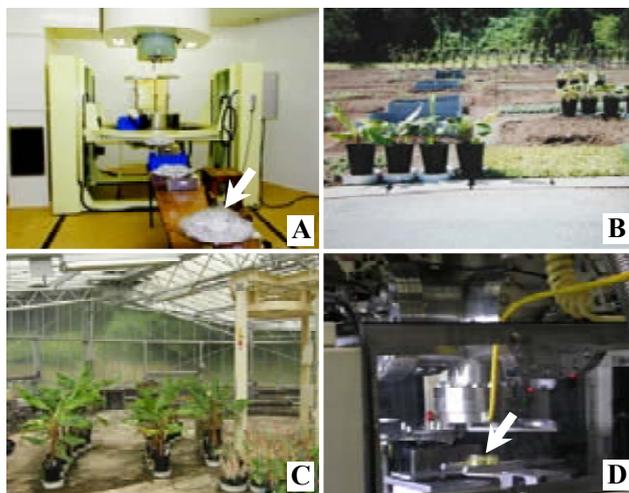
Values followed by the same letter in a column do not differ significantly according to Tukey-Kramer ( $p < 0.05$ ). Values in a row followed by the same capital letter do not differ significantly according to Tukey-Kramer ( $p < 0.05$ ). Values ± mean standard deviation.

**Table 4.2.** Factor of effectiveness-FE (%) and putative mutations produced by gamma-ray ( $^{60}\text{Co}$ ,  $^{137}\text{Cs}$ ) and carbon ion beam irradiation in four cultivars of banana

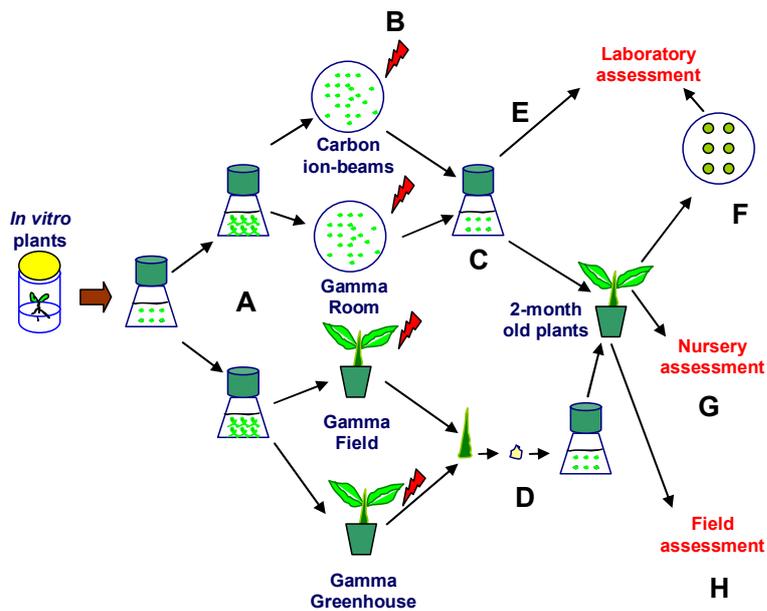
Facility/mutagen	Cultivar	Total no. of plants	No. of plants with variation	Putative mutation	Gy dose inducing mutation ( <i>n</i> )	FE (%)
Gamma room/ $^{60}\text{Co}$	‘C. Enano’	282	4	Tolerant of juglone	100 (3)	1.63
				Tolerant of juglone	150 (1)	0.35
Gamma field/ $^{60}\text{Co}$	‘C. Enano’	26	1	Tolerant of juglone	2 (1)	3.84
				AL <sup>1</sup>	0.25 (1)	20.0
Gamma greenhouse/ $^{137}\text{Cs}$	‘C. Enano’	5	5	AL, DL	0.25 (1)	20.0
				RL, LL,	0.5 (1)	20.0
				SL, YSL, LL	0.5 (1)	20.0
				YSL, LL	0.75 (1)	20.0
Gamma room/ $^{60}\text{Co}$	‘Williams’	156	7	Tolerant of juglone	50 (2)	1.28
				Tolerant of juglone	150 (5)	3.20
Gamma greenhouse/ $^{137}\text{Cs}$	‘Williams’	3	3	Tolerant of juglone	0.5 (1)	7.14
				AL	0.25 (1)	33.3
				AL, RSSL	0.5 (1)	33.3
Gamma room/ $^{60}\text{Co}$	‘Orito’	282	20	AL	0.75 (1)	33.3
				Tolerant of juglone	50 (8)	2.83
				Tolerant of juglone	100 (8)	2.83
Gamma greenhouse/ $^{137}\text{Cs}$	‘Orito’	5	5	Tolerant of juglone	150 (4)	1.41
				SSL, LL, YSL	0.25 (1)	20.0
				RSSL, AL	0.25 (1)	20.0
				LL, YSL	0.5 (1)	20.0
				LL, YSL, AL	0.5 (1)	20.0
Gamma room/ $^{60}\text{Co}$	‘FHIA-01’	120	18	LL, YSL, AL	0.75 (1)	20.0
				Low DNA content	200 (10)	8.33
				Low DNA content	300 (7)	5.83
Gamma room/ $^{60}\text{Co}$	‘FHIA-01’	120	1	Dwarfism	300 (1)	0.83

Facility/mutagen	Cultivar	Total no. of plants	No. of plants with variation	Putative mutation	Gy dose inducing mutation ( <i>n</i> )	FE (%)
Gamma room/ <sup>60</sup> Co	'FHIA-01'	120	1	Sigmoid drooping leaf	200 (1)	0.83
Gamma room/ <sup>60</sup> Co	'FHIA-01'	120	2	Fast growth of sucker	200 (1) 300 (1)	0.83 0.83
Gamma greenhouse/ <sup>137</sup> Cs	'FHIA-01'	5	5	YLS, LL	0.25 (1)	20.0
				YLS, LL	0.25 (1)	20.0
				YLS	0.5 (1)	20.0
				YLS	0.5 (1)	20.0
				YLS	0.75 (1)	20.0
Carbon ion beam	'C. Enano'	42	2	Good response to Black sigatoka disease	4 (1) 64 (1)	2.38 2.38
Carbon ion beam	'C. Enano'	85	1	Fast growth of plantlet	4 (1)	1.17
Carbon ion beam	'C. Enano'	29	5	Hexaploid	4 (5)	17.24
Carbon ion beam	'Williams'	40	6	Good response to Black sigatoka disease	1 (3) 8 (1) 16 (1) 128 (1)	7.5 2.5 2.5 2.5
				Hexaploid	4 (1)	1.69

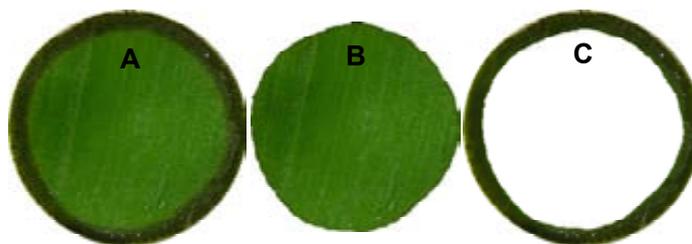
<sup>1</sup>LL = long leaf; YSL = yellow spotted leaf; SL = spindled leaf; SSL = spindled short leaf; RL = rudimentary leaf; AL = abnormal leaf; RSSL = right side short leaf; DL = double leaf.



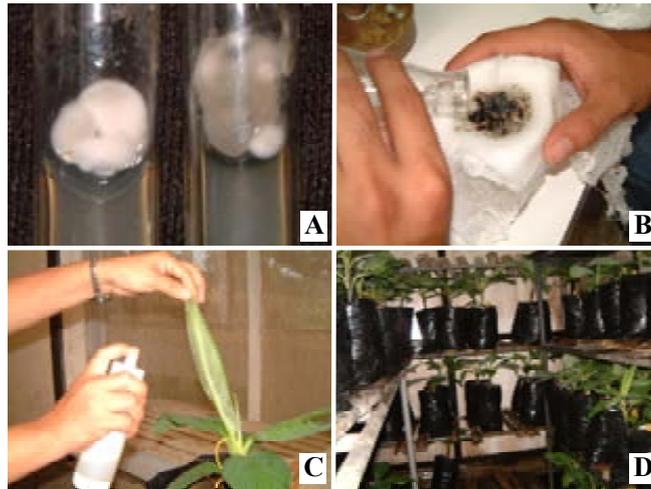
**Figure 4.1.** Irradiation of banana. (A) explants in 90 mm dishes (indicated by the arrow) in the “Gamma room” facility; (B) plants in the “Gamma field” facility; (C) plants in the “Gamma greenhouse” facility; and (D) a Petri dish (indicated by the arrow) containing the samples in the carbon ion beam irradiator.



**Figure 4.2.** Schematic diagram for the improvement of banana lines resistant to Black sigatoka disease through mutation induction with gamma-rays and carbon ion beams. (A) Propagation in liquid medium, solid medium for regeneration; (B) Induction of mutation through irradiation; (C) Propagation (three times) and regeneration; (D) Meristem propagation (three times) and regeneration; (E) Assessment of weight, height, survival rate and LD<sub>50</sub>; (F) Leaf disk toxin screening using juglone; (G) Assessment of relative DNA content by flow cytometry; (H) Assessment of Black sigatoka disease resistance: DDP (days), II (%) and putative mutations.



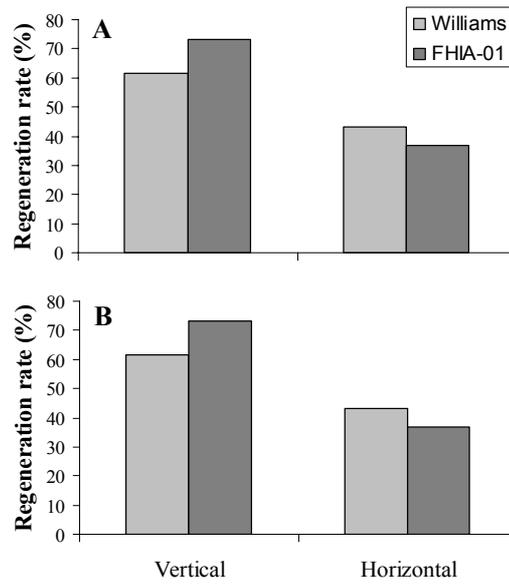
**Figure 4.3.** Digital pictures calculating LDNA (%) by using the GIMP 1.2 software, measuring the full disk area in pixels (A) minus the cut green area pixels by selecting hand-drawn regions (B), and obtaining the value for the necrotic area by subtraction (C).



**Figure 4.4.** *Mycosphaerella fijiensis* Morelet colonies (A), filtration (B) and inoculation of the irradiated banana plants (C) kept in an incubation room for 48 hours (D). A concentration of  $1.5 \times 10^6$  conidia/ml (inoculation of the first leaf) was used.



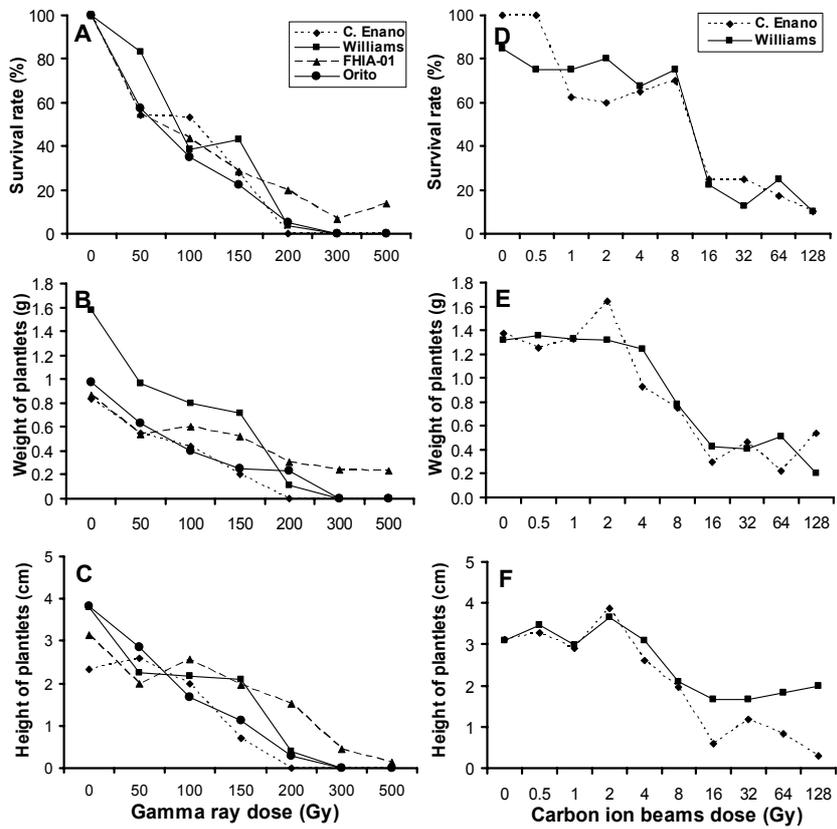
**Figure 4.5.** Inoculation method using diseased leaf fragments. (A) Collected leaves from a banana collection kept in the Estacion Experimental Tropical Pichilingue (INIAP), Ecuador. (B, C, D) Diseased leaf fragment moistening before placement among the plants to be inoculated. (E) Plants covered by a cotton sheet for 48 hours, and moistening three times a day. (F) A plant showing the inoculated leaves and inoculation dates.



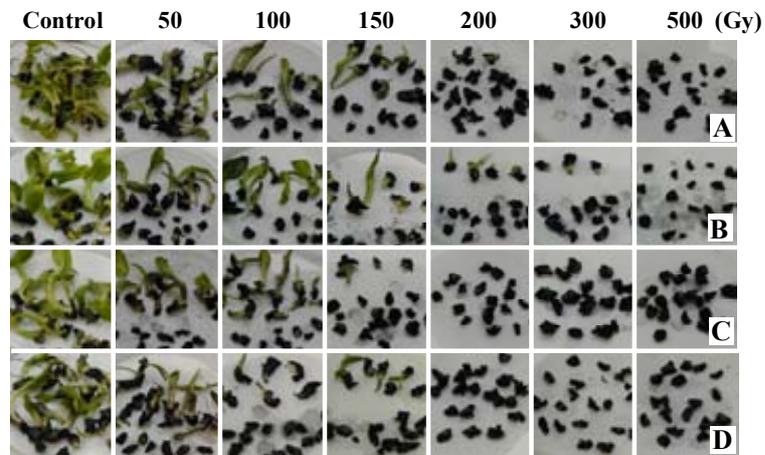
**Figure 4.6.** Relationships of the explants' weight (A) and regeneration rate (B) with the two slicing methods (vertical and horizontal) in 'Williams' and 'FHIA-01' 13 days after culture.



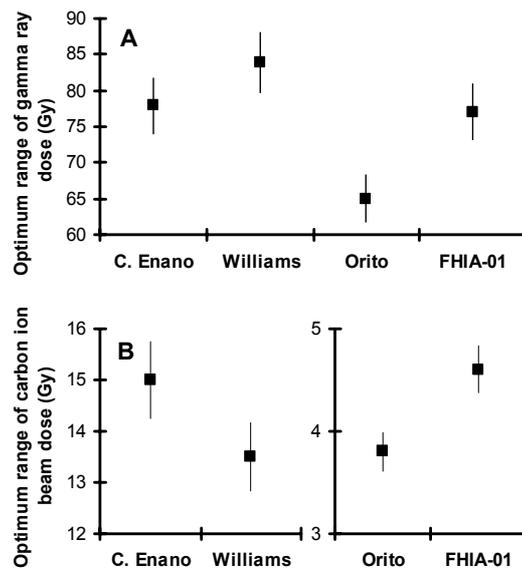
**Figure 4.7.** Regenerated plantlets of 'Williams' from both vertical (A) and horizontal (B) slicing methods 13 days after culture.



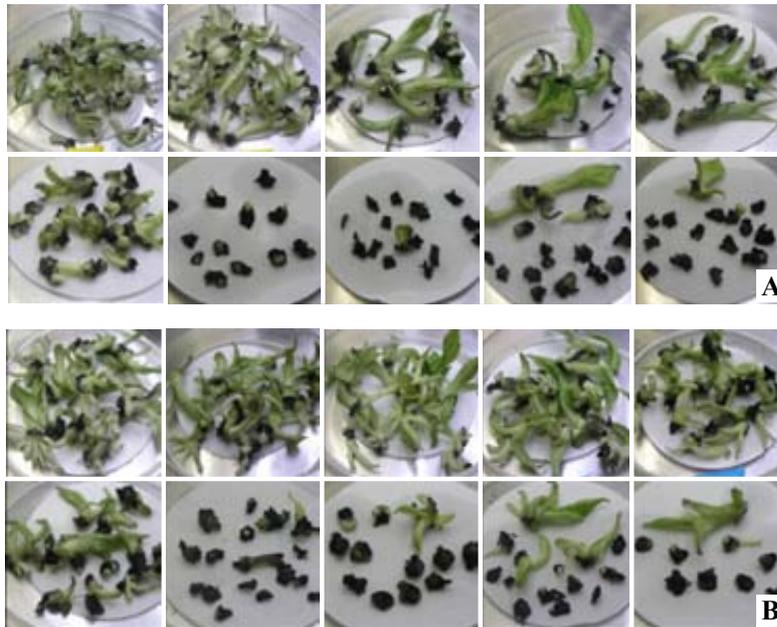
**Figure 4.8.** Survival rate (A, D), weight (B, E) and height (C, F) of the explants in banana cultivars one month after gamma-ray irradiation and 19 days after carbon ion beam irradiation.



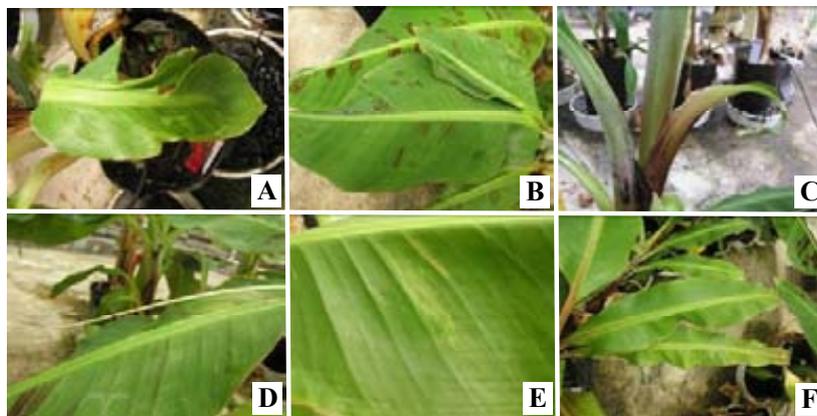
**Figure 4.9.** Biological effects produced by different gamma-ray doses (Gy) in four cultivars of banana. (A) ‘Williams’; (B) ‘FHIA-01’; (C) ‘Cavendish Enano’; (D) ‘Orito’.



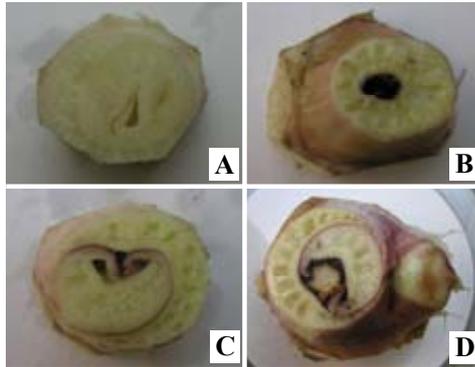
**Figure 4.10.** Optimum range of gamma-ray (A) and carbon ion beam (B) doses obtained with  $\pm 5\%$  of the  $LD_{50}$  values. Squares represent the  $LD_{50}$  values.



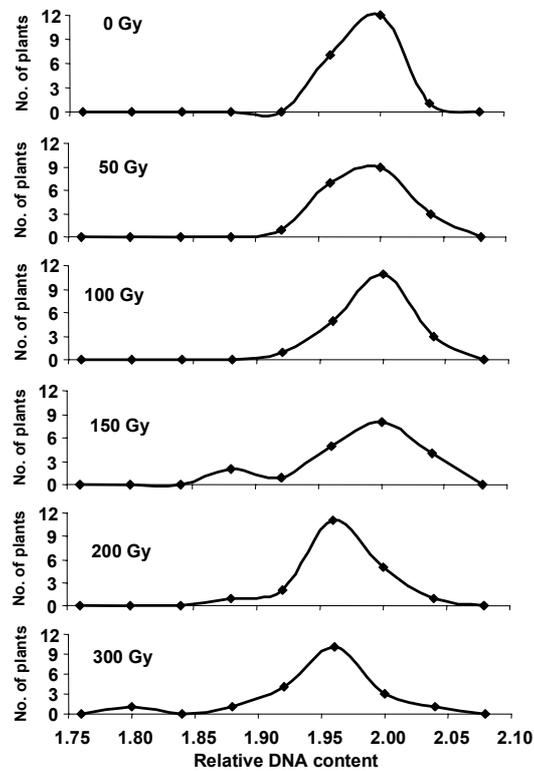
**Figure 4.11.** Biological effects produced by different carbon ion beam doses (Gy) in two cultivars of banana. (A) ‘Cavendish Enano’; (B) ‘Williams’. Carbon ion beam doses for each cultivar from left to right: 0 (control), 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 (Gy).



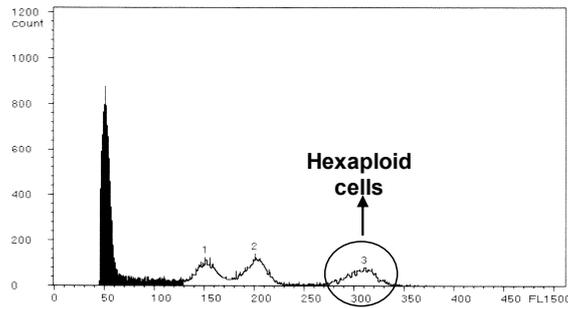
**Figure 4.12.** Unique characteristics observed at the “Gamma greenhouse” facility on irradiated plants after nine months. (A) abnormal leaf (AL); (B) double leaf (DL); (C) rudimentary leaf (RL); (D) spindled leaf (SL); (E) yellow spotted leaf (YSL); (F) long leaf (LL).



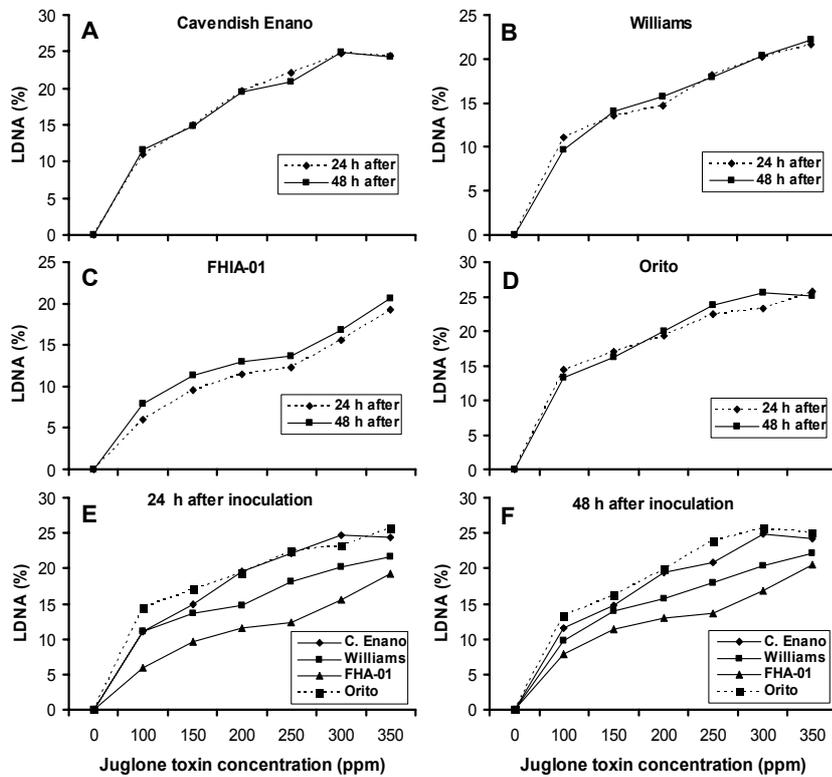
**Figure 4.13.** Banana meristems affected by long period-chronic irradiation ( $^{137}\text{Cs}$ ) applied in the “Gamma greenhouse” facility. ‘Cavendish Enano’ at 0.50 Gy (A, B), and ‘FHIA-01’ at 0.50 Gy (C, D).



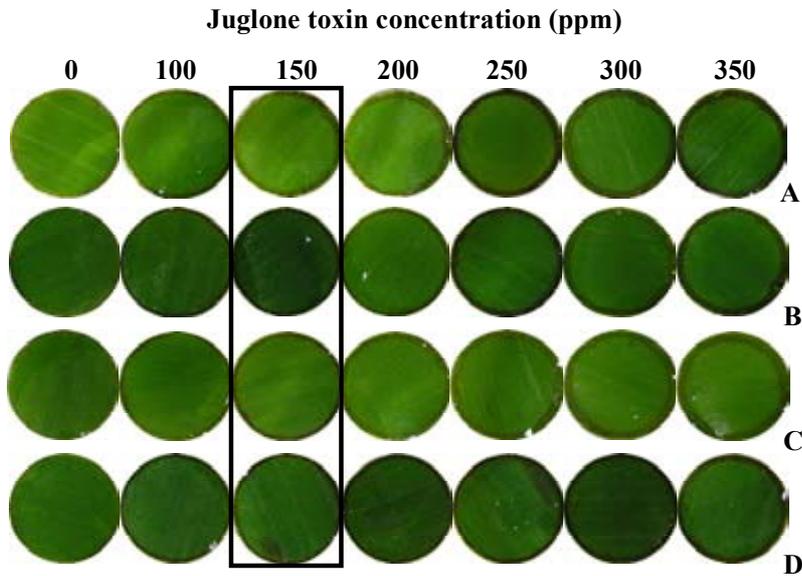
**Figure 4.14.** Frequency distributions of relative DNA contents in a population of ‘FHIA-01’ irradiated with different gamma-ray doses.



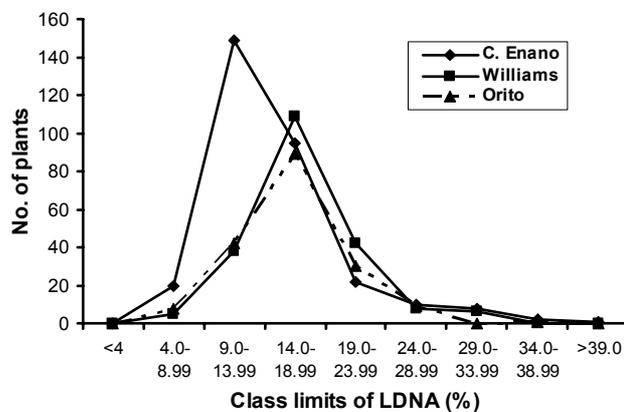
**Figure 4.15.** Relative DNA content measured by flow cytometry showed hexaploid cells in ‘Cavendish Enano’ (encircled, peak 3) produced by an ion beam dose of 4 Gy. ‘Cavendish Enano’, code no. CE4169 (peak 1) and ‘FHIA-01’ used as standard (peak 2).



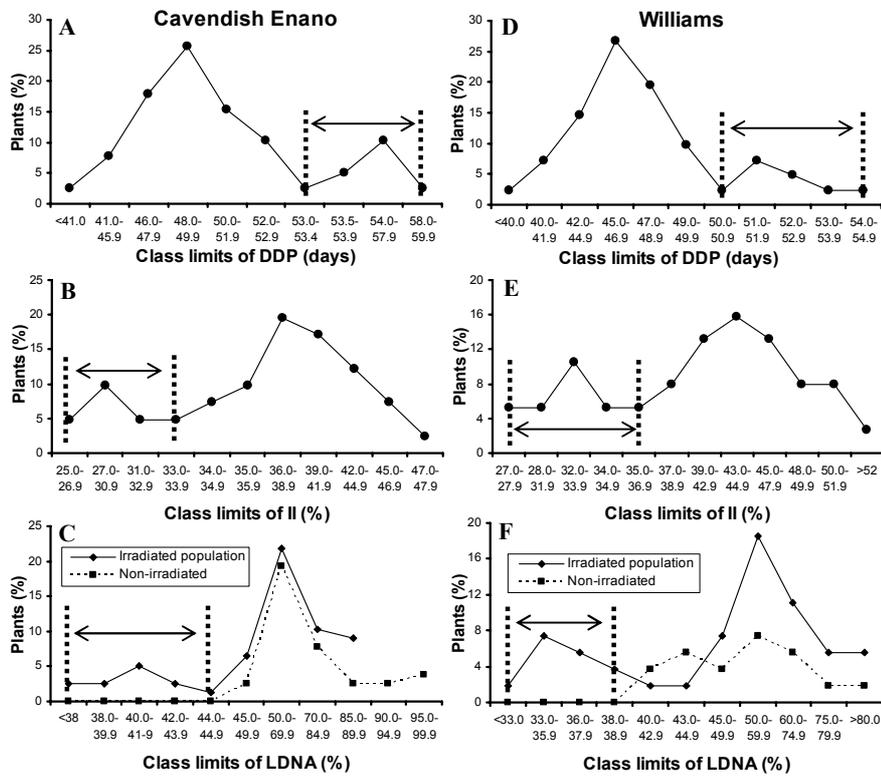
**Figure 4.16.** Relationship between LDNA (%) and juglone toxin concentration (ppm) in four cultivars of banana, 24 and 48 hours after inoculation.



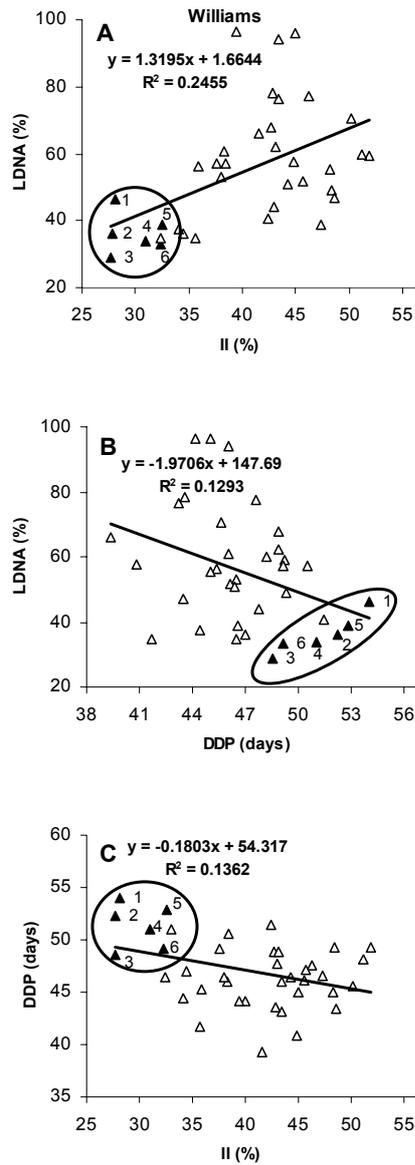
**Figure 4.17.** Leaf disk screening technique using juglone toxin in several concentrations (ppm) in four cultivars of banana. The highlighted disks (box) indicate the selected dose for subsequent screening. (A) ‘Cavendish Enano’; (B) ‘Williams’; (C) ‘Orito’; (D) ‘FHIA-01’.



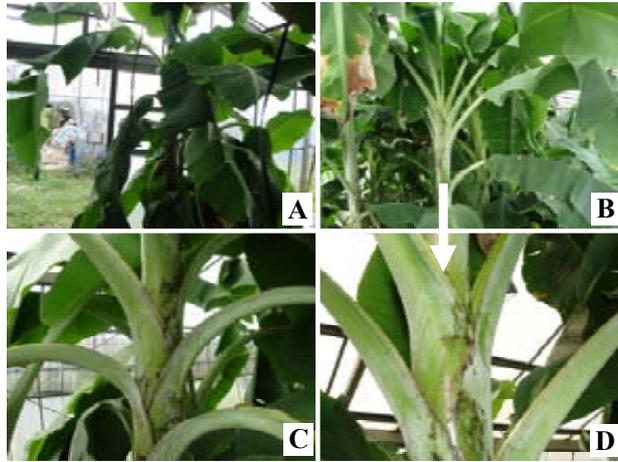
**Figure 4.18.** Mutagenesis efficiency in regenerated plants of banana after gamma-ray treatment. The frequency distribution was expressed by class limits of LDNA (%) in ‘Cavendish Enano’ (n = 208), ‘Williams’ (n = 179) and ‘Orito’ (n = 309). Plants in the lower values (encircled area) were selected.



**Figure 4.19.** Mutagenesis efficiency following carbon ion beam irradiation. Frequency distributions are expressed as class limits of DDP (days), II (%) and LDNA (%) in ‘Cavendish Enano’ (A-C) and ‘Williams’ (D-F). The range between dotted vertical lines indicates the best values of the three parameters.



**Figure 4.20.** Statistical analysis following irradiation of ‘Williams’ banana. (A) LDNA (%) regression versus II (%); (B) LDNA (%) regression versus DDP (days); (C) DDP (days) regression versus II (%). Encircled values with bold triangles belong to the code numbers ‘W 16 II 74’ (1), ‘W 128 I 67’ (2), ‘W 1 II 148’ (3), ‘W 8 II 13’ (4), ‘W 1 II 19’ (5) and ‘W 1 II 31’ (6) that clearly show relationships within the three combinations of the variables.



**Figure 4.21.** Putative mutant of 'FHIA-01' obtained by gamma-ray irradiation (A) and its petioles (B). Normal leaf shape of 'FHIA-01' and its petioles (C-D) growing under greenhouse conditions.



**Figure 4.22.** A 'Cavendish Enano' plant following carbon ion beam irradiation (4 Gy) showing fast growth (A-B in the centre) compared with the other plants in the group.



## Chapter 5

### Rapid and mass screening of banana and plantain for resistance to Black *sigatoka* disease using detached leaf and *in vitro* plantlets

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#### Abstract

We describe the use of *in vitro* plantlets in tubes and detached leaves in screening *Musa* genotypes against *Mycosphaerella fijiensis*, the causal agent of Black sigatoka disease. The utility of these two assays was evaluated using eight genotypes with different levels of disease resistance. Differences in disease response were observed when *in vitro* plantlets in tubes and detached leaves of selected *Musa* genotypes with different levels of disease resistance were inoculated with *M. fijiensis*. The response of genotypes depended on their level of disease resistance, with resistant genotypes having longer incubation times and smaller infected leaf areas compared to the susceptible cultivar ‘Agbagba’ and to relatively less resistant genotypes. A similar pattern in cultivar response was observed for symptom evolution time. Although symptom stages appeared earlier on in *in vitro* plants compared to detached leaves, genotypes were consistently ranked based on their level of disease resistance. Disease severity data from the two assays were strongly correlated, with higher severity being observed on the susceptible cultivar ‘Agbagba’ and lowest severity on resistant land race ‘Calcutta-4’. The remaining genotypes had disease severity levels corresponding to their levels of resistance to Black sigatoka disease. Based on these results, we conclude that these two rapid assays are reliable for screening banana and plantain for resistance to Black sigatoka disease.

#### INTRODUCTION

Black sigatoka disease, also known as Black leaf streak, is caused by *Mycosphaerella fijiensis* (teleomorph of *Pseudocercospora fijiensis*) and is regarded as the most economically important leaf disease of bananas and plantains worldwide (Carlier *et al.*, 2000; Marín *et al.*, 2003). The fungus reduces the green leaf area and impedes photosynthesis, which is needed for fruit filling. The disease results in heavy losses (Marín and Romero, 1992; Mobambo *et al.*, 1993; Craenen and Ortiz, 1998) and, in highly susceptible varieties, the disease can lead to the total collapse of the plant. Fungicides and planting of resistant genotypes are the main strategies for controlling the disease. In subsistence production of bananas and plantains, use of fungicides is impractical (Craenen and Ortiz, 1998). For commercial growers, banana genotypes resistant to Black sigatoka disease may not be a priority; however, they are more environmentally friendly.

Breeding for resistance to Black sigatoka disease in banana and plantain has taken a high priority worldwide. However, the evaluation of resistance has been slow due to the necessity of avoiding escapes by evaluating resistance in whole plants over several years and in a wide range of environments. Furthermore, effective field screening depends on the presence of proper environmental conditions, is time-consuming and expensive, and can often be carried out only once a year. Similarly,

early screening in a greenhouse (Mobambo *et al.*, 1994) depends on ensuring ambient conditions conducive for disease development, and a limited number of genotypes can be evaluated at a time. Screening for resistance requires methods to unambiguously discriminate resistant and susceptible genotypes at different stages of plant development. Such techniques should be robust, efficient and amenable to mass screening of genotypes. Thus, there has been interest in developing *in vitro* assays as methods for screening banana and plantain for Black sigatoka disease resistance. Direct inoculation of *in vitro* plantlets in tubes and inoculation of detached leaves in moist chambers are two assays that overcome the limitations associated with field screening. Recently, Twizeyimana *et al.* (2007) developed and evaluated the utility of *in vitro* techniques to screen *Musa* species for resistance to *M. fijiensis*. In that study, *M. fijiensis* inoculum concentrations, incubating conditions and plating medium for detached leaves were optimised. Our objective here is to provide a step-by-step protocol for rapid and mass screening of banana and plantain for resistance to Black sigatoka disease using detached leaves and *in vitro* plantlets in tubes.

## MATERIAL AND METHODS

### Screening banana and plantain genotypes using *in vitro* plantlets in tubes

#### *Preparation of tissue culture plants*

Tissue culture plants were prepared using standard shoot-tip culture protocols. In this procedure, we adopted a protocol described by Vuylsteke (1998). Briefly, to initiate cultures, 1 cm<sup>3</sup> pieces containing the apices were excised from suckers, surface-sterilised in 95% ethanol for 15 s, soaked for 15 minutes in 0.75% NaOCl solution to which a drop of Tween 20 was added per 50 ml, and then rinsed three times with sterile distilled water. Shoot tips 1-2 mm in length with 2-4 leaf primordia were isolated aseptically and placed on modified Murashige and Skoog (MS) medium basal salt mixture (Murashige and Skoog, 1962), supplemented with 30 g/l sucrose and 0.4 mg/l thiamine. Cultures were maintained at 28-30°C on a 12:12 hour light:dark cycle. Shoot tips and meristem were induced to proliferate by adding 0.18 mg/l indole acetic acid (IAA) and 4.5 mg/l benzylaminopurine (BAP) to the basal medium. Plant regeneration was accomplished on the same basal medium supplemented with 0.19 mg/l naphthalene acetic acid and 0.23 mg/l BAP. It takes 7 to 10 weeks to establish rooted plantlets from isolated shoot tips and about three weeks for *in vitro* plantlets to develop leaflets with enough material for inoculation.

#### *Inoculum preparation*

*M. fijiensis* inoculum was prepared by isolating the pathogen from diseased leaves as described by Stover (1976). Briefly, leaf fragments bearing Stage 6 necrosis were placed in moist bags for 48 hours to allow maturation of pseudothecia. Pieces of leaves with mature pseudothecia were stapled on 9 cm diameter filter paper disks, dipped in sterile water for 5 minutes and placed inside the lids of Petri dishes. The lids were then placed on Petri dishes containing 3% water agar. Within one hour, ascospores forcibly discharged naturally on the agar surface. Single germinating ascospores were transferred onto V8 juice agar after 24 hours. The V8 juice agar was prepared by mixing 100 ml of V8 juice, 0.2 g of CaCO<sub>3</sub>, 20 g of agar and 900 ml of sterile distilled water to make the resultant solution up to 1 l. The resultant solution was then autoclaved using standard procedures. Culture plates were incubated at 19-20°C under continuous UV light for pathogen development. Inoculated cultures started to produce conidia approximately 26 days later.

#### *Inoculation of plantlets in tubes*

Following sporulation of cultures, sterile distilled water was added to V8 agar on Petri dishes, and the spore suspension was filtered through two layers of cheesecloth. A drop of Tween 80 was added to the resulting spore suspension and using four hemocytometer counts, sterile distilled water was added to adjust spore concentrations to  $5 \times 10^5$  conidia/ml. This spore concentration was previously reported to

be optimum for inoculation in growth chamber assays (Twizeyimana *et al.*, 2007). Prior to inoculation, a drop of 1% Triton X-100 was added to the inoculum suspension to enable mycelia and spores to adhere to the leaf surface. The three largest leaves of each *in vitro* plantlet (one spot per leaf) were aseptically inoculated by pipetting 40 µl droplets of spore suspension. Droplets were placed carefully to avoid trickling from the leaves to the plant growth medium, on which the pathogen can grow well and kill the plantlets. Leaves touching the inner wall or the lid of the tubes or the medium were excluded from inoculation. Inoculated plantlets were incubated at 25°C with a 12:12 hour light:dark cycle for disease development (Figure 5.1).

#### *Disease assessment*

Observations on inoculated leaflets were made from 2 to 32 days after inoculation, and included incubation time, disease severity (% of leaf area infected), symptom evolution time and stages of symptoms. Incubation time is defined as the time between inoculation and appearance of the first symptoms (Stage 1), whereas symptom evolution time is defined as the number of days between Stage 1 and occurrence of mature lesions (Stage 6). Symptom stages were recorded as described by Fouré (1987): Stage 1 = first visible symptoms are yellowish specks <1 mm in diameter on the abaxial surface of the leaf; Stage 2 = initial speck elongates and widens to form a reddish brown streak up to 2 mm in length and streaks are visible on both sides of the leaf; Stage 3 = red-brown streaks reach a length of 20-30 mm and the colour starts to change from red to dark brown; Stage 4 = the streak broadens and develops into an elliptical spot, dark brown on the abaxial surface and black on the adaxial surface of the leaf; Stage 5 = the central area of the dark spot becomes totally black and necrotic, the lesion is slightly depressed and the black spot is surrounded by a bright yellow halo; Stage 6 = the centre of the spot dries out, fades and becomes whitish to grey, and the spot is surrounded by a dark brown to black border and further depressed.

A schematic representation of the above steps involved in the use of the *in vitro* plantlets in tubes for screening *Musa* genotypes is shown in Figure 5.2.

### **Screening banana and plantain genotypes using the detached leaf assay**

#### *Preparation of detached leaves*

Newly opened leaves were cut into large pieces that could easily be cleaned in a 1 l beaker. These leaf pieces were surface-sterilised in 1% NaOCl solution for 90 s, and washed in 5 to 6 changes of sterile distilled water. Thereafter, the pieces were cut into smaller segments, each measuring 4 cm in length by 3 cm in width.

#### *Medium preparation, plating and inoculation of leaf pieces*

Medium containing 1% agar technical (product number LP0013, Oxoid Ltd., Hampshire, England) was autoclaved, and 5 mg/l of gibberellic acid was added to the medium immediately before dispensing in Petri dishes. Gibberellic acid at this concentration is able to maintain a green colour in banana leaves for 45 to 52 days, which is needed to differentiate disease severity levels among cultivars (Twizeyimana *et al.*, 2007). Two of these pieces were placed in plastic Petri dishes with the adaxial side on 1% agar technical medium amended with gibberellic acid. Inoculum was prepared as described above; prior to inoculation, a drop of 1% Triton X-100 was added to the spore suspension. Leaf pieces were inoculated (two droplets per leaf piece) by pipetting 40 µl droplets of spore suspension ( $5 \times 10^5$  conidia/ml) onto the abaxial side of the leaf. Plating and inoculation of detached leaves were conducted under aseptic conditions. Petri dishes with inoculated leaf pieces on amended medium were sealed and incubated at 25°C with a 12:12 hour light:dark cycle.

### *Disease assessment*

Observations on inoculated detached leaves began two days after inoculation and lasted 32 days (Figure 5.3). Long days of assessment are required for genotypes that have higher levels of resistance to Black sigatoka disease. Data on incubation time, disease severity, symptom evolution time and symptom stages as described above were recorded.

A schematic representation of the above steps involved in using the detached leaf assay for screening *Musa* genotypes is depicted in Figure 5.4.

### **Utility of the rapid screening methods**

*Musa* genotypes with contrasting resistance levels under field conditions were evaluated using two rapid methods to determine the correspondence of resistance levels in detached leaves and *in vitro* plantlets. The genotypes (with field resistance reactions in parenthesis) were: ‘Agbagba’ (highly susceptible), ‘FHIA-25’ and ‘PITA-21’ (moderately susceptible), ‘PITA-17’ (resistant) and ‘Calcutta-4’ (highly resistant). Data on incubation time, symptom evolution time, time to Stage 4 and disease severity collected from the two assays were analysed as described previously (Twizeyimana *et al.*, 2007) to determine the utility of these assays as rapid screening methods for Black sigatoka disease.

## **RESULTS AND DISCUSSION**

Disease severity was significantly ( $p < 0.01$ ) affected by genotypes evaluated for resistance using both assays. For example, 32 days after inoculation, ‘Agbagba’ had disease severity levels of 81 and 31% when evaluated using *in vitro* plantlets and detached leaf assays, respectively, while ‘Calcutta-4’ had a corresponding severity level of 1.2 and 0.8% in the *in vitro* and detached leaf assays, respectively (Table 5.1). Moderately susceptible cultivars had intermediate levels of disease. In both assays, disease severity assessed 32 days after inoculation separated cultivars based on resistance. Similarly, evaluated genotypes significantly ( $p < 0.001$ ) affected incubation time, symptom evolution time, disease severity and symptom stages in both assays.

Resistant cultivars had longer incubation times than susceptible cultivars (Table 5.1) and much longer symptom evolution times compared to relatively more susceptible genotypes. Cultivars did not complete their symptom evolution time in the detached leaf assay, but a consistent pattern was observed in both assays, with susceptible cultivars having shorter symptom evolution times (e.g., 17 days for ‘Agbagba’) or days to Stage 4 (for the detached leaf assay) than the moderately resistant cultivars (e.g., 32 days for ‘PITA-21’). Disease severity was not significantly different ( $p = 0.2997$  for the two-tailed *t*-test) when assessed using the two assays. In addition, Spearman’s rank correlation showed that disease severity in the two assays was strongly correlated ( $r = 0.88$ ,  $p < 0.0001$ ). Although incubation time was significantly different ( $p = 0.0096$  for the two-tailed *t*-test) between the two assays, Spearman’s rank correlation indicated a strong correlation between the two assays ( $r = 0.70$ ,  $p < 0.0001$ ).

Development of symptoms was observed much earlier on in *in vitro* plantlets than on detached leaves (Table 5.2), irrespective of the cultivar evaluated. Highly significant differences ( $p < 0.0001$ ) in symptom stages were observed 16, 24 and 32 days after inoculation between the two assays. Ranking of cultivars based on symptom stage was similar for both assays, with susceptible and moderately resistant cultivars having correspondingly more advanced symptom stages compared to resistant cultivars, except at eight days after inoculation (Table 5.2). In the detached leaf assay, the genotypes evaluated did not show any symptoms after eight days of inoculation, except for the highly susceptible ‘Agbagba’ that had Stage 2 symptoms.

## CONCLUSIONS

The screening process has been one of the difficulties in breeding banana and plantain for black leaf streak resistance, with field evaluation being the principal procedure (Mobambo *et al.*, 1997; Nwauzoma *et al.*, 2002; Oluma *et al.*, 2003). However, field screening has limitations, since it depends on the presence of proper environmental conditions, can often be carried out only once a year and is time-consuming and expensive. Inoculation of *in vitro* plantlets in tubes and inoculation of detached leaves in moist chambers are two assays that overcome the limitations associated with field and other early screening (Mobambo *et al.*, 1994) of banana and plantain for resistance to Black sigatoka disease.

Detached leaves in moist chambers have been used primarily for screening for rusts (Asnaghi *et al.*, 2001; Herath *et al.*, 2001; Browne *et al.*, 2005) and other diseases (Xie and Mew, 1998; Foolad *et al.*, 2000; Huang *et al.*, 2005). Recently, Twizeyimana *et al.* (2007) conducted studies to evaluate the use of detached leaves for screening *Musa* species for resistance to black leaf streak. A critical aspect of this assay is the prevention of senescence of leaf pieces for the duration of the period required to express symptom stages and disease severity levels necessary to differentiate between cultivar responses (Xie and Mew, 1998). Benzimidazole (Asnaghi *et al.*, 2001) and cytokinin (Browne *et al.*, 2005) have been used in the incubation medium to prevent chlorophyll degradation in detached leaves of other plants. Based on studies by Twizeyimana *et al.* (2007), agar medium amended with 5 mg/l of gibberellic acid is able to prevent chlorophyll degradation up to 52 days after plating.

Development of Black sigatoka disease on *in vitro* plantlets and detached leaves was well correlated, and cultivars were consistently ranked using the two assays. Symptoms appeared much earlier when cultivars were screened using *in vitro* plantlets than with detached leaves as indicated by the significant difference in incubation time between the two assays. The shorter incubation times observed for *in vitro* plantlets may be related to the age of *in vitro* tissues, which are much more tender with limited secondary thickening as opposed to detached leaves from the field. The appearance of symptoms on *in vitro* plantlets differed from that observed on older plants in the field (Capó *et al.*, 2003). Primarily, spots were slightly circular, possibly because young plants derived from tissue culture have limited vein development and black leaf streak lesions tend to be spherical (Mourichon *et al.*, 2000). Although a paired analysis of disease severity indicated no significant difference between the two assays, this difference in morphology of the tissues may also explain the higher severity levels observed on *in vitro* plantlets compared to the detached leaves for a similar set of cultivars. Higher disease severity on young tissue culture plants has been reported previously (Mobambo *et al.*, 1997).

Disease development on young tissue culture plants has been reported to be highly correlated ( $r = 0.98$ ) with disease development on adult plants of the same genotype under field conditions (Mobambo *et al.*, 1997). A similar correlation has been reported by Romero and Sutton (1997) who observed that banana hybrids, 'FHIA-1' and 'FHIA-2', and the susceptible 'False Horn' cultivar expressed the same level of disease reaction in both growth chamber and field evaluations. As such, disease parameters from these two assays reported here are expected to correspond to disease reactions under field conditions. An added advantage of these two assays is that they allow for simultaneous testing of multiple fungal isolates on the same plant, which could shorten the time for development of broad-spectrum resistance to black leaf streak. The assays are reliable and efficient alternatives to greenhouse- and field-screening methods, and may be employed for mass screening of *Musa* species to facilitate breeding for Black sigatoka disease resistance. Resistant genotypes identified by these rapid screening methods must be evaluated under field conditions as the final confirmatory test.

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**Table 5.1.** Incubation time, symptom development and affected leaf area due to Black sigatoka disease on *Musa* genotypes evaluated using *in vitro* plantlets in tubes and detached leaves

Cultivar <sup>a</sup>	<i>In vitro</i> plantlets			Detached leaves		
	Incubation time (days)	Symptom development (days)	Affected leaf area (%)	Incubation time (days)	Days to Stage 4 <sup>b</sup>	Leaf area infected (%)
‘Agbagba’ (HS)	5.0	17.3	82.3	8.0	21.4	32.7
‘FHIA-25’ (MS)	6.3	31.2	29.8	14.0	29.1	14.5
‘PITA-21’ (MS)	6.8	32.0	30.8	12.3	28.9	11.0
‘PITA-17’ (R)	14.0	N/A <sup>c</sup>	5.7	23.1	N/A <sup>c</sup>	3.0
‘Calcutta-4’ (HR)	26.7	N/A <sup>c</sup>	1.2	29.5	N/A <sup>c</sup>	0.8
LSD ( $\alpha = 0.05$ )	7.0	4.8	16.2	2.7	5.8	10.2

<sup>a</sup>Disease reaction of cultivars under field conditions: HS = highly susceptible; MS = moderately susceptible; R = resistant; HR = highly resistant. <sup>b</sup>None of the cultivars evaluated using the detached leaf technique completed their symptom development and thus, number of days to Stage 4 were recorded instead. <sup>c</sup>‘Calcutta-4’ and ‘PITA-17’ did not complete their symptom development when evaluated using *in vitro* plantlets, and similarly symptoms did not reach Stage 4 when evaluated using the detached leaf technique. Within each column, mean values followed by similar letters are not significantly different ( $p \leq 0.05$ ) based on Fisher’s protected least significant difference (LSD) test.

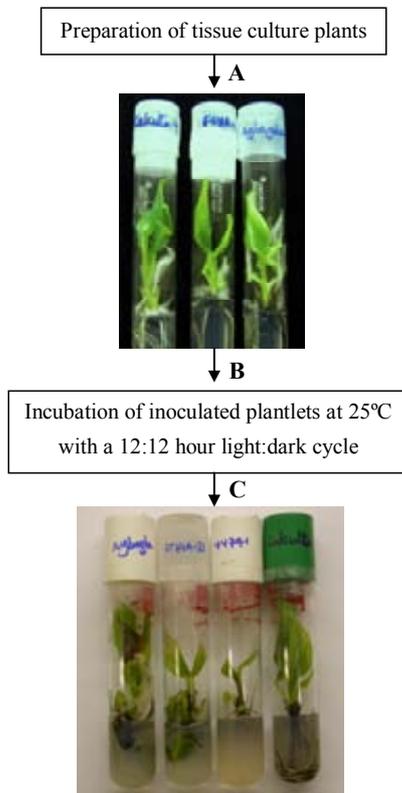
**Table 5.2.** Symptom stages of *Mycosphaerella fijiensis* on *Musa* cultivars on three assessment periods evaluated using *in vitro* plantlets in tubes and detached leaves

Cultivar <sup>a</sup>	<i>In vitro</i> plantlets in tubes				Detached leaves			
	8 days	16 days	24 days	32 days	8 days	16 days	24 days	32 days
‘Agbagba’ (HS)	4	6	6	6	2	3	5	5
‘FHIA-25’ (MS)	3	4	5	6	NS	1	3	5
‘PITA-21’ (MS)	2	4	5	6	NS	1	3	4
‘PITA-17’ (R)	1	1	2	3	NS	NS	1	3
‘Calcutta-4’ (HR)	NS <sup>b</sup>	NS	1	2	NS	NS	NS	1

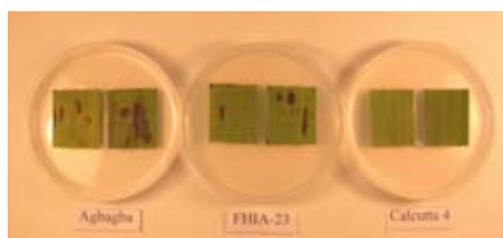
<sup>a</sup>Disease reaction of cultivars under field conditions: HS = highly susceptible; MS = moderately susceptible; R = resistant; HR = highly resistant. <sup>b</sup>NS denotes that no symptoms were observed on the respective cultivar. Symptom stages were based on the 1-6 Fouré scale (Fouré, 1987) in which Stage 1 = minute yellowish specks, <1 mm in length only seen on the lower leaf surface and not visible in translucent light; 2 = specks are 2-4 mm in length, brown and visible in translucent light; 3 = brown specks elongate, with streaks reaching 2-3 cm; 4 = broader, round or elliptical spots, brown underneath and black on the leaf surface; 5 = spots are totally black, surrounded by a yellow halo; and 6 = centre of spots dries out and fades to grey and a black ring surrounds the yellow halo.



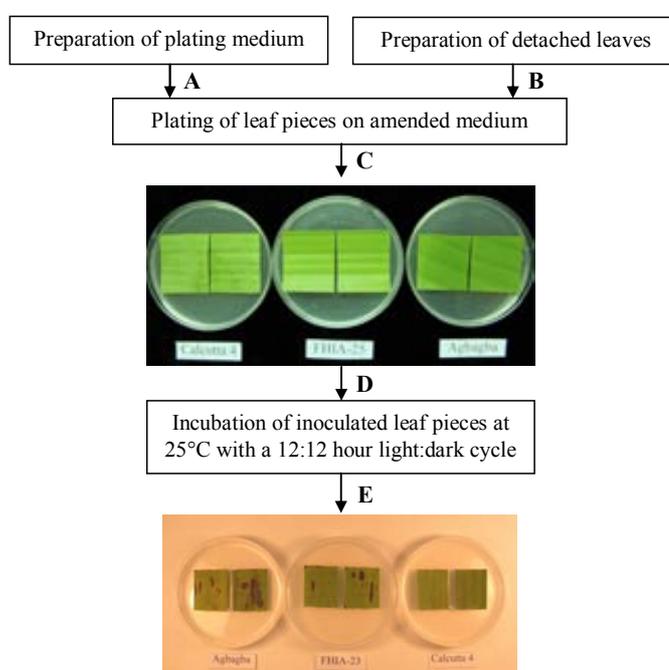
**Figure 5.1.** Symptoms of Black sigatoka disease on *in vitro* plantlets after being inoculated with *Mycosphaerella fijiensis*. ‘Agbagba’ and ‘Calcutta-4’ are highly susceptible and highly resistant to Black sigatoka disease, respectively, while ‘FHIA-23’ and ‘PITA-17’ (shown as 44791) have intermediate levels of disease resistance. No symptoms are visible on ‘Calcutta-4’, while ‘Agbagba’ has high disease severity.



**Figure 5.2.** Schematic representation of steps involved in the use of *in vitro* plants for screening banana and plantain for resistance to Black sigatoka disease caused by the fungus *Mycosphaerella fijiensis*. (A) Prepare tissue culture plantlets using standard protocols. (B) Prepare *M. fijiensis* conidial suspension and aseptically inoculate leaflets (one drop per leaflet) with 40  $\mu$ l of  $5 \times 10^5$  conidia/ml. Avoid leaflets touching the inner wall of the tube, lid or medium. (C) Observe disease symptoms starting 2-32 days after inoculation. Record data on incubation time, symptom evolution time, symptom stages and final disease severity.



**Figure 5.3.** Symptoms of Black sigatoka disease on detached leaves on medium amended with gibberellic acid 32 days after inoculation with *Mycosphaerella fijiensis*. ‘Aghabha’ and ‘Calcutta-4’ are highly susceptible and highly resistant to Black sigatoka disease, respectively, while ‘FHIA-23’ has an intermediate level of disease resistance. No symptoms are visible on ‘Calcutta-4’ as opposed to ‘Aghabha’ and ‘FHIA-23’.



**Figure 5.4.** Schematic representation of steps involved in the use of detached leaves for screening banana and plantain for resistance to Black sigatoka disease caused by the fungus *Mycosphaerella fijiensis*. (A) Prepare medium containing 1% agar technical and add 5 mg/l of gibberellic acid after autoclaving prior to dispensing into Petri dishes. (B) Collect clean, newly opened leaves, surface sterilise in 1% NaOCl, rinse in sterile distilled water and cut into pieces measuring 4 cm in length by 3 cm in width. (C) Aseptically place leaf pieces in Petri dishes with the adaxial side on the amended medium. (D) Prepare *M. fijiensis* conidial suspension and aseptically inoculate detached leaves (two drops per leaf piece) with 40  $\mu$ l of  $5 \times 10^5$  conidia/ml. (E) Observe disease symptoms starting 2-32 days after inoculation. Record data on incubation time, symptom evolution time, symptom stages and final disease severity.

## Chapter 6

### ***In vitro* selection for resistance to Fusarium wilt in Banana**

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#### **ABSTRACT**

Fusarium wilt or Panama disease, caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (FOC), is one of the most serious diseases of banana in tropical and subtropical countries. Although there are banana cultivars resistant to the disease, transfer of the resistance trait to susceptible cultivars by traditional cross-breeding is difficult. *In vitro* selections of banana mutants tolerant to race 1 of FOC were carried out with fusaric acid and culture filtrate on multiple bud clumps. Regenerated plants showed tolerance to the disease in the greenhouse, and some of the plants also showed resistance in the field. Although the level of tolerance observed was not sufficiently high, *in vitro* selection by toxic substances extracted from pathogenic fungi resulted in an improvement of plant tolerance to the pathogen. Other selection factors for *in vitro* selection were reviewed, and a protocol for obtaining disease tolerant plants of banana is suggested.

#### **INTRODUCTION**

Fusarium wilt or Panama disease is caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (FOC). The fungus can survive in the soil for a long period without host plants. Cultural practices for control of this disease, such as fungicide applications or culture rotations, are not economically practicable. Biological control is still in the initial phases of development but does have limited applications (Jaizme-Vega *et al.*, 1998; Ting, *et al.*, 2003). The only effective control available is based on the use of resistant or tolerant cultivars to the disease (Cordeiro, 1997; Cordeiro and Matos, 2000). Genetic improvement is, thus, indispensable for the sustainability of banana crop production. Genetic improvement by cross-breeding has been intensively conducted for the last three decades, and has produced some results in bananas of the 'Prata' subgroup (*Musa* sp. AAB group). However, the expansion of the disease and the emergence of a new fungal race in the 'Cavendish' subgroup (*Musa* sp. AAA group) still pose a threat to many banana plantations. Efforts to produce improved germplasm must be intensified and a larger number of new varieties must be obtained. Besides, the 'Cavendish' subgroup shows a high level of male and female sterility; thus, improving it by traditional cross-breeding is extremely difficult (Dantas *et al.*, 1997). Given these conditions, *in vitro* selection techniques coupled to mutation induction or somaclonal variation could complement conventional breeding methods.

*In vitro* selection is an *in vitro* cultivation technique under controlled stress conditions that favours or disfavors the growth of a cell or tissue type of interest. Compared with selection in an experimental field, *in vitro* selection can considerably reduce the space needed for improving crops. However, some factors influencing *in vitro* selection may differ from those in field selection, as for instance, resistance to the fungus FOC. In order to select plants resistant to Fusarium wilt, FOC can directly be applied to plants in field selections, but cannot be applied to plantlets *in vitro*. This is because the fungus grows much faster than plant tissues in *in vitro* culture conditions. The fungus quickly dominates the culture media and flasks, and the growth of the plant tissue is impeded by a lack of nutrients or space, rather than due to susceptibility to the disease. That is the biggest problem of *in vitro* selection. Furthermore,

the fungus does not attack *in vitro* tissues equally, thus allowing susceptible plantlets to escape from the selection pressure. To overcome this problem, techniques using several selection factors (agents) have been created, such as fusaric acid, culture filtrates of the pathogenic fungus and other substances that cause similar effects as the fungal attack (Švábová and Lebeda, 2005). In this study, techniques for *in vitro* selection using specific agents are reviewed.

## MATERIAL AND METHODS

### *In vitro* selection with fusaric acid

Fusaric acid is the most abundant toxin produced by several species of the genus *Fusarium*, and is responsible for the symptoms of Fusarium wilt disease (Davis, 1969). Once the fungus uses the toxin to destroy vegetable cells and to invade the interior of the plant, plants composed of toxin-resistant cells may block the invasion of the fungus and show tolerance to the disease. *In vitro* selection of mutants tolerant to fusaric acid seems to be a viable strategy for obtaining plants tolerant to Fusarium wilt disease.

The general process for *in vitro* selection is shown in Figure 6.1. We present the methods used and the results obtained in our laboratory. As the first step of *in vitro* selection, multiple bud clumps (see Figure 6.3A) were obtained by cultivating shoot tips of the banana plant cultivars ‘Maçã’ (*Musa* sp. AAB group) and ‘Nanicão’ (*Musa* sp. AAA group, ‘Cavendish’ subgroup) that are susceptible and resistant, respectively, to Fusarium wilt disease caused by race 1 of the fungus FOC. These bud clumps were maintained in a proliferation medium consisting of Murashige and Skoog (MS) medium with salts and vitamins (Murashige and Skoog, 1962), 5 mg/l 6-benzylaminopurine (BAP), 30 g/l sucrose, solidified by 2 g/l phytigel (Sigma Chemical Co., St. Louis, MO, USA). The cultures were maintained in a culture room ( $28 \pm 2^\circ\text{C}$ , 14 hour photoperiod,  $56 \mu\text{mol/m}^2/\text{s}$  light intensity). In the second step, concentrations of fusaric acid that inhibit growth of multiple buds were determined. The multiple bud clumps were cultured on the above mentioned proliferation medium supplemented with fusaric acid (Sigma Chemical Co.) at concentrations ranging from 0.05 to 1.6 mM. The fusaric acid was added to the autoclaved culture medium after filter-sterilisation (0.22 or 0.45  $\mu\text{m}$  pore size membrane filter). After one month of culture, the fresh weights of the bud clumps were measured.

The effect of fusaric acid on the growth of multiple bud clumps is shown in Figure 6.2. The growth of both cultivars (‘Maçã’ and ‘Nanicão’) was drastically inhibited by 0.1 mM fusaric acid. There was no significant difference in susceptibility to fusaric acid between the disease-susceptible ‘Maçã’ and disease-tolerant ‘Nanicão’, suggesting that the degree of disease resistance between these two cultivars cannot be distinguished by fusaric acid.

In the third step of selection, i.e., selection of tolerant Material, we chose 0.1 mM as the appropriate concentration of fusaric acid for the selection of resistant mutants of ‘Maçã’. Following treatment with the chemical mutagen ethyl methanesulfonate (EMS) for 2 hours to induce mutations (Matsumoto and Yamaguchi, 1984), multiple bud clumps were transferred into a selection medium, which consisted of proliferation medium supplemented with 0.1 mM fusaric acid. They were cultivated in selection medium for 3 months, with monthly transfers to fresh media. After the selection period, the multiple bud clumps were transferred to the culture medium for rooting (MS salts and vitamins, 0.25 mg/l  $\alpha$ -naphthalene acetic acid [NAA], 30 g/l sucrose and 2 g/l phytigel) without fusaric acid, and regenerated plantlets were transferred to polyethylene bags containing 2 l of soil in a greenhouse for acclimatisation (Matsumoto *et al.*, 1995).

Tests of tolerance to the disease, which were performed in the fourth and final step of the process, were carried out in a greenhouse by artificially inoculating plantlets (15-20 cm in height) acclimatised for one month or rhizomes (~50 cm in height) from plantlets acclimatised for four months with the fungus FOC. The artificial inoculation was performed by submerging the roots of uprooted plantlets or rhizomes in a spore suspension of the fungus FOC ( $10^7$  conidia/ml). They were then again transplanted

to polyethylene bags with soil previously infested by the fungus FOC (Sun and Su, 1984; Matsumoto *et al.*, 1995). The results of the tolerance test on selected 15-20 cm plantlets and rhizomes are shown in Tables 6.1 and 6.2, and in Figure 6.3. At the growth stage of the 15-20 cm plantlets, disease symptoms appeared in as many selected as in unselected plantlets. Thus, the selection did not seem to be effective. However, when rhizomes from 50 cm plantlets were inoculated with the fungus, the selected plantlets showed higher tolerance to the disease than those not selected. These results showed that selection by fusaric acid was effective: however, the acquired tolerance was not visible at the early stages of development of the acclimatised plantlets.

### ***In vitro* selection with culture filtrate**

The culture filtrate consists of a liquid culture medium in which microorganisms have been cultivated for a certain period of time and eliminated by filtration through a membrane filter. The culture filtrate is expected to contain several metabolic substances produced by the microorganisms, including several toxins besides the fusaric acid that was discussed previously. Microorganisms are usually eliminated by filtration rather than by autoclaving because some metabolic substances are unstable at high temperature. Compared with the use of fusaric acid, selection using culture filtrate may be more efficient for obtaining disease-tolerant plants, because the filtrate contains some toxins that have not yet been identified, but that may be responsible for defining the degree of disease susceptibility or tolerance in the host plants. In some species, *Fusarium*-tolerant plants have already been obtained using culture filtrates as a selection factor (Arcioni *et al.*, 1987; Binarova *et al.*, 1990; Borrás *et al.*, 1997). Therefore, we speculated that the technique could be useful for the assessment of resistance to *Fusarium* wilt in banana plants as well.

Details of the culture filtrate preparation are described in Matsumoto *et al.* (1999b). Race 1 of the fungus FOC was isolated from a disease-susceptible banana plant that showed symptoms of *Fusarium* wilt disease, propagated once in potato dextrose agar (PDA) medium and conserved in the refrigerator (4°C). A piece of the fungal colony with the PDA medium was inoculated in 100 ml of Czapek Dox Broth (CZD) medium in a 300 ml Erlenmeyer flask, with a multiple bud clump (~10 × 10 × 10 mm) of a disease-susceptible banana variety (co-cultivation technique). The culture was maintained in a culture room without agitation for 21 days. It was then filtered through a four-layer gauze (or cheesecloth) and centrifuged at 8000 rpm for 20 minutes. The supernatant was finally filtered through a 0.45 µm membrane filter to eliminate the fungus.

Figure 6.4 shows the growth inhibition caused by the culture filtrate on *in vitro* multiple bud clumps of disease-susceptible ‘Maçã’ and disease-tolerant ‘Nanicão’ cultivars. Inhibition of growth was higher in ‘Maçã’ than in ‘Nanicão’ (Figure 6.4). Since ‘Maçã’ is a susceptible cultivar and ‘Nanicão’ a resistant one, we could expect to obtain disease-tolerant plants using culture filtrate for selection. Therefore, in contrast to the experiment using fusaric acid, where no differences in growth inhibition were observed between the two cultivars (Figure 6.2), the use of FOC filtrates seems to be efficient for the selection of tolerance to *Fusarium* wilt.

To induce mutations, the same treatment was carried out as for selection with fusaric acid. After the mutagenic treatment, the multiple bud clumps were cultivated on proliferation medium (MS, 5 mg/l BAP, 30 g/l sucrose and 2 g/l phytagel) for 5 days, and then transferred to the medium for the first round of selection (MS, 5 mg/l BAP, 10% [v/v] culture filtrate, 30 g/l sucrose and 2 g/l Phytagel). After one month of cultivation, the growing bud clumps were subdivided and transferred to a new medium for the second round of selection (MS, 5 mg/l BAP, 15% [v/v] culture filtrate, 30 g/l sucrose and 2 g/l Phytagel). Another two rounds of selection were undertaken using the same medium. After the successive rounds of selection, the clumps of multiple buds tolerant to the culture filtrate were propagated, and plantlets were regenerated on rooting medium. The plantlets were acclimatised and their tolerance to the fungus was evaluated in the greenhouse as described previously.

Tables 6.3 and 6.4 show the results of the selection using the 10 to 15% (v/v) culture filtrate, after mutations were induced by the chemical mutagen EMS. When acclimatised plantlets (15-20 cm) were artificially infested with FOC and disease symptoms were observed in pseudostem, the selected plantlets did not show any significant increase in disease tolerance. However, symptom observation in rhizomes revealed that the selected plantlets were significantly more tolerant than those not selected (Table 6.3). The selection efficiency was also confirmed by evaluation of pseudostem height and root fresh weight (Table 6.4). Although selection with the culture filtrate was very efficient, the level of tolerance did not reach that of the 'Nanicão' cultivar, which is well-known for its resistance to Fusarium wilt (Table 6.3).

### **Evaluation methods for *in vitro*-selected plants**

The evaluation method for disease resistance is as important as the selection method itself for achieving successful results. As a rule, *in vitro* selection for Fusarium wilt tolerance does not utilise live FOC fungus. Consequently, an evaluation of the selected plants by the fungus itself is indispensable as a final step of the selection process. Such evaluation is carried out on field plants or acclimatised plantlets in the greenhouse. Although field tests are ideal for this purpose (Hwang, 1990; Smith *et al.*, 2006), they require a huge experimental area, and plants are frequently damaged by diseases not-targeted or other climatic factors. To overcome these problems, Sun and Su (1984) developed a method for acclimatised plantlets as described in section 2 (*in vitro* selection by fusaric acid). With some modification and adaptation, it was also applied for *ex vitro* selection (Bhagwat and Duncan, 1998; Bermúdez *et al.*, 2002; Mak *et al.*, 2004). Acclimatised small plantlets in the greenhouse or net-house were infested by the fungus FOC for the evaluation, and symptomless plantlets were selected to be transplanted in the field. This method can be used to analyse a limited number of plantlets and is feasible as a pre-field-selection for tolerance to the disease. Even though the method is very useful, care must be taken in its application, because the disease susceptibility or tolerance of the selected Material somewhat depends on the plantlet stage and evaluation parameter (see Tables 6.1-6.4).

Inoculation of fungus on both acclimatised plantlets and field plants may kill plants that, although susceptible to the disease, might have excellent agronomic characters. More recently, a non-destructive evaluation method for disease resistance was developed using fungal culture filtrate. It was based on concentrated culture-filtrate inoculation onto needle-mediated wounds of middle-aged banana leaves (Companiononi *et al.*, 2003, 2005). Evaluating seven banana clones, three of which were disease-susceptible and four of which were disease-resistant, the susceptible clones always showed major lesion areas after 48 hours of incubation. This evaluation method is of great interest not only because it is non-destructive but also due to the very short time needed for evaluation.

### **Protocol for *in vitro* selection of Fusarium wilt-tolerant plants**

The *in vitro* selection protocol used in our laboratory is as follows:

#### **Plant material preparation**

- (1) Small and apparently healthy suckers are collected from a Fusarium wilt-susceptible plant.
- (2) Suckers are washed with tap water and cut into  $10 \times 10 \times 10 \text{ mm}^3$  blocks with shoot tip.
- (3) The tissue blocks are surface-sterilised in a laminar flow cabinet with 70% alcohol for 15 s, followed by 1% sodium hypochloride with a few drops of Tween 20 for 15 minutes.
- (4) External tissues are removed leaving blocks of  $5 \times 5 \times 5 \text{ mm}^3$ .
- (5) They are transferred to banana proliferation medium consisting of MS (Murashige and Skoog, 1962) salts and vitamins, 5 mg/l BAP, 30 g/l sucrose, 2 g/l phytigel and maintained in a culture room ( $28 \pm 2^\circ\text{C}$ , 14 hour photoperiod, 45-60  $\mu\text{mol/m}^2/\text{s}$  light intensity).
- (6) After 3 to 8 months of culture (subcultures each of 30 to 45 days), multiple bud clumps will be obtained.

### Production of culture filtrates

- (7) FOC fungus is isolated on PDA medium from a disease-susceptible banana plant that shows disease symptoms.
- (8) The fungus is proliferated once on the same medium in Petri dishes for two weeks ( $28 \pm 2^\circ\text{C}$ , 45-60  $\mu\text{mol}/\text{m}^2/\text{s}$  of continuous illumination) and stored in a refrigerator ( $4^\circ\text{C}$ ) until use. Pathogenicity of the isolates should be checked using, for instance, the method of Sun and Su (1984).
- (9) A piece of the fungal colony (2-3 mm in diameter) and a tissue (~10 mm in diameter) of the banana multiple bud clump from Step 6 are inoculated into 100 ml of CZD medium in a 300 ml Erlenmeyer flask.
- (10) The culture is incubated at  $28 \pm 2^\circ\text{C}$  and 45-60  $\mu\text{mol}/\text{m}^2/\text{s}$  illumination with a 14 hour photoperiod for 21 days without shaking.
- (11) The cultured liquid medium is then filtered through a four-layer gauze or cheesecloth and centrifuged (8000 rpm, 20 minutes) to precipitate mycelium and conidia.
- (12) The supernatant is passed through a membrane filter (0.45 or 0.22  $\mu\text{m}$  pore size) to remove the remaining fungi. This cultured fungus-free medium is used as the culture filtrate for the following selection. The culture filtrate should be used immediately; it is not recommended to store it.

### Mutagen treatment and selection

- (13) At least 500 pieces, each  $\sim 3 \times 3 \times 3 \text{ mm}^3$ , of the banana multiple bud clumps from Step 6 are incubated in a 500 ml Erlenmeyer flask with 200 ml of aqueous solution supplemented with 4% (v/v) dimethylsulphoxide (DMSO) and 0.3% (v/v) EMS.
- (14) The flask is kept on a gyratory shaker (100 rpm) for two hours at  $28 \pm 2^\circ\text{C}$ .
- (15) After washing three times with sterile distilled water, the multiple bud clumps are transferred to banana proliferation medium (Step 5) in Petri dishes and cultured for one week.
- (16) The multiple bud clumps are then transferred onto the selection medium, which consists of proliferation medium supplemented with 10-15% (v/v) culture filtrate or 0.1 mM fusaric acid.
- (17) After 30 to 45 days of culture, growing multiple bud clumps are separated into smaller pieces ( $3 \times 3 \times 3 \text{ mm}^3$ ) and transferred to the newly prepared selection medium.
- (18) The above process is repeated once more.
- (19) The selected multiple bud clumps are propagated on proliferation medium for 2-3 months until multiple shoot formation.
- (20) The multiple shoots are separated and transferred to rooting medium composed of MS salts and vitamins, 0.25 mg/l NAA and 30 g/l sucrose solidified by 2 g/l of phytigel.
- (21) After 20 to 30 days of culture, regenerated plantlets are transferred to black polyethylene bags or containers with a substrate mixture and acclimatised in a greenhouse equipped with a mist system.
- (22) The disease resistance of the acclimatised plantlets is evaluated in the greenhouse and/or in the field.

## RESULTS AND DISCUSSION

### Perspectives of the *in vitro* selection for disease resistance

The results mentioned in this chapter show the applicability of fusaric acid and culture filtrate in the selection of plants tolerant to the fungus FOC. However, the level of tolerance obtained by both methods was always lower than the tolerance level of 'Nanicão' that was used as a resistant control for the disease. Some of selected lines of 'Maçã' plants showed resistance in the field. At the same time, however, they became more susceptible to another disease, namely Yellow sigatoka disease (Matsumoto *et al.*, 1999a). This effect might have been caused by secondary mutations or somaclonal variations induced by the successive rounds of selection, which were carried out with the aim of

avoiding occurrence of escapees and chimeras. Since many of the mycotoxins in the culture filtrates are mutagens (Jimenez *et al.*, 1997; Sewram *et al.*, 2005; Švábová and Lebeda, 2005), successive selections could induce secondary mutations. Undesirable mutations can be eliminated by new selection steps addressing the correction of the undesired character (Tang and Hwang, 1998). However, to avoid the secondary mutations in the first place, it is advisable to perform a single round or a few successive rounds of selection. The risk of occurrence of escapees and chimeras can be reduced by the use of smaller-sized explants, suspension cells or protoplasts. A reliable protocol of plant regeneration from the cells or protoplasts must become an important tool.

The history of plant breeding for disease resistance has shown that the acquired resistance will always be broken by the emergence of new microbial races. This means that we have to continuously breed new plants with different genes or mechanisms of disease resistance. *In vitro* selections with different selection factors make this strategy possible. Morpurgo *et al.* (1994; Chapter 7 of this book) showed that *in vitro* explants from disease-resistant and disease-susceptible banana cultivars were susceptible to both culture filtrate and fusaric acid irrespective of their known field resistance/susceptibility response. Therefore, they concluded that the use of culture filtrate or fusaric acid was not feasible for selecting a novel resistant genotype of *Musa* to FOC. However, contrary to their observations, the disease tolerances of susceptible banana cultivars were improved after selection with metabolic substances (Matsumoto *et al.*, 1995, 1999a; Cardenas *et al.*, 2003; Saravanan *et al.*, 2003). We could now draw the conclusion that the use of culture filtrates or fusaric acid for selecting a novel genotype of *Musa* resistant to FOC is quite feasible. The disease tolerance mechanism of *in vitro*-selected plants may then be different from that of pre-existing resistant cultivars, since both resistant and susceptible cultivars show equal susceptibility to the metabolic substances. Considering that the mechanisms of tolerance to one selection factor are different from another, tolerances to several selection factors can be accumulated in one genotype by cumulative selection processes (Tang and Hwang, 1998). The *in vitro* selection system may produce new plants that confer several resistance mechanisms regulated by several genes. The acquired tolerance would, then, be a horizontal resistance characteristic and would be difficult to be broken by the emergence of new races of the fungus. It could, therefore, be maintained for a longer period.

Besides pathogen-produced metabolic substances, many other substances or chemicals can be used for *in vitro* selection of tolerance to Fusarium wilt. New selection factors or gene markers can facilitate *in vitro* selections and increase their applicability. Elicitors from fungal cell wall fractions are promising substances, since plant defence responses were induced by the elicitor treatments (De Ascensao and Dubery, 2000). Although we still do not have supporting data, plants selected by a proline analogue, such as hydroxyproline (Matsumoto *et al.*, 1987), may over-produce proline and increase the absorption capacity of water from the soil. This could reduce damage caused by Fusarium wilt. Transgenic plants with an introduced glucanase gene have increased tolerance to Fusarium wilt (Mahmood *et al.*, 2003). Glucanase over-producing plants may also be obtained by selecting explants that grow on a culture medium containing glucan as a substitute for part of the sucrose.

## CONCLUSIONS

Reliable protocols for *in vitro* selection of banana plants resistant or tolerant to diseases are an important tool for the improvement of this crop, particularly in combination with *in vitro* mutagenesis. Our *in vitro* selection protocol based on the use of culture filtrates or fusaric acid for selecting novel genotypes of *Musa* resistant to FOC was shown to be quite feasible. In addition to the substances used in this study, many other substances or chemicals, such as fungal elicitors, may be useful for *in vitro* selection of tolerance to Fusarium wilt in bananas. Gene markers may also facilitate *in vitro* selections and increase their applicability.

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**Table 6.1.** Comparison of susceptibility of selected and unselected ‘Maçã’ banana plantlets to Fusarium wilt\*

Variety	Selection	Pseudostem height (mm)	Root fresh weight (g)	Fusarium wilt symptoms (%)	
				Pseudostem	Rhizome
‘Maçã’	Not selected	1435	11.88	76	88
‘Maçã’	Selected	1860	19.58	90	90
<i>t</i> -test **		0.0002	0.0003	0.2531	0.6332

\*15-20 cm plantlets of ‘Maçã’ bananas were infested artificially with race 1 of FOC in the greenhouse. 17 samples per treatment were analysed. \*\*Values from a two-sided *t*-test for the two values immediately above. Source: Matsumoto *et al.* (1995).

**Table 6.2.** Comparison of susceptibility of selected and unselected ‘Maçã’ banana rhizomes to Fusarium wilt\*

Variety	Selection	Pseudostem height (mm)	Root fresh weight (g)	Fusarium wilt symptoms (%)	
				Pseudostem	Rhizome
‘Maçã’	Not selected	264 <sup>a</sup>	0.46 <sup>A</sup>	57 <sup>a</sup>	100 <sup>A</sup>
‘Maçã’	Selected	825 <sup>b</sup>	2.48 <sup>B</sup>	14 <sup>b</sup>	57 <sup>B</sup>
‘Nanicao’	Not selected	852 <sup>b</sup>	3.69 <sup>B</sup>	0 <sup>b</sup>	29 <sup>B</sup>

\*Rhizomes from 50 cm plantlets were infested artificially with race 1 of FOC in the greenhouse. 14 rhizome samples were analysed. The ‘Nanicao’ variety was used as a control for the disease-tolerant variety. In each column, values followed by the same letter are not significantly different from each other at the 5% level using Duncan’s multiple-range test. Source: Matsumoto *et al.* (1995).

**Table 6.3.** Frequency of plants with symptoms of pseudostem and rhizome vascular discolouration caused by race 1 of FOC on banana plantlets of the ‘Maçã’ cultivar selected or not selected with culture filtrate in the greenhouse test

Variety	Selection	Number of plants observed	Fusarium wilt symptoms (%)	
			Pseudostem	Rhizome
‘Maçã’	Not selected	34	47.1	82.4
‘Maçã’	Selected	31	41.9	67.7
‘Nanicão’ <sup>1</sup>	Not selected	34	2.9	29.4

<sup>1</sup>Nanicão was tested as a control for a resistant plant to race 1 of FOC. A comparison between selected and not selected ‘Maçã’ showed a significance level of 0.4346 for pseudostem and 0.1406 for rhizome (*t*-test). A comparison between ‘Nanicão’ and other treatments showed a significance level <0.01. Source: Matsumoto *et al.* (1999a).

**Table 6.4.** Comparison of pseudostem and root growth between culture-filtrate selected and not selected ‘Maçã’ banana, infested by race 1 of FOC in greenhouse tests

Variety	Selection	Pseudostem height (mm)	Root fresh weight (mg)
‘Maçã’	Not selected	26.42 ± 6.25 <sup>1</sup>	460.71 ± 109.96
‘Maçã’	Selected	43.63 ± 10.83	1077.27 ± 313.43
two-sided significance level by <i>t</i> -test		0.1613	0.0536

<sup>1</sup>Mean ± standard error. Source: Matsumoto *et al.* (1999a).

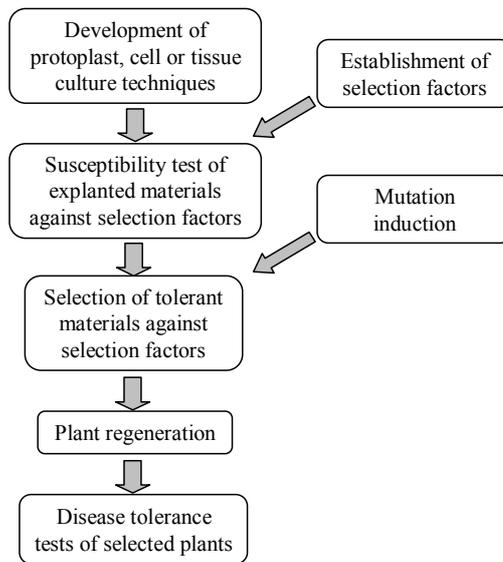


Figure 6.1. *In vitro* selection process to obtain disease-tolerant plants.

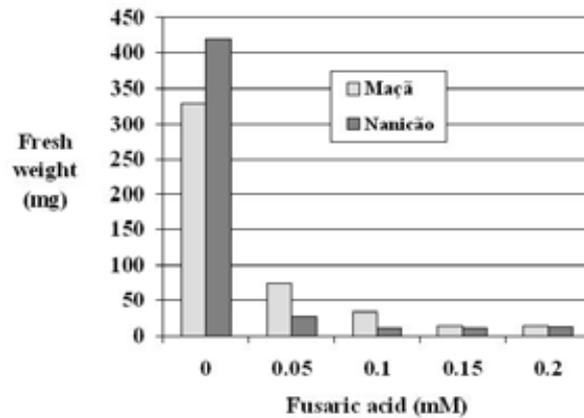
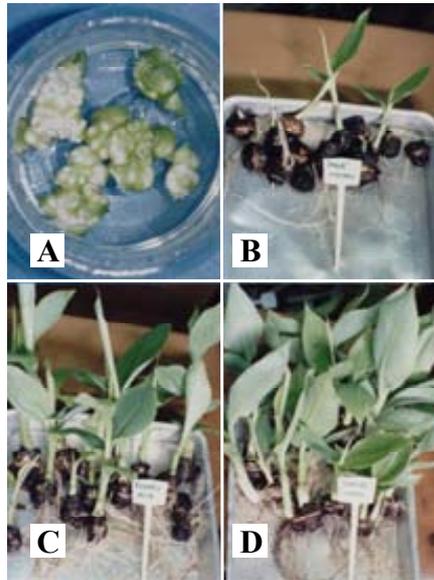
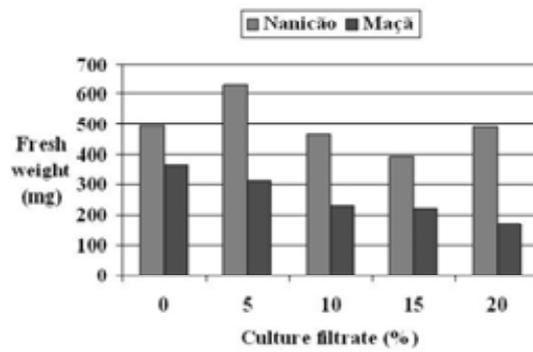


Figure 6.2. Effects of fusaric acid on growth of banana multiple bud clumps.



**Figure 6.3.** (A) Multiple bud clumps; (B) FOC-inoculated rhizomes from unselected 'Maçã' banana; (C) FOC-inoculated rhizomes from from 'Maçã' banana selected with fusaric acid; (D) FOC-inoculated rhizome of 'Nanicão' banana.



**Figure 6.4.** Effects of culture filtrate on growth of banana multiple bud clumps.



## Chapter 7

### Selection parameters for resistance to *Fusarium oxysporum* f. sp. *cubense* race 1 and race 4 on diploid banana (*Musa acuminata*)\*

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#### ABSTRACT

Shoot tip cultures from banana clones susceptible and resistant to *Fusarium oxysporum* f. sp. *cubense* (FOC) race 1 and race 4 were grown *in vitro* in the presence of different concentrations of fusaric acid and fungal crude filtrates or inoculated with a conidial suspension of FOC to assess correlation between *in vivo* and *in vitro* behaviour. Explants were susceptible to both filtrate and fusaric acid irrespective of their known field resistance/susceptibility response. No clear linkage between *in vivo* and *in vitro* behaviour was observed, and our results suggest that the use of crude filtrate or non-host-specific toxin (fusaric acid) in a screening programme for selecting a novel resistant genotype of *Musa* to FOC is not feasible. When peroxidase activity was used as a parameter to discriminate between susceptibility and tolerance, the results were in good agreement with the field response of host plant to pathogens. Early enzymatic activity increased in the incompatible host-pathogen interaction but not in the compatible interaction.

#### INTRODUCTION

According to the FAO Trade Yearbook 1990 (FAO, 1991), *Musa* (banana, plantain and cooking banana) is cultivated in 117 countries worldwide, mostly in the tropics and subtropics of South and Central America, Asia and Africa. Less than 10% of the whole production of 70 million tons of fruit enters world trade; the majority is locally consumed as staple starchy food. Several factors, edaphic, physical and biotic limit *Musa* production. Among the latter, the most important are two fungal diseases, the Sigatoka complex, caused by three related *Mycosphaerella* spp.; and Panama disease caused by FOC. The susceptible cultivar 'Gros Michel' was gradually replaced by the resistant 'Cavendish' clones but a new race of FOC has recently developed that has broken the resistance of 'Cavendish' to race 1. The new race 4 of FOC appeared, presumably as independent events, in four different regions of the world, namely the Canary Islands, Australia, Taiwan and South Africa (Stover, 1990) and is now spreading rapidly in other banana producing countries. Breeding for banana resistance is particularly difficult due to the sterile and polyploid nature of the plant and to the saprophytic – pathogenic nature of the fungus (Novak, 1992). The relationship of the pathogen with the plant host and the complex interplay occurring between them and environmental parameters results in an extremely complex situation (Beckman *et al.*, 1962; Marois, 1990). Selecting plant genotypes resistant to pathogens has become one of the major tools of combating agricultural losses and increasing productivity. However, traditional plant breeding is not able to keep pace with pathogen evolution. Field screening is time consuming and requires a large amount of space and intensive manpower inputs. Furthermore, disease symptoms develop only after a long period of incubation and the results can be misleading due to non-uniform distribution of pathogens in soil.

Plant tissue culture techniques have been used for investigating mechanisms of host pathogen relationship (reviewed in Chapter 2) and have been proposed as an aid in banana breeding programmes (Novak, 1992). The most widely used system for *in vitro* evaluation of

resistance/susceptibility is the use of purified toxins and/or fungal crude filtrate (Daub, 1986). However, the use of filtrate produced in culture by *Fusarium* spp. was questioned by Beckmann (1987). Results obtained by Buiatti *et al.* (1987) showed that in *F. oxysporum* f. sp. *dianthi* cell culture, a better correlation exists when biochemical parameters, i.e., phenolic metabolites, are considered. Peroxidase (EC 1.11.1.7) is a multi-purpose enzyme that has been correlated with active plant defence mechanisms by condensing phenolic compounds to form lignin. In this paper, we compared the effect of crude fungal filtrates on banana plants and the production of peroxidase after inoculation with FOC race 1 and race 4 conidial suspensions in order to identify suitable selection criteria.

## **MATERIAL AND METHODS**

### **Plant material**

Two diploid (AA) clones, namely 'SH-3362' and 'Pisang Mas' (syn. 'Sucrier'), showing different resistance to FOC race 1 and race 4 were used. 'SH-3362' is a product of cross-breeding with high resistance to both races of FOC (Rowe, 1991) while 'Pisang Mas' is a sterile diploid cultivar, widely cultivated for small sweet fruit, moderately resistant to race 1 and fully susceptible to race 4 of FOC.

### **Fungal culture**

FOC race 1 strain 2264 (vegetative compatibility group [VCG] 0125) and race 4 strain YO 604 (VCG 01290) were provided by Dr. K. Pegg, DPI, Queensland. Small plugs of mycelium were transferred to Petri dishes filled with solid Czapek-Dox medium (Difco, Becton Dickinson, Sparks, MD, USA) and incubated in the dark at 28°C. After a week, plugs of actively growing mycelium were transferred to a 250 ml Erlenmeyer flask containing 150 ml of liquid Czapek-Dox and further incubated under the same conditions as above for a maximum of 21 days. The culture broth was then filtered through a double layer of cheesecloth and sterilised through a 0.22 µm Millipore membrane. The crude filtrate was then stored at -20°C.

### ***In vitro* plant culture**

Shoot tips of *in vitro* grown banana plantlets were excised and transferred to 250 ml Erlenmeyer flasks containing 25 ml of culture medium composed of MS salts (Murashige and Skoog, 1962), supplemented with Gamborg's B5 vitamins (Gamborg *et al.*, 1968), 5 µM 2iP, 40 mg/l cysteine and 40 g/l sucrose. The pH was adjusted to pH5.8 and the medium was autoclaved at 120°C and 1 bar for 20 minutes. After a one week incubation at 25°C on a rotary shaker at 80 rpm under continuous light, the culture medium was removed and an equal amount of fresh medium was added in order to avoid toxic activity due to phenolic compounds produced by the explant.

### **Fusaric acid assay**

Shoot tips of *in vitro* grown banana were excised and transferred to the same culture medium supplemented with different concentrations (0.02, 0.045, 0.06, 0.09 mM) of fusaric acid (Sigma). The control and treated plants were screened after three weeks for growth parameters such as shoot and root fresh weight, shoot height and explant survival.

### ***In vitro* assay of crude filtrate**

Banana shoot tips were transferred to the same medium described above with or without different percentages (4.5, 6, 9 and 12% [v/v]) of crude filtrate obtained 21 days after fungal culture. Plants were screened after three weeks of culture for shoot and root fresh weight, shoot height, explant survival.

## **Ion leakage**

Leaf disks were obtained from fully expanded leaves of the same genotypes grown in the greenhouse using a cork borer. Leaf disks were held in pure crude filtrate of both races for 30 minutes under vacuum to facilitate filtrate penetration, washed three times with distilled water and placed in test tubes filled with 20 ml of an isotonic sucrose solution. Conductivity was measured (YSI Model 34, YSI, Yellow Springs, USA) at two minute intervals to follow ion release from the cell.

## **Plant inoculation**

Banana plantlets were grown and rooted under axenic conditions as described elsewhere (Novak *et al.*, 1987). Roots were trimmed and plantlets were treated with a microconidial suspension ( $5 \times 10^5$  conidia/ml) as described by Hwang and Ko (1987). Controls were treated with distilled water only. Treated and non-treated plants were transplanted in sterile vermiculite and placed in a growth chamber at 29°C. At weekly intervals, three plantlets were removed and assayed for peroxidase activity.

## **Peroxidase assay**

Corm tissue was ground in a mortar adding to the tissue 0.1 M sodium phosphate buffer pH 6.8 (1.2 [w/v]). The resulting homogenate was centrifuged at 14000 rpm in an Eppendorf centrifuge and assayed for protein content according to Bradford (1976). In addition, the method described by Garraway *et al.* (1989) was used to extract ionically bound peroxidase. Activity was measured in 1 ml final volume reaction mixtures. Five  $\mu$ l of corm extract were incubated in 0.3% guaiacol, 2 mM hydrogen peroxide in 0.01 M sodium phosphate buffer pH 6. After two minutes, absorbance was read at a wavelength of 470 nm and protein content assessed.

## **Isoelectrofocusing (IEF)**

Samples containing equal protein content were loaded on a polyacrylamide gel (T = 7.5%, C = 3%) containing Pharmalyte pH 3.5-9.5 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as a carrier ampholyte. The gel was prefocused for 30 minutes. Running conditions were 8 W fixed, 33 mA and a voltage up to 2500 V. After running, the gels were stained in sodium phosphate buffer pH 6, containing 0.3 % guaiacol and 2 mM hydrogen peroxide or 0.6 mg 4 chloro-1-naphtol per ml and 2 mM hydrogen peroxide (Ye *et al.*, 1990).

# **RESULTS AND DISCUSSION**

## **Effect of fusaric acid**

No conclusive results were obtained using fusaric acid as a selection agent in tissue culture media. A concentration of 0.45 mM of fusaric acid stimulated the growth of the resistant clone 'SH-3362' while at twice the concentration (90  $\mu$ M), plant growth was slightly inhibited. The clone 'Pisang Mas', susceptible to race 4, showed little response to different levels of fusaric acid (Figure 7.1).

## ***In vitro* assay of crude filtrate**

Production of toxic substances in the filtrate was assayed by reading 2 ml aliquots in a spectrophotometer set at 272 nm at weekly intervals. Absorbance was then correlated with biological effects of the same filtrate on banana shoot tip culture. A significant correlation ( $r = 0.92$ ) was found between these two methods. Crude filtrate obtained at weekly intervals exhibited maximum toxic activity three weeks after inoculation. Figures 7.2 shows the inhibition of shoot growth of both susceptible and tolerant clones cultured in media with different concentrations of crude FOC filtrate. The addition of the filtrate from both races of FOC in the medium at a concentration of 9% significantly reduced the growth of treated plants; at a concentration of 12%, explants were not able to

develop a new shoot and most died. A direct effect of the filtrate on the rate of mortality on the cultured plantlets of both genotypes was observed. Both susceptible and tolerant clones responded in the same way to the filtrate, i.e., inhibition of all the growth parameters (i.e., total fresh weight, plant height, root fresh weight and shoot fresh weight). No differential response was observed between banana clones with filtrates of race 1 or race 4. The same tendency was observed by determination of ion leakage (Figure 7.3). In this case, the addition of filtrate to detached leaf disks caused an increase in ion leakage as compared with the non-treated leaf tissue. The release of ions increased with time but it was not correlated with known fungal race pathogenicity. On the contrary, in 'SH-3362', the ion leakage after treatment with filtrate of race 1 was higher than that observed with race 4.

### **Growth of inoculated plants**

In all plants inoculated with FOC conidia from race 1 and 4, no wilting symptoms were observed up to 28 days of culture in vermiculite at 29°C. However, internal symptoms, i.e., browning of xylem vessels were observed on dissection of the susceptible clone 'Pisang Mas' inoculated with race 4. The same clone showed reduced root growth.

### **Peroxidase activity**

Different tissues, i.e., root, corm and leaf of both banana genotypes were analysed for constitutive peroxidase activity before fungal inoculation. In non-infected plants of both clones, the highest peroxidase activity was found in root tissue while the lowest activity was recorded in leaf tissue. However, the two clones showed marked differences in constitutive activity. This was at least five times higher in the root and corm tissues of the resistant 'SH-3362' clone than in the susceptible cultivar 'Pisang Mas' (Figure 7.4). In plants infected with FOC race 1, both clones showed a prompt increase in peroxidase activity with seven days of inoculation (see Figure 7.5). The general response of the two banana clones to race 1 was essentially similar, with fast induction and a high level of activity over the whole experimental period. The reaction of host plants to race 4 provided a completely different picture. After seven days, the resistant clone 'SH-3362' showed a 10-fold increase in peroxidase activity, declining slowly over the next 21 days (Figure 7.5A). The susceptible cultivar 'Pisang Mas', on the other hand, responded to the infections with a small increase, although not statistically significant, in enzymatic activity over the first seven days after infection (Figure 7.5B). Thereafter, the enzymatic activity in this clone either decreased to a lower level comparable with the non-inoculated plants (14 and 28 days after inoculation) or slightly increased (21 days after inoculation).

In addition to the qualitative aspects and the kinetics of induction, it was interesting to note that at any evaluation point there were marked differences between the resistant and the susceptible genotypes. In fact, the total peroxidase activity in the resistant clone 'SH-3362' was always higher at least by a factor of six than the enzymatic activity induced by the infection with FOC race 4 in the susceptible cultivar 'Pisang Mas'.

### **Isoelectrofocusing**

4 chloro-1-naphthol gave better band resolution than guaiacol and a higher number of isozymes were detected. Using this stain, it was possible to count at least 12 bands of isozymes, common to all the clones. Among them, seven anionic bands were present. We did not detect differences in isozyme patterns between induced and non-induced plants; intensity of bands increased in both control and treated plants with time. However, the intensity of some bands increased earlier in inoculated plants than in controls (Figure 7.6).

## CONCLUSIONS

Phytotoxins in crude fungal culture filtrates have previously been used as selection agents in *in vitro* cultures and a correlation between *in vivo* and *in vitro* responses has been reported in several plant-pathogen systems (Daub, 1986). However, the involvement of purified non-host-specific toxins or crude filtrate in wilt disease has been questioned (Beckmann, 1987; Buiatti and Ingram, 1991). In our work, crude filtrate reduced all growth parameters tested and finally led to the death of explants without typical wilting symptoms in both tolerant and resistant *Musa* genotypes. Production of toxic secondary metabolites during culture of *Fusarium* spp. depends not only on the species of the *forma specialis* but also on culture and growth conditions such as temperature, inoculum concentration and age of culture as well as on the physiological stage of the pathogenic organism itself (Durbin, 1983). Usually, the activity of toxic compounds present in a crude filtrate is measured by bioassay of living material. A major drawback in this procedure is the long time needed to observe effects. The spectrophotometric method described in this work provides a faster way of determining both the biological effects and the filtrate concentration to be added in the medium.

Our results are in agreement with those described by Epp (1987), who was not able to discriminate between resistant and susceptible banana plants using fusaric acid as a selection agent in culture media. Resistance to FOC, therefore, does not seem to be correlated with resistance to a non-host-specific toxin. No conclusive response was obtained using banana shoot tips in culture on a medium containing crude filtrate; this suggests that toxic compounds excreted by the fungus while in culture are not responsible for the breakdown in resistance caused by FOC race 4 in previously resistant banana clones. Since in the literature (e.g., Daub, 1986) no host-specific toxins have been reported for the genus *Fusarium*, it appears quite unlikely that toxins could act as determinants of virulence. Toxic effects suggest that crude filtrate may play some role in pathogenesis but this should act rather in the post-infection processes. This assumption is in agreement with the *in vitro* behaviour of alfalfa where virulence of *Fusarium* could not be correlated with the toxic activity shown by the filtrate on explants (Binarova *et al.*, 1990).

Previous work conducted on the *Dianthus caryophyllus*-*Fusarium oxysporum* f. sp. *dianthi* interaction using two different systems, i.e., culture filtrate and fungal cell wall elicitor, showed that the latter is a more efficient experimental system for the identification and characterisation of susceptible and tolerant responses in the host plant (Buiatti *et al.*, 1985, 1987).

Resistance to FOC can be accomplished by the whole plants' defence mechanism. Indeed, the only reported mechanism that acts as a defence against FOC in banana is a prompt response to infection mediated by the build-up of a mechanical barrier that may prevent the spread of the pathogen into the plants' vascular system (Beckman *et al.*, 1961; Beckman, 1990).

Peroxidase is a multi-purpose enzyme that catalyses the condensation of phenolic compounds into lignin. The current model that involves peroxidase in defence mechanisms considers the condensation of phenolic monomers derived from the phenylpropanoid pathway into insoluble polymers (Robb *et al.*, 1991). This step is catalysed by anionic peroxidase. It has been demonstrated by different authors (Seever *et al.*, 1971; Graham and Graham, 1991; Peng and Kuc, 1992) that peroxidase plays an important, early and specific role in the hypersensitive containment of the pathogen. The results presented in this work demonstrate that the resistant plant responded actively to infection of both FOC races. The fact that in the compatible interaction, peroxidase activity increases rapidly suggests that in banana this enzyme might be involved in the defence response. These observations are in agreement with the model proposed by Beckman (1990), which involves the rapid build-up of mechanical barriers and with the role of phenolic metabolite infusion as proposed by Strobel and Sinclair (1991). The speed and magnitude for activation of the defence mechanism appear critical for the expression of resistance (Reuveni *et al.*, 1992). Moreover, the measurement of constitutive peroxidase activity shows a positive correlation between high enzymatic activity in non-infected plants and the resistance to FOC. This fact, jointly with the activation of enzymatic activity in tissues that are not the primary

site of infection, is in agreement with the systemic defence mechanism proposed by Hammerschmidt and Kuc (1982) and by Hammerschmidt *et al.* (1982). To be effective, a defence mechanism based on peroxidase activity should be constitutively present or rapidly elicited in response to pathogen infection. In the case of the race 4 susceptible cultivar 'Pisang Mas', this threshold is probably not reached, while in the resistant 'SH-3362' this is already present in uninfected plantlets. On the contrary, 'Pisang Mas' is able to reach the threshold level when infected with the incompatible race 1. However, this work was done using only two clones and the actual role of peroxidase in the active containment of *Fusarium* should be confirmed by a more extensive screening using different clones of *Musa*. To conclude, whereas this work shows that peroxidase activity can be used as a parameter to discriminate between susceptible and tolerant clones of banana, it emphasises the need for more extensive research on the physiological mechanism that underlines the resistance/susceptibility response of the banana to FOC.

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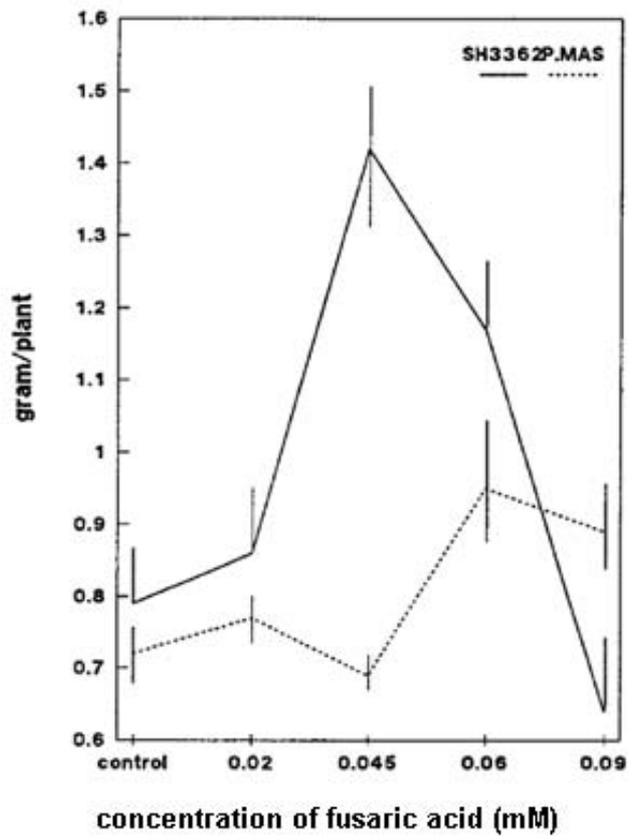


Fig. 1. Effect of fusaric acid on shoot tip growth of a diploid banana resistant clone 'SH-3362' and a susceptible cultivar 'Pisang Mas' to *Fusarium oxysporum* f. sp. *cubense*. Each point represents the average of 5 replicates and three independent experiments. Bars represent the standard deviation of the average.

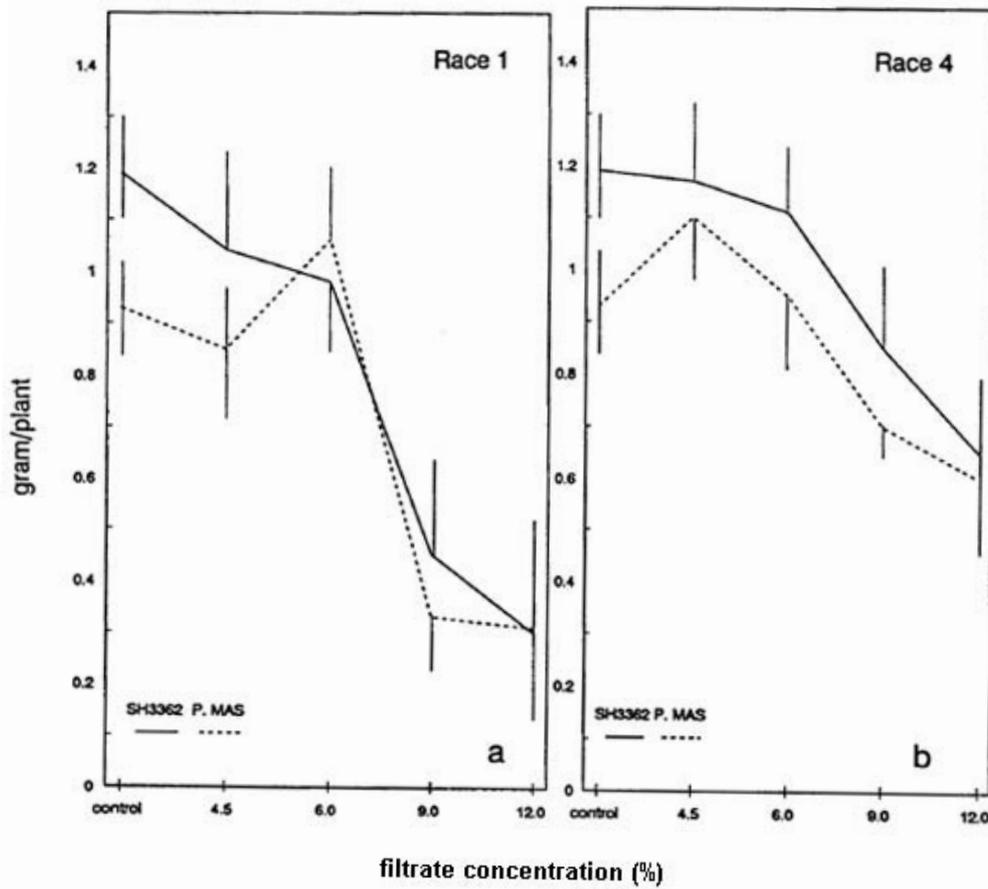


Fig. 2. Effect of different concentration of crude filtrate obtained from 21 day old *Fusarium oxysporum* f. sp. *cubense* race 4 and 1 on shoot tip growth of a 'SH-3362' and 'Pisang Mas'. Each point represents the average of 10 replicates and three independent experiments. Bars show the standard deviation of the average.

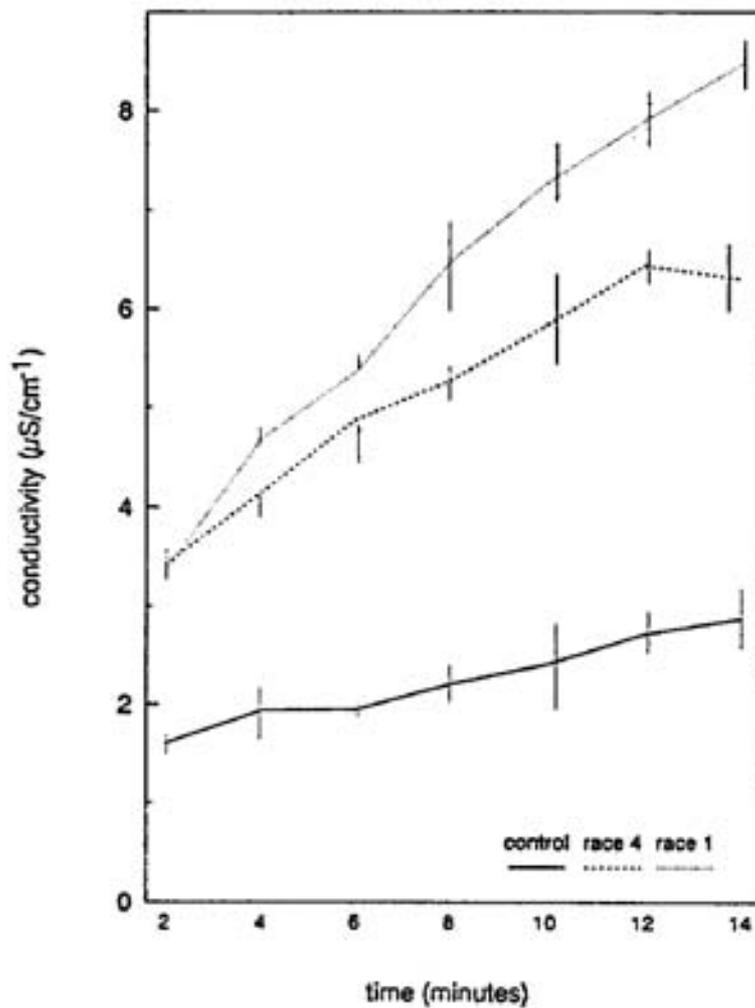


Fig. 3. Effect of 21 day old *Fusarium oxysporum* f. sp. *cubense* culture filtrate of race 4 and 1 on ion leakage leaf discs of mature plant of a diploid banana clone resistant 'SH-3362'. Each point represents the average of 10 replicates and two independent experiments. Bars show the standard deviation of the average.

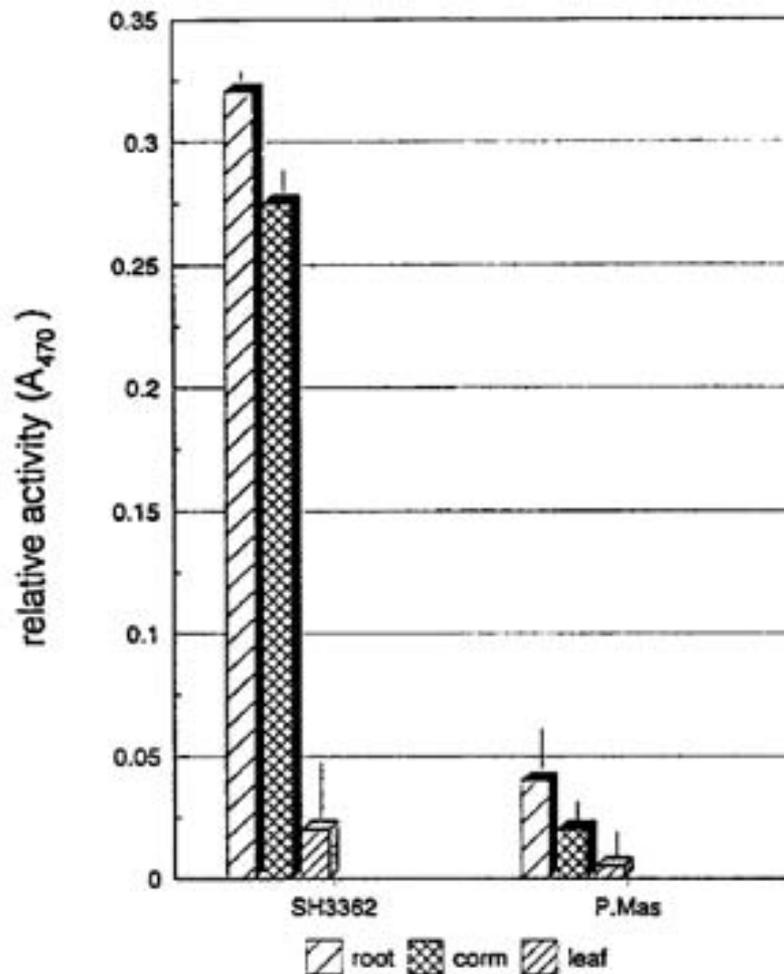


Fig. 4. Constitutive peroxidase relative activity in root, corm and leaf tissues of non-inoculated resistant banana clone 'SH-3362' and susceptible cultivar 'Pisang Mas' to *Fusarium oxysporum* f. sp. *cubense*. Relative activity was described in terms of the change of absorbance per min per 5  $\mu$ l of extract. Each point represents the average of two independent experiments. Bars show the standard deviation of the average.

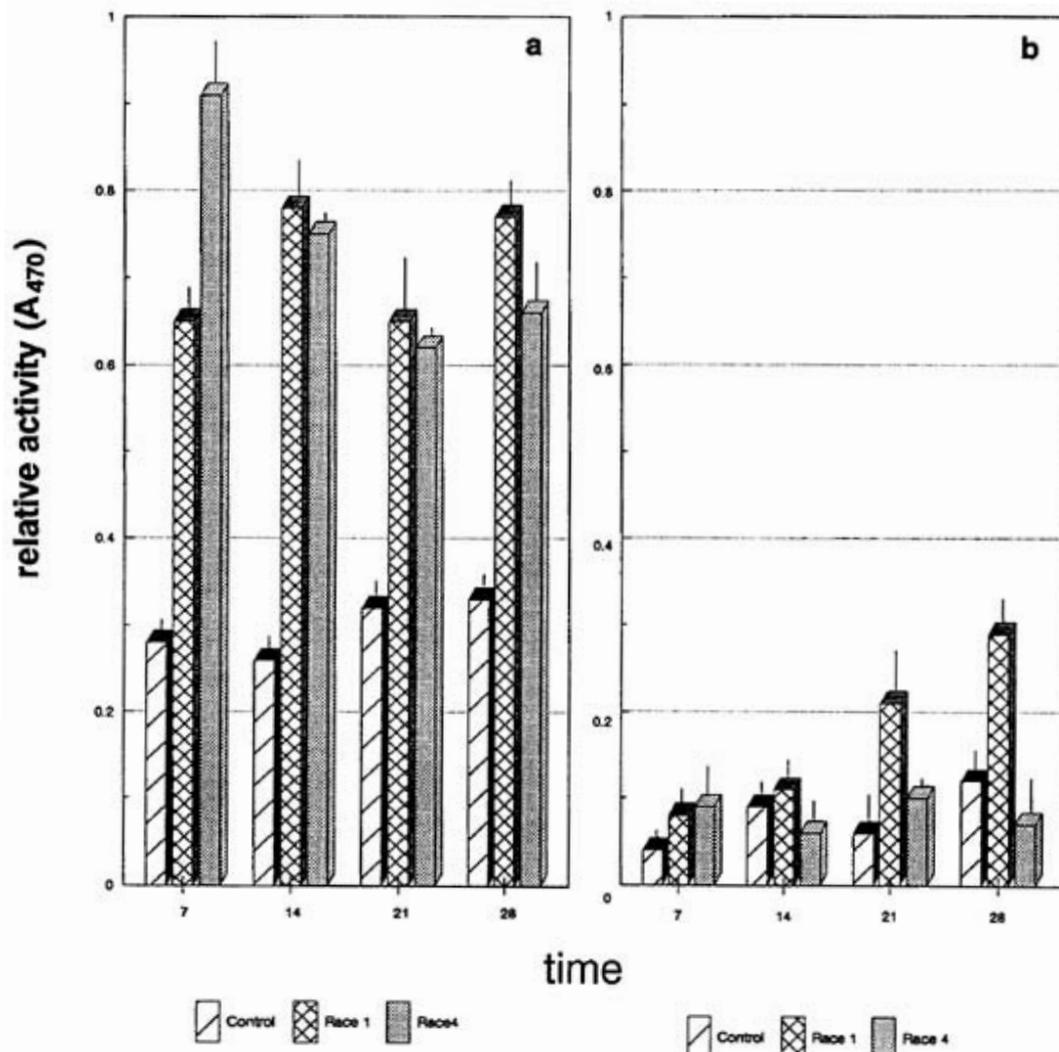


Fig. 5. Time course of peroxidase relative activity in corn tissue extract of diploid resistant banana clones 'SH-3362' (a) and susceptible 'Pisang Mas' (b), non-inoculated and inoculated with a conidial suspension ( $5 \times 10^6/ml$ ) of *Fusarium oxysporum* f. sp. *cubense* race 1 and 4. Relative activity was described in terms of the change of absorbance per min per  $5 \mu l$  of extract. Each point represents the average of 3 replicates and two independent experiments. Bars show the standard deviation of the average.



## Chapter 8

### Differential *chitinase* activity in banana cultivars as a response to *Fusarium oxysporum* f. sp. *cubense* infection

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#### Abstract

Six banana clones with varying levels of resistance were inoculated with conidial suspension of races 1 and 4 of *Fusarium oxysporum* f. sp. *cubense* (FOC). Chitinase activity in the corm and root tissues was monitored before and after infection to relate with the field resistance or susceptibility of banana cultivars. Resistant clones showed high constitutive chitinase activity in roots and a rapid response to infection. The results suggest that chitinase could be considered as part of a complex mechanism leading to disease resistance.

#### INTRODUCTION

Bananas (*Musa* spp.) are important crops, providing carbohydrate-rich food to large populations in the tropics and subtropics. In South and Central America, bananas are also important export crops. Protection against fungal diseases represents a major challenge to banana production. In many areas of the world, losses from diseases reduce income and pose a threat to the economic survival of small-holding farmers. At present, disease control is based mainly on three strategies: application of pesticides, breeding for disease resistance and improvement of agronomic practices. However, chemical control of *Fusarium* wilt is not yet available. Banana breeding is time consuming, expensive, and constrained by the sterility of most cultivated bananas. Thus, a better understanding of the resistance mechanisms is required to develop a reliable early screening method.

Like many other species, bananas employ a diverse array of defence mechanisms to resist pathogen attack. For example, callose deposition and phenolic compounds have been associated with resistance to *Fusarium oxysporum* f. sp. *cubense* (FOC) (Wardlaw, 1930; Beckman, 1990), but little is known about the physiology of the defence response.

Chitinases hydrolyse chitin, a homopolymer of 6-1-4 linked N-acetyl-D-glucosamine, which is a major component of the cell wall of most fungi. Chitinases have been isolated and purified from bacteria, fungi and plants, and are able to inhibit fungal growth by degrading the newly synthesised chitin at the hyphal tips. Chitinases are synthesised in low amount in many plants species and crops. Most evidence for the role of chitinases in the plant defence response is based on data from *in vitro* systems or from complementation tests involving plant transformation and scoring of resistance. Purified enzymes have been shown to hydrolyse isolated fungal cell walls and inhibit the growth of pathogen in culture.

The present investigations were undertaken to study the chitinase response of bananas clones with different ploidy levels and resistance to *F. oxysporum*. Previous studies showed that in several banana clones, chitinase was expressed constitutively. In this paper, we report evidence on the induction of chitinase following infection of banana plants with FOC conidial culture.

## MATERIAL AND METHODS

### Plants, pathogen and inoculation

FOC race 1 strain 2264 (VCG 0125) and race 4 strain Yo 604 (VCG 01290) were kindly provided by Dr. K. Pegg, Queensland Department of Primary Industries (QDPI), Indooroopilly, Australia. The strains were cultivated on Czapek-Dox medium (Difco, Becton Dickinson, Sparks, MD, USA) for one week. Actively growing hyphae were removed, transferred to Potato Dextrose Agar (PDA), and incubated at 28°C under constant light to promote conidia formation. Conidial suspensions in distilled water were collected by scratching the mycelial surface.

The following banana clones which differ in genome, ploidy, and susceptibility to FOC race 1 and 4 were investigated: 'SH-3362' (AA) resistant to race 1 and 4; 'Grand Naine' (AAA), 'Pisang Mas' (AA), 'SH-3142' (AA), 'Dwarf Parfitt' (AAA), susceptible to race 4 and resistant to race 1, 'Highgate' (AAA) susceptible to both races. Plants were multiplied *in vitro* and rooted as described elsewhere (Novák *et al.*, 1989). After one month, rooted plantlets were obtained and agar was washed off with tap water, the roots were trimmed and the plants were inoculated by dipping the roots in spore suspensions containing  $10^5$  conidia/ml for 10 minutes under air flow to facilitate the mechanical uptake of conidia by root vessels. The controls were treated in the same manner but inoculated only with distilled water. Immediately after inoculation, the plants were transferred to pots in a greenhouse and samples were taken at intervals for chitinase activity.

### Chitinase analysis

Plantlets were sampled at random and the corm tissue collected under cold water by trimming the roots and the pseudostem. The tissue was weighed and ground with a mortar and pestle in liquid nitrogen. Phosphate buffer pH6.8 was added to the resulting powder, and after homogenisation incubated for 30 minutes at 4°C. The homogenate was collected with a micropipette, centrifuged for 15 minutes at 14000 rpm at 4°C, and the supernatant was assayed immediately for protein content according to the method of Bradford (Bradford, 1976). Extracts with equal amount of protein were incubated in 100 ml of glycol chitin, and variable amounts of distilled water were added to bring the incubation mixture to 200 ml and incubated overnight at 40°C. The final reaction mixture was brought to 1 ml and read at 510 nm using a spectrophotometer, and expressed as unit protein content.

### Isoelectrofocusing (IEF)

Equal amounts of protein were applied on a 4.6% polyacrylamide gel containing 5% Pharmalyte as a carrier ampholyte. Running conditions were as follows: 8 W fixed, 12-33 mA, 2500 V. After running, the gel was washed, and the bands were differentiated using an overlay gel (7.5%) with 1.1 ml of 3 M NaAc buffer pH5 and 1.3 ml glycol chitin in a final volume of 33 ml.

## RESULTS AND DISCUSSION

No wilting symptoms were observed in the inoculated material up to 28 days of culture. However, internal symptoms, i.e., tissue discolouration, were observed on dissection of the susceptible clones inoculated with both FOC races. The clones were analysed for constitutive chitinase activity before fungal inoculation. No differences were observed between susceptible and resistant clones in constitutive chitinase.

After inoculation with a conidial suspension of FOC race 1, all clones except 'Highgate' showed increased chitinase activity but with a different time course (Figure 8.1). The clone 'Dwarf Parfitt' showed a rapid and sustained increase in enzymatic activity during the first two weeks, while in 'Highgate' chitinase did not increase. The clone 'Pisang Mas' exhibited a slow increase in enzymatic

activity with time. When the same banana clones were incubated with FOC race 4, clone 'SH-3142' showed a higher increase in chitinase activity (Figure 8.2). An increase in chitinase activity was particularly evident in 'Pisang Mas', with a peak activity after five weeks. Clone 'SH-3142' showed a different pattern of enzymatic activity, with a burst induction after 48 hours, followed by another peak 5 weeks after infection (Figures 8.3 and 8.4).

The resistant clone 'SH3142', infected with race 1 and 4 showed root chitinase activity comparable to corm tissue (Figures 8.5 and 8.6). However, in plants inoculated with race 4, there was a three- to four-fold increase when compared with plants inoculated with race 1. Moreover, root chitinase activity was already evident 24 hours after infection, while in corm tissue the activity increase was evident after 48 hours (Figures 8.7 and 8.8). In 'Pisang Mas', the enzymatic activity increased both after infection with race 1 and 4 but in a transient manner, i.e., after 48 hours when infected with race 1 and after 5 weeks when inoculated with race 4; however, the increase was not statistically significant. As far as the response to race 1 is concerned, a four-fold increase over the control was observed but only at a single point. In the highly susceptible clone 'Highgate', the chitinase activity did not increase at all. Clone 'SH-3142' showed a more prompt response than the other clones when inoculated with race 4.

At least 12 isochitinases have been recorded in diploid bananas (Morpurgo *et al.*, 1994). To determine which isozymes contribute to the rise in total activity, IEF was used to separate the isochitinases extracted from the banana clones. At least four bands, two cationic and two anionic, concomitantly increased in the incompatible reaction between race 4 and 'SH-3362'. However, this increase was not observed in the incompatible reaction of the same clone with race 1. The activity of most of the other isozymes remained unchanged with time (data not shown).

The current model on a chitinase-based defence mechanism considers that the enzyme acts as an anti-fungal compound as well as a producer of chitin fragments, which act as elicitors or messengers to activate other metabolic compounds for defence against the pathogen. The results presented in this paper confirm that the resistant clones of banana respond actively to infection (Morpurgo *et al.*, 1994) with a dramatic increase in the chitinase activity, but this response is more tissue-specific than the previously studied peroxidase. However, total chitinase activity by itself should not be considered an absolute parameter. The rate of production appears to be more important, i.e., the activation response of the host to the infection. The speed and magnitude of the activation of the mechanism appear to be critical for expression of resistance.

Resistance of plants to invasion by potential pathogens is the result of a multiple defence reaction comprising both constitutive and inducible mechanisms. The use of biochemical markers to select for resistance may expedite breeding programmes by reducing the number of field trials. The main criteria for practical use of such markers are reliability in predicting resistance and the ease of handling the assay. This would have a comparative advantage over traditional field screening. For any marker, a number of prerequisites must be fulfilled. Firstly, it should positively correlate with the level of resistance or susceptibility of the breeding population. Secondly, it should be able to distinguish between F<sub>2</sub> segregants or between individuals among a mutagenised population. Thirdly, it should be applicable to a large breeding population.

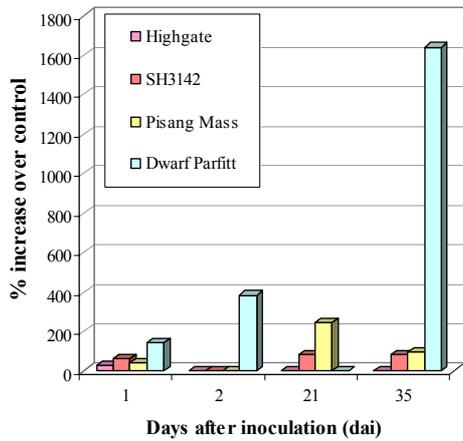
The results of this study showed that the plant reaction to pathogens can be estimated by the inducible chitinase activity during the time course of active chitinase production after infection. However, to follow the enzymatic activity over a long time involves intensive work and careful control of environmental conditions. Thus, predicting plant response on the basis of inducible chitinase becomes a long procedure.

## CONCLUSIONS

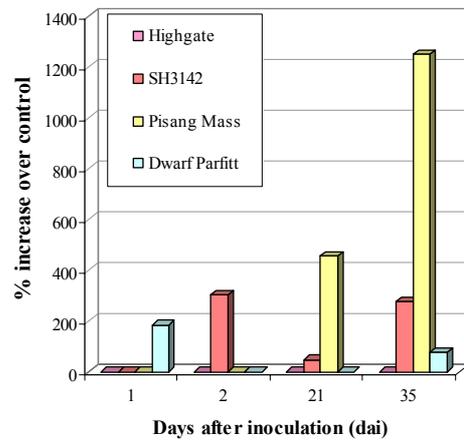
Based on the present study, it was possible to divide the experimental population into two broad classes of resistant and susceptible sub-populations. On the basis of chitinase activity in root tissues, it would be possible to identify suitable parents for breeding for resistance to *Fusarium*. The differential response based on chitinase activity confirms our previous results (Morpurgo *et al.*, 1994) obtained with peroxidases, and suggests that such an approach could be useful in screening for variation among the cultivars, wild species and sub-species to identify sources of resistance.

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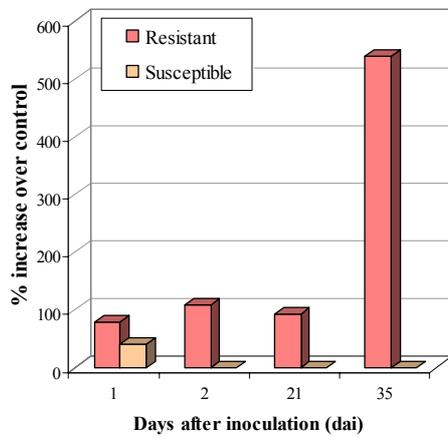
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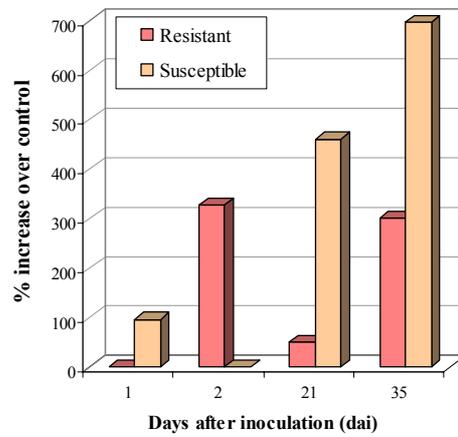
**Figure 8.1.** Differential response of banana clones to inoculation with *Fusarium oxysporum* f. sp. *cubense* race 1 measured as chitinase activity (A470) increase over the control. Relative activity is described in terms of the change of absorbance per minute per 5  $\mu$ l of extract. Each point represents the average of three replicates and two independent experiments.



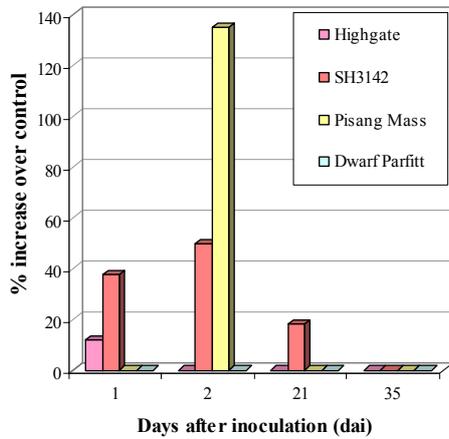
**Figure 8.2.** Differential response of banana clones to inoculation with *Fusarium oxysporum* f. sp. *cubense* race 4 measured as chitinase activity (A470) increase over the control. Relative activity is described in terms of the change of absorbance per minute per 5  $\mu$ l of extract. Each point represents the average of three replicates and two independent experiments.



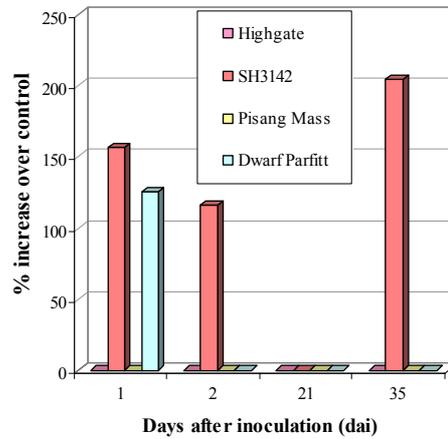
**Figure 8.3.** Chitinase activity in corm tissue of susceptible and resistant bananas after infection with FOC race 1.



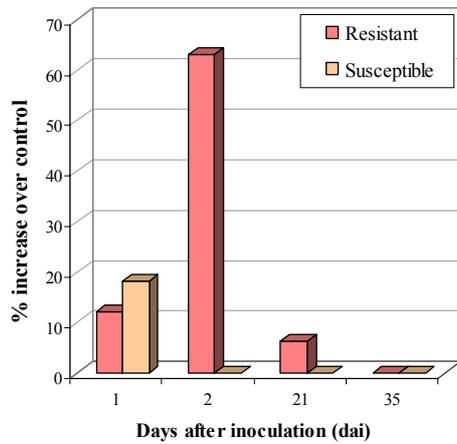
**Figure 8.4.** Chitinase activity in corm tissue of susceptible and resistant bananas after infection with FOC race 4.



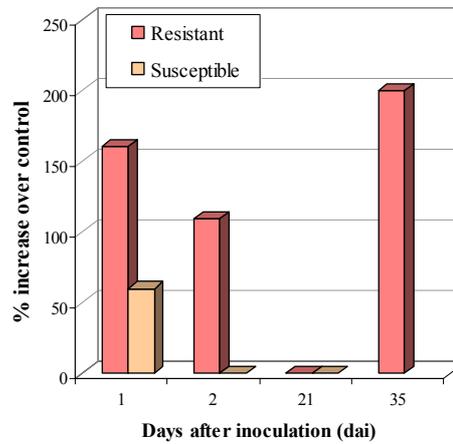
**Figure 8.5.** Chitinase activity in root tissue of 'Highgate', 'SH-3142', 'Pisang Mas' and 'Dwarf Parfitt' bananas after infection with FOC race 1.



**Figure 8.6.** Chitinase activity in root tissue of 'Highgate', 'SH-3142', 'Pisang Mas' and 'Dwarf Parfitt' bananas after infection with FOC race 4.



**Figure 8.7.** Chitinase activity in root tissue of susceptible and resistant bananas after infection with FOC race 1.



**Figure 8.8.** Chitinase activity in root tissue of susceptible and resistant bananas after infection with FOC race 4.

## Chapter 9

### Mass-screening of mutants resistant to *Alternaria* blotch from *in vitro*-cultured apple shoots irradiated with X-rays

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#### Abstract

We have established mass screening methods for producing mutants resistant to *Alternaria* blotch disease in several cultivars of apple (*Malus domestica*) by irradiation with X-rays. An *in vitro* assay system using chemically-synthesised AM-toxin I of *Alternaria alternata* (Fr.) Keissler is successful for screening the resistant mutants. The degree of resistance to the disease was assayed by the number of necrotic lesions induced by applying various concentrations of AM-toxin I to leaf disks of the first, third and fifth leaves from the shoot apex of plants. Following the establishment of the optimal screening conditions, we produced several disease-resistant mutants by irradiating *in vitro* shoots with various doses of X-rays. After resistant mutants were selected by the first and second screening tests with AM-toxin I, they were tested by spraying them with a spore suspension of the pathogen. The resistance to the disease was maintained for five years during field cultivation of the mutants, implying that the resistance was not due to environmental factors but due to genetic fixation. No differences were observed in the number of fruiting trees or colouration of fruits between mutants and the original plants.

#### INTRODUCTION

Inducing mutation by exposure to radiation is one of the most significant tools in breeding, especially for vegetatively propagated crops and perennial orchard trees. When breeding of a tree is performed via crossing, a long time is needed to eliminate unnecessary traits by back-crossing. Compared with crossing, only a few traits are usually affected by breeding through mutation induction. Many cultivars of orchard trees have been produced by natural mutation. Most of these are derived from mutants of bud mutation (Shamel, 1943; Soost and Cameron, 1975). Since Stadler (1930) utilised X-rays and radium irradiation for inducing artificial mutations, irradiation of seeds or dormant scions has been commonly attempted for producing useful mutants. When the genetic background of the traits is clear, the efficiency of mutation breeding by irradiation is enhanced. Traits controlled by polygenes are not easily altered, but traits controlled by a single gene are good candidates for genetic improvement. In orchard trees, disease resistance against scab and rust in apples is known to be controlled by a single dominant gene; whilst, canker in peach, black spot in pears and *Alternaria* blotch in apples are each controlled by a single recessive gene.

As irradiation with gamma- or X-rays usually induces recessive mutations, mutation breeding with irradiation is effective for diseases controlled by recessive genes. One such example is the breeding of the Japanese pear (*Pyrus pyrifolia* Nakai) cv. 'Nijisseiki' resistant to black spot disease, which was first carried out by chronic irradiation with gamma-rays (Sanada *et al.*, 1988). Black spot disease is caused by the Japanese pear pathotype of *A. alternata* (Fr.) Keissler (Kozaki, 1973), and homozygous

recessive genes result in resistance to the disease. Masuda *et al.* (1997) also produced mutants of the Japanese pear cultivar 'Osanijisseiki' that were resistant to this disease.

In apples, attempts were also made to select mutants resistant to *Alternaria* blotch in the susceptible cultivar 'Indo' (Tabira *et al.*, 1993a; Sanada *et al.*, 1994; Masuda and Yoshioka, 1996). *Alternaria* blotch is a very serious disease of apple in Japan caused by the apple pathotype of *A. alternata* (Fr.) Keissler (previously described as *A. mali* Roberts). Upon infection by this fungus, necrotic lesions appear on leaves followed by defoliation as the disease progresses, resulting in a decreased fruit yield. The disease can usually be controlled by spraying the apple trees with fungicides many times during the growing season. However, controlling the disease is difficult if the primary leaf infections are already abundant by the time of the first spraying. Furthermore, spraying many times is laborious, represents a health hazard to farmers and is detrimental to the environment. Saito and Takeda (1984) found that susceptibility to the disease is controlled by a single dominant gene; susceptible cultivars are heterozygous and resistant cultivars are homozygous recessive. This mode of inheritance is similar to that of black spot disease resistance in Japanese pear.

It is known that host-specific toxins, which are produced by the pathogen at the time of spore formation, cause damage to the plant cytoplasm and chloroplasts (Park *et al.*, 1981). These toxins can be purified (Ueno *et al.*, 1975) and classified into three groups, namely AM-toxin I, II and III. In order to evaluate the degree of plant susceptibility to *Alternaria* blotch, filtrates of fungal cultures or crude toxins partially purified from filtrates have been previously used. However, these are sometimes troublesome since the titre of the toxins or filtrates may vary among cultures. In addition, intermediate types of resistance are not easily distinguished when using a culture filtrate or crude toxin (Tabira *et al.*, 1993a). Thus, to eliminate the variability of filtrates, we decided to use a chemically-synthesised toxin (AM-toxin I).

The classification of cultivars according to degree of susceptibility to a pathogenic toxin is directly correlated to the actual degree of susceptibility to the live pathogen in plants grown outside (Tabira *et al.*, 1993a; Masuda, 1995). Therefore, we usually use *in vitro* shoots as testing material, because these can be prepared throughout the year and are easy to handle as compared with shoots grown outside. When the screening is first carried out with *in vitro*-grown shoots, the correlation between susceptible and intermediate resistant cultivars may not be as clearly observed as on *in vivo* material. It is, therefore, important to establish an assay method for determining the degree of susceptibility when using *in vitro*-grown shoots for the selection of resistant mutants.

Until recently, acute or chronic gamma-ray irradiation was generally used for inducing mutation as was done for Japanese pear. However, in our experience, gamma-ray irradiation may not always be the best choice. For instance, gamma-irradiation requires facilities that may not be available; in Japan, for example, gamma-ray irradiation facilities are only available at the Institute of Radiation Breeding in the National Institute for Agrobiological Resources (NIAR) under the Ministry of Agriculture, Forestry and Fisheries (MAFF). Moreover, in acute gamma-ray irradiation, in addition to the desired mutations, the frequency of unfavourable mutations may be much higher than that of chronic gamma-ray irradiation. On the other hand, for the induction of mutations, chronic gamma-ray irradiation requires a longer period of time than acute gamma-ray irradiation. Moreover, in the case of a gamma-field such as the one used in Japan, in order to obtain mutants exhibiting useful traits, it is important to produce mutants on a mass scale, which requires a large area for selection of the mutants with desirable traits after irradiation. Therefore, we chose a more convenient system, and used X-ray irradiation as an alternative tool to gamma-ray irradiation. X-ray facilities are small and the irradiation can be performed in small laboratories and can be carried out on a smaller scale.

As for the irradiation doses, the sensitivity to irradiation varies between different kinds of fruit trees. Generally, persimmons and chestnuts show high sensitivity, whereas citrus fruits and loquats show high resistance (Shamel and Pomeroy, 1936). Some traits, such as skin colour and fruit size, tend to be affected more easily by irradiation, whereas fruit shape and ripening time are more recalcitrant to

mutation induction (Shamel and Pomeroy, 1936). In apples, the sensitivity also differs between cultivars; therefore, we first had to establish the optimal dose and conditions for irradiation for each cultivar. As a comparison to X-ray irradiation, we also performed gamma-ray irradiation of shoots from several cultivars. In parallel, we established an optimal screening method for *Alternaria* blotch disease (Saito *et al.*, 2001). After optimisation of both irradiation conditions and screening protocol, we produced apple mutants resistant to *Alternaria* blotch disease from several susceptible cultivars through a highly efficient regeneration procedure for apple shoots (Saito and Suzuki, 1999).

## **MATERIAL AND METHODS**

### **Irradiation of shoots with various doses**

*In vitro*-grown apical shoots were cut into 5 mm pieces and sub-cultured at intervals on shoot proliferation medium (Murashige and Skoog [MS] medium containing 1.0 mg/l 6-benzylaminopurine [BAP], 30 g/l sucrose and 0.8% agar, pH5.8) (Murashige and Skoog, 1962). 10 shoots were inoculated onto a fresh proliferation medium 30 days after subculture. These shoots were irradiated with various doses of X-ray in a soft X-ray irradiation unit (Soft X-ray unit, OHMICRON OM-100RAL, Ohmic Ltd., Tokyo, Japan). Shoots of cv. 'Hokuto' were irradiated with doses of 5, 6, 8, 10, 12, 15 and 20 KR at a dose rate of 0.01, 0.1, 0.5 and 1 KR/min, whereas, shoots of cv. 'Aori 10' were irradiated with the same dose at 0.01, 0.1 and 1 KR/min.

Concerning gamma-ray irradiation, irradiation with doses of 60, 80 or 120 Gy at a dose rate of 5 Gy/hour was carried out in the gamma-room of the Institute of Radiation Breeding, NIAR, MAFF. After irradiation, Petri dishes containing shoots were cultured at 25°C with a 16:8 hour light:dark photoperiod. The numbers of surviving shoots and morphological properties were investigated 30 days after irradiation.

### **Method of assaying susceptibility to pathogenic toxins on leaf disks and apical shoots with AM-toxin**

The degree of susceptibility to AM-toxin was evaluated for several apple cultivars including resistant, intermediately resistant and susceptible cultivars. 10 leaves of the first, third and fifth leaves from the top shoot were collected, respectively. Leaves were washed first with detergent and then with tap water. After drying off the water, disks (diameter: 8 mm) were cut out with a cork-borer from the central parts of the leaf.

Chemically-synthesised AM-toxin I (Hashimoto *et al.*, 1996) was dissolved in 1 ml dimethylsulfoxide (DMSO), suspended in sterile water and stocked at 100 µM. This solution was sequentially diluted to 10, 1 and 0.1 µM, and sterilised by filtration through a 0.45 µM membrane filter. The toxin solution was diluted and 0.5 ml was dispensed into 24 hole-titre plates. Leaf disks were placed into each solution and immersed for 48 hours in darkness at 28°C. Thereafter, the degree of necrotic lesions was evaluated.

### **The first screening test of susceptibility in irradiated shoots**

After irradiation, shoots of the surviving plants were allowed to elongate, and each shoot was divided into 10 segments and transferred onto a proliferation medium. Multiple shoots were produced one month after culture. These shoots were each separated into 10 pieces, and sub-cultured onto a fresh medium. This procedure was repeated 3 times to propagate shoots and eliminate chimeras. Finally, 10 shoots (~2 cm long) were cut off and transferred onto a rooting medium (MS medium containing 1.0 mg/l 3-indolebutyric acid [IBA], 30 g/l sucrose and 0.8% agar, pH5.8).

Rooted plantlets were produced after ~1 month, and were then acclimatised and transferred to pots containing soil (Prime Mix TKS-2, Sakata Seed Ltd., Yokohama, Japan) after eliminating agar

attached to roots with tap water. Pots were covered with polystyrene bags to maintain humidity and plants were cultivated in the shade for two weeks. After new leaves developed, the polystyrene bags were removed to acclimatise plants to the open air; the plants were cultivated in a sunny place for ~1 month.

Thereafter, these plants (~15 cm) were screened with toxin to evaluate the degree of susceptibility. The third leaf of each shoot was cut into 8 mm diameter disks with a cork borer. Two disks from each leaf were immersed with the toxin solution and incubated at 28°C in darkness for two days, and the occurrence of necrotic lesions observed.

### **The second and third screening tests in selected resistant mutants**

Resistant mutants selected by the first screening test were grown in a greenhouse and the third leaves from the tops of shoots of elongated plants (~30 to 40 cm) were collected. Leaf disks (diameter: 8 mm) from the central region of these leaves were cut out with a cork borer. To re-evaluate the degree of resistance, the leaf disks were immersed in the high concentration (100 µM) of toxin solution used for the titre plates, and incubated for two days at 28°C in darkness. Highly resistant plants showing no necrosis were selected.

After selection by the second screening test, the third screening by spores of the pathogen was carried out. Pathogens (*A. alternata* strain FIV-C-45) were cultured on solid potato dextrose agar medium (Difco, MD USA) for 5-7 days. Proliferated pathogens were collected with a brush and suspended in small amounts of distilled water. The suspension was then filtered through gauze to eliminate cell debris and agar. The suspension containing spores of the pathogen was adjusted to  $3.3 \times 10^4$ /ml with distilled water. Spore suspension was then sprayed onto the leaves of selected mutant plants. Leaves inoculated with pathogen were kept for two days at 25°C in the inoculation box, in which humidity was maintained at 90-95%. Thereafter, plants were transferred to a greenhouse, and disease symptoms and number of necrotic lesions were investigated for the upper 10 leaves from the top shoots seven days after inoculation. The procedure of producing mutants resistant to *Alternaria* blotch is illustrated in Figure 9.1.

### **Establishment of an assay method for evaluating the degree of susceptibility**

The degree of susceptibility to AM-toxin I was different among cultivars (Figure 9.2A). A resistant cultivar ('Jonathan'), an intermediate cultivar ('Fuji'), a susceptible cultivar ('Oorin') and a highly susceptible cultivar ('Indo') were used as standard material for evaluating the degree of susceptibility (Tables 9.1 and 9.2). Samples were classified into four groups by the degree of necrosis as follows: grade 0 = no necrotic lesions; grade 1 = necrotic lesions on the leaf disk periphery; grade 2 = necrotic lesions on <50% of the leaf disk; grade 3 = necrotic lesions on 50-75% of the leaf disk; grade 4 = necrotic lesions on 75-100% of the leaf disk. Thereafter, the average grades were calculated based on the degree of necrotic lesions on each leaf. The results of the screening with AM-toxin were mostly consistent with those obtained using the culture filtrate (Saito *et al.*, 1989) and the grade of necrotic lesions of leaf disks properly corresponded to the degree of susceptibility as previously ascertained from field tests, when applying toxin concentrations of 0.1-1 µM. The degree of susceptibility for the resistant cultivar ('Jonathan') was from grade 0 to 0.5, for the intermediate cultivar ('Fuji') from grade 0.5 to 3.5, and for the susceptible cultivars ('Oorin' and 'Indo') from grades 2.0 to 4.0. Thus, the degree of susceptibility to *Alternaria* blotch was successfully evaluated *in vitro* by measuring necrotic lesions of leaf disks exposed to chemically-synthesised toxin.

For the screening test, another important factor was the age of the leaves. Young leaves were more susceptible to toxin or fungal filtrate than older leaves (Figure 9.2B) (Sawamura and Yanase, 1963; Saito and Takeda, 1984). The first leaves from the top of the shoot were too susceptible to the toxin,

whereas the upper 3rd and 5th leaves from the top of the shoots were appropriate Material for the evaluation of the degree of susceptibility. Therefore, the upper 3rd leaves were usually used for the evaluation with toxin at the concentrations of 1 and 10  $\mu\text{M}$ .

### **Survival of irradiated shoots with various dose rates of irradiation**

Cultivars 'Hokuto' and 'Aori 10' were mainly used as material for production of resistant mutants. They varied in their percentage survival for various dose rates of irradiation (Figure 9.3). Up to 12 KR, no plant died at a dose rate of 0.01 KR/min irradiation. However, the survival rate decreased gradually as the dose rate increased above 0.1 KR/min. In cv. 'Hokuto', no plant died at a dose rate of 0.01 KR/min and at doses of up to 12 KR, whereas the survival rate declined to 50% at a radiation dose of 13 KR with a dose rate of 0.1 KR/min (Figure 9.3A). In cv. 'Aori 10', no plant died at the dose rate of 0.01 KR/min, whereas only 50% survived a radiation dose of 6 KR with a dose rate of 0.1 KR/min (Figure 9.3B). This means that the extent of irradiation damage varied between the two cultivars. The survival rate was always higher in cv. 'Hokuto' than in cv. 'Aori 10' (Figure 9.3C). Under conditions of high irradiation doses, many of the surviving plants showed rosette-type shoots or abnormal leaf morphology (Figure 9.4D). Survival rate increased when chronic radiation was given at low dose rates compared with those at high dose rates for the same radiation dose. The survival rate also decreased with an increase in total dose.

### **Efficient radiation dose and dose rate for production of mutants**

Leaf disks of cultivars 'Hokuto' and 'Aori 10' were screened with the toxin and the corresponding resistant shoots were selected. Subsequently, shoots were elongated and rooted, resulting in the formation of plantlets. In cv. 'Hokuto', non-irradiated plants showed severe necrotic lesions (>grade 3) at a toxin concentration of 10  $\mu\text{M}$  (Table 9.3). At radiation doses < 4 KR, almost all of the irradiated plants showed the same number of necrotic lesions as non-irradiated plants. A radiation dose of 4 KR was, thus, insufficient for the production of resistant mutants. The number of necrotic lesions decreased on several plants irradiated with a radiation dose of 6 KR and some of them showed no necrotic lesions, the same as for the resistant cultivar 'Jonathan', which was graded as 0. This implies that irradiation with a 6 KR dose was efficient for mutant production.

Thus, the first screening of resistant mutants was carried out in plants irradiated with radiation doses between 6 and 12 KR. During the first screening, plants whose leaf disks remained green in solution containing 10  $\mu\text{M}$  of the toxin (Figure 9.5A) were selected for the second screening test. Leaf disks were prepared from the third leaves from the top of the shoots of juvenile plants selected in the first screening. These leaf disks were screened by immersion in a solution containing 100  $\mu\text{M}$  toxin (Figure 9.5B). Highly resistant mutants, showing grades 0 or 1, were obtained from plants irradiated with 8 and 10 KR. These plants were more resistant than the resistant cultivar, 'Jonathan'.

Besides the susceptible cultivars 'Hokuto' and 'Aori 10', other susceptible cultivars, namely 'Fuji' and 'Oorin', were also used as Material for the production of resistant mutants (Table 9.4). In the first screening of cv. 'Fuji', the frequency of occurrence of resistant mutants did not differ greatly in irradiated plants at radiation doses between 8 and 10 KR. However, more resistant mutants were obtained in plants irradiated with a 0.1 KR/min dose rate than with a 0.01 KR/min dose rate. Resistant mutants were also obtained in cv. 'Oorin' irradiated with 8 and 10 KR, whereas only a few resistant plants were obtained with a radiation dose of 6 KR. In the gamma-ray irradiated plants, resistant plants were also obtained from cultivars 'Hokuto' and 'Oorin' in the first screening with irradiation doses of 60, 80 and 120 Gy (Table 9.5). No resistant mutants were obtained for cv. 'Aori 10' at any irradiation dose of either X- or gamma-rays.

## Evaluation of phenotypes of resistant mutants

In the second screening, resistant mutants showing the highly resistant grades of 0 and 1 appeared in cv. 'Fuji' plants irradiated with 6, 8 and 10 KR at a dose rate of 0.1 KR/min, whereas no resistant mutants were obtained at 0.01 KR/min. As for cv. 'Oorin', the same tendency was observed as for cv. 'Fuji', although more resistant mutants were obtained compared to cv. 'Fuji'. After the second screening selection, the frequency of disease symptoms was tested in resistant mutants by inoculation with a spore suspension of the pathogenic fungus (Table 9.6). Many of these mutants were more resistant than the original plants (Figure 9.6), showing a degree of resistance the same as that of the resistant cultivar, 'Jonagold' or 'Jonathan', and the highly resistant cultivar 'Sansa', which showed evaluation grades of 0. Four resistant mutants were obtained in cv. 'Hokuto' by X-ray irradiation (dose rate 0.1 KR/min, radiation dose 10 KR); 13 and 20 resistant mutants were obtained by X- and gamma-ray irradiation, respectively, in cv. 'Fuji'; and 12 resistant mutants were obtained by X-ray irradiation in cv. 'Oorin'. Additionally, several intermediate resistant mutants were obtained from cv. 'Aori 10' (data not shown).

In resistant mutants of cv. 'Hokuto', the frequency of the occurrence of *Alternaria* blotch was investigated for five years to test the variability of disease resistance. Five scions of each of the four mutants and original plants were grafted onto rootstocks of the dwarf cultivar 'EM 26' and cultivated in the farm. The disease occurrence was greatly repressed in these mutants compared with the original plant over five years (Table 9.7). No morphological abnormality has so far been observed in the mutants (Figure 9.7). Moreover, there was no difference in the number of fruiting trees and colouration of fruits between these mutants and the original plants. As for diseases including 'stem end cracking', 'fruit splitting' and 'core rot', the occurrence was less in the mutants than in the original plants (Table 9.7). The observation that there was no alteration in fruit properties except for disease resistance coupled to the stability of the disease resistance opens the possibility that these mutants could be candidates for new cultivars. This study has demonstrated the usefulness of X-ray irradiation, besides gamma-ray irradiation, in breeding of orchard trees.

## Calculating the severity of symptoms

The severity of symptoms was calculated based on the conversion formula below:

$$\text{Severity of symptoms} = \frac{\sum(G \times n)}{N} \times 6 \times 100$$

Where: G = Disease grade is calculated as follows:

Disease Grade (G)	Number of disease spots per leaf						defoliation
	0	1-5	6-10	11-30	31-50	>51	
	0	1	2	3	4	5	6

n = Number of leaves corresponding to the disease grade (G)

N = Number of leaves used in tests (10 leaves per plant)

## RESULTS AND DISCUSSION

In Japanese pears, mutants resistant to Black spot disease have been obtained for cultivars 'Nijisseiki' (Sanada, 1986; Kotobuki *et al.*, 1992) and 'Osanijisseiki' (Masuda *et al.*, 1997) by chronic gamma-ray irradiation, and for cultivars 'Shinsui' and 'Osanijisseiki' (Murata *et al.*, 1994) by acute gamma-ray irradiation (dose rate 2.5 Gy/hour, total dose 80 Gy). Of 2168 and 2335 dormant shoots of 'Shinsui' and 'Osanijisseiki', respectively, one resistant mutant each was obtained by acute irradiation. One resistant mutant was also produced from 671 *in vitro*-grown shoots of 'Osanijisseiki' by acute gamma-ray irradiation (dose rate 5 Gy/hour, total dose 80 Gy) (Tabira *et al.*, 1993b). In apple, Tabira *et al.* (1993a) produced one resistant mutant from 453 *in vitro* shoots of cv. 'Indo' by acute gamma-ray

irradiation (dose rate 5 Gy/hour, total dose 120 Gy) and this resistant mutant showed 10000-fold higher resistance than did the original plants. Using almost the same procedure of acute gamma-ray irradiation at a dose rate of 5 Gy/hour (total dose 80 Gy), Masuda and Yoshioka (1996) selected one resistant mutant from 3602 *in vitro* shoots by screening with a pathogenic toxin. A mutant of 'Nijisseiki' resistant to black spot disease was registered as 'Gold Nijisseiki' and commercialised. Subsequently, a self-compatible and resistant cultivar called 'Gold Osanijisseiki' was developed and registered, for which the cultivated area in Tottori prefecture, the major district of its production, in 2005 was up to 999 ha and the yield was 23300 tones. This implies that half of the total production of 'Nijisseiki' was converted to cultivars derived from mutants.

In this study, we demonstrated that X-ray irradiation was effective in apple breeding as well as gamma-ray irradiation. We obtained mutants resistant to *Alternaria* blotch of the cultivars 'Hokuto', 'Fuji' and 'Oorin' through screenings with the toxin and spore suspension of pathogenic fungus. Five mutants of 1153 plants were obtained for cv. 'Hokuto' at a dose rate of 0.1 KR/min (Table 9.3), whereas for cv. 'Fuji', 27 resistant mutants were selected from 1340 plants by X-ray irradiation (Table 9.4). In the case of gamma-ray irradiation, 33 mutants were obtained from 1017 plants (Table 9.5), indicating that the frequency of mutation was almost of the same order between X- and gamma-ray irradiation. For 'Oorin', 39 resistant mutants were obtained from 530 plants by X-ray irradiation. Among them, highly resistant mutants appeared that had the same or higher resistance compared with the resistant cultivars 'Jonathan' and 'Jonagold'.

The frequency of occurrence of resistant mutants of cv. 'Oorin' was on the order of  $10^{-1}$  in the first screening, although the general frequency of mutation is  $10^{-3}$  to  $10^{-4}$ , demonstrating that the frequencies of this study are higher. Whereas, the frequency of resistant mutants in Japanese pears produced by acute gamma-ray irradiation is estimated at  $10^{-3}$ , which is almost the same as that of chronic gamma-ray irradiation (Sanada *et al.*, 1988), the frequency of mutation generation by acute gamma-ray irradiation is generally suggested to be higher than that by chronic irradiation (Sparrow *et al.*, 1961). However, irradiation at high dose rates tended to cause chromosomal rearrangements and chimeras, resulting in the occurrence of inferior traits. In apples, shoot mutations occurred so frequently that they could have been induced by an intrinsic mutator. A retrotransposon was reported to be an intrinsic mutator in plants (Hirochika, 1993; Kobayashi *et al.*, 2004). If irradiation induced the activation of a retrotransposon in apples, these retrotransposons might move into chromosomal breaks resulting in a mutation.

In the case of resistant mutants of Japanese pear, all the mutants that have been obtained until now are intermediate resistant types. A possible reason to explain these partially resistant mutants in Japanese pear is that the L-2 layer of the apical dome of shoots has been genetically changed by irradiation and became resistant, whereas the L-1 layer remained unchanged and susceptible. The resulting plants would, therefore, be chimeric having different genetic composition in the L-1 and L-2 cells. These chimeras could not be separated and were transmitted to progeny. In orchard trees, the operation called 'cutting back' has been carried out to eliminate chimeras and produce stable mutants; however, this is a long-term process. To avoid the formation of chimeras in combination with X- or gamma-ray irradiation, plant tissue culture techniques are utilised (Daub, 1986; Novak, 1991). In this study, we obtained no chimeric mutants by using *in vitro* shoot culture.

Tabira *et al.* (1998) compared resistant mutants of *Alternaria* blotch and the original apple cultivar by 2D protein gel electrophoresis. They found that among 1400 proteins, a specific spot of molecular mass 60 kDa (PI 5.5) was lost in resistant mutants. In their results, 9 susceptible cultivars, excluding 'Oorin', had this spot, whereas, it was not present in any of 12 resistant cultivars. This spot seems to be correlated with the susceptibility, and resistant mutants produced by irradiation were derived from the loss of this protein. The data presented in this paper showed that the gene responsible for susceptibility to *Alternaria* blotch might be lost by gamma-ray irradiation, which resulted in the production of resistant mutants. In our study, Random Amplification of Polymorphic DNA (RAPD) markers linked to susceptibility to *Alternaria* blotch were absent in resistant mutants of irradiated cv.

'Hokuto' although these markers were found in the original cv. 'Hokuto' (Fukasawa-Akada *et al.*, 1999). In the case that target traits are controlled by a single recessive gene, X-ray irradiation is demonstrated to be a useful tool in mutation breeding, owing to its property of gene destruction. However, if aimed traits are controlled by polygenes or a single dominant gene, another approach will be necessary.

## CONCLUSIONS

A simple mass screening technique was developed for the selection of disease resistance to *Alternaria* blotch in apples using a small X-ray irradiation unit and *in vitro*-cultured shoots. The frequency of mutation was nearly identical to that caused by gamma-ray irradiation. Mutants exhibited stable disease resistance to *Alternaria* blotch for five years and no abnormal phenotype was observed, demonstrating that this technique is effective for the production of induced mutants if the desired traits are controlled by recessive genes.

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**Table 9.1.** Effect of AM-toxin I on leaf disks of cultivars with various degrees of susceptibility to *Alternaria blotch*

Cultivar	Degree of susceptibility <sup>a</sup>	Leaf position	Concentration of toxin			
			100 $\mu$ M	10 $\mu$ M	1 $\mu$ M	0.1 $\mu$ M
'Indo'	Highly susceptible	1	4.0 $\pm$ 0.00*a	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a
		3	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	3.4 $\pm$ 0.48b
		5	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	3.2 $\pm$ 0.32b
'Oorin'	Susceptible	1	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00 a	3.6 $\pm$ 0.48a
		3	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	3.6 $\pm$ 0.48a	2.7 $\pm$ 0.42d
		5	3.8 $\pm$ 0.32a	3.8 $\pm$ 0.32a	3.2 $\pm$ 0.48b	2.0 $\pm$ 0.20e
'Fuji'	Intermediate	1	4.0 $\pm$ 0.00a	4.0 $\pm$ 0.00a	3.5 $\pm$ 0.50b	3.3 $\pm$ 0.42b
		3	4.0 $\pm$ 0.00a	3.4 $\pm$ 0.48b	2.6 $\pm$ 0.48d	1.2 $\pm$ 0.32g
		5	3.2 $\pm$ 0.32b	2.1 $\pm$ 0.18e	1.1 $\pm$ 0.36g	0.5 $\pm$ 0.50i
'Jonathan'	Resistant	1	4.0 $\pm$ 0.00a	3.2 $\pm$ 0.64b	0.5 $\pm$ 0.50i	0.0 $\pm$ 0.00j
		3	2.8 $\pm$ 0.48c	0.4 $\pm$ 0.48i	0.0 $\pm$ 0.00j	0.0 $\pm$ 0.00j
		5	2.1 $\pm$ 0.36e	0.2 $\pm$ 0.32i	0.0 $\pm$ 0.00j	0.0 $\pm$ 0.00j

<sup>a</sup>The grade of susceptibility is calculated based on the occurrence of necrotic lesions on leaf disks as follows: 0 = no necrotic lesion; 1 = a few necrotic lesions at the cut end of leaf disks; 2 = necrotic lesions on <50% of a leaf disk; 3 = necrotic lesions on 50-75% of a leaf disk; 4 = necrotic lesions on 75-100% of a leaf disk. Each grade was a mean of 10 leaves. Values are means  $\pm$  SE (n = 10 leaves). Means followed by the same letter are not significantly different (p < 0.05).

**Table 9.2.** Effect of AM-toxin I on *in vitro* shoots of cultivars with various degrees of susceptibility to Alternaria blotch

Cultivar	Degree of susceptibility <sup>a</sup>	Concentration of toxin			
		10 µM	1 µM	0.1 µM	0 µM
'Starking Delicious'	Highly susceptible	4.0 ± 0.00*a	3.5 ± 0.50a	3.0 ± 0.00a	0.0 ± 0.00c
'Aori 10'	Susceptible	3.5 ± 0.50a	2.5 ± 0.50a	2.0 ± 1.00b	0.0 ± 0.00c
'Hokuto'	Susceptible	2.0 ± 0.00b	2.0 ± 0.00b	1.0 ± 0.00b	0.0 ± 0.00c
'Fuji'	Intermediate	1.0 ± 0.00b	0.0 ± 0.00c	0.0 ± 0.00c	0.0 ± 0.00c
'Jonagold'	Resistant	0.0 ± 0.00c	0.0 ± 0.00c	0.0 ± 0.00c	0.0 ± 0.00c

<sup>a</sup>The grade of susceptibility is calculated based on the occurrence of necrotic lesions on *in vitro* shoots as follows: 0 = no necrotic lesion; 1 = a few necrotic lesions near the end of the shoots; 2 = necrotic lesions on <50% of the shoot; 3 = necrotic lesions on 50-75% of the shoot; 4 = necrotic lesions on 75-100% of the shoot. Each grade was a mean of two shoots. Values are means ± SE (n = 2 shoots). Means followed by the same letter are not significantly different (p < 0.05).

**Table 9.3.** Production of resistant mutants in cv. 'Hokuto' by X-ray irradiation and selection with *Alternaria alternata* toxin

Cultivar	Dose rate (KR/min)	Radiation dose (KR)	Toxin concentration ( $\mu$ M)	No. of shoots screened	No. of shoots according to the degree of susceptibility <sup>a</sup>				No. of shoots 1st screening test <sup>b</sup>	No. of shoots 2nd screening test <sup>c</sup>	
					0	1	2	3			4
'Hokuto'	Non-irradiated	-	10	49	0	0	0	6	43	-	-
		0.01	10	263	1	33	86	63	80	1	0
	0.1	8	10	223	3	25	58	52	85	3	0
		12	10	232	5	48	51	63	65	5	0
	Non-irradiated	1	10	92	0	0	0	3	89	0	-
		2	10	88	0	0	8	21	59	0	-
		4	10	82	0	1	17	29	35	0	-
		6	10	276	4	55	54	79	84	4	0
		8	10	312	5	30	87	103	87	5	1
		10	10	303	8	38	116	78	63	8	4
'Jonathan' (resistant)		-	100	10	0	2	2	2	4	-	-
		Non-irradiated	10	10	10	8	2	0	0	0	-

<sup>a</sup>Same as described in Table 9.1; <sup>b</sup>Shoots evaluated as grade 0 after immersion in 10  $\mu$ M toxin in the first screening test; <sup>c</sup>Shoots evaluated as grade 0 or 1 after immersion in 100  $\mu$ M toxin in the second screening test.

**Table 9.4.** Production of resistant mutants in cultivars 'Fuji', 'Oorin' and 'Aori 10' by X-ray irradiation and selection with *Alternaria alternata* toxin

Cultivar	Dose rate (KR/min)	Radiation dose (KR)	No. of screened shoots	No. of shoots according to the degree of susceptibility <sup>a</sup>					No. of shoots 1st screening <sup>b</sup>	No. of shoots 2nd screening <sup>c</sup>
				0	1	2	3	4		
'Fuji'	Non-irradiated		38	0	0	0	13	25	-	-
	0.01	6	99	2	11	33	28	25	2	0
		8	111	1	32	29	18	31	1	0
		10	102	2	33	29	27	11	2	0
	0.1	6	280	22	67	73	66	52	22	6
		8	527	53	164	132	81	97	53	12
'Oorin'	Non-irradiated		20	0	0	0	1	19	-	-
	0.1	6	189	12	41	66	52	18	12	10
		8	185	27	70	64	10	14	27	18
		10	156	21	61	57	11	6	21	11
	Non-irradiated		40	0	0	0	0	40	-	-
	0.01	6	123	0	0	0	0	123	0	-
'Aori 10'	Non-irradiated		125	0	0	0	0	125	0	-
	0.1	6	121	0	0	0	0	121	0	-
		8	110	0	0	0	1	109	0	-
		10	98	0	0	0	0	98	0	-
	Non-irradiated		10	8	2	0	0	0	-	-
	(resistant)									

<sup>a</sup>Evaluation of susceptibility marked with grades from 0 to 4 was the same as described in Table 9.1; <sup>b</sup>Shoots evaluated grade 0 after immersion in 10 µM toxin in the first leaf disk screening test; <sup>c</sup>Shoots evaluated grade 0 or 1 after immersion of 100 µM toxin in the second leaf disk screening test.

**Table 9.5.** Production of resistant mutants in cultivars 'Hokuto', 'Fuji' and 'Aori 10' by gamma-ray irradiation and selection with *Alternaria alternata* toxin

Cultivar	Dose rate (Gy/hour)	Radiation dose (Gy)	Toxin concentration (µM)	No. of screened shoots	No. of shoots according to the degree of susceptibility <sup>a</sup>							No. of shoots 2 <sup>nd</sup> screening <sup>c</sup>
					No. of shoots according to the degree of susceptibility <sup>a</sup>				No. of shoots 1st screening <sup>b</sup>	No. of shoots 2 <sup>nd</sup> screening <sup>c</sup>		
					0	1	2	3			4	
'Hokuto'	Non-irradiated	-	10	49	0	0	0	6	43	-	-	
	5	60	10	181	3	14	26	29	109	3	0	
			1		7	23	31	32	88			
		80	10	168	5	19	38	35	71	5	0	
		120	10	203	13	27	42	35	51	4	0	
'Fuji'	Non-irradiated	-	10	38	0	0	0	13	25	-	-	
	5	60	10	116	5	15	33	38	25	5	1	
		80	10	410	35	115	93	81	86	35	12	
		120	10	491	46	164	165	71	45	46	20	
	Non-irradiated	-	10	40	0	0	0	0	40	-	-	
'Aori 10'	5	60	10	96	0	0	0	0	96	-	-	
		80	10	87	0	0	0	1	86	-	-	
		120	10	45	0	0	0	0	45	-	-	
	Non-irradiated	-	100	10	0	2	2	2	4	-	-	
		-	10	10	8	2	0	0	0	-	-	
'Jonathan' (resistant)	Non-irradiated	-	1	10	8	2	0	0	0	-	-	
		-	1	10	8	2	0	0	0	-	-	
		-	1	10	8	2	0	0	0	-	-	

<sup>a</sup>Same as described in Table 9.1; <sup>b</sup>Shoots evaluated as grade 0 after immersion in 10 µM toxin in the first screening test; <sup>c</sup> Shoots evaluated as grade 0 or 1 after immersion in 100 µM toxin in the second screening test.

**Table 9.6.** Symptoms after inoculation of control and resistant mutant plants with a spore suspension\*

Cultivar	Susceptibility level	Radiation	Dose rate	Dose	No. of plants	Rate of leaf symptoms (%) <sup>a</sup>	Severity of symptoms <sup>b</sup>	No. of resistant mutants								
'Hokuto'	Susceptible control	Non-irradiated	-	-	5	84.0 ± 4.8	45.5 ± 5.3	-								
	Selected mutant	X-rays	-	10 KR	4	20.0 ± 5.0	6.7 ± 4.2	4								
'Oorin'	Susceptible control	Non-irradiated	-	-	2	75.0 ± 5.0	49.2 ± 0.9	-								
	Selected mutant		0.1	6 KR	5	56.0 ± 12.8	15.3 ± 7.7	3								
'Fuji'	Susceptible control	Non-irradiated	-	-	5	54.0 ± 4.8	19.3 ± 3.5	-								
									Selected mutant		0.1	6 KR	2	40.0 ± 0.0	6.7 ± 0.0	2
									Selected mutant		0.1	8 KR	8	25.0 ± 22.5	4.8 ± 4.4	7
'Fuji'	Susceptible control	Non-irradiated	-	-	5	54.0 ± 4.8	19.3 ± 3.5	-								
									Selected mutant		0.1	8 KR	4	5.0 ± 5.0	0.9 ± 0.9	4
									Selected mutant		0.1	10 KR	4	10.0 ± 0.0	1.7 ± 0.0	1
'Jonagold'	Resistant control	Non-irradiated	-	-	4	15.0 ± 10.0	3.3 ± 1.7	-								
									Selected mutant		0.1	80 Gy	8	16.3 ± 17.8	4.2 ± 4.8	5
									Selected mutant		0.1	120 Gy	15	20.0 ± 12.9	4.1 ± 3.1	14
'Jonathan'	Resistant control	Non-irradiated	-	-	3	3.3 ± 4.4	0.6 ± 0.8	-								
									Selected mutant		0.1	80 Gy	8	16.3 ± 17.8	4.2 ± 4.8	5
'Sansa'	Resistant control	Non-irradiated	-	-	3	0.0 ± 0.0	0.0 ± 0.0	-								
									Selected mutant		0.1	80 Gy	8	16.3 ± 17.8	4.2 ± 4.8	5

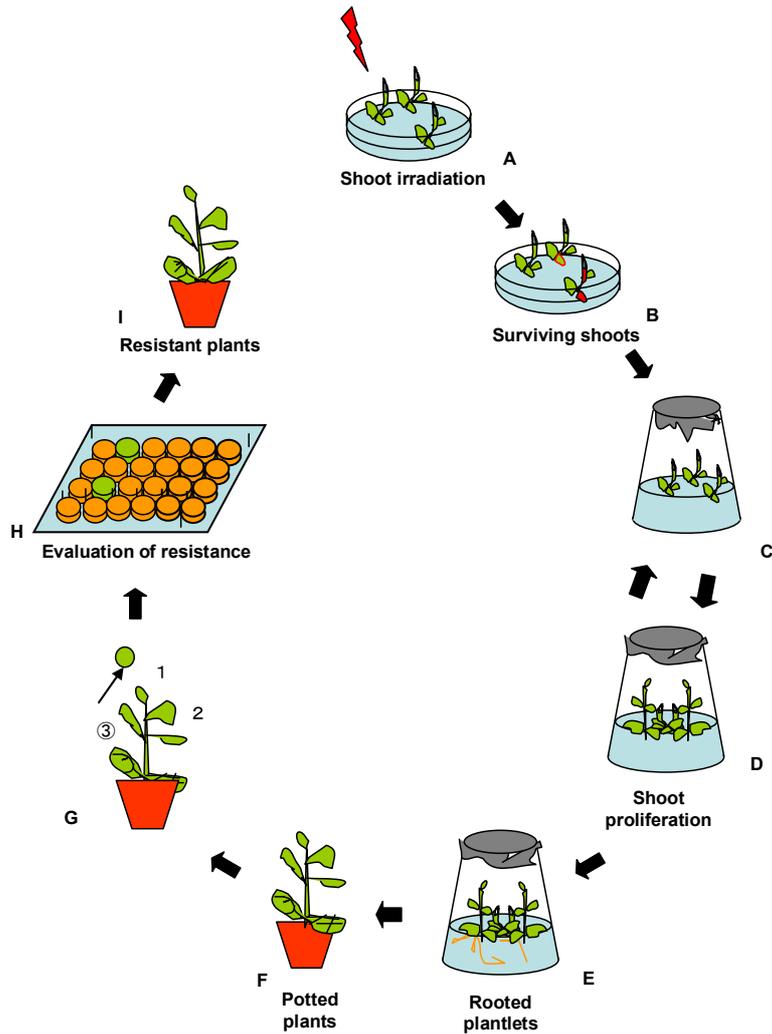
\*The mutant plants shown here had been screened with the toxin method. <sup>a</sup>Rate of symptoms appeared within group after the inoculation of spore suspension; <sup>b</sup>Severity of symptoms appeared within group after the inoculation of spore suspension; 'In cvs. 'Hokuto' and 'Oorin', we selected resistant mutants showing <40% infected leaf symptoms and severity of symptoms <15. In cv. 'Fuji', we selected resistant mutants showing <40% rate of infected leaf symptoms and severity of symptoms <10. Values are means ± SE (n = 1-15 plants).

**Table 9.7.** Fruit characteristics of the mutants and original cv. 'Hokuto'

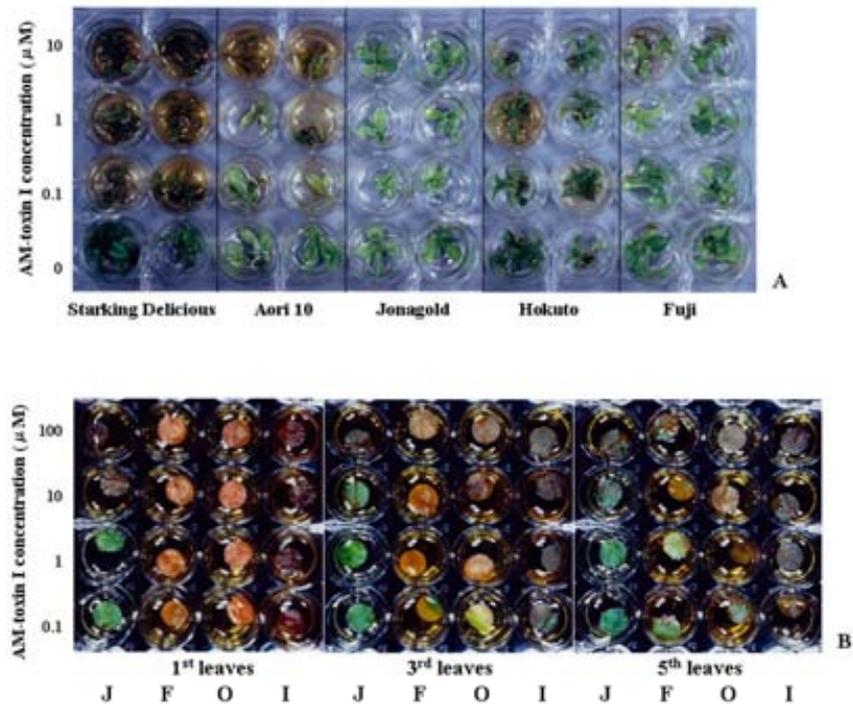
Mutant/ original line	No. of fruit samples	No. of damaged fruits			Fruit colouration (1-5) <sup>a</sup>	Core rot (0-3) <sup>b</sup>
		Stem end cracking	Fruit splitting	Fruit drop		
5-1	11	0	1	0	4.0	0.7
5-3	7	0	0	1	4.0	1.0
3-1	6	0	0	1	4.3	0.2
3-2	9	0	0	0	4.2	0.8
3-3	47	0	1	0	4.1	0.9
3-4	10	0	0	3	4.9	0.4
19-1	35	0	0	2	4.0	0.9
18-2	3	0	0	0	4.7	1.3
18-3	7	0	0	0	4.0	0.7
original 1	59	2	3	4	4.1	1.1
original 3	3	0	1	0	3.0	0.7
original 4	4	0	0	0	4.3	2.0

<sup>a</sup>Samples were rated from 1-5 according to the degree of fruit colouration from lowest to highest colouration;

<sup>b</sup>Samples were rated from 1-3 according to the degree of core rot from lowest to highest core rot.

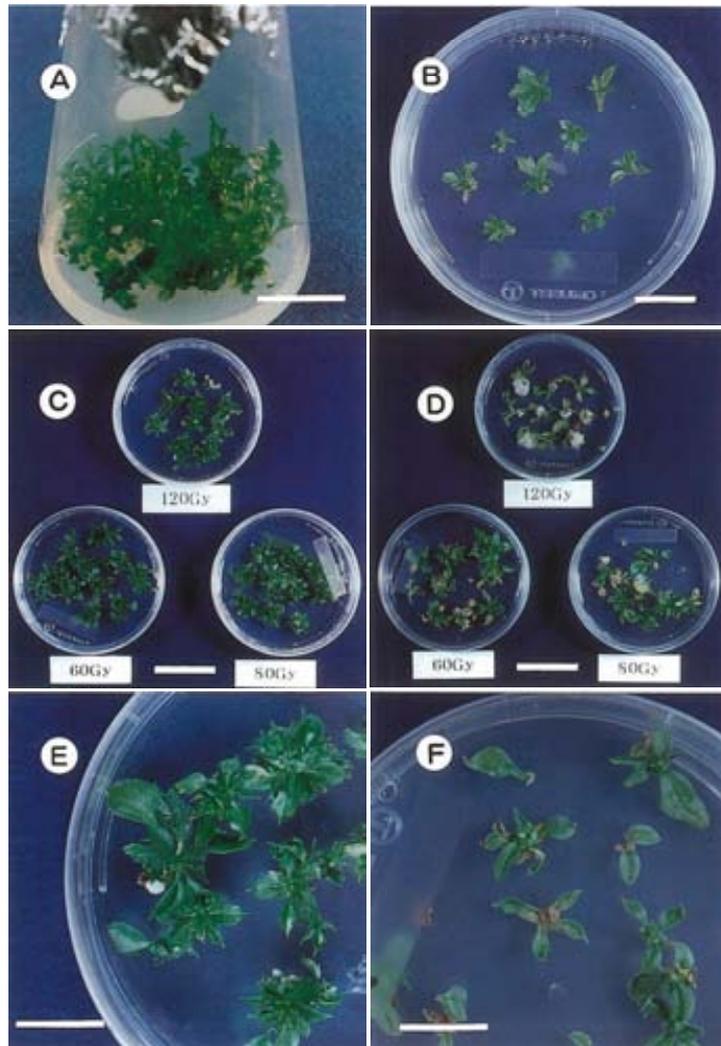


**Figure 9.1.** Schematic representation of the procedure of producing mutants resistant to *Alternaria* blotch. (A) Irradiation to shoots with various doses and dose rates. (B) Investigation of surviving shoots. (C) Culture of surviving shoots in proliferation medium. (D) Proliferation of surviving plantlets. Shoot clumps were divided and transferred into fresh medium to proliferate (repeated at least three times). (E) Elongated shoots were transferred to rooting medium. (F) Plants were acclimatised. (G) The third leaves (from the top) were collected and disks were cut out with a cork borer from the central parts of the leaf. (H) Evaluation of resistance was carried out by immersing the leaf disks in AM-toxin solutions and evaluating the degree of necrosis on the leaf disks. Plants whose disks remained green were selected for a second screening with the AM-toxin. (J) Selection of resistant plants.

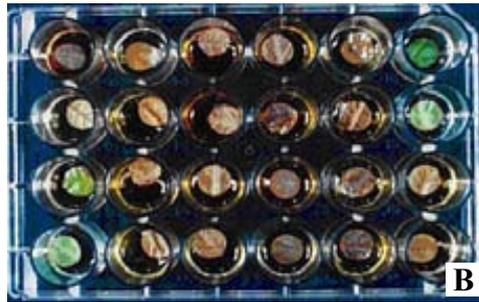
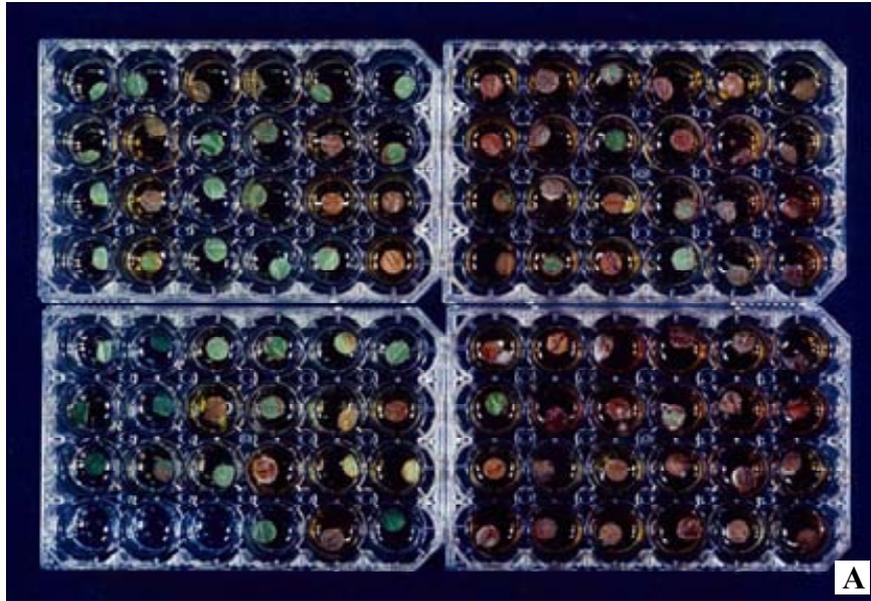


**Figure 9.2.** Susceptibility of cultivars to Alternaria blotch AM-toxin I. (A) *In vitro* shoots of cultivars ‘Starking Delicious’, ‘Aori 10’, ‘Jonagold’, ‘Hokuto’ and ‘Fuji’ were directly immersed in an AM-toxin solution. Browning of shoots of susceptible cultivars is the result of necrosis, whereas shoots of resistant cultivars remained green. The AM-toxin I solution was used at concentrations of 0 (control), 0.1, 1 and 10  $\mu\text{M}$ . (B) Leaf disks of the first, third and fifth leaves of cultivars ‘Jonathan’ (J), ‘Fuji’ (F), ‘Oorin’ (O) and ‘Indo’ (I) immersed in AM-toxin solution. The susceptibility to AM-toxin I varied among cultivars and leaf age, i.e., older leaves were slightly more resistant to the toxin. The AM-toxin I solution was used at concentrations of 0.1, 1, 10 and 100  $\mu\text{M}$ .

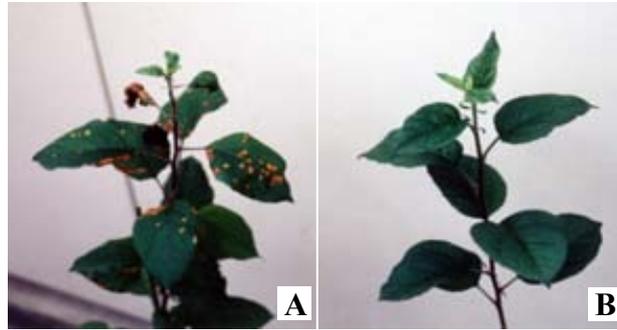




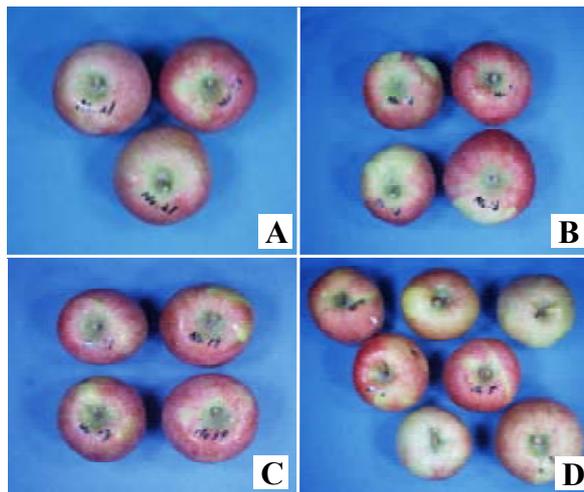
**Figure 9.4.** Appearance of *in vitro*-cultured shoots after gamma-ray irradiation. (A) *In vitro*-cultured shoots of cv. 'Oorin' (after being cultured for 30 days on shoot proliferation medium). (B) Non-irradiated *in vitro* cultured shoots of cv. 'Hokuto'. (C) Appearance of *in vitro*-cultured shoots one month after irradiation on cv. 'Hokuto' (radiation doses of 60, 80 and 120 Gy). Shoots appeared as normal as non-irradiated ones. (D) Appearance of *in vitro*-cultured shoots one month after irradiation on cv. 'Aori 10' (radiation doses of 60, 80 and 120 Gy). Callus formation from shoots appeared in parts of the irradiated shoots. (E) Magnified detail of (C). Normal phenotypes were seen in 120 Gy-irradiated cv. 'Hokuto'. (F) Magnified detail of (D). Rosette-type shoots and abnormal leaf morphology appeared one month after gamma-ray irradiation on cv. 'Aori 10' (radiation doses of 120 Gy). Bar: 2 cm (A, B, E, F). Bar: 3 cm (C, D).



**Figure 9.5.** Screening tests of mutants with AM-toxin I solution. (A) First screening test for selecting 'Fuji' mutants resistant to *Alternaria* blotch. Leaf disks were cut from the third leaves and immersed in 10  $\mu$ M AM-toxin I solution. Leaf disks of resistant mutants remained green, whereas necrotic lesions appeared on the susceptible ones. (B) Second screening test for mutants selected in the first screening. Leaf disks from the third leaves of each selected mutant were immersed in 10 and 100  $\mu$ M AM-toxin I solution. Leaf disks of 12 mutants were treated per plate.



**Figure 9.6.** Regenerated plant of the original plant of cv. 'Fuji' (A), which showed necrotic lesions 10 days after inoculation with a spore suspension of the apple pathotype of *Alternaria alternata*, and of a mutant resistant to Alternaria blotch (B).



**Figure 9.7.** Evaluation of fruits of mutants resistant to Alternaria blotch. Fruits of mutant lines 21 (A), 3 (B) and 19 (C) of cv. 'Hokuto' compared to fruits of the original cv. 'Hokuto' (D). All fruits were harvested in 2005. No morphological changes were observed between the mutants and the original cv. 'Hokuto'.



## Chapter 10

### The pineapple-*Fusarium subglutinans* interaction: an early selection system for disease resistance

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#### Abstract

This chapter summarises a study on the effects of selective agents from *Fusarium subglutinans* on seedlings, calli and *in vitro* plantlets of pineapple (*Ananas comosus* [L.] Merr) as well as their applications for breeding programmes in order to increase the resistance to fusariose disease in pineapple. The phytotoxic effects of culture filtrates from *F. subglutinans*, the causal agent of fusariose in pineapple, were examined in several pineapple cultivars differing in resistance level. The phytotoxic effects were assessed by electrolyte leakage and by placing the culture filtrate on wounds in seedlings, by inhibition of growth and regeneration of calli, and by inhibition of multiplication of *in vitro* plantlets. As a proof of concept, the susceptible cultivars were the most sensitive in each test, whereas resistant cultivars showed resistance to the culture filtrate and their calli grew well even in the presence of high concentrations of culture filtrate, which were highly toxic to the susceptible cultivars. In addition, culture filtrates reduced shoot formation in *in vitro* plantlets and when plants were regenerated from calli derived from the susceptible cultivars but not from resistant ones. These results show the potential of culture filtrates of *F. subglutinans* for *in vitro* screening of pineapple for resistance to the pathogen. Furthermore, the possibility of using culture filtrates as a selection agent for resistance in F<sub>1</sub> hybrids was also evaluated. Using culture filtrate as a selection criterion allowed resistant plants to be obtained that behaved quite similarly to those when the fungus itself was used as a selection agent.

#### INTRODUCTION

Fusariose, caused by *Fusarium subglutinans*, constitutes the most serious pineapple disease in Brazil where it was first reported in the State of Sao Paulo (Kimati and Tokeshi, 1964). Besides causing losses as high as 80% of marketable pineapple fruits (Robbs *et al.*, 1965), the pathogen infects approximately 40% of the asexually propagated material and kills about 20% of the pineapple plants prior to harvesting (Matos, 1995; Matos *et al.*, 1997).

The pathogen is able to infect all parts of the pineapple plant and the development of the disease results in a characteristic gum exudation (Matos, 1995). A pineapple plant propagated from an infected asexual propagative part can show, besides gum exudation, one or more of the following alterations: bending of the stem, usually to the side where the lesion is located; changes in plant phyllotaxis, increasing the number of leaves per spiral; changes in plant architecture, looking like a funnel; shortening of the stem; death of the apical meristem; chlorosis (Pissarra *et al.*, 1979). Due to the disease development in the stem and base of the leaves, water flow is reduced, the plant stops growing and the leaves show a reddish colour (Matos, 1995). In a later stage of infection, the plant wilts and dies.

The control of pineapple fusariosis has been mainly based on the use of pathogen-free propagating material, evading the pathogen and direct protection by chemical control (Matos, 1995). Chemical control is costly and often ineffective, and in time may lead to the occurrence of pathogen isolates resistant to fungicides. Moreover, the application of chemicals for crop protection should be reduced for environmental and health reasons (Pinho *et al.*, 1997).

The potential for disease resistance as a control measure for pineapple fusariosis has been suggested in studies based on observations carried out either under field conditions or with artificial inoculation techniques (Matos *et al.*, 1991). Considering that growing resistant cultivars constitutes one of the cheapest and most efficient control measures of plant disease, the identification of sources of resistance to *F. subglutinans* is a very important step in obtaining commercial pineapple cultivars resistant to the pathogen (Cabral and Matos, 1995).

Many plant pathogens produce toxins related to a number of different diseases (Wood *et al.*, 1972; Durbin, 1981). For example, race T of *Bipolaris maydis* (Nisikado and Miyake) Shoemaker (syn. *Helminthosporium maydis*), the causal agent of southern corn leaf blight, produces T-toxin, which acts specifically on the mitochondria of susceptible cells (Miller and Koeppel, 1971). Fungi of the genus *Fusarium* produce a variety of biologically-active metabolites in culture filtrates reported to be toxic to cotyledons, germinating seeds and plants of many vegetal species (Jin *et al.*, 1996; Matsumoto *et al.*, 1999a). Fusaric acid (5-butylpyridine-2-carboxylic acid), produced by several fungi of the genus *Fusarium*, is associated with wilt of banana, cotton, pea, tomato, and other plants, and affects membrane permeability, increasing leakage of potassium and other electrolytes and inhibits respiration in this interaction (McLean, 1996; Kuzniak *et al.*, 1999). Treatment of tomato cells with fusaric acid resulted in decreased cell viability, but in increased activities of intracellular superoxide dismutase, catalase, guajacol peroxidase and ascorbate peroxidase. An increase in extracellular pH and oxygen production could reflect both the phytotoxic effect as well as fusaric acid action as a plant-pathogen recognition factor (Kuzniak, 2001).

Experiments with pathogen culture filtrates have shown that the *in vitro* tissue response correlates with the disease reaction of the host variety. Thus, in these cases, selection using a phytotoxin might offer an alternative to field selection as reported for a number of host-pathogen interactions, and may allow selection of important traits in disease resistance (Daub, 1986). Advantages of using *in vitro* plantlets and calli rather than whole plants for assaying phytotoxins have been reviewed elsewhere (Chapter 2; Botta *et al.*, 1994; Remotti *et al.*, 1997). Screening for disease resistance in the field is time-consuming, costly and dependent upon natural fluctuations in the inoculum abundance and weather factors that influence pathogen spread, infection, disease development and disease expression (Ostry, 1997). The creation of genetic variability and a suitable procedure for reliably identifying resistant genotypes constitute two prerequisites for disease resistance improvement.

Progress in the field of *in vitro* selections is often hampered by the fact that: (1) the substances contained in filtrates may not yet have been completely characterised; (2) the expression of resistance to the toxin *in vitro* may vary from that shown to the pathogen *in planta*; and (3) the level of desired resistance is not obtainable via toxin influence (Chapter 2; Švábová and Lebeda, 2005).

The aim of this chapter is to summarise our main results and knowledge about toxins and other substances produced during the interaction between pineapple and *F. subglutinans*, their potential role in pathogenesis mechanisms, and possible screening and selection for *in vitro* and *in vivo* resistance. The chapter covers basic research studies and methods that elucidate the phytotoxic effects of culture filtrate and toxins produced by *F. subglutinans* on *in vitro* and *in vivo* plants, but also try to develop practical applications to obtain resistant breeding lines. Their application successfully resulted in resistant lines. Nowadays, these techniques are an important complement to classical breeding methods.

## MATERIAL AND METHODS

### Culture filtrates and fusaric acid production

An isolate of *F. subglutinans* (isolated by the Bioplants Centre, Ciego de Avila, Cuba) was grown on potato dextrose agar (PDA) dishes for seven days at  $26 \pm 2^\circ\text{C}$  with a 12 hour photoperiod at 4000 lux, provided by warm white fluorescent tubes. Dishes of fungal mycelium were inoculated in Czapek-Dox broth (1 l) supplemented with 2 g of  $\text{NaNO}_3$ , 1 g of  $\text{K}_2\text{HPO}_4$ , 0.5 g of  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ , 0.5 g of KCl, 10 mg of  $\text{FeSO}_4$ , 30 g of sucrose, 1 ml of 1% (w/v)  $\text{ZnSO}_4$  and 1 ml of 0.5% (w/v)  $\text{CuSO}_4$ . The cultures were grown in 250 ml Erlenmeyer flasks with 100 ml of medium at  $26 \pm 2^\circ\text{C}$  under the light conditions mentioned above. After three weeks of stationary incubation, mycelia and conidia were filtered through Whatman No.1 filter paper (Whatman, Clifton, NJ, USA) and a 0.2  $\mu\text{m}$  Millipore membrane (Sartorius AG, Goettingen, Germany). The culture filtrate was evaporated under reduced pressure at  $40^\circ\text{C}$  to 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (v/v) using a rotary evaporator (Heidolph, Bioblock Scientific, Strasbourg, France). The isolation, extraction and purification of fusaric acid were performed following the methodology described by Bacon *et al.* (1996). Alternatively, commercial fusaric acid may also be used (e.g., product number F6513, Sigma Chemical, St. Louis, MO, USA).

### Electrolyte leakage bioassay

Ion leakage was used as an indicator of cell membrane damage in response to the presence of phytotoxic culture filtrate on pineapple seedling (30 days old) of resistant ('Perolera') and susceptible ('Smooth Cayenne') cultivars. Leaf disks (diameter: 10 mm) of each cultivar were taken using a cork borer, and placed in a tube with 1 ml of 80% culture filtrate. Disks were infiltrated under a vacuum for 24 hours at room temperature, rinsed with distilled water several times and placed in 20 ml of distilled water for 24 hours to allow electrolyte leakage. The conductivity of this latter solution was measured with a 660 Metrohm conductivity meter (Metrohm AG, Herisau, Switzerland) equipped with a temperature-compensating electrode ( $K = 0.77$ ). Leaf disks infiltrated with distilled water and fungal culture media were used as controls. Each test was repeated five times.

### Applying the culture filtrate or fusaric acid on wounded leaf segments

To determine the phytotoxic effects of the culture filtrate or fusaric acid, they were applied at different concentrations onto wounded leaf segments. Fusaric acid concentrations of each culture filtrate concentration were used. The cultivars 'Perolera' (resistant), 'Spring' (resistant), 'Perola' (susceptible) and 'Smooth Cayenne' (susceptible) were used. Leaf segments (~20 mm) of 30 day old seedlings were excised, placed on moist filter paper in Petri dishes, and punctured with a fine needle, and 5  $\mu\text{l}$  of the culture filtrate or fusaric acid (in different concentrations) were placed onto the wound area. All leaf segments were incubated on moist filter paper in Petri dishes (continuous fluorescent light, approximately  $22 \pm 2^\circ\text{C}$ ). Sterile water and fungal culture medium were used as negative and positive controls, respectively, and the assays were performed using five leaf segments per plant and 25 plants per cultivar. The reactions of leaves were noted after 35 hours of incubation and necrotic spots were measured using the following equation:

$$A = \pi/4 a \times b$$

Where: A = Necrosis spot area ( $\text{mm}^2$ ), a = radius longer (mm), b = radius shorter (mm) and  $\pi = 3.1415$ .

## Effect of culture filtrate on calli and *in vitro* plantlets

### *Effect of culture filtrate on growth of calli*

Calli were established aseptically from two pineapple cultivars according to Daquinta *et al.* (1996), and placed on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 2.5 mg/l of Dicamba (3,6-dichloro-o-anisic acid) and 0.5 mg/l of 6-benzylaminopurine (BAP) (medium A) per litre and incubated for five to six weeks under dark conditions at  $26 \pm 2^\circ\text{C}$ . The induced friable calli were subcultured monthly. Culture filtrates (without concentrate) adjusted to pH5.8 were added to autoclaved medium A at three concentrations (10, 20 and 30%) and poured into 100 mm diameter dishes.

Ten pieces (~10 mg) of calli from each cultivar were transferred to medium A supplemented with culture filtrate. The experiment consisted of three dishes (replicates) for each cultivar in a completely randomised design. Dishes were incubated under the conditions mentioned above and two weeks later, calli were visually ranked in five categories: 1 = no browning; 2 = callus surface slightly brown; 3 = whole tissue brown; 4 = whole tissue deeply brown with restricted growth; and 5 = whole tissue deeply brown with no growth. In addition, callus growth rate was measured as recommended by Arai and Takeuchi (1993). This experiment was repeated once.

### *Effect of culture filtrate on plant regeneration and shoot multiplication from calli and in vitro plantlets*

Two cultivars resistant to *F. subglutinans*, 'Perolera' and 'Spring', and two susceptible ones, 'Smooth Cayenne' and 'Perola', representing a range of disease reactions, were used in all the experiments. Calli and *in vitro* plantlets were established aseptically according to Daquinta *et al.* (1997). The *in vitro* plantlets were placed on MS basal medium supplemented with 2.1 mg/l BAP and 0.3 mg/l naphthaleneacetic acid (NAA) (medium for shoot multiplication) and incubated with a photoperiod of 12 hours at  $26^\circ\text{C}$ . For plant regeneration, the calli were placed on MS basal medium supplemented with 0.5 mg/l BAP (plant regeneration medium) and incubated under the same conditions.

Culture filtrates adjusted to pH5.8 were added to autoclaved medium at five concentrations (4, 8, 12, 16 and 20% [v/v]) to assess their effect on shoot multiplication and plant regeneration. The plant regeneration medium was poured into 100 mm diameter Petri dishes and the shoot multiplication medium was transferred to 250 ml Erlenmeyer flasks. 100 shoots of *in vitro* plantlets (~240 mg) from each cultivar were transferred to the shoot multiplication medium supplemented with the different concentrations of the culture filtrate. The incubation conditions were as described above. The multiplication coefficient was determined from the mean number of shoots per explant. A similar number of calli (~100 mg) from each cultivar was placed on the plant regeneration medium and regeneration was calculated from the mean number of shoots per callus. The multiplication coefficient was recorded after two months of culture, and plant regeneration was determined after three months. All the experiments were repeated twice.

## Selection procedures

The culture filtrate was applied onto wounded leaves to select susceptible and resistant  $F_1$  hybrids. The culture filtrate and fusaric acid concentrations used were 80% (v/v) and 0.75 mg/ml, respectively.  $F_1$  hybrids of 'Spring'  $\times$  'Smooth Cayenne'; 'Smooth Cayenne'  $\times$  'Spring'; 'Smooth Cayenne'  $\times$  'Perolera' and 'Perolera'  $\times$  'Smooth Cayenne' were used. The resistance level of the  $F_1$  hybrids and cultivars under natural conditions was measured in greenhouse field tests. 25 plantlets per cultivar and  $F_1$  hybrid were wounded with a stainless needle. A puncture wound was produced at the basal region. Inoculation was performed by dipping wounded plantlets in an inoculum of  $10^5$  conidia/ml for three minutes. After inoculation, the plantlets were kept under greenhouse conditions at  $25\text{-}30^\circ\text{C}$ , for two months, and then inspected for disease development.

Evaluation was based on a 0-6 numerical rating system in which 0 = no disease development in the stem; 1 = 2% of the stem infected by the pathogen; 2 = 3-5%; 3 = 6-10%; 4 = 11-20%; 5 = 21-50%; and 6 = 51-100% (Disease Index [DI]). A resistant reaction is defined as no disease development in inoculated plantlets. F<sub>1</sub> hybrids and cultivars in which infection takes place but the colonisation of the host tissue proceeds slowly, reaching a DI of 3 or less two months after inoculation and showing significantly less disease severity than the susceptible control are considered as tolerant of the pathogen (Matos *et al.*, 1991). The experiment was conducted with a completely randomised design.

### Data analysis

The experimental data were analysed by analysis of variance conducted according to Duncan's new multiple range test using SPSS/PC (Statistics Package for Social Science) (1992). Means were compared by least significant differences at  $p < 0.05$  unless otherwise stated.

## RESULTS AND DISCUSSION

The results of the studies presented here indicate that *F. subglutinans* isolates that cause fusariose disease produce one or more phytotoxins in culture that are also phytotoxic to leaves, calli and *in vitro* plantlets of pineapple. The assays shown here provide a good measure for determining the phytotoxic effects of *F. subglutinans* culture filtrate and the potential of using the filtrate for *in vitro* screening of pineapple for resistance to this pathogen.

### Electrolyte leakage bioassay

The change in the conductivity ( $\mu\text{S}/\text{cm}$ ) of the solution over a 24 hour incubation period was used as the measure of electrolyte leakage. Table 10.1 shows how electrolyte leakage depends on the cultivar used. 'Smooth Cayenne' (susceptible to fusariose) showed a highly significant electrolyte leakage in the presence of the culture filtrate. In contrast, 'Perolera' (resistant) showed no significant electrolyte leakage, even in the presence of culture filtrate. The distilled water and fungus culture medium did not induce significant electrolyte leakage in the washing solution (Borrás *et al.*, 1998).

One of the most vulnerable parts of the cell is the plasma membrane, the first subcellular component likely to encounter the toxin (Batchvarova *et al.*, 1992). A rapid and nearly universal response of susceptible plants or tissue to toxin treatment is an alteration of membrane permeability, which is usually detected as an efflux of solutes or as a depolarisation of electropotential across the plasma membrane (Dunkle and Wolpert, 1981). Regardless of the primary site of action of host-specific toxins, the results of studies in which electrolyte leakage is taken as an indication of toxicity must be carefully interpreted, and the relationship of electrolyte leakage to other disease symptoms induced by the toxin must be clearly established (Dunkle and Wolpert, 1981).

### Applying the culture filtrate or fusaric acid onto wounded leaves

Figure 10.1A describes the effect of different culture filtrate concentrations on the development of necrotic spots from pineapple leaves. *F. subglutinans* filtrate induced necrotic spots on pineapple leaves from resistant and susceptible cultivars after a 35 hour treatment. The necrotic spot areas in susceptible pineapple leaves treated with the filtrate were larger than those of the resistant cultivars. 'Smooth Cayenne' and 'Perola' (both susceptible) developed necrotic lesions with an area of up to 10 mm<sup>2</sup> (Borrás *et al.*, 2001).

A significant increase in necrotic spot area was directly correlated to an increase in the concentration of culture filtrate in susceptible cultivars. The 'Perolera' and 'Spring' resistant cultivars showed a lower degree of necrotic spot area when treated with culture filtrate in comparison to susceptible cultivars. Significant differences between susceptible and resistant cultivars were observed when the culture filtrate was applied at a 50% concentration and higher (Borrás *et al.*, 2001).

On the other hand, all cultivars were sensitive to fusaric acid regardless of their resistance level and the sensitivity increased as the concentration used increased (Figure 10.1B). These results corroborate our observations that fusaric acid has a non-specific behaviour in the response of susceptible and resistant pineapple cultivars (Borrás *et al.*, 2001).

This study represents the first attempt to assess the potential of using culture filtrate as a selection agent for fusariosis resistance. The role of toxins in pathogenesis is usually assessed by evaluating the correlation between toxin production and pathogenicity of the organism, and between the sensitivity of different genotypes to the toxins and their susceptibility to the disease (Yoder, 1980). Our results have shown that the cultivars' responses to the culture filtrate of *F. subglutinans* are related to the responses of the cultivars to the pathogen. This behaviour was also found by Jin *et al.* (1996) who used a phytotoxic culture filtrate from *F. solani* on wounded leaves and showed that the susceptible cultivars were more sensitive to culture filtrate than the resistant ones. Furthermore, their assay showed that the response of soybean plants to crude fungal culture filtrate was correlated with the severity of Sudden Death Syndrome in inoculated soybean (*Glycine max* L.) plants grown in a greenhouse.

In our case, fusaric acid produced phytotoxic effects on all cultivars. Therefore, the response of different pineapple genotypes did not correlate with the response to *F. subglutinans* under natural conditions. However, since fusaric acid has been classified as a non-selective toxin (Gäumann, 1957; Matsumoto *et al.*, 1995), it is likely that there must be other components present in the culture filtrate that are responsible for the selectivity observed. Extracellular molecules such as fungal elicitors of plant resistance have been reported in a number of studies related to host-pathogen interactions (Knogge, 1996; Abad *et al.*, 1997; Bailey *et al.*, 1997; Ellis *et al.*, 2000).

### **Effect of culture filtrate on calli and *in vitro* plantlets**

#### *Effect of culture filtrate on growth of calli*

Calli of 'Smooth Cayenne' and 'Perolera' grew well on the subculture medium and their growth was not affected by fungal culture media. However, there was a general trend for increased calli browning in the callus pieces of 'Smooth Cayenne' at higher levels of culture filtrate in medium A (Figure 10.2). The mean calli browning rate increased from 2.2 to 4 and 5 when the amount of culture filtrate in the medium was 10, 20 and 30%, respectively. In contrast, 'Perolera' calli had a lower browning rate, especially when higher levels of culture filtrate were added to medium A. On the other hand, the growth of 'Smooth Cayenne' calli decreased on subculture medium containing a high concentration of the culture filtrates. Compared to the other cultivars, 'Perolera' calli were not greatly affected by the culture filtrate (Table 10.2). In our study, culture filtrates of *F. subglutinans* were toxic to susceptible pineapple calli, as they caused a decrease in callus growth and caused browning. It was also observed that resistant calli had lower browning rates, even at higher concentrations of *F. solani* culture filtrate (Jin *et al.*, 1996).

As in our previous study (Borrás *et al.*, 1998), callus browning rate and callus fresh weight were found to be good measures of callus sensitivity to culture filtrate. The correlation between pineapple cultivar susceptibility and the toxicity of culture filtrates suggests that filtrates could be used for *in vitro* screening of disease resistance. Tissue culture systems can provide a means of rapid screening for disease resistance when a toxin is involved in disease development. However, it would still be premature to conclude that any of these characteristics could be used for *in vitro* screening of germplasms for fusarioid disease as has already been attempted in several host-parasite systems (Kaur *et al.*, 1987).

#### *Effect of culture filtrate on plant regeneration and shoot multiplication from calli and *in vitro* plantlets*

The regeneration of plantlets from calli of the susceptible cultivars 'Smooth Cayenne' and 'Perola' was inhibited at high concentrations of culture filtrate in the regeneration medium. Even at the lowest

culture filtrate concentration, regeneration was considerably reduced (Table 10.3). Calli from the resistant cultivars 'Perolera' and 'Spring' were less affected at the same concentrations of culture filtrate (Hidalgo *et al.*, 1999).

*In vitro* shoot multiplication from plantlets of the susceptible cultivars 'Smooth Cayenne' and 'Perola' was highly inhibited in media containing high concentrations of culture filtrate. Resistant *in vitro* plantlets ('Perolera' and 'Spring') were less affected by the same concentrations, demonstrating a higher tolerance compared with untreated controls (Table 10.4).

The present results support previous observations that *F. subglutinans* culture filtrates applied at various concentrations can reduce growth and development of pineapple tissue cultures and that the effect is correlated with the resistance of the cultivars from which the tissue cultures were derived (Hidalgo *et al.*, 1999). Mendes *et al.* (1993) found that the addition of filtrates of *F. oxysporum* f. sp. *cubense* to culture medium had toxic effects on the growth of banana shoot tips. Ahmed *et al.* (1991) tested wheat calli for tolerance to toxic metabolites of *F. graminearum* and *F. culmorum*, and observed that the regeneration ability of the tolerant calli was lower than that of the unselected calli. A similar response was observed when susceptible soybean calli were exposed to high levels of *F. solani* culture filtrate, showing reduced growth and regeneration (Jin *et al.*, 1996).

In contrast, in tests on the effect of *Alternaria solani* culture filtrate on adventitious shoot regeneration from tuber disks of five potato cultivars, which had been selected on the basis of their field reaction to *A. solani* and represented a range of disease reactions, the filtrate actually stimulated regeneration (Lynch *et al.*, 1991).

The toxicity of the *F. subglutinans* culture filtrate on *in vitro* plantlets and calli of the selected cultivars of pineapple reflected the performance of the isolate in greenhouse tests on whole plants, suggesting a possible role for fungal extracellular toxic compounds in this disease. The results indicate that these characteristics can be used for *in vitro* screening of germplasm for resistant lines, as has already been attempted in several other host-parasite systems (Ludwig *et al.*, 1992; Song *et al.*, 1994).

## Selection procedures

Susceptible F<sub>1</sub> hybrid pineapple leaves treated with culture filtrate developed necrotic spots of between 8 and 10 mm<sup>2</sup> (Table 10.5). The culture filtrate produced the highest phytotoxic effects on the susceptible F<sub>1</sub> hybrids. Accordingly, the mean DI registered in pineapple plants inoculated with the *F. subglutinans* pathogen was significantly lower in F<sub>1</sub> hybrids resistant to the culture filtrate than in susceptible ones. In total, eight F<sub>1</sub> hybrids showed resistance to the culture filtrate, one F<sub>1</sub> hybrid had partial resistance and four were sensitive to it. The fusaric acid had phytotoxic effects on all the cultivars and F<sub>1</sub> hybrids, producing necrotic spot areas of 14 mm<sup>2</sup> without any significant genotypic difference (Borrás *et al.*, 2001).

There are no effective chemicals for preventing fusariose disease. The only and best way to eliminate this disease is to develop cultivars that are resistant to or at least tolerant of *F. subglutinans*. In cross breeding programmes, new resistant cultivars can be developed using resistant cultivars or wild species as breeding material. The reaction of pineapple plants to *F. subglutinans* infection can vary within and among cultivars, indicating that differences in resistance to fusariose disease exist in pineapple genotypes under some conditions (Cabral and Coppens, 1997).

Our results show that it is possible to select resistant plants using the culture filtrate of *F. subglutinans* and this method reflects infections under natural conditions. A number of efficient protocols have been developed to select for resistance in plants to various pathogens by using culture filtrate and purified toxins in selection experiments (Litz and Lavi, 1997; Alarcon *et al.*, 1998; Matsumoto *et al.*, 1999b). A prerequisite for success is the sensitivity of the plant to the toxin, reflecting the susceptibility of the intact plant to the pathogen. Resistance selection can be performed in several ways. One method is

one-step selection, in which a lethal concentration of the selective agent is used; another method is stepwise selection, in which the concentration of the selective agent is increased gradually until it reaches a lethal concentration (McLean, 1996).

## CONCLUSIONS

The basic advantages of using *in vitro* cultures as compared to natural conditions are: (1) unfavourable weather and climate conditions are avoided, which enables the assessment of quantitative differences in polygenic traits more easily and precisely; (2) a large number of individuals can be tested in a small space; (3) it is easier to manipulate mutants, haploids, somaclones with higher variability in the genome; and (4) mass screening of mutants for resistance is facilitated (Nedelnik and Repkova, 1998).

Although there are not many reports of cultivars whose resistance is based on *in vitro* selections (although many resistant breeding lines were obtained), interest in utilising *in vitro* methods for improving resistance to plant pathogens remains (Upadhyay and Mukerji, 1997). Thus, selection of resistance *in vitro* must be considered as one of the methods which, in combination with conventional resistance screening and plant breeding methods including biotechnological procedures, may offer plant breeders a new approach to accelerate the development of disease-resistant plants (Crino, 1997).

These results suggest that the selection approach could have two major limitations: (1) the lack of knowledge concerning the genotypic dependence of the characterised toxins or extra-cellular metabolites that confer host-pathogen selectivity and (2) no assurance that the susceptibility and/or resistance of cultured tissues to the culture filtrate or toxins reflect those of the whole plant. The evaluation of genotypes produced within pineapple breeding programmes using culture filtrate could, therefore, offer a first step towards a simplification of selection procedures, which should be complemented by a second step of selection using artificial inoculation with the pathogen as previously reported by Matos *et al.* (1991).

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**Table 10.1.** Electrolyte leakage from leaf dishes of cultivars ‘Smooth Cayenne’ and ‘Perolera’

Treatment	Conductivity ( $\mu\text{S}/\text{cm}$ )	
	‘Perolera’	‘Smooth Cayenne’
80% culture filtrate	3.9a	9.8b
Water (control)	2.1a	2.2a
Fungal culture medium (control)	3.4a	3.7a
Least significant difference (0.05)	0.7	5.7
<b>Coefficient of variation (%)</b>	20	23

a: Treatments not significantly different at  $p < 0.05$ ; b: Treatment significantly different at  $p < 0.01$ .

**Table 10.2.** Pineapple calli browning rate and calli fresh weight of two pineapple cultivars grown on *Fusarium subglutinans* culture filtrate-amended medium A for two weeks

Cultivars	Concentration of culture filtrate (%)	Calli browning rate <sup>1</sup>	Calli fresh weight (g) <sup>2</sup>
‘Perolera’	0	1a	16.8a
	10	1a	15.9a
	20	1.1a	15.3a
	30	1.2a	14.9a
‘Smooth Cayenne’	0	1a	14.2a
	10	2.2b	10.9b
	20	4.5c	6.4c
	30	5c	3.9d
<b>Coefficient of variation (%)</b>		23	32

<sup>1</sup>Browning rate: 1 = no browning; 2 = callus surface slightly brown; 3 = whole tissue brown; 4 = deeply brown with restricted growth; 5 = deeply brown with no growth. <sup>2</sup>Measured according to Arai and Takeuchi (1993). a: Treatments not significantly different at  $p < 0.05$ ; b: Treatment significantly different at  $p < 0.01$ .

**Table 10.3.** Effect of different concentrations of *Fusarium subglutinans* culture filtrates on pineapple plant regeneration from callus\*

Concentration of culture filtrate (%)	Susceptible cultivars		Resistant cultivars	
	‘Smooth Cayenne’	‘Perola’	‘Perolera’	‘Spring’
0	9.6a	7.1a	8.6a	6.8a
4	3.3b	2.8b	7.4ab	5.1ab
8	1.5c	0.4c	5.0bc	3.7bc
12	0.0c	0.0c	3.3cd	2.0cd
16	0.0c	0.0c	1.6de	1.1de
20	0.0c	0.0c	0.0e	0.0e
<b>Standard error</b>	0.78	0.95	1.40	1.34

\*Calculated as the mean number of shoots per callus. Data within a column followed by the same letter are not significantly different at  $p < 0.05$  using Duncan’s new multiple range test.

**Table 10.4.** Effect of different concentrations of *Fusarium subglutinans* culture filtrates on pineapple plant multiplication *in vitro*\*

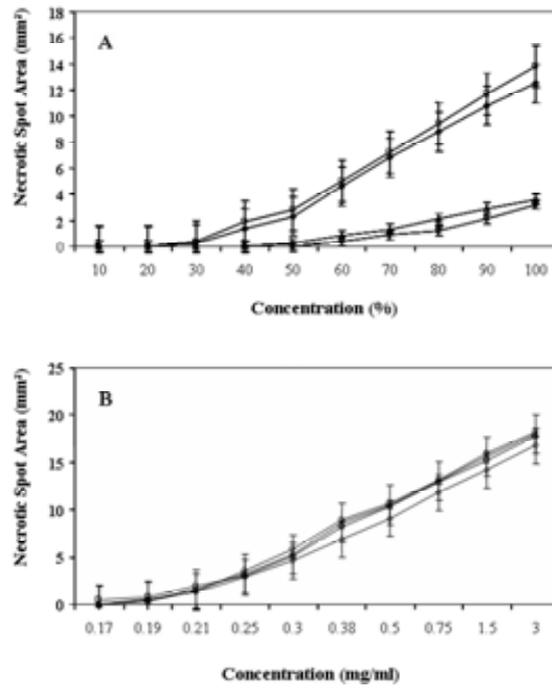
Concentration of culture filtrate (%)	Susceptible cultivars		Resistant cultivars	
	'Smooth Cayenne'	'Perola'	'Perolera'	'Spring'
0	7.5a	7.9a	7.2a	6.2a
4	2.9b	4.1b	6.4b	5.8b
8	2.1c	2.8c	5.4c	4.6c
12	1.5d	1.7d	4.7d	2.4d
16	0.8e	0.8e	2.7e	1.5e
20	0.3f	0.2f	1.9f	0.9f
<b>Standard error</b>	0.24	0.20	0.21	0.19

\*Calculated as the mean number of shoots per explant. Data within a column followed by the same letter are not significantly different at  $p < 0.05$  using Duncan's new multiple range test.

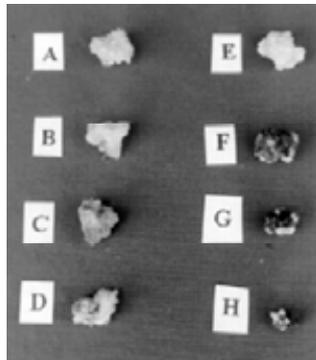
**Table 10.5.** Behaviour of F<sub>1</sub> hybrids and cultivars treated with culture filtrate, fusaric acid and *Fusarium subglutinans* pathogen under laboratory and greenhouse conditions

F <sub>1</sub> hybrid and cultivars	Necrotic spot area (mm <sup>2</sup> )		Disease Index
	Culture filtrate (80%)	Fusaric acid (0.75 mg/ml)	
'Spring' × 'Smooth Cayenne' no. 24	0.3c <sup>1</sup>	14.2	0c
'Perolera' × 'Smooth Cayenne' no. 52	1.2c	12.8	0c
'Perolera' × 'Smooth Cayenne' no. 56	0.2c	12.7	0c
'Perolera' × 'Smooth Cayenne' no. 58	0.7c	13.4	0c
'Perolera' × 'Smooth Cayenne' no. 60	0.6c	13.8	0c
'Smooth Cayenne' × 'Spring' no. 02	4.6b	12.1	3.9b
'Spring' × 'Smooth Cayenne' no. 08	8.7a	14.1	5.4a
'Spring' × 'Smooth Cayenne' no. 23	9.6a	13.6	6.0a
'Smooth Cayenne' × 'Perolera' no. 03	0.8c	14.5	0c
'Smooth Cayenne' × 'Perolera' no. 12	1.5c	13.7	0c
'Smooth Cayenne' × 'Perolera' no. 22	9.5a	12.6	6.0a
'Smooth Cayenne' × 'Perolera' no. 32	9.1a	12.3	5.5a
'Smooth Cayenne' × 'Perolera' no. 15	0.4c	14.5	0c
'Perolera'	1.2c	13.1	0c
'Spring'	1.8c	13.0	0c
'Smooth Cayenne'	8.8a	13.8	6.0a
'Perola'	9.4a	12.9	5.6a
<b>Standard deviation</b>	0.98	NS	0.46

<sup>1</sup>Data followed by the same letter within a column are not significantly different. The data were analysed by single analysis of variance (Duncan,  $p < 0.05$ ). NS: not significant.



**Figure 10.1.** Effect of culture filtrate (A) and fusaric acid (B) produced by *Fusarium subglutinans* on pineapple leaves of 'Smooth Cayenne' (◇), 'Perola' (□), 'Perolera' (○) and 'Spring' (△) cultivars under laboratory conditions. The data points represent the mean value of five leaf segments and the vertical bars the standard deviation.



**Figure 10.2.** Phytotoxic effects of different concentrations of culture filtrate from *Fusarium subglutinans* on pineapple calli. (A-D) Resistant cultivar 'Perolera'; (E-H) Susceptible cultivar 'Smooth Cayenne'. (A, E) No culture filtrate (control); (B, F) 10% culture filtrate; (C, G) 20% culture filtrate; (D, H) 30% culture filtrate.

## Chapter 11

### Mass-screening techniques of some tropical crops for resistance to anthracnose diseases using phytotoxic metabolites

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#### Abstract

Toxic metabolites, also known as chemopathogens, are one of the weapons used by pathogens to induce a disease condition in susceptible host plants. Many pathogens are known to produce toxins both *in vitro* and *in vivo*, and these toxins have been implicated in the development of disease symptoms in the host tissues. Among such pathogens are various *Colletotrichum* spp., the causal agents of anthracnose. Anthracnose is one of the most devastating diseases of many tropical crops such as cereals, legumes, tuber/root crops and fruits. *Colletotrichum* f. sp. *manihotis*, *C. gloeosporioides*, *C. lindemuthianum*, *C. truncatum* and *C. graminicola* are the causal agents of cassava, yam, cowpea, soybean and maize anthracnose, respectively, and produce toxic metabolites in culture that fluoresce between 254 and 366 nm. Disease symptoms induced on these crops by the extracted metabolites of the respective pathogens are similar to those induced by the pathogens during natural infections. Bioassays of the purified culture filtrate of these pathogens using the respective host plants produced different sizes of lesions. Results of the *in vitro* screening of these crops using toxic metabolites produced by the respective pathogens showed a positive correlation with field screenings based on natural epidemics. Toxic metabolites appear to be a more effective technique for screening crops for resistance to anthracnose and other diseases caused by toxigenic pathogens than screening methods based on natural disease infections.

#### INTRODUCTION

Diseases constitute a major set back in crop production worldwide and especially in the tropics. Diseases affect plants from the planting stage through harvesting, as well as during the storage of their produce. Sinclair (1988) reported that diseases accounted for an estimated loss of 7 million tons of agricultural produce worldwide. Anthracnose diseases of cassava and yam have resulted in an annual loss of 30-70% in both crops (Nwankiti, 1982; Ikotun and Hahn, 1991). The effect of plant diseases is not only on the market value of the produce but also on the availability of planting material. Hahn *et al.* (1989) reported that the most significant effect of cassava anthracnose disease is the reduction of healthy planting material available to the farmers.

Several methods have been employed to manage plant diseases in crops (Hahn *et al.*, 1988, 1989), but the most effective approach has been to breed and select for disease-resistant cultivars (Nwankiti *et al.*, 1987). Most of the existing techniques for selecting resistant cultivars include evaluation for disease incidence and severity in the field and in the greenhouse. However, these screening procedures are very cumbersome, time consuming and labour intensive. In addition, field screening requires the availability of large planting areas.

Symptoms produced by some pathogen species of *Colletotrichum*, *Curvularia*, *Helminthosporium*, *Pseudomonas*, *Cochliobolus*, *Alternaria* and *Mycosphaerella* include spots and blights with a limiting pathogen-free yellow halo in leaves (Byther and Steiner, 1972; Daub, 1982; Nwankiti, 1982; Adebitan, 1984; Theberge, 1985; Hartman *et al.*, 1986); dark-brown to black lesions in cotyledons (CIAT, 1980); symptoms like die-bark, canker and stalk rot in stems; and black canker in pods (Sharma and Sharma, 1969; Chandraskhanran and Ramakrishnan, 1973; Singh and Allen, 1979; Theberge, 1985; Singh and Rachie 1985). These symptoms suggest a role for toxic metabolites secreted by the pathogen during the development of the disease.

Toxic metabolites isolated from some species of *Colletotrichum* cause symptoms similar to those caused by the live pathogens. Such toxic metabolites include colletotin from *C. fuscum* (Goodman, 1960; Lewis and Goodman, 1962), colletotrichin and colletopyrone from *C. nicotianae* (Masatoshi *et al.*, 1976, 1978). Identified metabolites from other pathogens include piricularin from *Piricularia oryzae*, victorin from *Cochliobolus victoriae*, phaseolotoxin from *Pseudomonas syringae* pv. *phaseolicola*, cercosporin from *Cercospora* spp. and toxins from *Periconia circinata* and *Helminthosporium sacchari* (Samddar and Scheffer, 1968; Byther and Steiner, 1971, 1972; Strobel and Hapner, 1978; Daub, 1982).

The phytotoxic metabolites of most of these pathogens have been reported to play a significant role in pathogenesis (Chandraskhanran and Ramakrishnan, 1973; Walker and Templeton, 1978; Amusa 1991, Amusa *et al.*, 1993). Some of these metabolites, also known as pathotoxins, are chemopathogens of biological origin that can be used to replace the producing pathogens in studies to investigate the nature and the development of pathogenesis (Wheeler and Luke, 1963). This is because the pathotoxins play a causal role in the onset of diseases and produce symptoms characteristic of the disease in susceptible plants. Thus, phytotoxic metabolites have been used to screen crops for disease resistance (Wheeler and Luke, 1955; Hartman *et al.*, 1986; Amusa, *et al.*, 1994; Amusa, 1998, 2000).

This chapter reports the development of a method using toxic metabolites of *Colletotrichum* species that infect some economic crops in Africa. This method allows for rapid screening of disease resistance in cultivars of the host crops. It is believed that, if properly employed, this method could be used as a reliable tool for the rapid screening of large numbers of clones produced by plant breeders every year and also to predict possible reactions of plants to infections by pathogens.

## **MATERIAL AND METHODS**

### **Plant material**

Cowpea, soybean, maize, cassava and yam plants showing anthracnose symptoms were obtained from various demonstration plots at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Infected sorghum plants were obtained from a private garden located at the University of Ibadan. Stems and leaves of cassava and yam exhibiting symptoms of anthracnose were also collected from the Tuber Root Improvement (TRIP) experimental research field located at IITA.

The infected plant parts were excised, cut into 2 mm pieces and surface-sterilised with 10% sodium hypochloride for 30 s. They were then plated on Acidified Potato Dextrose Agar (APDA) and incubated for 6 days at 26°C.

The pathogens were identified using cultural, morphological, pathogenicity tests as well as comparing them with confirmed representatives of the different species. Further confirmation of the identity of the pathogens was undertaken by the International Mycological Institute, New Surrey, UK.

## **Toxin production in culture and extraction**

Two media were used for toxin production: modified Richard's medium (Sharma and Sharma, 1969) and Czapek-Dox medium (Lewis and Goodman, 1962). 100 ml of each of the media were dispensed into 250 ml Erlenmeyer flasks and autoclaved. A total of 10 flasks (250 ml Erlenmeyer) were used for each medium. Each flask was inoculated with three mycelial disks (diameter: 5 mm) cut from the margin of young growing colonies of the pathogen. Half of the flasks were incubated in a shaker at 100 rpm for 30 days at 26°C with a 12:12 hour photoperiod (Masatoshi *et al.*, 1976). The other half of the inoculated flasks was incubated statically for 30 days at 26°C with a 12:12 hour photoperiod. Three culture flasks were removed from the incubator every seven days and the contents were filtered using six layers of cheesecloth to remove the mycelial mat. The culture filtrates were adjusted to pH3 using 1 N HCl and were then extracted three times with a 1:1 (v/v) of ethylacetate for 48 hours. The pH of the extracted metabolites was readjusted to pH7 using 1 N NaOH after which they were dried out at 40°C under reduced pressure using a rotary evaporator. The extracts were then weighed, and the weight of extractable metabolites from each pathogen and culture medium was recorded.

## **Partial purification of the toxic metabolites**

The extracted phytotoxic metabolites of each pathogen were dissolved in a small quantity of ethylacetate and then spotted on preparative thin layer chromatography (TLC) on Merck silica gel 60 F254 plates (0.5 mm) that were pre-coated using ethylacetate, acetic acid and water (3:3:1) as the solvent system (Lewis and Goodman, 1962; Frantzen *et al.*, 1982). The phytotoxic components were detected on TLC plates under ultraviolet (UV) light at 254 and 366 nm. The zones that fluoresced under the UV light were marked and scraped from the plates, and eluted with 250 ml of ethylacetate. After evaporation of the ethylacetate, the resulting metabolites became an oily brownish semi-solid substance, which was then tested for bioactivity. Non-fluorescing zones were marked, scraped and subjected to the same test for bioactivity.

## **Detached leaf bioassay**

Cowpea, soybean, sorghum and maize plants were grown in 1.2 dm<sup>3</sup> pots for six weeks in the greenhouse. These plates were brought to the laboratory 12 hours before being used. Mature leaves were excised with a sterile razor blade at the petiole and then covered with wet sanitary cotton. 10 µl of the partially purified toxic metabolites were individually spotted on one half of the leaf. Sterile Richard's medium was spotted as a control on the other leaf half. A pin was used to prick through the centre of each spot to allow infiltration of the solutions. Treated leaves were placed in sterile Petri dishes and incubated in a high humidity chamber at 28°C and observed after 24 hours. Three leaves of each test plant were used for each bioassay, and replicated three times.

## **Seed bioassay**

3 ml of the partially purified toxic metabolites at different concentrations (100 µg/ml, 10 µg/ml, 1 µg/ml and 0.1 µg/ml) were poured onto sheets of sterile filter paper placed in Petri dishes. The solvent was allowed to evaporate completely by leaving the plate open for 12 hours, and then 1 ml of sterile distilled water was added to each dish. 20 seeds each of cowpea, soybean, maize, sorghum and millet were placed on the sterile filter papers impregnated with toxic metabolites. After incubation at 30°C for 24, 48, 72 and 96 hours under continuous light, the number of germinated seeds was counted. Control experiments were also conducted in the same manner but without the *Colletotrichum* toxic metabolites.

## **Seedling bioassay**

3 ml of the toxic *Colletotrichum* metabolites at concentrations of 100, 1 and 0.1 µg/ml were gently spotted onto filter papers placed in Petri dishes. After complete evaporation of the solvent, 1 ml of distilled water was poured into each of the Petri dishes. 10 seedlings each of cowpea, soybean and

maize and 24 of sorghum and millet were transferred onto the filter papers containing the toxic metabolites. After incubation at 30°C for 3 days under continuous light, the lengths of the hypocotyls and roots were measured. The seedlings used in these experiments were obtained by germinating seeds on wet filter paper at 30°C for a period of two (sorghum and millet) or four days (cowpea, maize and soybean).

### **Whole plant bioassay**

Test plants were grown on vermiculite for 21 days in a greenhouse. After carefully uprooting, test plants were placed in test tubes containing 10 ml of the toxic metabolites (100 µg/ml) of the respective *Colletotrichum* species pathogenic to the crop. This assay was replicated three times. In the control experiments, uninoculated sterile Richard's medium was used. The experiment continued for 24 hours and observations were made and recorded every 12 hours.

### ***In vitro* screening of legumes (cowpea and soybean) for anthracnose disease resistance**

14 cultivars of cowpea and soybean were screened with the toxic metabolites of *C. lindemuthianum* and *C. truncatum*. Screening for resistance to anthracnose and brown blotch in both cowpea and soybean was carried out using leaf, stem and pod puncture bioassay techniques. Eight week old cowpea and soybean leaves, stems and pods from the greenhouse showing no symptoms of infection were excised from the shoots and brought to the laboratory. They were rinsed in running tap water, dipped in 10% NaOCl for 30 s and then rinsed in five changes of sterile distilled water. The plant parts were individually placed in sterile Petri dishes lined with moistened sterile filter paper. Subsequently, three 10 µl droplets of the concentrated phytotoxic metabolites were placed on these plant parts, which were then punctured with sterile needles at the centre of each droplet to allow infiltration. Ten leaves, stems and pods were inoculated per cultivar of each crop type and these were replicated three times.

### ***In vitro* screening of maize (*Zea mays*) for anthracnose diseases using toxic metabolites**

To establish an *in vitro* screening method for anthracnose resistance in cereals, the maize inbred line 1787 obtained from IITA germplasm that is known to be susceptible to *C. graminicola*, i.e., the causative agent of anthracnose leaf blight (ALB) and anthracnose stalk rot (ASR), was used. When plants were 12 weeks old, the leaves were excised with razor blades, cut into 8 cm long pieces, and covered with wet sanitary cotton. 10 µl of the metabolites of the pathogen were spot inoculated on one half of each leaf piece. On the other half, spots of uninoculated Richard's medium were placed as a control. A sterile pin was used to prick through the centre of each spot. The treated leaves were placed in sterile, moist Petri dishes and incubated for 24 hours. Three leaves were inoculated per plant, a total of five plants were selected and these were replicated three times.

Subsequently, seven other maize inbred lines raised in pots in the greenhouse were used for the bioassay. Six week old maize leaves from the greenhouse showing no symptoms of infection were excised from the shoot and brought to the laboratory. The leaves were cut into 8 cm long pieces, rinsed in running tap water, dipped in 10% NaOCl for 30 s and then rinsed in five changes of sterile distilled water. The surface-sterilised maize leaves were individually placed in sterile Petri dishes lined with moistened sterile filter paper. Three 10 µl droplets of the concentrated phytotoxic metabolites were placed on these plant parts, which were then punctured with sterile needles at the centre of each droplet. Five leaves were inoculated per maize inbred line and these were replicated four times. This experiment was repeated three times.

### ***In vitro* screening of tuber root crops (cassava and yam cultivars) for disease resistance**

Screening for resistance in both cassava and yam clones was performed using leaf and stem puncture bioassay techniques. 45 clones of cassava and 24 clones of yam were evaluated using toxic metabolites of the pathogens. Stems and leaves from two month old plants of cassava and yam

showing no symptoms of infection were excised from the main shoots and taken to the laboratory. They were rinsed in running tap water, dipped in 10% NaOCl for 30 s, and then rinsed again in sterile distilled water. Cassava and yam stems were cut into pieces (10 cm). Excised leaves and stems were placed in sterile Petri dishes lined with moistened sterile filter papers. The partially purified phytotoxic metabolites (10 µg/ml) were spotted on the stem cuttings and excised leaves. A total of four droplets were placed on each leaf or stem. Five leaves and stems per clone were used, and these were replicated three times. The punctured leaf surfaces were encircled with petroleum jelly to avoid the spread of the metabolites and incubated for 12 hours. Inoculated stems were incubated for 10 days. Distilled water and uninoculated Richard's medium were used as controls.

### **Rating resistance and susceptibility levels in the *in vitro* toxic metabolite assays**

The reaction to the toxic metabolites was rated according to a modified lesion diameter scale (Goodie, 1958; Sudi and Podhardizky, 1959) to determine the level of resistance of the plant material to anthracnose. The diameter of the induced necrotic lesions was recorded and transformed to log values. The degree of resistance or susceptibility was determined using the following scale: <7.0 mm = highly resistant; 7.1-11.0 mm = resistant; 11.1-15.0 mm = moderately susceptible; and >15.1 mm = susceptible. The data were then subjected to analysis of variance (ANOVA) and the Duncan's multiple range (DMR) test to separate the means at  $p < 0.05$ .

### **Field screening of tuber root crops (cassava and yam cultivars)**

45 cassava and 24 yam clones were planted in 1996 and 1997 in the Tuber Root Improvement Programme (TRIP) experimental research field located at the IITA, Ibadan, Nigeria. The institute lies in the lowlands rain forest belt with a mean annual rainfall of 1000-1500 mm, and temperatures ranging around 23-32°C and 19-35°C during the wet (April to October) and dry (November to March) seasons, respectively. The IITA is located to the north of Ibadan at latitude of 7° 31' N and a longitude of 3° 45' E, and at an altitude of 210 m above sea level.

### **Cultivation of cassava germplasm**

Each cassava cultivar was planted in a 10 × 10 m sized plot and spaced at 1 × 1 m between and within rows. A cassava stem cutting ~12 cm in length was planted by partial burial of the stem on the ground, leaving about two nodes unburied. The design used was the completely randomised block design with four replicates for each cultivar, with each plot containing 100 plants. The plots were weeded manually three times during the course of the experiments.

All the cassava plants in each plot, except those at the edge, were assessed at 3, 6 and 9 months after planting (MAP) for the incidence and severity of cassava anthracnose disease (CAD). The cassava plants were examined from top to bottom for symptoms of CAD infection. The severity score was based on a scale of 1-5 (Ikotun and Hahn, 1991): 1 = no visible symptoms; 2 = development of shallow canker on the lower part of the plant; 3 = development of successive canker higher up the plant with the older canker becoming larger and deeper; 4 = development of a dark brown lesion on green shoot, petiole and leaves, young shoot and young leaves; 5 = death of part of or whole plant.

Disease scores for field tests were calculated as the mean value between the severity score as follows: < 2.0 = resistant; 2.0-3.0 = moderately susceptible; > 3.0 = susceptible. The data collected were subject to ANOVA, with inferences drawn from the analysed data using DMR tests.

### **Cultivation of yam germplasm**

Each yam cultivar was planted in a 10 × 10 m sized plot and spaced at 1 × 1 m between and within rows. In each plot, mounts were made and on each mount, a yam miniset was planted by burying it under the soil within the mount. The design used was the completely randomised block design with

four replicates for each cultivar, with each plot containing 100 plants. The plots were weeded manually three times during the course of the experiments.

All the yam plants in each plot, except those at the edge, were assessed at 3, and 6 MAP for the incidence and severity of yam anthracnose disease (YAD). The yam foliage and the stems were examined for symptoms of infection. The severity score were based on a scale of 1-5 (IITA, 1993): 1 = no symptoms (highly resistant); 2 = necrotic spots 1.00-2.00 mm in diameter (resistant); 3 = necrotic spots 2.01-3.00 mm in diameter (moderately susceptible); 4 = necrotic spots of 3.01-4.00 mm in diameter (susceptible); 5 = necrotic spots >4.00 mm in diameter (highly susceptible).

The results were pooled and subjected to ANOVA. Comparisons were made using the DMR test.

## RESULTS AND DISCUSSION

### Conditions for the production of toxic metabolites *in vitro*

Isolates of *C. truncatum*, *C. lindemuthianum*, *C. gloeosporioides*, *C. gloeosporioides* f. sp. *manihotis* and *C. graminicola* used in this study were tested for the production of toxic metabolites in both Richard's and Czapek-Dox media. *Colletotrichum* cultured in Richard's medium under constant shaking produced the highest concentrations of toxic metabolites, whereas cultures in this medium under still incubation ranked second in the production of toxic metabolites. Cultures in Czapek-Dox medium incubated with and without shaking ranked third and fourth, respectively, as shown in Table 11.1. *Colletotrichum* species have been previously reported to produce phytotoxic metabolites when cultured *in vitro* (Goodman, 1960; Sharma and Sharma, 1969; Masatoshi *et al.*, 1976). Richard's medium was found to support higher production of toxic metabolites than the Czapek-Dox medium. This might be connected to the nutrients present in the Richard's medium (Frantzen *et al.*, 1982). The presence of these nutrients, at the required concentration, is necessary for effective toxin production in culture (Amusa, 1991). Greater concentrations of toxic metabolites were produced when cultures were shaken. Shaking incubations provide suitable aeration to the culture medium, supporting growth of fungi and, as such, this condition has been well exploited for toxin production (Masatochi *et al.*, 1976, 1978; Frantzen *et al.*, 1982; Amusa, 1991).

When subjected to TLC chromatography, the bands containing the phytotoxic metabolites fluoresced under ultraviolet light at 254 and 366 nm. The toxic metabolites of *C. truncatum* and *C. lindemuthianum* had a band corresponding to a retention factor (RF) of 0.70-0.75; toxic metabolites of *C. gloeosporioides* and *C. gloeosporioides* f. sp. *manihotis* had a band at RF 0.65-0.70; the RF band obtained for *C. graminicola* was at 0.75-0.80. Metabolites from the non-fluorescent bands induced neither chlorotic nor necrotic lesions on test plants.

Toxic metabolites were produced in seven day old cultures of all *Colletotrichum* species whereas, 28 day old cultures were found to be more effective in inducing symptoms on susceptible hosts because they contained larger amounts of extractable metabolites of the pathogens (Figure 11.1A-C). There were no significant differences ( $p < 0.05$ ) between the amounts of the extractable metabolites obtained from 21 and 28 day old cultures. Phytotoxic metabolite activity detected in seven day old cultures suggests that the production of toxic metabolites probably commences early during infection and may play an important role in pathogenicity and expression of symptoms. The production of fluorescent compounds such as colletotrichin and colletopyrone produced by *C. nicotianae* (Goodman, 1960; Lewis and Goodman, 1962; Masatochi *et al.*, 1976, 1978), and collectotin, a glycopeptide from *C. fuscum*, has been previously reported (Goodman, 1960).

### Toxic activity of *Colletotrichum* metabolites

In order to assess the activity of the partially purified metabolites produced by the *Colletotrichum* species, tests with detached leaves and stems were conducted. The toxic metabolites induced necrotic

lesions of varying sizes on leaves and stems of susceptible hosts. Chlorotic lesions were induced and became visible on the leaves of test plants within 3 hours of inoculation. About 10 hours after inoculation, the chlorotic lesions become necrotic surrounded by a yellow halo (Figure 11.2). The toxic metabolites of these *Colletotrichum* species also affected the germination of the test crops as shown in Table 11.2. No germination was recorded when seeds were treated with 100 µg/ml of the metabolites, while at 10 µg/ml of the toxic metabolites, germination of 7-30% was recorded. The percentage of germination increased when lower concentrations of metabolites were used, suggesting that the toxic metabolites are responsible for the inhibition of seed germination and prevention of seedling growth. It is speculated that the phytotoxins produced by these *Colletotrichum* species have a lethal effect on the seeds and seedling tissues. *Colletotrichum* species are known to infect seeds and prevent germination and, in cases where germination occurs, the seedlings soon die.

Results of the seedling bioassay revealed that seedlings treated with a 100 µg/ml solution showed symptoms of blight disease and ceased to grow. Although the severity of symptoms caused by the metabolites decreased with increased dilutions, at a concentration of 0.1 µg/ml, there was a slight increase in the length of both roots and shoots of the cowpea and soybean seedlings as compared to controls without the phytotoxins (Figure 11.3A, B). These observations suggest increased seedling vigour at low concentrations of the phytotoxins for reasons not yet understood.

The effect of the *Colletotrichum* toxic metabolites (100 µg/ml) on the test crops was very rapid and could be observed already within 12 hours of exposure. The first observable symptom on these young test crops was the yellowing of the leaves, especially those at the base. This was followed by drooping and wilting of the leaves; the plants then collapsed and eventually died. At 100 µg/ml, the metabolites kill young plant shoots, perhaps by affecting the function of the vascular tissues. A high degree of correlation was seen between the response of cassava clones to natural infection by *C. gloeosporioides* f. sp. *manihotis* and to the toxic metabolites *in vitro* (Table 11.3).

#### ***In vitro* screening technique to determine resistance and susceptibility of legumes to anthracnose**

The metabolites of *C. truncatum* and *C. lindemuthianum* in culture were also phytotoxic to cowpea and soybean explants *in vitro*, and the symptoms produced by these metabolites were similar to those induced by the pathogens themselves. Leaf explants *in vitro* showed much larger lesions than stem cuttings and pods (Table 11.4). Due to the large variation in lesion sizes between these explants, the leaf lesion size was found to be more suitable for calculating the level of resistance/susceptibility of the cowpea cultivars. Among the 14 cowpea cultivars treated with *C. truncatum* phytotoxins, three cultivars ('IT82D-994', 'IT82D-699' and 'IT81D-773') exhibited induced necrotic lesions averaging between 11.6 and 13.9 mm in diameter in the leaves, and were considered to be moderately susceptible to the toxic metabolites. The remaining 11 cultivars had leaf lesions >15.1 mm and were considered to be susceptible. Mean lesion values between leaves, stems and pods of the cultivars 'IT82D-994', 'IT82D-699' and 'IT81D-773' were found to be statistically ( $p < 0.05$ ) lower than for the other cultivars (Table 11.4).

All 14 cowpea cultivars displayed large leaf necrotic lesions and were considered susceptible to the metabolites of *C. lindemuthianum*. Averages between leaf, stem and pods lesions suggest that the cultivars 'IT82D-994', 'IT81D-773', 'IT82D-699', 'TVU-3232', '848-2245-4', 'IT82D-60' and 'IFE BROWN' may be slightly less susceptible than the other cultivars; however, these findings were not statistically significant (Table 11.4).

The cowpea cultivars 'IT82D-994', 'IT82E-32' are reportedly susceptible to anthracnose and brown blotch diseases (Adebitan, 1991). This corresponds well to the results of *in vitro* tests in this study. Similarly, field and screen-house studies showed that 'IFE BROWN' and 'IT82D-60' are susceptible to *C. lindemuthianum* and *C. truncatum* (Emechebe and Soyinka, 1985), which also corresponds to the reaction of these two cowpea cultivars to the metabolites of the pathogens *in vitro*.

Explants of soybean treated with toxic metabolites showed less variance in the lesion sizes in leaves and pods; therefore, mean lesion values between these two types of explants were suitable to estimate level of resistance and susceptibility. 'PI-17144' and 'TGM236' had mean necrotic lesions of 5.3 and 10.3 mm, respectively. 'PI-17144' was considered highly resistant, and 'TGM236' was resistant to soybean anthracnose. The remaining 12 cultivars were found to be susceptible with mean lesions >15.1 mm (Table 11.5). The soybean cultivars 'PI-17144' and 'TGM236' have been reported to be resistant to anthracnose (IITA, 1987), whereas the reaction of the susceptible cultivars to the *in vitro* test with toxic metabolites of *C. truncatum* corresponded to the greenhouse and field-screening experiments (IITA, 1987). Therefore, the results of the present study show that *in vitro* screening for disease resistance using toxic metabolites obtained from *C. truncatum* and *C. lindemuthianum* is practicable and a good indicator of resistance to these pathogens. Also, the use of these metabolites allows for the rapid screening of a large population of crops and cultivars within a short period of time (24 hours), as also shown by Hell and Weber (1986).

### ***In vitro* screening technique to determine anthracnose resistance in maize and cereal crops**

Initial *in vitro* tests using the maize inbred line 1787, which is known to be susceptible to anthracnose, and the toxic metabolites of *C. graminicola* revealed the induction of necrotic lesions on leaves within 24 hours of inoculation. On the other hand, in control experiments, the leaves of line 1787 were inoculated without the toxic metabolites and did not show any symptoms.

Similar tests were then performed with six other maize inbred lines in comparison to 1787. Inoculation of lines 5057, 1787, 9030, 1201 and 5012 with the toxic metabolites of *C. graminicola* induced the development of necrotic lesions ranging from 18.8 to 22.6 mm in diameter (Table 11.6). These maize lines were rated as susceptible to the development of necrotic lesions caused by toxic metabolites of *C. graminicola*. On the other hand, Ku 1414 and 9450 exhibited necrotic lesions of 11.2 and 12.9 mm, respectively, and were considered moderately susceptible to the toxic metabolites of *C. graminicola* (Table 11.6). In 1988, IITA reported the high susceptibility of maize inbred lines 9030, 1787, 1201 and 5057 among others developed at the IITA to anthracnose stalk rot, while Ku 1414 and 9450 were found to be resistant to both stalk rot and leaf blight phases of the disease in the field and in greenhouse experiments (IITA, 1988). Based on the similar responses of these seven maize inbred lines to *in vitro* inoculation with toxic metabolites of *C. graminicola* and to the causal agents (i.e., live pathogens), we conclude that it is possible to screen for resistance to anthracnose disease in maize using a fast and reliable *in vitro* method with toxic metabolites of this pathogen. Likewise, toxic metabolites of some fungal pathogens have been used as a tool for rapid screening of a large number of cereals, such as rice, oats, sorghum and maize (Wheeler and Luke, 1955; Schertz and Tai, 1969; Byther and Steiner, 1971, 1972; Gengenbach *et al.*, 1977) for resistance against some fungal diseases of these cereal crops.

### **Tuber and root crops (*in vitro* screening technique)**

The 24 clones of yam (*Dioscorea* sp.) reacted differentially to the induction of necrotic lesions by the toxic metabolites of *C. gloeosporioides*. Based on these reactions, yam clones were categorised as susceptible, moderately susceptible and resistant (Table 11.7). Out of the 24 clones, only TDr750, TDr179 and TDr89/01750 were completely resistant. TDe, TDa87/01117 and TDr87/00211 were moderately susceptible, with necrotic lesions <15.0 mm. The remaining 18 clones exhibited varying degrees of susceptibility (Table 11.7). Yam clones TDr 89/01750, TDr 179 and TDr 750, with scores between 1.0 and 1.60, were regarded as resistant based on the field disease scores. The other 18 clones, with disease scores of between 2.00 and 3.62, were susceptible to varying degrees (Table 11.7). Moreover, there was a positive and significant correlation ( $r = 0.890$ ) between the reactions of the yam clones to natural infection in the field and to the toxic metabolites of the pathogen.

11 of the 45 clones of cassava (*Manihot esculenta*) treated with toxic metabolites of *C. gloeosporioides* f. sp. *manihotis* had necrotic lesions <7.0 mm and were considered to be highly

resistant (Table 11.8). Eight other clones were considered resistant, with lesion sizes ranging between 7.1 and 11.0 mm. Nine other clones were considered moderately susceptible, while 17 clones exhibited varying degrees of susceptibility (Table 11.8). Based on the field disease scores of the same 45 cassava clones, 17 clones were found to be resistant, 10 moderately susceptible and the remaining 17 clones were susceptible to varying degrees (Table 11.8). The resistant clones in the field included those that were rated resistant and highly resistant in the *in vitro* assay. Likewise, the 17 susceptible clones in the field were also found to be susceptible *in vitro*. Therefore, a positive and significant correlation existed between the results of the field and the *in vitro* screening tests of the 45 cassava clones (Table 11.8).

Taken together, the present results reveal a strong positive correlation between field disease score, based on natural infections, and necrotic lesion sizes induced on the plant tissue using toxic metabolites *in vitro*. Moreover, the disease reactions of these cassava and yam clones were similar to those obtained from several other field screenings using natural infection in previous studies (IITA, 1993). For instance, yam clones TDr89/01750 and TDr179 were reported as resistant to yam scorch (anthracnose), while TDa289 and TDa291 were regarded as very susceptible (IITA, 1993). These results correspond well with the results of this study. Similarly, in field and screen-house studies, the cassava clones Tms30211, Tms63391 and Tms30572 were found to be resistant to cassava anthracnose (Ikotun and Hahn, 1991), while 4(2)1443 was reported to be susceptible (IITA, 1993). Furthermore, the *in vitro* screening experiment was completed within 24 hours of inoculation. Hence, several thousand cassava and yam germplasms can be screened within a short period of time by this method.

In cases where toxins act as the sole determinants of the diseases in plants, knowledge of such toxins may be used to control the diseases. Quchi *et al.* (1989) described the detoxification of fusaric acid produced by *Fusarium* spp., that cause wilt of tomato plants, with the use of *Pseudomonas solanacearum* A-16 and *Cladosporium werneckii*. These two organisms produced detoxifying compounds and these, in combination with the organisms, have been used to protect young tomato plants against Fusarium wilt. Since then, this method has been useful in other crops for disease screening (Wheeler and Luke, 1955; Sudy and Podharszky, 1959; Rines and Luke, 1985; Hartman *et al.*, 1986; Amusa, 1991). The results of this experiment suggest that screening for disease resistance with toxic metabolites extracted from *Colletotrichum* spp. is feasible. This method also has the potential to be more efficient and less expensive than field screening.

## CONCLUSIONS

Effective field screenings using the causative pathogens of anthracnose disease take, on average, two to three years of activities, and depend on several environmental factors that may affect the pathogenicity level of the inoculum. Therefore, field screenings are not only very cumbersome and time consuming, but also highly variable.

The production of phytotoxic metabolites by the five *Colletotrichum* species in this study has given an insight into one of their modes of attack on plant tissues and, hence, could help breeders in breeding for resistance. Since there are variations in the mode of reaction of the host plant tissues to phytotoxic metabolites of the pathogens, the phytotoxin-tolerant host crops could serve as a marker in breeding for resistance. However, since the metabolites of the pathogen affect plant tissues directly, and resistance and susceptibility to diseases in plants is usually genetically controlled, environmental factors that normally affect conventional field-screening methods are totally eliminated. Positive and significant correlation was found to exist between the results of the field screening of the 45 cassava and 24 yam clones using natural infections and necrotic lesion sizes induced on the plant tissues using toxic metabolites of the pathogen. The *in vitro* screening experiment was completed within 36 hours of inoculation. Hence, several thousand cowpea, soybean, maize, cassava and yam germplasms can be screened within a short period of time by this method. The success of this experiment may, however, depend on the virulence of the strains of the pathogen used.

The future looks bright as new, more effective and safer management techniques find their way into plant disease management. Some of these new techniques actually involve the development of new cultivars with high and dependable levels of resistance to important diseases within a very short time. Traditional breeding techniques will continue to play a role in varietal development, but the use of new and rapid techniques of evaluation promise to accelerate the development of resistant cultivars. The chemical industry should devote more time and resources to producing some of these phytotoxins, which are environmentally friendly, highly dependable bio-control Material that will greatly improve our ability to manage some of the more important diseases affecting important crops.

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**Table 11.1.** Effect of media and incubation on the production of toxic metabolites by some species of *Colletotrichum*

Pathogen <i>Colletotrichum</i> spp.	Extractable metabolite (mg)		
	Medium	Shaking	Still
<i>C. graminicola</i>	Richard's	590	360
	Czapek Dox	298	200
<i>C. lindemuthianum</i>	Richard's	540	325
	Czapek Dox	285	210
<i>C. truncatum</i>	Richard's	390	275
	Czapek Dox	290	210
<i>C. gloeosporioides</i> f. sp. <i>manihotis</i>	Richard's	500	300
	Czapek Dox	260	205
<i>C. gloeosporioides</i>	Richard's	410	295
	Czapek Dox	235	190

The amount of toxic metabolite recorded above was obtained from a pool of 750 ml of each of the five pathogens. Source: Amusa (1991).

**Table 11.2.** Percentage of germination of cowpea, soybean, maize, sorghum and millet seeds with varying concentration of metabolites from *Colletotrichum* species

Test plant	Pathogen producing toxic metabolites	Germination (%)				
		Concentration of toxic metabolite (µg/ml)				
		100	10	1	0.1	Control
Cowpea (IT82-60)	<i>C. lindemuthianum</i>	0	10	60	100	100
Cowpea (IT82-60)	<i>C. truncatum</i>	0	7	50	95	100
Cowpea (TVx-2336)	<i>C. lindemuthianum</i>	0	8	58	100	100
Cowpea (TVx-2336)	<i>C. truncatum</i>	0	8	52	100	100
Soybean (Samsoy)	<i>C. truncatum</i>	0	10	49	93	100
Maize	<i>C. graminicola</i>	0	20	60	100	100
Sorghum	<i>C. graminicola</i>	0	38	68	100	100
Millet	<i>C. graminicola</i>	0	21	64	100	100

Source: Amusa (1991).

**Table 11.3.** Pearson correlation coefficient of the response of cassava clones to natural infection of *Colletotrichum gloeosporioides* f. sp. *manihotis* and to the toxic metabolites

	Field disease score	Necrotic lesion sizes on leaves ( <i>in vitro</i> )	Necrotic lesion sizes on stem ( <i>in vitro</i> )
Field disease score	-	0.9038	0.9586
Necrotic lesion sizes on leaves ( <i>in vitro</i> )		-	0.9053
Necrotic lesion sizes on stem ( <i>in vitro</i> )			-

Values are significant at  $p < 0.001$  ( $n = 45$ ). Source: Amusa (2000).

**Table 11.4.** Response of 14 cowpea cultivars to *in vitro* inoculation with phytotoxic metabolites of *Colletotrichum truncatum* and *C. lindemuthianum*

Cultivar	Mean size of necrotic lesion (mm)							
	<i>C. truncatum</i>				<i>C. lindemuthianum</i>			
	Leaves	Stems	Pods	$\bar{X}$	Leaves	Stems	Pods	$\bar{X}$
IT282E-16	22.2	16.2	14.7	17.7a	20.7	16.3	14.6	17.2a
TVU3236	21.7	16.0	13.4	17.0a	21.6	15.2	14.1	16.9ab
TVU-300	20.4	15.4	14.6	16.2ab	17.5	15.9	14.9	16.1bc
IT82E-32	19.2	15.4	14.4	16.3ab	15.9	15.5	14.6	15.0cd
TVU-1994	18.3	16.2	14.9	16.4ab	18.3	15.2	13.9	15.8cd
TVU-1990	18.2	15.5	14.4	16.2ab	18.5	14.1	12.8	15.3cd
IT81D-1137	16.2	15.7	13.8	15.2bc	16.9	16.9	14.1	15.3cd
IFE BROWN	16.5	16.4	14.7	15.9bc	18.6	15.6	13.9	14.7d
IT82D-60	16.4	15.9	14.7	15.7bc	18.2	15.3	13.9	14.5d
848-2245-4	16.6	14.7	13.7	15.0bc	16.2	14.4	14.1	14.2d
TVU-3232	15.4	14.5	13.4	14.4c	15.2	14.2	13.8	14.0d
IT82D-699	13.9	13.7	11.1	12.9d	17.2	14.9	13.1	14.0d
IT81D-773	13.5	13.0	11.5	12.7d	15.5	14.8	13.7	14.2d
IT82D-994	11.6	12.1	11.2	11.6d	15.7	14.2	13.3	13.7d

Each value is a mean of five replicates (5 measurements/plant) and is a transformation from the log<sub>e</sub> value used for the analysis. Mean values between leaves, stems and pods ( $\bar{X}$ ) followed by the same letters are not significant at  $p < 0.05$  by Duncan's multiple range test. Source: Amusa *et al.* (1994).

**Table 11.5.** Response of 14 soybean cultivars to *in vitro* inoculation with phytotoxic metabolites of *Colletotrichum truncatum*

Cultivars	Mean necrotic lesion sizes (mm)		
	Leaves	Pod	$\bar{X}$
536D	23.6	19.4	21.5a
932-2E	23.6	18.9	21.3a
TGX923D	22.9	18.8	20.9ab
SAMSOY	25.6	18.3	20.3ab
1614-1E	21.9	19.3	20.6ab
TGM-337	20.7	18.9	19.8bc
TGM-298	20.3	18.5	19.4bc
1-851D	19.2	16.7	18.2cd
TGM-297-2	18.1	17.2	17.7de
TGM-623	18.3	15.3	16.8def
TGX-536-02D	17.8	15.2	16.5ef
TGM-705	15.2	15.0	15.4f
TGM-236	10.6	10.1	10.3g
PI-17144	3.5	7.2	5.3h

Each value is a mean of five replicates (5 measurements/plant) and is a transformation from the log<sub>e</sub> value used for the analysis. Each value within the same column is a mean of three replicates (30 plants/clone). Mean values between leaves and pods ( $\bar{X}$ ) followed by the same letters are not significant at  $p < 0.05$  by Duncan's multiple range test. Source: Amusa *et al.* (1994).

**Table 11.6.** Response of seven maize inbred lines to *in vitro* inoculation with phytotoxic metabolites of *Colletotrichum graminicola*

Maize inbred lines	Mean necrotic size (mm) <sup>1</sup>
5057	22.6a
1787	22.1ab
9030	21.9b
1201	20.9b
5012	18.8c
9450	12.9d
Ku1414	11.0d

<sup>1</sup>Mean values followed by the same letters are not significant at  $p < 0.05$  by Duncan's multiple range test. Source: Amusa (1996).

**Table 11.7.** Response of 24 yam clones to *in vitro* inoculation with phytotoxic metabolites of *Colletotrichum gloeosporioides* in comparison with field screening

Clone	<i>In vitro</i> screening		Field screening	
	Necrotic lesion size on leaves	Disease score	Necrotic lesion size on leaves	Disease score
TDa87/01116	22.62a	S	3.61a	S
TDc289	22.49a	S	3.61a	S
TDa86/001115	22.49a	S	3.60a	S
TDa86/00258	22.45a	S	3.57a	S
TDa85/01093	22.11b	S	3.43a	S
TDa85/00272	20.11b	S	3.32ab	S
TDa85/00601	20.07b	S	3.19abc	S
TDr85/00272	19.91b	S	2.54cd	MS
TDr87/00203	18.87c	S	2.51cd	MS
TDa291	18.81c	S	2.39de	MS
TDr86/00057	18.56c	S	2.47de	MS
TDa87/00293	18.447c	S	2.16de	MS
TDa297	17.23d	S	2.26de	MS
TDa86/00057	17.01d	S	2.08ef	MS
TDr87/00340	16.79d	S	2.12e	MS
TDr293	15.77e	S	2.10e	MS
TDa294	15.35e	S	2.01ef	MS
TDe	15.08e	S	2.00ef	MS
TDa87/01117	12.58f	MS	1.60fg	R
TDr87/00211	12.58f	MS	1.58fg	R
TDa5	12.35f	MS	1.58fg	R
TDc750	9.86gh	R	1.54g	R
TDr179	8.75h	R	1.41g	R
TDr89/01750	8.59h	R	1.30g	R

Each value is a mean of five replicates (% measurement/plant), each is a transformation from the log<sub>e</sub> value used for the analysis. Each value within the same column is a mean of three replicates (30 – plants/clone). Mean value followed by the same letters are not significant at  $p < 0.05$  by Duncan's multiple range test. S = susceptible; MS = moderately susceptible; R = resistant. TDa = Tropical *Dioscorea alata*; TDr = Tropical *Dioscorea rotundata*; TDe = Tropical *Dioscorea esculenta*; TDc = Tropical *Dioscorea cayenensis*. Source: Amusa (2000).

**Table 11.8.** Response of 45 cassava clones to *in vitro* inoculation with phytotoxic metabolites of *Colletotrichum gloeosporioides* f. sp. *manihotis*

Cassava clone	<i>In vitro</i> screening				Field screening	
	Stem	Leaves	Average size	Disease score	Average size	Disease score
87/00004	24.98	21.47	23.22a	S	3.85ab	S
87/00038	23.33	20.89	22.11ab	S	3.72ab	S
87/00010	22.94	20.78	21.86ab	S	3.51ab	S
84/00065	23.90	19.68	21.79ab	S	3.51ab	S
87/00011	22.01	20.33	21.79ab	S	3.45ab	S
87/00028	21.27	19.51	20.39bcd	S	3.40ab	S
84/00460	20.17	20.55	20.36bcd	S	3.40ab	S
87/00052	20.64	18.47	19.55cde	S	3.38ab	S
83/01162	20.01	18.15	19.08def	S	3.36ab	S
84/00535	17.84	19.45	18.38def	S	3.38ab	S
87/00015	17.28	19.48	18.38ef	S	3.42ab	S
4(2)1443	23.41	13.09	18.23ef	S	3.35ab	S
87/00133	20.58	15.67	18.12ef	S	3.28ab	S
86/00079	16.81	17.84	17.33fg	S	3.18b	S
84/00083	15.85	16.91	16.38gh	S	3.12b	S
83/01438	16.17	16.00	16.08gh	S	3.04bc	S
87/00069	15.26	16.16	15.71ghij	S	3.00bc	S
86/00368	14.90	15.11	15.01hijk	MS	2.71bc	MS
85/00537	14.82	14.88	14.85hijklm	MS	2.64c	MS
80/02496	14.25	14.81	14.53ijklm	MS	2.51c	MS
CB/71693	14.34	13.54	13.94ijklm	MS	2.48c	MS
84/00115	13.76	13.73	13.47klm	MS	2.32c	MS
81/00065	12.81	13.76	13.29imn	NIS	2.45c	MS
83/01137	12.80	12.40	12.60mno	MS	2.34cd	MS
86/00133	12.91	11.49	12.20mnop	MS	3.21d	MS
87/00007	11.47	12.27	11.27nop	MS	2.25d	MS
82/01258	10.83	11.16	11.00opq	R	2.15d	MS
84/00182	10.69	10.65	10.67pq	R	2.04d	MS
84/00064	11.74	9.38	10.56pq	R	1.64d	R
87/00043	9.88	11.21	10.54q	R	1.61de	R
87/00050	8.57	11.17	9.87qr	R	1.55de	R
87/00072	8.41	10.17	9.24qrs	R	1.59de	R
87/00038	4.12	12.73	8.43rs	R	1.60de	R
30572	3.21	12.97	8.09t	R	1.61de	R
81/0006S	5.07	3.58	4.32t	HR	1.52de	R
63397	5.26	3.19	4.23t	HR	1.41e	R
87/00017	4.28	4.16	4.22t	HR	1.26e	R
87/00110	3.31	4.81	4.06t	HR	1.15c	R
87/00146	3.71	4.30	4.00t	HR	1.12e	R
86/00143	4.31	3.76	3.95t	HR	1.12de	R
89/00072	3.74	3.01	3.82t	HR	1.13de	R
CBRS-10-80411	3.42	3.99	3.7t	HR	1.12de	R
30211	3.03	4.03	3.67t	HR	1.14de	R
87/00072	4.21	2.90	3.56t	HR	1.12de	R
CB/81/00202	3.15	3.96	3.56t	HR	1.12de	R

Each value is a mean of five replicates (five measurements/plant) transformed to  $\log_e$  values. Each value within the same column is a mean of three replicates (30 plants/clone). Mean values followed by the same letters are not significant ( $p = 0.05$ ) by Duncan's multiple range test. S = susceptible; MS = moderately susceptible; R = resistant; HR = highly resistant. Source: Amusa (2000).

## Chapter 12

### Screening of peas for resistance to *Fusarium* wilt and root rot (*Fusarium oxysporum*, *Fusarium solani*)

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#### Abstract

In the introduction to this chapter, two fungal pathogens (*Fusarium solani* and *F. oxysporum*) are reviewed from the viewpoint of their incidence, importance, symptoms, mode of infection, harmfulness for peas, and known resistance resources. Further, this chapter gives detailed information about the screening of germplasm and plant breeding material, including the methods of pathogen isolation, cultivation and maintenance, preparation of plant material for screening and inoculum preparation. Three types of laboratory screening methods (tube tests, seed soaking test, root submersion test) are described. The results of testing are assessed via root and shoot symptom expression and further evaluated as a degree of infection. In *F. oxysporum* f. sp. *pisi*, six races are described, including the reaction to them on a set of differential genotypes. Finally, practical applications of these screening methods are summarised.

#### INTRODUCTION

*Fusarium* is an anamorph genus with worldwide distribution and a remarkable degree of diversity. Its species are common in soil and have been found to be important pathogens on a very wide range of agricultural crops (Zemánková and Lebeda, 2001). *F. solani* and *F. oxysporum* are both cosmopolitan species (Backhouse *et al.*, 2001). Root rot, caused by *F. solani* (Mart.) Sacc. is a serious problem for many plants, and is a troublesome and often serious disease in both dryland and irrigated areas (Kraft, 2000). Fusarium root rot of peas, caused by *F. solani* f. sp. *pisi*, was first reported as a serious pathogen in the USA (Bisby, 1918; Jones, 1923). This disease is distinct from Fusarium wilt, caused by *F. oxysporum* f. sp. *pisi*, but sometimes occurs in conjunction with other diseases of peas (Zaumeyer and Thomas, 1957). Fusarium wilts are among the most important diseases affecting grain legumes throughout the world (Allen and Lenné, 1998). Fusarium wilt diseases are caused by formae speciales of *F. oxysporum* which are pathogenic to selected genera or species of crop plants. Furthermore, Fusarium wilt pathogens are specialised in the process of systemic infection and colonisation of the plant vascular system (Jiménez-Díaz, 2000). *F. oxysporum* Schlecht. f. sp. *pisi* (Van Hall) Snyd. et Hans. causes vascular wilt of pea, because of its mycelium plugs in the tracheas. *F. solani* (Mart.) App. et Wr. causes root and stem rot followed by necrosis and death of leaves (Kraft, 1994). Both pathogens very often occur simultaneously. Pathogenic forms of *Fusarium* spp. penetrate a host root either through wounds or directly through root apices, and the fungus moves into the

vascular tissue. The pathogen is spread throughout the plant by means of mycelia growth or conidia, primarily microconidia, produced in infected xylem vessel elements. One of the earliest responses to *Fusarium* spp. infection is the deposition of additional wall callose material (papillae) within contact cells. The vascular plugging was found to seal off xylem elements of resistant pea cultivars in the same way as physical barriers (lignification) can retard or prevent vascular invasion (Kraft, 1994).

Breeding of grain legumes and peas for disease resistance is considered to be a basic prerequisite for improving and stabilising yield (Ranalli, 2003). The most prominent diseases are root fungal diseases; however, most of these cannot be controlled by chemicals. Some previous literature data concerning the variation in resistance/susceptibility of peas to *Fusarium* spp. have been summarised by several authors (e.g., Hagedorn, 1984; Jacobsen, 1992). Ali *et al.* (1994) summarised the available information on valuable sources of resistance to soil-borne root disease of peas. From this review, it is evident that sources of resistance in peas are rather limited. Most of these sources have been reported in cultivated forms and cultivars of *Pisum sativum*. Evaluations of current pea cultivars show that resistance to *Fusarium* root rot and vascular wilt is not very common (Hwang *et al.*, 1995; Jedryczka, 1995). Detailed data about sources of resistance to *Fusarium* diseases in wild *Pisum* species and accessions are not available. A set of 10 accessions of wild *P. sativum* subspecies and varieties was studied for resistance to *F. solani* and *F. oxysporum* under controlled inoculation (Lebeda and Švábová, 1997). The observation of visual symptoms on roots and stems showed substantial differences in the response among the *P. sativum* accessions under study. Complete resistance to both pathogens was not recorded. A very high level of resistance was observed in four accessions (W 1957, W 1824, W 1828, W 1872) (Lebeda and Švábová, 1997). Screening for resistance to *Fusarium* wilt race 2 of a *P. sativum* core collection (altogether 452 accessions, including two wild progenitors: *P. sativum* ssp. *abyssinicum* and *P. sativum* ssp. *elatius*) was undertaken in the USA (McPhee *et al.*, 1999). However, only 62 (14%) of the accessions were resistant. The resistant accessions included accessions from *P. sativum* ssp. *elatius* originating from 24 countries. Of the screened accessions, 39 were also resistant to race 1. One wild progenitor of *P. sativum* ssp. *elatius* (PI 344012) possessed resistance to races 1 and 2 (McPhee *et al.*, 1999).

Resistance to *Fusarium* wilt in peas is race-specific. There are some data available about the genetics of resistance to four US races (1, 2, 5 and 6), while the genetics of resistance to races 3 and 4 is not understood. The genetics of resistance to races 1, 2, 5 and 6 is conferred by different single dominant genes (Hagedorn, 1984; Muehlbauer, 1992) and is available in numerous germplasms (McPhee *et al.*, 1999). Grajal-Martin and Muehlbauer (1992) studied the link between the resistance genes for races 1 (*Fw*) and 2 (*Fnw*), and reported a recombination frequency of 46% indicating independent assortment. Recent advances in pea genetics and genetic mapping have been summarised by Jing *et al.* (2005) and Loridon *et al.* (2005).

## **MATERIAL AND METHODS**

### **Pathogen isolation, cultivation and maintenance**

The isolation of *Fusarium* spp. from soils and plant debris is carried out on various selective media, e.g., potato peptone agar (PPA), peptone-pentachloronitrobenzene (PCNB) or selective *Fusarium* agar (SFA) media (Brayford, 1993; Summerell *et al.*, 2003), containing antibiotics to prevent bacterial contamination. The Petri dishes and flasks with medium are sterilised by autoclaving and the hot content of the flask with agar medium is evenly poured into Petri dishes under sterile conditions (flow-box). After cooling and solidification, the Petri dishes can be packed, marked and stored in a refrigerator until further use. The samples of soils or plant debris with symptoms of fusarioses are placed onto the surface of selective media and further incubated in the dark at room temperature (20–22°C) for 8–10 days. The colonies of *Fusarium* spp. are further subcultured (purified) until uniform growth and expected mycelia phenotypes are attained. Pure cultures are placed on media for the induction of sporulation, e.g., carnation leaf agar (CLA) or potato dextrose agar (PDA) (Summerell *et al.*, 2003), Komada's medium (Dhingra and Sinclair, 1985), sucrose nutrient agar (SNA) or potato

sucrose agar (PSA) (Brayford, 1993), and checked under the microscope to determine the species. The isolates are then multiplied and maintained on various media in Petri dishes (mostly on Czapek-Dox Agar [CDA]) depending on how they will be used.

Cultivation of *F. oxysporum* and *F. solani* for fungal identification must be done on a nutrient-poor medium such as CLA (Nelson *et al.*, 1983). Optimal development of sporodochia and sporodochial macroconidia requires exposure of colonies, at least, to fluorescent light, and preferably also to near UV or black light. Culturing the fungus on media with high sugar levels (i.e., PDA, PSA) should be avoided as such media tend to promote mutations leading to degeneration of strains. Degenerated strains may show slower growth, abundance of aerial mycelium, reduced pigmentation, paucity of macroconidia (Jiménez-Díaz, 2000) or reduced (lost) pathogenicity. For long-term preservation of cultures, lyophilisation or storage in liquid nitrogen is recommended.

### **Preparation of plant material for screening**

Seeds are surface-sterilised with 96% ethanol for 30 s and subsequently for 20 minutes in 5-10% chloramine (Bochemie Ltd., Bohumín, Czech Republic), or by another chemical (hypochlorite) used for seed surface-sterilisation. The seeds are then rinsed three times in sterile deionised water and germinated aseptically in the dark at room temperature (20-22°C) in flasks on a layer of cellulose wadding, soaked with sterile water. Seedlings about eight days old, with well developed roots, are transplanted into glass tubes (for the tube test) containing CDA with Knop's solution (1 g of Ca(NO<sub>3</sub>)<sub>2</sub>, 0.25 g of KH<sub>2</sub>PO<sub>4</sub>, a trace amount of FeCl<sub>3</sub>, 1000 ml of H<sub>2</sub>O) (Lebeda and Buczkowski, 1986).

### **Inoculum preparation, inoculation and incubation**

#### *Tube test*

Isolates of *Fusarium* spp. are cultivated on the surface of CDA in glass tubes (diameter: 16 mm). Inoculated glass tubes are incubated in the dark at room temperature. A seven day old pathogen culture is used for resistance screening. Pre-germinated seeds are transferred to tubes with a fungal culture and ~10 ml of Knop's solution in sterile conditions (Johnston and Booth, 1983). The plants in tubes are cultivated for 21 days at a 20/22°C day/night temperature with a 12 hour photoperiod and an irradiance of 100 µmol/m<sup>2</sup>/s in a growth chamber. During the incubation, Knop's solution must be regularly supplemented into the tubes.

An adapted tube resistance screening test, according to Lebeda and Buczkowski (1986), was used for resistance screening of peas (Lebeda and Švábová, 1997). Isolates of *F. solani* and *F. oxysporum* were cultivated on the surface of CDA in glass tubes (diameter: 16 mm) in the dark at 23°C. Glass tubes with seven day old pathogen cultures were used for growing and screening pea seedlings. The control plants were grown in glass tubes with CDA and Knop's solution; however, without pathogen. At least 10 pea plants of one genotype were used for screening and disease evaluation (see Figure 12.6).

Whaley (1984) developed a similar test for rapid *in vitro* screening of peas for resistance to *F. solani* f. sp. *pisi*. Seeds are surface-disinfected and suspended in 0.1% water agar containing 1 × 10<sup>6</sup> conidia/ml. Seeds are germinated on moist filter paper until the plumules are 30 mm long, and the seedlings are transferred to the test tubes. Inoculation is conducted by filling the test tube with a conidial suspension. Peas are incubated in the inoculum for 14 days in a growth chamber at 24°C. The reaction of seedlings was evaluated according to the scale described below.

#### *Seed soaking test*

Another screening procedure was described by Kraft and Kaiser (1993). It involves soaking high vigour seed for 4 hours in a conidial suspension adjusted to 1 × 10<sup>6</sup> conidia/ml. Inoculated seeds are

then planted into perlite in plastic boxes and incubated in the growth chamber or greenhouse. The perlite is maintained in wet conditions throughout the two week incubation. The plants examined (tested) are scored for disease resistance. In addition, a technique of evaluating resistance of peas in artificially-infested soil has been developed (Kraft, 1975).

#### *Root submersion test*

Several screening methods have been described for evaluating pea seedlings for resistance to *F. oxysporum* f. sp. *pisi*. In general, these methods are based on the following steps: (1) uprooting of seedlings; (2) root washing and cutting; (3) submersion of roots in a spore suspension and cutting; and (4) transplantation of inoculated seedlings into a growing medium (Dixon and Doodson, 1970; Haglund, 1974; Kraft and Haglund, 1978). Haglund (1989) described a new submersion “tray method” of screening pea seedlings for resistance to *F. oxysporum* f. sp. *pisi*. This method involves growing pea seedlings on Styrofoam trays containing 72 cells filled with vermiculite (or agropelrite). Six seeds were planted in each cell for 16-20 days at 16-18°C until the peas were at the 4-5 node stage of development. At this time, the plants were removed from individual cells, around one-third of the root plugs were cut off, the remaining roots were submerged into the inoculum for ~5 s and the plugs were transplanted into 10 cm diameter plastic pots filled with vermiculite (or agropelrite). The reaction of the roots was evaluated ~21 days after transplanting using a 0-3 scale (see below) or a 0-4 scale modified from Vishnyakova (2000). The modified “tray method” of Haglund (1989) is also used in the laboratory of the authors of this chapter (Figures 12.1-12.5).

#### **Disease assessment**

In the case of the tube test, evaluation of the degree of infection (DI) could be carried out continuously at two-day intervals (until 21 days after inoculation); for other tests (seed soaking, root submersion), evaluation is performed at the end (21 days after inoculation). Infected and control plants are mostly grown in three replications (each replication has 10 plants). The symptoms on roots and shoots (stems and leaves) are assessed on a 0-3 scale (Lebeda and Buczkowski, 1986; Luhová *et al.*, 2002).

Roots 0-3 scale (Lebeda and Buczkowski, 1986; Luhová *et al.*, 2002): 0 = symptomless, roots free of any visual symptoms; 1 = limited occurrence of local necrosis, discolouration (browning) on the main and lateral roots; 2 = mild necrotisation and reduced development of main and lateral roots; 3 = severe necrotisation and growth depression of main root, no development of lateral roots, complete collapse of roots (Figures 12.6 and 12.7).

Roots 0-4 scale (Vishnyakova, 2000): 0 = symptomless or very weak symptoms ( $\leq 10\%$  of tissue is affected); 1 = weak symptoms (10-25% of tissue is affected); 2 = medium symptoms (26-50% of tissue is affected); 3 = strong symptoms (51-75% of tissue is affected); 4 = very strong symptoms ( $>75\%$  of tissue is affected).

Shoots (above ground): 0 = symptomless, stems and leaves free of any visual symptoms; 1 = limited growth depression and wilting, plant is more or less turgid; 2 = mild growth depression, wilting and chlorosis of leaves; 3 = severe wilting and chlorosis, complete collapse of plant (Lebeda and Buczkowski, 1986). The final degree of infection (DI = P) was expressed in percentage (0-100%) according to Townsend and Heuberger (1943) using the following formula:

$$P = \frac{\sum(n \times v)}{x \times N} \times 100$$

Where: P = the total degree of infection (DI); n = number of plants in each assessed category (infection degree); v = infection degree (0-4); x = scale range (in this case = 4) and N = total number of assessed plants.

## Determination of pathogenic variability

Altogether, six races of *F. oxysporum* f. sp. *pisi* have been reported until now (Haglund, 1974; Kraft, 2000). Race 1 was identified in the USA in 1924. Since then, a further five races have been recognised: race 2 in the Netherlands; races 3 and 3A in continental Europe and England; race 4 in central Canada (Haglund, 1984); races 5 and 6 in Washington State in the USA in the 1970s (Haglund and Kraft, 1970, 1979). Races 1 and 2 occur in all growing areas of the world, whereas races 5 and 6 pose a threat only in the western USA and Canada (Haglund and Anderson, 1987). The differentiation of the most important races is shown in Table 12.1.

The symptoms of races 1, 5 and 6 include discolouration of the roots and basal parts of stems, yellow to orange colour in vascular tissues, and progressive yellowing of lower leaves. Race 2 has similar symptoms, but moreover with curling of leaves and stipules, and yellowing of the leaves from the stem base to the top part of the plant (apex) (Haglund, 1984).

## CONCLUSIONS

Resistance screening methods described in this chapter have broad applications not only for resistance studies of peas (*P. sativum*) to *Fusarium* spp., but also for other crops as the methodology may be easily adapted. Resistance to *Fusarium* spp. is an important characteristic for the creation of germplasm core collections of pea genetic resources, as well as in breeding programmes focused on the improvement of resistance to root pathogens. Lines with confirmed resistance are used as standards during: (1) the selection process for determination of the resistance level in hybrid populations and their selection for production of resistant varieties; (2) testing of commercial cultivars from the List of Declared Cultivars that are regularly tested for resistance to the complex of root pathogens (including *Fusarium* spp.) in laboratory and field tests; and (3) determination of *F. oxysporum* f. sp. *pisi* races as a part of studies focused on virulence structure of pathogen populations using the differential set of pea lines (Table 12.1; Haglund, 1974; Kraft, 1994). The methods described here allow for the simultaneous screening of large sets of genotypes and are reproducible.

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**Table 12.1.** Reaction of *Fusarium oxysporum* f. sp. *pisii* races

<b>Line</b>	<b><i>F. oxysporum</i> f. sp. <i>pisii</i> races</b>			
	<b>1</b>	<b>2</b>	<b>5</b>	<b>6</b>
M 410	S	S	S	S
Vantage	R	S	S	S
Mini	S	R	S	S
Mini 93	R	R	S	S
Sundance II	R	S	R	S
Grant	R	S	S	R
WSU 23	R	R	R	S
WSU 28	R	S	R	R
74 SN 5	R	R	R	R

R = resistant; S = susceptible.



**Figure 12.1.** Reduction of the roots before inoculation with *Fusarium oxysporum* races 1 and 2 according to Haglund's method. *Photograph:* R. Dostálová.



**Figure 12.2.** Evaluation scale (0-4) of root symptoms for *Fusarium oxysporum* f. sp. *pisii*. *Photograph:* R. Dostálová.



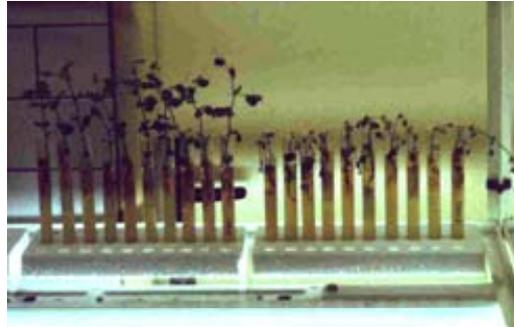
**Figure 12.3.** Detail of cv. 'Gotik' with a tolerant response (left), 'Terno' with complete collapse (middle) and 'Concorde' with a sensitive response (right). *Photograph:* R. Dostálová.



**Figure 12.4.** Control and inoculated pea plants – *Fusarium oxysporum* f. sp. *pisi* race 2. *Photograph:* R. Dostálová.



**Figure 12.5.** Resistance screening of pea plants in perlite and culture boxes, cultivation room. *Photograph:* R. Dostálová.



**Figure 12.6.** Tube test. Response of pea plants to *Fusarium oxysporum* f. sp. *pisii* (left: resistant response; right: susceptible response). *Photograph:* A. Lebeda.



**Figure 12.7.** Tube test – response of pea plants to *Fusarium solani* (from left to right: control, filtrate (10% [v/v]), inoculation by pathogen culture). *Photograph:* M. Griga.

## Chapter 13

### Mass-screening techniques for the early selection of disease resistance in chickpea (*Cicer arietinum*)

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#### Abstract

Host plant resistance offers the most sustainable and effective disease management option to combat diseases in chickpea. In this chapter, we briefly describe the distribution, economic importance and symptoms of the most important diseases of chickpea including *Ascochyta* blight, *Botrytis* grey mould, *Fusarium* wilt, dry root rot, collar rot, black root rot and stem rot. Screening methods developed by different research institutes to select germplasm and breeding lines resistant against these diseases are reviewed. A variety of field, greenhouse, growth room and laboratory screening techniques along with disease rating scale to facilitate the identification of resistant breeding material are discussed in detail. Coloured illustrations for each technique are provided as well as extensive lists of available resistant sources against all diseases. For the selection of sources of resistance to various diseases in chickpea, and for breeding high yielding cultivars with improved levels of resistance, this chapter describes current techniques for the simultaneous screening of a large number of chickpea plants.

#### INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important food legume crop of the semi-arid tropics, particularly in the rainfed ecology of the Indian subcontinent, the Mediterranean region, the West Asian and North African region (WANA), Eastern Africa and Latin America. In the recent past, this crop has experienced an export-driven expansion into new niches such as Australia and Canada. Globally, chickpea is cultivated on an area of about 11.12 million ha, adding 8.62 million tons of grains to the global food basket (FAO, 2005). As many as 45 countries grow chickpea but a dozen countries, viz., India, Turkey, Pakistan, Iran, Mexico, Myanmar, Ethiopia, Australia, Spain, Canada, Syria and Morocco, contribute 96% to global production. Chickpea is an important source of protein for millions of people; it especially provides essential amino acids for vegetarian populations and for those for whom meat is a scarce luxury. It is also important in cropping systems because of its ability to fix atmospheric nitrogen and to build sustainability in soil fertility.

Chickpea is attacked by 172 pathogens including 67 species of fungi, 3 bacterial species, 22 viruses and mycoplasma, and 80 species of nematodes (Haware, 1998). Some of the major diseases of global importance are: *Ascochyta* blight (*Ascochyta rabiei* [Pass.] Labr.), *Fusarium* wilt (*Fusarium oxysporum* Schlecht: Fries emend Syd. and Hans. f. sp. *ciceri* [Padwick] Matuo and Sato), *Botrytis* grey mould (*Botrytis cinerea* Pers. Ex Fr.), dry root rot (*Rhizoctonia bataticola* [Taub.] Butler), black root rot (*Fusarium solani* [Mart.] Sacc.), collar rot (*Sclerotium rolfsii* Sacc.), stem rot (*Sclerotinia sclerotiorum* [Lib.] de Bary) and foot rot (*Operculella padwickii* Kheswalla). The incorporation of

resistance against these diseases in host plants is the most economical and effective method of combating these diseases, and could be utilised in hybridisation programmes for the development of resistant cultivars for commercial cultivation of chickpea. While effective resistance to some of these diseases is available in some areas, adequate levels of resistance against all of these diseases is not available in the cultivated germplasm and breeding material of chickpea. However, high levels of resistance are available in wild *Cicer* species, and these can be identified as sources of useful resistance characters with the use of the reliable screening techniques described in this chapter.

Host plant resistance is the most economical, environmentally acceptable and sustainable means of controlling most biotic constraints, especially diseases. For exploitation of host plant resistance, the first step is to develop a reliable screening technique for large-scale screening of germplasm and breeding material. Techniques to screen chickpea germplasm and breeding material against various diseases have been developed by different research institutions, and are being used to screen for resistance in cultivated and wild *Cicer* species, and their segregating breeding material across the world.

Since the late 1970s, pathologists at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India, have focused their attention on developing techniques to screen germplasm and breeding material, to develop disease-resistant cultivars and to assist breeders. Most of these early approaches have been briefly reported (Reddy and Nene, 1978; Nene and Haware, 1980; Singh *et al.*, 1981). Procedural details of these techniques were reported comprehensively by Nene *et al.* (1981). In general, the field techniques are used for large-scale screenings of germplasm and breeding material, and greenhouse/laboratory techniques are used to confirm resistance identified in the field screening as well as to carry out inheritance and race identification studies, and studies on plant material for which supply is restricted or limited.

## **I. Ascochyta blight (*Ascochyta rabiei*)**

### **Distribution and economic importance**

Ascochyta blight (AB) of chickpea is the most devastating disease in many chickpea-growing areas of the world. It has been reported from 35 countries across six continents, i.e., Asia (Bangladesh, China, India, Iran, Iraq, Israel, Jordan, Lebanon, Pakistan, Syria and Turkey); Africa (Algeria, Egypt, Ethiopia, Kenya, Libya, Morocco, Sudan, Tanzania and Tunisia); Europe (Bulgaria, Cyprus, France, Greece, Hungary, Italy, Portugal, Romania, Spain and the Ukraine); North America (Canada and the USA); South America (Columbia and Mexico); and Australia (Nene *et al.*, 1996). The economic importance of the disease is evident from the frequent occurrence of epidemics in the past in several chickpea-growing areas of the world. More than 20 epidemics have been reported and most of these epidemics have occurred in Pakistan, India and European countries. The disease occurred in epidemic form in 1981-1983 in the north-western states of India and Pakistan, resulting in total loss of the crop (Singh *et al.*, 1982, 1984). The occurrence of severe epidemics of AB has also caused substantial losses in yield in the Mediterranean region (Hawtin and Singh, 1984). More than \$1 million US dollars of financial losses in the Palouse region of the USA were reported by Kaiser and Muehlbauer (1988). Elsewhere, the disease is currently the most important yield-limiting factor, potentially affecting 95% of the chickpea area in Australia (Knights and Siddique, 2002).

### **Symptoms of Ascochyta blight**

The initial symptoms appear as water soaked lesions on upper leaves. Later, these lesions become dark brown spots and spread rapidly on aerial parts of the plant: leaves, petioles, flowers, pods, branches and stem (Figure 13.1). Pycnidia are arranged in concentric rings in the lesions, which is the characteristic symptom of the disease. The spots on leaves and pods are circular, while on the stem and branches they are elongated. The apical twigs, branches and stem often show girdling, and the plant parts above the girdled portion are killed or break off even before drying. On the seed coat, dark

lesions are formed with pycnidia, which often lead to seed infection through testa as well as the cotyledons.

## **Methodology**

Different screening techniques have been developed and modified at various research centres for artificial resistance screening of chickpea germplasm against *A. rabiei* in controlled environment and field conditions.

### ***Pot culture***

Chickpea plants are grown in polythene pots (15 cm) in the greenhouse. The pots containing one month old test plants and susceptible controls are placed in an 8-10 cm deep circular pit. Water is added to the pots before inoculation. The plants are inoculated by spraying spore suspension ( $1 \times 10^5$  spores/ml) and are covered with a moist muslin cloth chamber. After 48 hours, cloth chambers are removed and the plants are kept wet during the day time by spraying water daily for up to 13 days from 10 am to 4 pm. Leaf wetness is maintained for 21 days to ensure maximum disease severity (Singh *et al.*, 1982).

### ***Controlled environment screening techniques (CEST)***

A controlled environment facility (CEF) with adjustable temperature, humidity and photoperiod was developed at ICRISAT for screening chickpea germplasm and breeding material for AB resistance (Figure 13.2). Several screening techniques were developed and standardised using the CEF and are explained below.

#### ***Whole plant screening technique (WPST)***

Chickpea seedlings are grown in plastic trays (35 × 25 × 8 cm) filled with a mixture of sterilised river sand and vermiculite (3:1) in a greenhouse for 10 days. The susceptible control 'Pb 7' is sown in each tray along with test entries (Figure 13.3A). Trays are transferred to the CEF, maintained at  $20 \pm 1^\circ\text{C}$  and ~1500 lux light intensity for 12 hours a day and allowed to acclimatise for 24 hours. The seedlings are inoculated by spraying a conidial suspension ( $5 \times 10^4$  conidia/ml) of *A. rabiei*. The conidia are produced on chickpea seed and harvested into sterile distilled water. After inoculation, the seedlings are allowed to partially dry for 30 minutes to avoid dislodging of spores, and 100% relative humidity (RH) is maintained for four days, and thereafter, 6-8 hours a day until the completion of the experiment (Pande *et al.*, 2005). Disease severity is scored on a 1-9 rating scale at 14 DAI (Haware *et al.*, 1995a).

#### ***Cut twig screening techniques (CTST)***

In comparison to the above method, the CTST is relatively simple and requires less time, resources and effort although it is ultimately less reliable since it does not evaluate effects on whole plants. Furthermore, test plants are used when damaged and this may induce chemical changes in the test material that could affect the results or perhaps exaggerate the effect of defence chemicals such as the phytoalexins, medicarpin and maackiain, that are known to be produced in chickpea (Stevenson *et al.*, 1997; Stevenson and Aslam, 2006). In this technique, the tender shoots of chickpea plants (30-60 days after sowing) are cut with a sharp edge blade in the evening and inoculated by keeping them alive in water and sand. Details of the technique are given below.

#### ***Cut twig screening technique in water (CTST-W)***

In the CTST-W, the lower portion of the detached twigs is wrapped with a cotton plug and transferred to a test tube (15 × 100 mm) containing fresh water. These tubes are placed in a test tube stand,

transferred to the CEF maintained at  $20 \pm 1^\circ\text{C}$  and  $\sim 1500$  lux light intensity for 12 hours a day, and allowed to acclimatise for 24 hours (Figure 13.3B). The seedlings are inoculated by spraying a conidial suspension ( $5 \times 10^4$  conidia/ml) of *A. rabiei*. The conidia are produced on chickpea seed and harvested into sterile distilled water. After inoculation, the seedlings are allowed to partially dry for 30 minutes to avoid dislodging of spores, and 100% relative RH is maintained for four days, and thereafter, 6-8 hours a day until the completion of the experiment. The disease symptoms appear on the susceptible control six days after inoculation. This method of screening is very useful in a wide hybridisation programme, where every plant may be valuable for other parameters. Resistant plants identified by this method can be further used in crosses and back crosses in the same crop season (Sharma *et al.*, 1995). Disease severity is scored on a 1-9 rating scale at 10 and 14 DAI (Pande *et al.*, 2005).

#### *Cut twig screening technique in sand (CTST-S)*

In this technique, instead of placing the detached twigs in tap water in test tubes, they are planted in sterilised moist sand filled in plastic trays ( $35 \times 25 \times 8$  cm) (Figure 13.3C). Twigs of susceptible cultivars along with test entries are also kept in each tray for comparison. Trays are transferred to the CEF, maintained at  $20 \pm 1^\circ\text{C}$ , allowed to acclimatise for 24 hours and inoculated following standard procedures as mentioned earlier. Disease severity is scored on a 1-9 rating scale at 10 and 14 DAI (Pande *et al.*, 2005).

#### *Detached leaf/leaflet technique*

The leaves of chickpea plants are detached from the test plants, sterilised with sodium hypochlorite solution (5%) or mercuric chloride (0.1%) and aseptically transferred to Petri dishes containing water agar. These leaves are inoculated by spraying spore suspension ( $1 \times 10^5$  spores/ml) of *A. rabiei*. The lids of the Petri dishes are sealed with paraffin wax and incubated at  $20 \pm 1^\circ\text{C}$  with a 12 hour photoperiod. The development of lesions is initiated in 2-3 days. Lesions become apparent in 4-6 days and the observations on disease development are recorded on day 8 (Singh and Sharma, 1998).

Leaflets from the most recent fully expanded leaves are collected from 15 day old chickpea plants. The detached leaflets are allowed to float; lower surface down on tap water inside 90 mm Petri dishes, and the upper surfaces of the leaflets are inoculated with 5  $\mu\text{l}$  of *A. rabiei*. The leaflets are incubated for 14 days at  $20 \pm 1^\circ\text{C}$ , with a 12 hour photoperiod. Disease severity scores are based on the number of leaflets infected and lesion size (Dolar *et al.*, 1994). The disease ratings are scored as described previously for whole plant screening methods.

#### **Field screening**

The field screening technique consists of planting test material in 3 to 5 m rows, spaced 40 cm apart in replicated trials. Indicator-*cum*-infecter rows of a highly susceptible variety ('L 550' or 'Pb 7' or 'ILC 1929') are planted after 4-8 rows, depending upon the material to be tested. At the flowering stage, plants are inoculated in the evening by spraying with a spore suspension ( $1 \times 10^5$  spores/ml). Inoculum is multiplied on potato dextrose broth or chickpea extract dextrose broth medium. High RH ( $>85\%$ ) is maintained by running a perfo-spray system for 10-15 minutes every hour from 10 am to 4 pm daily. There should be 100% mortality in susceptible material/control 15 days after inoculation. The observations are recorded 21 days after inoculation (Singh *et al.*, 1982; Reddy *et al.*, 1984).

Nene *et al.* (1981) gave a detailed account for developing screening techniques for chickpea germplasm against AB (Figure 13.4A). The procedures included:

- (1) Collecting debris of infected chickpea plants and storing it dry for use in the following season. For 1 ha, six bags ( $100 \times 75$  cm) should be sufficient.

- (2) Planting 2-4 rows of a susceptible cultivar ('ICC 460', 'Syrian Local' and 'Pb 7') all around the plot.
- (3) Planting test lines following normal agronomic operations. Ensure that a susceptible cultivar is planted after every 2-4 test rows. These rows will serve as indicator-cum-spreader rows.
- (4) Identifying the normal time of infection. In many countries, favourable (cool and wet) weather is common around flowering time. At such time, scatter the infected plant debris (step 1) all over the plot.
- (5) Arranging for a sprinkler irrigation system as a standby. This must be used if dry weather prevails at the normal time of infection.
- (6) If the disease development is not uniform, spraying spore suspension prepared either from infected plants from the field itself or from a pure culture of the fungus. For a 1 ha plot, prepare 25-40 flasks (250 ml) of inoculum; 150-250 l of diluted inoculum will be enough for 1 ha. (Prepare chickpea flour-dextrose broth by mixing 40 g of chickpea flour and 20 g of dextrose in 1000 ml of water. Pour 30 ml of broth into each flask. Autoclave at 15 lb for 20 minutes. Inoculate with a pure culture of *A. rabiei* and incubate for 10 days with 12 hours of light at 20-25°C. Dilute as indicated above).
- (7) Recording disease rating when the susceptible control lines show the maximum rating (9 on the 1-9 rating scale). Record the disease rating again when the crop is close to maturity.

Note: Closer spacing, with rows across the normal wind direction, enhances uniform disease development.

### ***Isolation plant propagator***

Nene *et al.* (1981) gave a detailed account of this technique for screening chickpea germplasm against AB (Figure 13.4B). The procedures included:

- (1) Using units of the isolation plant propagator (Burkard Manufacturing Co. Ltd., Rickmansworth, Herts, England).
- (2) Whenever necessary, operating evaporative coolers around the propagators to maintain temperatures below 30°C.
- (3) Arranging for artificial light (cool day light; eight 120 cm tubes, 40 W each) in the lower sections of the propagators.
- (4) Filling pots with autoclaved fine riverbed sand and vermiculite (3:1).
- (5) Growing at least 10 seedlings of one accession in one pot. In addition, raising seedlings of a susceptible control (ICC 460) in one pot in each subsection of the propagator.
- (6) Obtaining a pure culture of an aggressive isolate of *A. rabiei*. Prepare chickpea flour-dextrose broth (40 g chickpea flour and 20 g dextrose in 1000 ml distilled water) and place 30 ml in each of the 250 ml flasks. Autoclave at 15 lb for 20 minutes. Inoculate the medium with the fungus. Incubate at 20-25°C for 10 days with 12 hours of artificial light per day.
- (7) Removing fungal growth from the flasks and diluting with sterile distilled water to  $2 \times 10^4$  spores/ml.
- (8) Using a hand sprayer to spray inoculate on the two week old seedlings with the fungus inoculum. Cover plants with a plastic cover for 10 days.
- (9) Recording the incubation period, percentage infection and percentage mortality.
- (10) Scoring the disease severity on a 1-9 rating scale twice, once when the susceptible check shows a rating of 9, and again 10 days later.

### **Disease rating scale**

The rating scale for AB on chickpea seedlings is given in Table 13.1. Based on the disease score, the test lines are categorised for their reaction to AB infection as follows: 1 = immune (I); 1.1-3 =

resistant (R); 3.1-5 = moderately resistant (MR); 5.1-7 = susceptible (S); and 7.1-9 = highly susceptible (HS).

Test lines showing a rating of 1-3 are considered acceptable for the breeding programme; ratings of 3.1-5 are only acceptable if lines with a rating of 1-3 are not available; ratings of 5.1-9 are not acceptable.

### **Sources of resistance**

Several sources of resistance to AB have been identified in studies conducted in different chickpea-growing areas of the world (Table 13.2).

## **II. Botrytis grey mould (*Botrytis cinerea*)**

### **Distribution and economic importance**

Botrytis grey mould (BGM) of chickpea is an important disease in the South Asian countries, Bangladesh, Nepal, India and Pakistan. It has also been reported from Argentina, Australia, Canada, Columbia, Hungary, Mexico, Myanmar, Spain, Turkey, the USA and Vietnam. The grey mould appeared in epidemic form in 1968 in the chickpea crop of the sub-mountainous region of Uttar Pradesh, Terai area (Joshi and Singh, 1969), and again in 1981-1983 along with AB in north-western states of India, viz. Punjab, Haryana, Himachal Pradesh, Jammu and Kashmir, Uttar Pradesh, Rajasthan, parts of Bihar and West Bengal, where the crop was completely destroyed (Singh *et al.*, 1982; Garewal and Laha, 1983). This disease caused a 100% loss in yield in the Terai region of Nepal (Reddy *et al.*, 1988). Since the epidemic in the early 1980s, the disease has occurred regularly in a moderate to severe form depending upon the environmental conditions and has become a major production constraint where winters are characterised by cool, wet and foggy days.

### **Symptoms**

All aerial plant parts are attacked by this disease. Initial symptoms are water soaking and softening of affected plant parts, viz., leaf, flowers and tender shoots. On these plant parts, brown spots are produced, which are readily covered with dense fungal growth in the form of sporophores and mycelium (Figure 13.5). Plant parts covered by dense foliage and in wet conditions are heavily covered with sporophores. On stem, the grey mould symptoms are gradually replaced by dark grey to black sporodochia. When the relative humidity is low, irregular brown spots on leaves appear without any fungal growth. Occasionally, small and tiny black sclerotia are produced on dead tissues and on water soaked lesions on pods. Under congenial environmental conditions, all the flowers are attacked, resulting in the complete failure of the crop. The infected pods either do not produce any seeds or produce only small, shrivelled seeds (Haware and McDonald, 1992). The disease can appear at any time during plant growth, but maximum development of the disease is observed during the reproductive phase.

### **Methodology**

Techniques to screen chickpea germplasm and breeding material for BGM resistance have been developed by different research institutes. The screening techniques, viz., growth room and field are being used for screening germplasm and breeding material for BGM resistance.

#### ***Growth room screening technique***

This technique was developed at ICRISAT, Patancheru. Seedlings of test genotypes, along with the susceptible control 'H 208' are raised in 10 cm diameter plastic pots (5 seedlings/pot) filled with a

sand and vermiculite mixture (4:1) in a greenhouse (Figure 13.6). The pathogen, *B. cinerea*, is multiplied on potato dextrose broth and incubated at 25°C with a 12 hour photoperiod in a Percival incubator. 10 day old seedlings are transplanted to a plant growth room and inoculated with a 10 day old conidial suspension ( $5 \times 10^4$  conidia/ml) of the pathogen. The growth room is maintained at  $24 \pm 2^\circ\text{C}$  and 95-100% RH with a 12 hour photoperiod until the end of the experiment. Disease symptoms start appearing 24 hours after inoculation. BGM severity is scored on a 1-9 rating scale (1 = no infection and 9 = all plants killed) three days after inoculation and subsequently every alternate day for nine days (Pande *et al.*, 2002).

### ***Growth chamber screening technique***

This screening technique was developed by Punjab Agricultural University (PAU), Ludhiana, Punjab, India. The test lines are planted in polythene bags ( $15 \times 10$  cm) containing sandy-loam soil in a greenhouse. Test plants along with susceptible controls ('G 543' or 'H 208') are transferred, 25 days after sowing, to a growth chamber with controlled environmental conditions (approximately 20°C, >90% RH, and alternate light and dark periods). After watering the pots, plants are inoculated by spraying spore suspension ( $5 \times 10^4$  spores/ml) of *B. cinerea*, and are enclosed for six days within a moist chamber prepared with polythene sheets supported by iron frames ( $46 \times 46$  cm). During the incubation period, an 8:16 hour light: dark photoperiod is provided. The disease symptoms appear after 24 hours, and 100% mortality of susceptible lines and the control are recorded six days after inoculation (Singh *et al.*, 1982).

### ***Controlled environment screening techniques (CTST)***

Using the same CEF mentioned for AB, the following screening techniques have been developed for screening resistance against BGM.

#### ***Whole plant screening technique (WPST)***

This technique was developed at ICRISAT, Patancheru. Seedlings of the test material are grown in plastic trays ( $35 \times 25 \times 8$  cm) filled with a mixture of sterilised river sand and vermiculite (4:1) in a greenhouse for 10 days (Figure 13.7A). A susceptible control, 'JG 62'/'H 208', is sown as an indicator in each tray along with test entries. Trays are transferred to a CEF, maintained at  $15 \pm 1^\circ\text{C}$  and ~1500 lux light intensity for 12 hours a day, and allowed to acclimatise for 24 hours. The seedlings are inoculated by spraying a conidial suspension ( $3 \times 10^5$  conidia/ml) of *B. cinerea*. The conidia are produced on autoclaved marigold flowers and harvested into sterile distilled water. After inoculation, the seedlings are allowed to partially dry for 30 minutes to avoid dislodging of spores and 100% RH is maintained until the end of the experiment. Disease severity is scored on a 1-9 rating scale at 14 and 20 DAI.

#### ***Cut twig screening technique (CTST)***

The cut twig screening technique (CTST) proved very effective and efficient for screening breeding material derived from a wide range of hybridisation programmes, particularly for back crossing (Singh *et al.*, 1997, 1998), even though this technique may affect plant chemistry and, thus, resistance as mentioned above. Details of the techniques are given below.

#### **Cut twig screening technique in water (CTST-W)**

This technique involves cutting 10-15 cm long tender chickpea shoots in the evening with a sharp-edged blade. The lower portion of the single twigs are wrapped with a cotton plug and transferred to a test tube ( $15 \times 100$  cm) filled with water. The tubes are placed in a test tube stand and transferred to a CEF at  $15 \pm 1^\circ\text{C}$  and a 12 hour photoperiod one day prior to inoculation for acclimatisation (Figure 13.7B). Twigs are then spray inoculated with the inoculum ( $3 \times 10^5$  conidia/ml) of *B. cinerea*. Twigs

of a susceptible variety ('H 208', 'G 543' or 'L 550') are used as susceptible controls. Post-inoculation incubation conditions are similar to those used for the whole plant screening technique. Disease symptoms appear 24 hours after inoculation. Complete mortality is observed six days after inoculation in the susceptible lines (Singh *et al.*, 1998).

#### Cut twig screening technique in sand (CTST-S)

The procedure for screening BGM using CTST-S (trays) is similar to that described for AB CTST-S screening (Figure 13.7C). Inoculum concentration, inoculation method and post-inoculation incubation conditions are those described for the whole plant screening technique for BGM. Data on disease severity is recorded eight days after inoculation (Pande *et al.*, unpublished).

#### **Field screening technique**

This technique is efficient for large-scale screening of germplasm and breeding material in segregating generations. The test lines are sown in 2-3 m long rows spaced at 30 × 10 cm. Indicator-*cum*-infecter rows of the susceptible cultivar 'H 208' are sown after every two test rows. When the plants are 70-80 days old (at the onset of flowering), the field is irrigated in the morning and plants are inoculated by spraying a spore suspension (50000 spores m/l) of 10 day old culture of *B. cinerea*. From the morning of the following day, sprinkler irrigation or the perfo-spray system is run every day for about 15 minutes every 1 or 2 hours from 9 am to 5 pm depending upon the environmental conditions (Figure 13.8). The inoculation of plants is repeated twice at 10-day intervals after the first inoculation. The disease severity is scored on a 1-9 rating scale in mid-February, and in the first and last weeks of March (Pande *et al.*, 2002).

#### **Disease rating scale**

A 1-9 rating scale is used at ICRISAT for scoring BGM. The interpretation of the scale is given in Tables 13.3 and 13.4.

#### **Sources of resistance**

Host-plant resistance available to BGM infection in chickpea, determined by screening programmes conducted at various locations in India and other countries, is given in Table 13.5.

### **III. Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceris*)**

#### **Distribution and economic importance**

Fusarium wilt (FW) is a serious disease in many chickpea-growing parts of the world. It has been reported from 32 countries on six continents, i.e., Asia (Bangladesh, China, India, Iran, Iraq, Myanmar, Nepal, Pakistan, Sri Lanka, Syria and Turkey); Africa (Algeria, Egypt, Ethiopia, Kenya, Malawi, Morocco, Sudan, Tunisia, Uganda and Zambia); Europe (Hungary, Italy, Spain and the former USSR); North America (the USA); South America (Argentina, Chile, Columbia, Mexico and Peru); and Australia.

It is estimated to cause a 10-15% yield loss annually in India, but can result in 100% loss under specific conditions (Jalali and Chand, 1992). In Spain, a 12-15% loss due to wilt and root rot was reported (Trapero-Cases and Jimenez-Diaz, 1985). The production of chickpea in California has declined in recent years and FW is a major constraint in the production of kabuli chickpea in Mediterranean areas (Haware, 1990).

## Symptoms

The disease can affect the crop at any growth stage. The whole seedlings collapse and lie flat on the ground. Affected seedlings do not show rotting on the root. The actual affected plants show typical wilting, i.e., drooping of petiole, rachis and leaflets. The lower leaves are chlorotic, gradually turn yellow and then light brown or straw coloured (Figure 13.9A). Dried leaflets of the infected plants are not shed at maturity. When the stem of the infected plant is split open vertically, black discolouration of xylem vessels can be seen (Figure 13.9B). Sometimes, only a few branches are affected, resulting in partial wilt. Drooping of petioles and vascular discolouration are characteristic symptoms of the disease.

## Methodology

Chickpea wilt pathogen is mainly soil-borne and for successful management of the disease, it is important to exploit host plant resistance since control with fungicidal spray into the soil is impractical and good resistance is available to this pathogen. To identify sources of resistance and development of wilt-resistant genotypes, wilt sick plots are required. Techniques to develop these are described below along with techniques for screening against wilt.

### *Field screening*

Test lines are planted in 5 m long rows, 40 cm apart, in a wilt sick plot using a randomised block design. A susceptible control, 'JG 62' ('ICC 4951'), is planted after every two test rows (Figure 13.10A). Observations on seed germination are recorded 20 days after sowing and on wilt incidence after a one month interval and until harvesting. Detailed procedures for development of a wilt sick plot and identification of sources of resistance have been given by Nene *et al.* (1981, 1989). The procedures included:

- (1) Selecting a plot of adequate size and ensuring that it is isolated from other chickpea fields to avoid spread of the fungus inoculum from this plot to others. The plot should have been cropped in the previous year with chickpea, and at least traces of wilt incidence should have been observed.
- (2) Collecting as many wilted plants from other fields as possible, chopping them into small pieces and incorporating these uniformly into the surface soil of the plot.
- (3) Planting a sole crop of a highly susceptible cultivar ('JG 62/T-3') in this plot. Ensure a good plant population and carry out normal agronomic operations.
- (4) By the end of the season, at least 20% of the plants should show wilt symptoms. After harvesting and threshing, scatter the debris uniformly all over the plot and incorporate it by dicing. In addition, repeat step 2; this will help to increase the level of the inoculum to make the soil "sick".
- (5) Repeating steps 3 and 4 in the next season. By the end of this season, you should see >90% wilt incidence. If the incidence is <70%, repeat steps 3 and 4 once more.
- (6) Initiating screening in the next season. Plant a susceptible cultivar after every two test rows in the whole field. These rows will serve as controls, and will help in monitoring and maintaining the wilt sickness of the plot. The susceptible control rows should show >90% wilt.
- (7) From the 4th or 5th year onwards, plant a susceptible control in every fifth row. This will provide space for more breeding material and, at the same time, maintain the level of sickness.
- (8) Planting any other crop in this plot is not recommended.

It must be emphasised here that by following all of these steps, a sick plot in which *F. oxysporum* f. sp. *ciceris* will be the most predominant pathogen can be developed. However, the presence of other soil-borne pathogens cannot be avoided.

### **Pot culture**

Wilt resistant lines identified by the field screening technique are further tested in pot culture under controlled conditions to confirm resistance. The inoculum of the fungus is multiplied on sand-maize medium (9:1) in 250 ml flasks for 15 days at 25°C. Half of the content of the flask is mixed in a pot containing 2 kg of sterilised soil. The fungus is allowed to become established in the soil mixture in clay pots for four days and chickpea seeds are then planted in the infested soil. In a modification of this method, 20 day old seedlings are transplanted into the inoculated pots after dipping their roots in the spore suspension of *F. oxysporum* f. sp. *ciceris*. Observations on the percentage of plant mortality are recorded 15 and 45 days after inoculation (Singh *et al.*, 1987).

Nene *et al.* (1981) gave a detailed account for developing the pot screening technique and screening chickpea germplasm against wilt (Figure 13.10B):

- (1) Obtain a pure culture of *F. oxysporum* f. sp. *ciceris* from infected chickpeas in your area by following standard isolation procedures.
- (2) Prepare a sand-maize meal medium by placing 90 g of riverbed sand, 10 g of maize meal and 20 ml of distilled water in each 250 ml Erlenmeyer flask. Autoclave the medium in the flasks at 15 lb for 20 minutes. Inoculate each flask with a bit of fungal growth from tubes and incubate at 25°C for 15 days.
- (3) Prepare a fungus-soil mixture by hand-mixing contents of each flask with 2 kg of non-autoclaved field soil. The soil must come from a chickpea field where wilt normally occurs; for example, the field test plot (sick plot).
- (4) Fill large (diameter: 30 cm) earthen pots with the inoculated soil from step 3. Approximately 10 kg of soil will be required to fill each pot. Water the pots and wait for four days before proceeding to the next step.
- (5) Sow 40-50 seeds of a highly susceptible cultivar in each pot at a 2-3 cm depth. Water adequately and regularly. Most plants should show wilting after 10 days.
- (6) Remove healthy plants after 30 days. Chop and incorporate all the wilted plants into the soil.
- (7) Repeat steps 5 and 6 until 90% wilt is observed. These pots are then ready for screening.
- (8) Divide a pot into two sections. Plant 10 seeds of a test line in one section and 10 seeds of a susceptible control in the other.
- (9) These pots can be used for several successive screenings.

Note: Screening in pots requires up to only 60 days at any time of the year, compared with 4-6 months of the season in a sick plot.

### **Water culture**

Nene *et al.* (1981) gave a detailed account for developing this technique and screening chickpea germplasm against wilt (Figure 13.11):

- (1) Obtain a pure culture of *F. oxysporum* f. sp. *ciceris* from infected chickpeas in your area.
- (2) Sow 15 surface-sterilised (5 minutes in 2.5% sodium hypochlorite) seeds of the test as well as susceptible control lines in autoclaved riverbed sand placed in 15 cm pots. Use one pot for each line. Approximately 1 kg of sand will be required for each pot. Nurse the seedlings until these are required for transplanting.
- (3) Prepare potato dextrose broth (peeled and sliced potato: 200 g; dextrose: 20 g; distilled water: 1000 ml). Place 100 ml of broth in a 250 ml flask and prepare as many flasks as needed. One flask of inoculum will be sufficient for testing 18 lines (10 seedlings per line). Autoclave at 15 lb for 20 minutes.
- (4) Inoculate the medium (step 3) with a bit of the fungal growth from tubes. Incubate on a shaker (eight hours each day) at room temperature (25-30°C) for 10 days.

- (5) Dilute the entire contents of a flask with sterile distilled water to get a final inoculum dilution of 2.5% (~3.5 l of water is needed to attain the desired dilution of the contents of one flask). This will ensure approximately  $6.5 \times 10^5$  spores/ml.
- (6) Pour 20 ml of inoculum into each sterilised 150 × 15 mm glass tube (step 5).
- (7) Remove the 10 day old (from sowing) seedling from the sand (step 2). Wash the root system in running water and then rinse in sterile distilled water.
- (8) Transplant one seedling into each tube (step 6) and hold it in position with a cotton plug.
- (9) Add sterile distilled water to the tubes every two days to make up the loss.
- (10) Use 10 seedlings for each line. With each batch of test line, use seedlings of a susceptible line ('JG 62') as a control. In addition, keep a non-inoculated seedling as another control for each line.
- (11) Keep tubes in a specially designed box.
- (12) The susceptible control usually wilts in 7-10 days. Record data 15 days after inoculation (step 8). Non-inoculated seedlings should remain green for up to three weeks.

### ***Root-dip technique***

- (1) Obtain a culture of *F. oxysporum* f. sp. *ciceris* from infected chickpea plants and purify by single spore isolation.
- (2) Sow surface-sterilised seeds of the test plants and a susceptible control in sterilised polythene bags for eight days.
- (3) For multiplication of inoculum, a 7 mm disk of actively growing culture of *F. oxysporum* f. sp. *ciceris* is inoculated into 100 ml of potato dextrose broth in 250 ml flasks and incubated for five days at 25°C in a shaker at 125 rpm.
- (4) Dilute the entire contents of a flask with sterile distilled water to get a final inoculum concentration of  $6.5 \times 10^5$  conidia/ml for use as inoculum.
- (5) Eight day old seedlings grown in sterile sand from step 2 are uprooted, the lowermost portion of the roots cut and the roots dipped for 30 s in inoculum (step 4) (Figure 13.12).
- (6) Inoculated seedlings are transplanted to pre-irrigated vertisol and sand (4:1) in plastic pots (diameter: 15 cm) and the temperature is maintained at  $25 \pm 3^\circ\text{C}$  in a greenhouse. Inoculated seedlings are observed for mortality from 15 to 30 DAI (Nene *et al.*, 1981).
- (7) 15 seedlings of each accession are tested in three replications with five seedlings in each pot.

### **Disease rating scale**

The disease rating scale presented in Table 13.6 is used. Test lines showing a disease incidence of  $\leq 10\%$  are considered acceptable for the breeding programme, while test lines showing 10.1-20.0% are only considered if lines with  $\leq 10\%$  are not available.

### **Sources of resistance**

Seven races of *Fusarium* are known to cause wilt of chickpea. Races 1, 2, 3 and 4 were identified from India (Haware and Nene, 1982c); race 0 and 5 from Spain (Jimenez-Díaz *et al.*, 1989); and race 6 from California (Phillips, 1998). A large number of good sources of resistance have been identified (Haware *et al.*, 1992a, 1992b; Pundhir *et al.*, 1998; Dua *et al.*, 2001). The two most important sources of resistance are the germplasm line, 'WR 315' ('ICC 8933'), and the cultivar, 'JG 74'. The former is resistant to all races except race 3, while the latter is resistant to all races except race 2 (Haware, 1998). A twin-podded variety, 'JG 62', is highly susceptible to all races, except race 0, and widely used as a susceptible control for races 1 to 4 in India. The availability of a good source of resistance to FW and the availability of easy and effective field screening methods have made breeding for resistance to FW an easy task.

Using these techniques, over 150 wilt-resistant lines at ICRISAT and 100 lines at PAU, Ludhiana, India have been identified (Singh *et al.*, 1984, 1986, 1991; Nene, 1988; Haware *et al.*, 1990). Some of the sources of resistance to FW identified in different countries are listed in Table 13.7.

Resistance to wilt is more common in desi chickpea germplasm (Haware *et al.*, 1990). A large number of chickpea genotypes having a moderate to high level of resistance against wilt have been identified through multi-location testing in wilt sick plots under the All India Coordinated Pulses Improvement Project since 1985 (Table 13.8). Some of these have been utilised to develop wilt-resistant varieties.

#### **IV. Dry root rot (*Rhizoctonia bataticola*)**

##### **Distribution and economic importance**

Dry root rot (DRR) has been recorded in Egypt, Ethiopia, India, Iran, Kenya, Lebanon, Mexico, Myanmar, Pakistan, Spain, Sri Lanka, Sudan, Syria, Tanzania, Turkey, Uganda, the USA and Zambia (Nene *et al.*, 1996). It causes considerable yield losses that vary from 5% to >50% in badly infested fields. The disease has been reported from all the chickpea-growing areas of India, but is more serious in central and southern parts of the country, particularly in rainfed and poor soils.

##### **Symptoms**

The petioles and leaflets of the affected plants droop only at the top of the plant. The tap root turns black, shows sign of rotting, and is devoid of lateral and finer roots (Figure 13.13). A greyish mycelium can sometimes be seen on the tap root. The dead roots are quite brittle and show shredding of bark. The tip of the root is easily broken on touching. With the aid of a magnifying glass, minute sclerotia can be seen on the exposed wood of the root and inner side of the bark or whenever split open at the collar region vertically.

##### **Methodology**

The field and pot screening techniques described for FW are also applicable for screening against this disease. Nene *et al.* (1981) gave a detailed account for developing and screening chickpea germplasm against this disease. The steps mentioned for the development of a wilt sick plot also hold good for DRR. The only difference is to plant a cultivar that is susceptible to *R. bataticola*, but resistant to *F. oxysporum* f. sp. *ciceris*, i.e., 'BG 212' and 'ICC 229'. As pointed out in step 9 under wilt screening techniques, it will not be possible to have a sick plot only for *R. bataticola*. We can, however, encourage its multiplication to ensure its predominance over other soil-borne pathogens. To confirm resistance specifically to *R. bataticola*, it would be necessary to follow the blotter paper technique (see below).

##### **Blotter paper technique**

- (1) Obtain a pure culture of *R. bataticola* from infected chickpea plants in your area.
- (2) Sow 20 surface-sterilised (5 minutes in 2.5% sodium hypochlorite) seeds of the test lines in autoclaved riverbed sand placed in 15 cm pots. Use one pot for each line. Sow seeds of control lines as well. Approximately 1 kg of sand will be required for one pot. Nurse the seedlings until five days after sowing.
- (3) Prepare potato dextrose broth (peeled and sliced potato: 200 g; dextrose: 20 g; distilled water: 1000 ml). Place 100 ml of broth into one 250 ml flask and prepare as many flasks as needed. Autoclave at 15 lb for 20 minutes. One flask of inoculum will be needed to test 10 lines at once.
- (4) Inoculate the medium (step 3) with the fungus. Incubate for five days at 25°C.

- (5) Remove the mycelial mats from the flasks at the end of the incubation period. Add two mycelial mats to 100 ml of sterile distilled water and macerate these in a blender for 1 minute (operate the blender intermittently). Place this inoculum in a beaker of a suitable size.
- (6) Uproot the five day old seedlings of the test lines (step 2). Wash the root system in running water and rinse in sterile distilled water.
- (7) Hold all seedlings of a test line in your hand and dip the roots into the inoculum (step 5) with an up and downwards movement for about 30 s. Remove excess inoculum by touching the edge of the beaker.
- (8) Place 20 seedlings of the test line side by side on a blotter paper (size 45 × 25 cm with one fold; any colour; thin) so that only the cotyledons and roots are covered, and the green tops of the seedlings remain outside the blotter paper after it is folded. Fold the blotter paper and moisten it adequately but not excessively. One folded blotter paper will have seedlings of one test line (Figure 13.14).
- (9) Inoculate seedlings of a susceptible control ('BG 212') with each batch of test seedlings.
- (10) Keep the folded blotters, one on top of the other, in heaps of 10 in a tray. One of these 10 blotters should have the seedlings of the susceptible control (step 9).
- (11) Place the trays in an incubator at 35°C for eight days. Provide 12 hours of artificial light. Moisten the blotters adequately every day.
- (12) At the end of the incubation period (eight days), examine the seedlings for the extent of root damage, and score for the disease.

The DRR severity is recorded on a 1-9 rating scale as shown in Table 13.9.

### Sources of resistance

ICRISAT and NARS in India screened >10000 chickpea accessions and breeding lines in various segregating generations. These centres have identified several sources of resistance such as 'ICC 2867', '9023', '9032', '1003', '10803', '11550' and '11551', having combined resistance against DRR and FW (Nene *et al.*, 1989).

Several chickpea lines, viz., 'GG 588', '589', '609', 'GL 782', 'GF 734', 'ICC 4969', 'G 543', 'F 61', 'GL 84254', '85058', '86059', '86071', 'PPL 41', '41-1', '57', 'PGL 81-1', 'GG 763', '773' and '774', have been found to be resistant to root rot. These lines also had multiple disease resistance to wilt and foot rot, and some of them were also resistant to AB. The mechanisms responsible for this activity may well be the same as those for medicarpin and maackiain that have been found to be responsible for resistance to both AB (Daniel and Barz, 1990) and Fusarium wilt (Stevenson *et al.*, 1997). In addition, several wilt-resistant lines, viz., 'GL 84170', '84200', '84254', '85058', '86059', '86071', '86072', '90134', '90145', 'PPL 41', '41-1', '57', '146', '155', 'GG 773' and '774', were also found to be resistant to root rot (Singh *et al.*, 1987, 1991a, 1991b). Although the genotypes 'Kranti' ('ICCC 37'), 'Bharti' ('ICCV 10'), 'JG 130' and 'Sadbhawana' ('WCG 1') are tolerant of DRR disease in chickpea (Dhar *et al.*, 2004), stable sources of resistance to DRR have not yet been found and are eagerly being sought.

## V. Collar rot (*Sclerotium rolfsii*)

### Distribution and economic importance

Collar rot (CR) of chickpea is widespread in moist tropics and warm countries including Bangladesh, Colombia, Egypt, Ethiopia, India, Kenya, Mexico, Nepal, Pakistan, the Philippines, Sudan, Syria, Uganda and Zambia (Nene *et al.*, 1996).

## Symptoms

The disease usually occurs at the seedling stage, particularly in wet soil conditions. The affected plants turn yellow and show signs of rotting at the collar region (Figure 13.15A). Whitish mycelial strands can be seen on dried tap root (Figure 13.15B). On seedlings uprooted from wet soil in the early stages of infection, rapeseed-like sclerotia (diameter: 1 mm) can be observed. The disease is usually seen in patches in the field.

## Methodology

### *Greenhouse screening technique*

- (1) Isolate a culture of *S. rolfsii* from CR-infected chickpea plants following standard isolation procedures and purify by single sclerotial culture on potato dextrose agar (PDA).
- (2) For multiplication of *S. rolfsii*, prepare a groundnut (*Arachis hypogea*) shell medium (soak partially broken groundnut shells in water for two hours separately and autoclave at 121°C for 45 minutes). Inoculate each flask with a mycelial bit (1 cm<sup>2</sup>) from a 10 day old culture of *S. rolfsii* grown on PDA and incubate at 25 ± 1°C with a 12 hour photoperiod for 20 days.
- (3) The potting medium is prepared by mixing groundnut shell inoculum from step 2 with autoclaved soil (medium vertisol, pH7) at a rate of 100 g of inoculum per 4 kg of soil.
- (4) The potting medium from step 3 is filled in metal trays (70 × 30 × 16 cm).
- (5) 10 seeds of each cultivar are sown in infested soil in a completely randomised block design in three replications at 2-3 cm depths. Water adequately and regularly.
- (6) Observations are recorded on collar rot incidence 30 days after inoculation.
- (7) The temperature in the greenhouse should range between 28-30°C.

### Sources of resistance

Resistance in available germplasm seems to be scarce; however, tolerance has been reported (Gurha *et al.*, 1982). Germplasm accessions 'ICC 1696', 'ICC 4709' and 'ICC 14391' (S. D. Singh, personal communication); breeding lines 'RSG 130', '132' and '191' (Chitale *et al.*, 1990); and cultivar 'SAKI 9516' (Dua *et al.*, 2001) have shown low incidence of CR.

## VI. Black root rot (*Fusarium solani*)

### Distribution and economic importance

Black root rot (BRR) is a minor disease reported from Argentina, Chile, India, Mexico, Spain, Syria and the USA. However, losses are significant and there is a need to identify sources of resistance to this disease.

### Symptoms

The disease can occur at any stage but more often at the seedling stage. The affected plants turn yellow and wilt. Dead plants are seen scattered in the field. The root system is rotten, most of the finer roots are shed and the remaining roots turn black (Figure 13.16A). Affected plants dry prematurely but may go on producing new roots if sufficient moisture is available. Excessive moisture and moderately high temperatures (25-30°C) encourage disease development (Nene and Reddy, 1987).

## Methodology

### *Greenhouse screening*

- (1) *F. solani* isolated from chickpea roots is multiplied on potato dextrose broth (100 ml in a 250 ml flask) for seven days at 25°C in a shaker at 125 rpm.
- (2) Seedlings are raised in plastic pots (15 cm) in an autoclaved sand-soil (vertisol) mixture (1:1).
- (3) The inoculum is diluted by adding 100 ml of sterile water and mixed thoroughly.
- (4) For inoculation, about 3 cm of soil around the seedlings is removed and 5 ml of inoculum is poured near the collar region. The soil surface is re-levelled. The soil is kept moist before and after inoculation.
- (5) 25 days after inoculation, the seedlings are carefully removed from each pot and the soil is washed from the roots. Data is recorded on a 1-9 rating scale.

### *Root-dip technique*

The root-dip technique described for Fusarium wilt is also applicable for screening against this disease. The steps mentioned for growing the seedlings and inoculum preparation are the same. The only difference is that the culture used is *F. solani* and the disease score is recorded on a 1-9 rating scale based on root blackening.

The severity of BRR is recorded on a 1-9 rating scale as shown in Table 13.10.

## VII. Stem rot (*Sclerotinia sclerotiorum*)

### **Distribution and economic importance**

Stem rot (SR) of chickpea is a devastating disease in many chickpea-growing parts of the world. It has been reported from Algeria, Australia, Bangladesh, Chile, Egypt, Hungary, India, Iran and Morocco (Nene *et al.*, 1996).

It is particularly severe in the north-western states of India. It appeared in epidemic form and caused heavy damage in 1947-1948, 1948-1949, 1953-1954, 1977-1978, 1980-1981 and 1982-1983, and the crop was completely damaged in some of the chickpea-growing areas (Singh and Singh, 1979; Singh *et al.*, 1989). It again appeared in a severe form in 1986-1987 and 1987-1988 (Singh *et al.*, 1989).

### **Symptoms**

The disease rarely attacks younger plants. It normally attacks adult plants near flowering and podding stages, when they produce a dense canopy and the soil remains wet for a longer period due to rain. The characteristic symptom of the disease is the production of a web of white mycelial strands at the collar region and above (Figure 13.16B). Black irregular sclerotial bodies can be seen mingled with mycelial strands. Sometimes, lesions and fungal growth can be seen on aerial parts of the plant. This is due to aerial infection by the pathogen. The leaves of affected plants turn yellow, dry up and turn straw coloured.

### **Methodology**

Several workers have tried different screening techniques using various types of inocula such as SR sick pots, stem base inoculation with colonised chickpea seeds, stem pieces, carrot slices, PDA disks, sclerotia and ascospores. The technique consists of inoculating chickpea plants with any of the inocula mentioned above and maintaining high moisture/RH in a greenhouse or covering with polythene bags or *dasuti* cloth chambers for about 144 hours at a temperature of around 20-25°C. Fresh inoculum

(four days old) is more virulent than older samples. The screening technique consists of inoculation of soil with 14 day old chickpea meal medium (4:1) in the ratio of 1:16 in polythene bags (75 × 10 cm). The seeds of test lines are planted in these pots in a greenhouse.

Chickpea plants, 25-30 days old, are placed outside the greenhouse in 8 cm deep circular pits. Water is added in and around the pit, and plants are covered with moist *dasuti* cloth chambers for 10 days. High moisture/RH is maintained by daily spraying the chamber with water from 10 am to 4 pm. There is substantial production of apothecia on the base of the plants that release ascospores inside the chamber and rapidly cause secondary infection. The disease can effectively be produced with the application of ascospores in place of sick pots as described above (Kapila and Singh, 1995).

### Sources of resistance

In view of the nature of the disease, it is difficult to identify sources of resistance. Under natural epiphytotic conditions, 'GL 84102', 'GL 88223', 'GLK 88114' and 'GF 89-75' were moderately resistant to SR, in addition to a few breeding lines which escaped attack by the disease (Singh *et al.*, 1987). Among 11 accessions of eight *Cicer* species screened, *Cicer judaicum* ('ILWC 19-2'), *C. reticulatum* ('JM 200'), *C. pinnatifidum* ('199') and *C. yamashitae* ('210') were found to be moderately resistant to SR.

### CONCLUSIONS

Cultivation of disease-resistant chickpea cultivars is the most effective method of disease control. Host plant resistance provides economical, environmentally acceptable and sustainable disease management. For identification and development of sources of host plant resistance in the available germplasm and breeding material, reliable and reproducible screening techniques are essential. Techniques to screen chickpea germplasm and breeding material against various chickpea diseases have been developed by different research institutions and are described here. However, there is a need to correlate field, greenhouse, growth room and laboratory screening techniques for disease resistance in chickpea.

Although chickpea research workers have tried to generate adequate information on chickpea diseases and their management, efficient and complete control measures are not yet available for use by farmers poor in resources in South Asia and elsewhere. Substantial progress has been made in the development of screening techniques to identify sources of resistance, but there has been little success, especially in identifying high levels of resistance in cultivated *Cicer* species. However, high and stable levels of resistance have been identified in wild *Cicer* species. It is expected that chickpea disease-resistant and high yielding cultivars endowed with other desirable characteristics will be made available to farmers in the near future. Meanwhile, high priority should be given to participating in farm validation of the available components (including moderate levels of host plant resistance with high yield) of disease management and their integration.

### REFERENCES TO CHAPTER 13

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**Table 13.1.** Disease rating scale for Ascochyta blight

<b>Rating</b>	<b>Symptoms</b>
1	No symptoms
2	Minute lesions prominent on the apical stem
3	Lesions up to 5 mm in size and slight drooping of the apical stem
4	Lesions obvious on all plant parts and clear drooping of apical stem
5	Lesions obvious on all plants parts; defoliation initiated; breaking and drying of branches slight to moderate
6	Lesions as in 5; defoliation; broken, dry branches common; some plants killed
7	Lesions as in 5; defoliation; broken, dry branches very common; up to 25% of the plants killed
8	Symptoms as in 7 but up to 50% of the plants killed
9	Symptoms as in 7 but up to 100% of the plants killed

**Table 13.2.** Sources of resistance to Ascochyta blight in chickpea germplasm

Genotype	Remarks	Reference
ICC 3634, ICC 4200, ICC 4248, ICC 5124, ICC 6981, ILC 196, ILC 3346, ILC 3956 and ILC 4421		Reddy and Singh (1984)
ILC 72, ILC 191, ILC 3279 and ILC 3856	Resistant in eight chickpea-growing countries (including India, Pakistan and the Mediterranean region)	Singh <i>et al.</i> (1984)
ICC 76, ICC 187, ICC 607, ICC 1121, ICC 1136, ICC 1416, ICC 1754, ICC 1762, ICC 1903, ICC 7773, ILC 236, ILC 482, ILC 484, ILC 2548 and ILC 2956	ILC 482 and ICC 1903 were always rated 1 on a 1-9 scale	Katiyar and Sood (1985)
ICC 4000 and 4014	Both foliage and pods of ICC 4000 were resistant to Ascochyta blight	Singh and Kapoor (1985)
ILC 3864, ILC 3870 and ILC 4421		Pal and Singh (1990)
ILC 190, ILC 201, ILC 202, ILC 2506, ILC 3856, ILC 5928, ICC 3996 and FLIP 83-48	Resistant to 3 to 6 races of <i>A. rabiei</i>	Singh and Reddy (1990)
ILC 5586, ILC 5894, ILC 5926, ILC 6482, ILC 7795, ICC 4475, ICC 6328 and ICC 12004	Resistant both in greenhouse and field	Singh and Reddy (1992)
ILC 3287	Rate-reducing phenomenon of Ascochyta blight observed	Reddy and Singh (1993)
CG 715, ACC 76, H 86-8, H 86-100 and HK 86-120		Singh and Pal (1993)
ILC 3896, ICC 7514, NEC 123, P 1279-2 and P 4268-1		Gaur and Singh (1996)
ICC 8161		Shukla and Pandya (1988)
ICC 1278, ICC 1284, ICC 1285 and ICC 1304		Wadud and Riaz (1988)
FLIP 92-262C, FLIP 92-110C and FLIP 92-154C		Toker <i>et al.</i> (1999)

**Table 13.3.** Description of different parameters of the Botrytis grey mould scoring scale

Rating	Leaf (%)	Stem and branches	Flower (%)	Pod (%)
1	No infection	No infection	No infection	No infection
2	Up to 1	No infection	Up to 1	Up to 1
3	2-5	No infection	2-5	2-5
4	6-15	Small lesion on few branches	6-15	6-15
5	16-25	Moderate lesions on stem and branches	16-25	16-25
6	26-40 and defoliation is common	Expanding lesions on stem and branches	26-40	26-40
7	41-60 and defoliation is very common	Large lesions; stem girdling and drying of branches	41-60	41-60
8	61-80 and nearly complete defoliation	Very large lesions; stem girdling; extensive drying of branches	61-80	61-80
9	81-100 and complete defoliation	Complete drying of stem and branches	81-100	81-100

**Table 13.4.** Rating scale of Botrytis grey mould of chickpea for field and controlled environment screening

Rating	Field screening	Controlled environment screening
1	No infection on any part of the plant	No infection on any part of the plant
2	Minute lesions on lower leaves, flowers and pods covered under dense plant canopy; usually not visible	Minute water soaked lesions on emerging tender leaves; usually not seen
3	Lesions on <5% of the leaves, flowers and pods covered under dense plant canopy	Minute water soaked lesions on 1-5% of emerging and uppermost tender leaves; usually seen after careful examination
4	Lesions and some fungal growth (conidiophores and conidia) can be seen on up to 15% of the leaves, flowers, pods and branches covered under dense plant canopy	Water soaked lesions on 6-10% of uppermost tender leaves and tender shoots
5	Lesions and slight fungal growth on up to 25% of the leaves, flowers, pods, stems and branches covered under dense plant canopy	Water soaked lesions; soft rotting of 11-25% of tender leaves and shoots
6	Lesions and fungal growth on up to 40% of the leaves, flowers, pods, stems and branches; defoliation; 25% of the plants killed	Water soaked lesions; soft rotting of 26-40% of top leaves and shoots
7	Large lesions and good fungal growth on up to 60% of the leaves, flowers, pods, stem and branches; defoliation common; drying of branches; 50% of the plants killed	Soft rotting; fungal growth on 41-55% of the leaves and branches
8	Large lesions and profuse fungal growth on up to 80% of the leaves, flowers, pods, stems and branches; severe defoliation; drying of branches; 75% of the plants killed	Soft rotting; fungal growth on 56-70% of the leaves, branches and stems
9	Large lesions; very profuse fungal growth on up to 100% of the flowers, pods, stems and branches; almost complete defoliation; drying of plants; 100% of the plants killed	Extensive soft rotting; fungal growth on >70% of the leaves, branches and stems

**Table 13.5.** Sources of resistance to Botrytis grey mould resistance in chickpea

<b>Resistance</b>	<b>Genotype</b>	<b>Reference</b>
Wild species	ILWC 35/S-1 ( <i>C. echinospermum</i> ) and ILWC 9/S-1 ( <i>C. pinnatifidum</i> )	Singh <i>et al.</i> (1991a)
	<i>C. judaicum</i> 182, <i>C. judaicum</i> ILWC 19-2, <i>C. pinnatifidum</i> 188, <i>C. pinnatifidum</i> 189, <i>C. pinnatifidum</i> 199, <i>C. pinnatifidum</i> ILWC 9/S-1, <i>C. bijugum</i> ILWC 9/S-1, <i>C. bijugum</i> ILWC 7/S-1, <i>C. echinospermum</i> ILWC 35/S-1 and <i>C. echinospermum</i> ILWC 39	Singh <i>et al.</i> (1998)
Land races	GPC 14, HIMA and P 6223 ICC 1069, 6250, 7574 and 10302	Singh and Kant (1999) Rathi <i>et al.</i> (1984a; 1984b)
	ICC 466, ICC 478, ICC 662, ICC 755, ICC 756, ICC 799, ICC 800, ICC 1069, ICC 1591, ICC 7574, ICC 10302, ICCL 87322	Tripathi and Rathi (2000)
	GL 84212 and ICC 1905 GNG-3, C-235 and BG-249 P 919, CPI 56566, JM 995 and E 100 Y	Singh and Kaur (1989) Pandey <i>et al.</i> (1982) Singh and Kapoor (1985)

**Table 13.6.** Disease rating scale for Fusarium wilt

<b>Disease incidence (%)</b>	<b>Disease reaction</b>
0-10	Resistant
10.1-20.0	Moderately resistant
20.1-40.0	Moderately susceptible
40.1-100	Susceptible

**Table 13.7.** Sources of resistance to *Fusarium* wilt in chickpea germplasm

Genotype	Remarks	Reference
GL 84170, 84200, 84254, 85058, 86059, 86071, 86072, 90134, 90145, PPL 41, 41-1, 57, 146, 155, GG 773 and 774	Moderately resistant in India	Singh <i>et al.</i> (1991b)
Avarodhi (ICC 14344), BG 246, ICC 32, ICC 42	Resistant at several locations in India in multi-location testing	Nene <i>et al.</i> (1989)
Kabuli cultivars (Surutato 77, Senora, UC 15 and UC 27)	Resistant in Mexico and California	Haware <i>et al.</i> (1990)
FLIP 84-43 (ILC 5411, FLIP 85-20, FLIP 85-29C, ILC 127, 219, 237, 267 and 513	Highly resistant in Spain	Jimenez-Diaz <i>et al.</i> (1989)
IC 10149, ICC 9023, ICC 11550, ICC 902, ICC 42, ICCV 10, Phule G 95007, KWR 108, GPF 2, IPC 99-13, IPC 99-1, IPC 2000-14, IPC 2000-41, IPC 99-10, IPC 2000-18, IPC 2000-52, IPCK 9-3, CPS 1, WR 315, JG 74, JG 1265, GL 8834, GL 87079, GL 91061, GL 86123, H 86-72, H 86-18, KPG 259-4	Resistant source reported from India against FW	Gaur and Chaturvedi (2004)
Accessions of several wild species including <i>C. bijugum</i> , <i>C. echinospermum</i> , <i>C. judaicum</i> and <i>C. pinnatifidum</i>	Resistant	Haware <i>et al.</i> (1992a)

**Table 13.8.** Chickpea genotypes with a moderate to high level of resistance against *Fusarium oxysporum* f. sp. *ciceris*

Chickpea type	Genotype
Desi	Avarodhi (ICC 14344), BG 209, 246, 313, 307, BGM 418, 419, 432, 436, 439, 443, 444, 451, GF 89-36, GL 86114, 86123, GNG 468, 469, H 82-2, 83-18, 83-23, 83-60, 83-84, 84-8, 85-65, 85-124, 86-8, 86-18, 86-20, 86-21, 86-39, 86-42, 86-72, 86-73, 86-91, 86-100, 86-142, 86-143, 86-156, 86-158, 86-170, 88-123, 89-50, JG 315, 317, 322, KPG 63, 142-1, 143-1, PDG 83-39, 84-10, 84-14, 85-7, 85-18, Phule G 87207
Kabuli	ICCC 32, ICC 42, BG 287

Source: Consolidated Report on Rabi Pulses, Plant Pathology, AICRP, Kanpur (1985-1997).

**Table 13.9.** Rating scale for dry root rot disease

<b>Rating</b>	<b>Observation</b>	<b>Disease reaction</b>
1	No infection on roots	Immune
>1 and <3	Very few small lesions on roots	Resistant
>3 and <5	Lesions on roots clear but small; new roots free from infection	Moderately resistant
>5 and <7	Lesions on roots; many new roots generally free from lesions	Susceptible
>7	Roots infected and completely discoloured	Highly susceptible

**Table 13.10.** Rating scale for black root rot disease

<b>Disease rating</b>	<b>Symptoms</b>
1	Plant healthy; no root infection
3	Plant healthy; slight infection in hypocotyl region along with restricted lesions on few roots
5	Plant stunted; black root rotting on 50% of roots
7	Plant stunted accompanied by yellowing of leaves; 75% of roots affected
9	Plants with severe stunting and yellowing of leaves; completely rotted roots



**Figure 13.1.** Ascochyta blight lesions on stems (A), leaves (B) and pods (C).



**Figure 13.2.** Controlled environment facility at ICRISAT, Patancheru, Andhra Pradesh, India.



**Figure 13.3.** Controlled environment screening techniques. (A) Whole plant screening technique; (B) Cut twig screening technique in water; (C) Cut twig screening technique in sand.



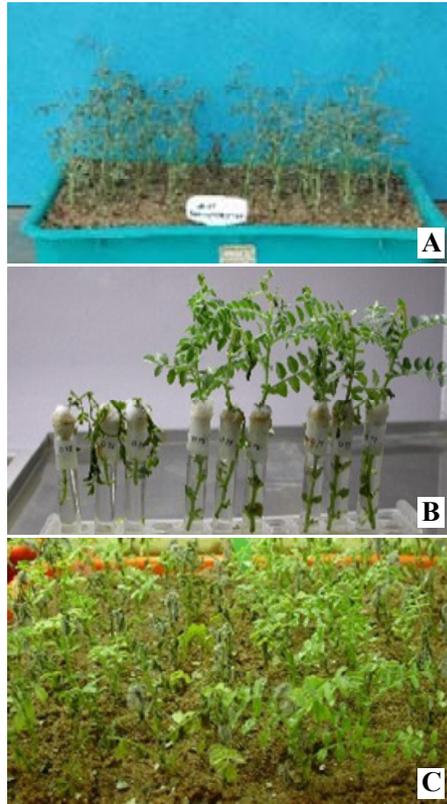
**Figure 13.4.** Field screening (A) and plant propagator screening technique (B) for *Ascochyta* blight.



**Figure 13.5.** *Botrytis* grey mould on flowers, twigs (A) and pods (B).



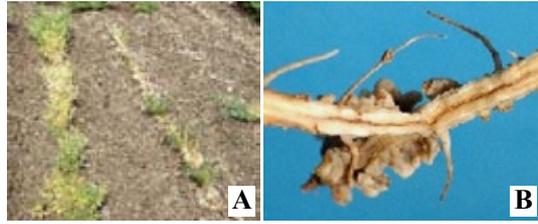
**Figure 13.6.** Growth room screening technique (pots) for *Botrytis* grey mould.



**Figure 13.7.** Controlled environment screening techniques at ICRISAT, Patancheru 502 324, Andhra Pradesh, India. (A) Whole plant screening technique; (B) Cut twig screening technique in water; (C) Cut twig screening technique in sand.



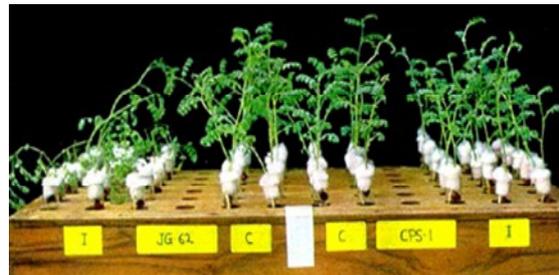
**Figure 13.8.** Field screening technique using the perforated irrigation system for Botrytis grey mould.



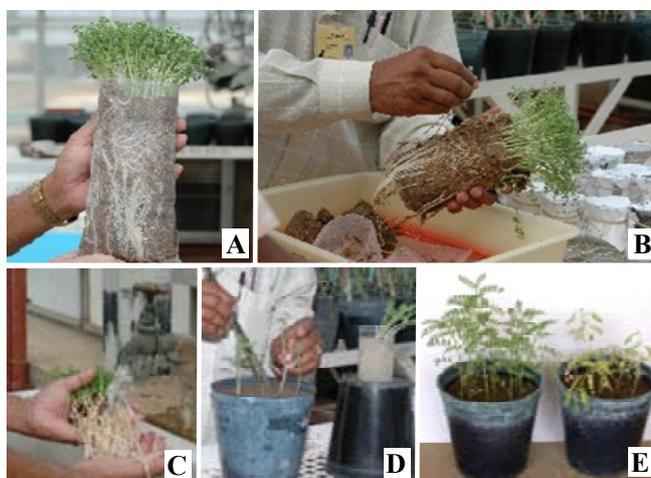
**Figure 13.9.** Fusarium wilt. (A) Cowpea plants killed by fusariosis; (B) Internal browning of the root.



**Figure 13.10.** Screening for tolerance to Fusarium wilt in the field (A) and pot (B).



**Figure 13.11.** Water culture technique for Fusarium wilt.



**Figure 13.12.** Root-dip technique for *Fusarium* wilt screening. Eight day old seedlings are grown in sterile sand (A); uprooted (B); washed and the lowermost portion of the roots cut (C); roots are dipped for 30 s in the *Fusarium* inoculum and transplanted into plastic pots (diameter: 15 cm) containing pre-irrigated vertisol and sand (4:1 [v/v]) and maintained at  $25 \pm 3^\circ\text{C}$  in a greenhouse (D). Inoculated seedlings are observed for mortality from 15 to 30 days after inoculation (DAI) (E).



**Figure 13.13.** Dry root rot symptoms in detail.



**Figure 13.14.** Blotter paper technique for dry root rot screening.



**Figure 13.15.** Collar rot. (A) Field symptoms; (B) White mycelial growth at the collar region.



**Figure 13.16.** Symptoms of black root rot (A) and *Sclerotinia* stem rot (B).



## Chapter 14

### Improving tolerance to *Fusarium oxysporum* f. sp. *melonis* in melon using tissue culture and mutation techniques

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#### Abstract

Fusarium wilt is a vascular disease of the Cucurbitaceae family, especially in muskmelon (*Cucumis melo* L.), caused by the soil fungus *Fusarium oxysporum* f. sp. *melonis* (FOM). This pathogen persists in the soil for extended periods of time, and the only effective control is the use of resistant cultivars. During the last three decades, tissue culture techniques have been utilised in crop improvement to generate changes in the genetic material of plants via *in vitro* somaclonal variations (by organogenesis or somatic embryogenesis) and induced mutagenesis. More recently, researchers have been using *in vitro* techniques to investigate the effects of fungal culture filtrates or toxins on susceptible and resistant genotypes of different plant species or cultivars to assess disease resistance. This method is effectively used for cucumber and melon. There are various *in vitro* culture techniques that can be used for cucumber (Malepszy, 1988). In this chapter, we show a method for mass-selection of melon mutants resistant to Fusarium wilt. *In vitro* selection of resistant cells, from both irradiated and non-irradiated explants, is performed using culture filtrates of different FOM races. This research could lead to the development of new melon cultivars resistant to Fusarium wilt.

#### INTRODUCTION

Fusarium wilt is a vascular disease of the Cucurbitaceae family caused by the soil fungus FOM, which is detrimental to muskmelons (*C. melo* L.). Fusarium wilt of melon is prevalent in temperate and tropical regions and is a problem worldwide. FOM can survive in the soil for extended periods of time as chlamydospores, and is capable of colonising crop residues and the roots of most crops grown in rotation with melon. The only effective control is the use of resistant cultivars. Four races of FOM have been identified, namely 0, 1, 2 and 1.2 (Risser *et al.*, 1976; Mas *et al.*, 1981). Race 1.2 has been further subdivided into races 1.2y and 1.2w, which cause yellowing and wilt symptoms, respectively. Two resistance genes (*Fom-1* and *Fom-2*) have been identified in melons (Mas *et al.*, 1981; Martyn and Gordon, 1996; Joobeur *et al.*, 2004). *Fom-1* confers resistance to FOM races 0 and 2, and *Fom-2* confers resistance to races 0 and 1. These two genes are extensively used in breeding programmes, which can be assisted by marker assisted selection using markers linked to these resistance genes

(Wang *et al.*, 2000; Zheng and Wolff, 2000; Burger *et al.*, 2003). No genes have been identified that confer resistance to race 1.2 (Zink and Thomas, 1990; Wechter *et al.*, 1995). However, polygenic recessive genes have been found to confer resistance to race 1.2 in Piboule genotypes (Messiaen *et al.*, 1962; Risser *et al.*, 1976). Table 14.1 shows some sources of resistance to FOM among melon genotypes.

Turkey is the second largest world producer of cantaloupes and other melons, behind China only. In 2004, Turkey produced 1700000 tons and accounted for 6.1% of the worldwide production of melons (FAO, 2005), but its production is declining year after year because of Fusarium wilt. Therefore, combating Fusarium wilt in the cultivation of melons is of high economic importance for Turkey. In some parts of Turkey, the prevalent races of this pathogen have been determined. Fantino and Zengin (1974) isolated race 1.2 from wilted plants showing intensive root rot in Eastern Thrace. In the Aegean region, Yildiz (1977) recovered three races of the pathogen, race 1 being the most common (57%), followed by race 1.2 (35%) and race 0 (6%). Yücel *et al.* (1994) obtained races 0, and 1.2 in the East Mediterranean region. Erzurum *et al.* (1999) isolated races 0, 1.2 and 2 in Central Anatolia. Based on these results, Fusarium wilt is a widespread disease all over Turkey. FOM has caused severe losses for farmers as our native cultivars are not resistant to this disease. It is believed that our native cultivars will disappear if resistance to FOM is not introduced into the cultivated material. For this reason, many scientists in Turkey are focusing on research to develop new resistant cultivars via conventional and biotechnological breeding methods.

*In vitro* techniques became widely spread during the 20th century, and their potential to make important contributions to plant breeding was quickly understood. *In vitro* techniques for crop improvement first consisted of micropropagation and plant regeneration, and then *in vitro* methods were also found to be useful for eliminating disease and selecting for resistant cells or explants.

Over the last three decades, *in vitro* tissue culture techniques have been used to generate genetic changes via somaclonal variations (by organogenesis or somatic embryogenesis) that can be used for breeding purposes. *In vitro* selection using specific chemical compounds and pathogens is another useful aspect of tissue culture (Figure 14.1). Selection with phytotoxins and culture filtrates appears to be more effective than the use of the pathogen itself (Van Harten, 1998). Researchers now use fungal culture filtrates or toxins to investigate the response of susceptible and resistant genotypes of different plant species or cultivars to disease factors. The use of *in vitro* methods for the evaluation of resistance is dependent upon a positive correlation between *in vitro* culture filtrate resistance and whole plant disease resistance. Gray *et al.* (1986), Chawla and Wenzel (1987), Connell *et al.* (1990), Malepszy and El-Kazzaz (1990), and El-Kazzaz and Malepszy (1992) developed protocols for *in vitro* determination of resistance. In comparison with field screening and other biotechnological methods, selection techniques are more cost and labour effective, and do not require large experimental fields.

More recently, *in vitro* techniques have been combined with mutation induction for generating genetic variation, including novel disease-resistant mutants. Mutation induction can be caused by chemical or physical mutagens that alter the structure of the DNA (Smith, 1985). Treatment of *in vitro* tissues with physical or chemical mutagens may increase the frequency of genetic variation considerably. The physical mutagens most commonly used are X-rays, gamma rays and UV light, whereas ethyl methanesulfonate (EMS) is the chemical mutagen most used in crop improvement. Irradiation treatments may be a suitable choice of mutagen for a number of reasons including the fact that application is fast and that, in contrast to chemical mutagens, there is no risk that residues remain in the medium. The *in vitro* mutation frequencies are much higher than for somaclonal variation. Somaclonal variation is also important for *in vitro* plant breeding and desirable cells or plantlets, which are disease resistant, can be obtained by this method. Finally, *in vitro* techniques are also useful in classical mutation breeding programmes by vegetative propagation before or after mutagenic treatment, by *in vitro* selection or by clonal propagation of selected mutants (Figure 14.1).

According to Malepszy and El-Kazzaz (1990), *in vitro* selection using FOM filtrates can be effectively used for the selection of cucumber and melon (Megnegneau and Branchard, 1991). This method can also be applied for the selection of various mutants, which are disease resistant. In this chapter, we describe a method to screen for resistance in melon, from both irradiated and non-irradiated explants by using FOM culture filtrates of various races in *in vitro* conditions. If successfully applied, this methodology can lead to the identification of new melon cultivars resistant to FOM within a relatively short period of time. The same methodology can also be adapted for screening other crop species for resistance to *Fusarium* spp.

## METHODOLOGY

The present work was performed with *in vitro* plantlets obtained from seeds of melon cv. ‘Yuva’ which is an important commercial cultivar in Turkey. Two types of explants (cotyledon and hypocotyls with leaf and cotyledon explants) were used for callus and suspension culture initiation (Taner *et al.*, 2004).

### Preparation of fungal culture filtrate and selection medium

In this research, we followed the procedure developed by Megnegneau and Branchard (1991). Petri dishes containing potato dextrose agar (PDA) medium were inoculated with FOM isolates (A-1)<sub>4</sub> and (A-6)<sub>4</sub> obtained from the Plant Protection Department at Ankara University in Turkey (Erzurum *et al.*, 1999). Petri dishes were incubated at 26°C in the dark. 15 days later,  $2 \times 10^6$  conidia were transferred to 200 ml of liquid Richard’s medium. Cultures were kept at 26°C in the dark. After 20 days, fungal cultures were filtered twice through filter paper to remove mycelia. The pH of the filtrate was adjusted to pH5.7 with 1 N HCl or 0.1 N NaOH. Subsequently, the cultures were sterilised through a 0.22 µm filter unit attached to a syringe. During filtrate preparation, it is very important to avoid thermal degradation of toxic compounds in the fungal culture filtrate. Filtrates should, therefore, be aliquoted and frozen for long-term storage. The working filtrate aliquot can be kept in the refrigerator. Filtrates were added to autoclaved modified Murashige and Skoog (MS) basal medium. According to Taner (2002) and Taner *et al.* (2004), non-irradiated and irradiated explants were transferred to Petri dishes containing half strength MS medium with basal salts (Murashige and Skoog, 1962) supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin, 250 mg/l casein enzymatic hydrolysate (Sigma Chemical Co., St. Louis, MO, USA) and 15% sucrose (for cotyledon explants) and MS basal medium, which was supplemented with 0.5 mg/l indole-3-acetic acid (IAA), 0.5 mg/l 6-benzylaminopurine (BAP) and 15% sucrose (for hypocotyls with leaf and cotyledon explants). For both media, the pH was adjusted to pH5.6 and the fungal culture filtrate was added at different percentages (4, 6, 8, 10, 12, 14, 16, 18 and 20% [v/v]). Control plates contained only the modified MS basal medium without fungal culture filtrate. The steps for fungal culture filtrate preparation are shown in Figure 14.2 and described in detail elsewhere in this book.

### Preparation of *in vitro* plant material

Figure 14.3 shows the steps for the initiation of the callus cultures. Seeds of melon genotypes were surface-sterilised, rinsed and germinated *in vitro* as previously described by Çürük (1999) and Taner (2002). Briefly, seeds were surface-sterilised for 20 minutes in 20% sodium hypochloride solution containing 1% (v/v) Tween 20, and washed three times in sterile distilled water. After removal of the seed coats, seeds were placed in “baby food culture jars” with Magenta B-caps (product numbers V8630 and B8648, Sigma) that contained 50 ml of solid MS medium with 0.7% Difco agar and 15% sucrose. The cultures were maintained at 25°C, under fluorescent illuminance with a light intensity of 10000 lux and a 16 hour photoperiod. For resistance screening, two kinds of non-irradiated and irradiated explants (i.e., cotyledons and hypocotyls containing leaves and cotyledons) were used according to Taner *et al.* (2004). Cotyledon explants were transferred to Petri dishes containing half strength MS medium with basal salts (Murashige and Skoog, 1962) supplemented with 0.5 mg/l (2,4-D), 0.5 mg/l kinetin and 15% sucrose. Hypocotyls with leaf and cotyledon explants were transferred to

MS basal medium, which was supplemented with 0.5 mg/l IAA, 0.5 mg/l BAP and 15% sucrose. For both media, the pH was adjusted to pH5.6 with 1 N HCl or 0.1 N NaOH. It is very important that the experiments contain >50 explants for each combination.

### ***In vitro* mutagenic treatment**

Irradiation doses and their effects on plants are known to be genotype-dependent. Even with the same genetic material, the optimal dose for mutation induction depends on the type of plant material to be irradiated. In the case of *in vitro* irradiation, the optimal dose is typically lower than that used for seed irradiation. Therefore, every experiment should start with radiosensitivity tests to determine the optimal irradiation dose for the plant material being used. The LD<sub>50</sub> (i.e., the dose that will kill 50% of the test organisms within a designated period) is often used as the optimal dose for mutation induction. The lower the irradiation LD<sub>50</sub>, the more sensitive the plant material is.

After seed germination, seven day old *in vitro* plantlets which contained true leaves and cotyledons were irradiated with a <sup>60</sup>Co gamma source. Five different doses of gamma rays were used (10, 20, 30, 40 and 50 Gy) for the radiosensitivity assays. In our experiments, a gamma irradiation dose of 25 Gy was found to be the most effective dose for *in vitro* plantlets of the ‘Yuva’ cultivar. Moreover, after irradiation, it is important to transfer the explants onto fresh regeneration medium containing the fungal culture filtrate as described in the “Preparation of *in vitro* plant material” section of this chapter to avoid any toxicity of the medium components due to the irradiation.

### ***In vitro* screening and evaluation**

The fungal culture filtrate was added to the media at different concentrations as indicated above. Cotyledon cultures were incubated at 26°C in the dark for three weeks. Hypocotyl explants with leaves and cotyledons were incubated with a light period of 16 hours at 15000 lux and at 26°C for three weeks according to Taner *et al.* (2004). At the end of this three week period, explants were assessed according to their regeneration capacity (Figure 14.4). Formation of white/yellow callus from the explants indicated survival capacity of the explants in the presence of the filtrate, suggesting that the plant material was resistant to the disease. On the other hand, a brownish colour indicated that the calli were dead, pointing to susceptibility.

The resistant calli were selected and subcultured for an additional two weeks on the same medium containing the filtrate for further confirmation of resistance. The resistant calli were then transferred to hormone-free MS medium supplemented with the fungal filtrate for induction of somatic embryogenesis (Taner and Yanmaz, 2003). For plantlet regeneration, MS medium supplemented with 1.0 mg/l IAA was used according to Taner (2002). After this period, the rate of mortality of the explants was estimated. The regeneration of shoots and roots, growth performance and number of shoots were used as indicators for measuring the degree of resistance. Plantlets were then transferred to greenhouses and advanced to the M<sub>2</sub> generation for further selection and evaluation.

## **CONCLUSIONS**

Seven doubled-haploid (DH) melon lines, originating from parthenogenesis using irradiated pollen were produced. Two promising DH lines were selected for resistance/tolerance to Fusarium wilt following inoculations with race 1.2w. These two DH lines represent a source of resistance/tolerance that is commercially exploitable either as rootstocks or as lines for conventional breeding (Ficcadenti *et al.*, 2002).

Table 14.2 shows the results of previous studies using *in vitro* methods for the selection of melon and cucumber resistant to Fusarium wilt. This technique is very effective for the rapid screening of melon and cucumber. We are currently applying *in vitro* mutagenesis combined to *in vitro* selection for the

improvement of the commercial melon cultivar 'Yuva' to expand the genetic variation of muskmelon in Turkey and to select resistant types of this cultivar.

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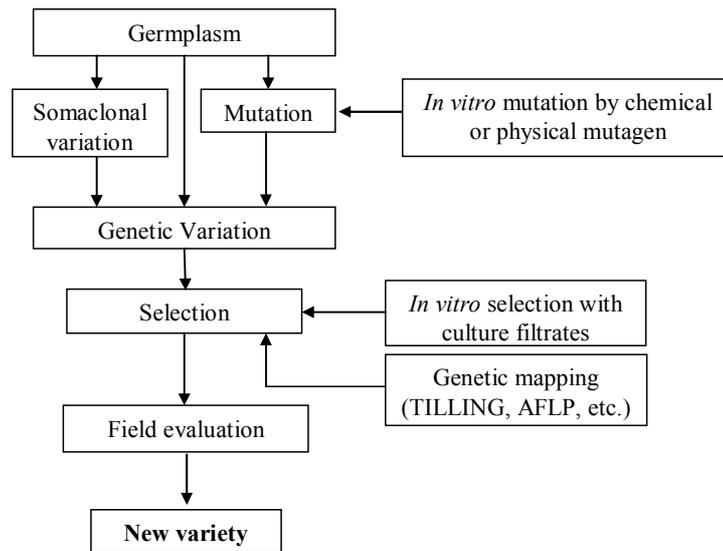
**Table 14.1.** Muskmelon genotypes resistant to *Fusarium oxysporum* f. sp. *melonis*\*

Genotype	Resistance to FOM races	Type and origin
‘Védrantais’	0 and 2	Cultivar (INRA, France)
‘Dulce’	0 and 2	Cultivar (USA)
FM 025	0 and 2	Breeding line (ARO, Israel)
FM 018	0 and 2	Breeding line (ARO, Israel)
FM 004	0 and 2	Breeding line (ARO, Israel)
FM 023	0 and 2	Breeding line (ARO, Israel)
FM 014	0 and 2	Breeding line (ARO, Israel)
FM 024	0 and 2	Breeding line (ARO, Israel)
‘Doublon’	0 and 2	Cultivar (INRA, France)
‘Hemed’	0 and 2	Commercial cultivar (Hazera, Israel)
‘Freeman cucumber’	0 and 1	Breeding line (ARO, Israel)
PI 161375	0 and 1	Breeding line (ARO, Israel)
F65	0 and 1	Breeding line (ARO, Israel)
I4-6-2-B	0 and 1	Breeding line (ARO, Israel)
‘Maqdimon F <sub>1</sub> Hybrid’	0 and 1	Commercial cultivar (Hazera, Israel)
‘Omega (5080) F <sub>1</sub> Hybrid’	0, 1 and 2	Commercial cultivar (Nunheru Zaden, The Netherlands)
‘Caruso (5093) F <sub>1</sub> Hybrid’	0, 1 and 2	Commercial cultivar (Nunheru Zaden, The Netherlands)
‘Piboule’	1.2	Cultivar (France)
‘Dinero’	1.2	Commercial cultivar (Syngenta Seeds, Milano, Italy)
ASR04993033	1.2	Commercial cultivar (Asgrow Seeds, Latina, Italy)

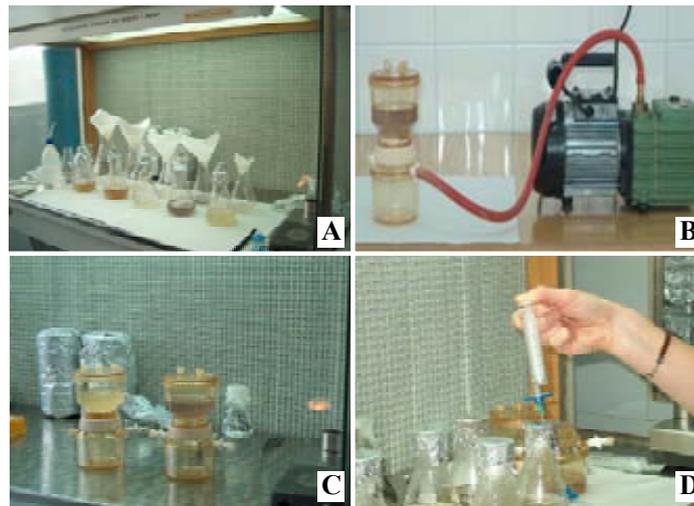
\*Modified from Burger *et al.* (2003).

**Table 14.2.** *In vitro* screening for resistance to *Fusarium* wilt in *Cucumis* spp

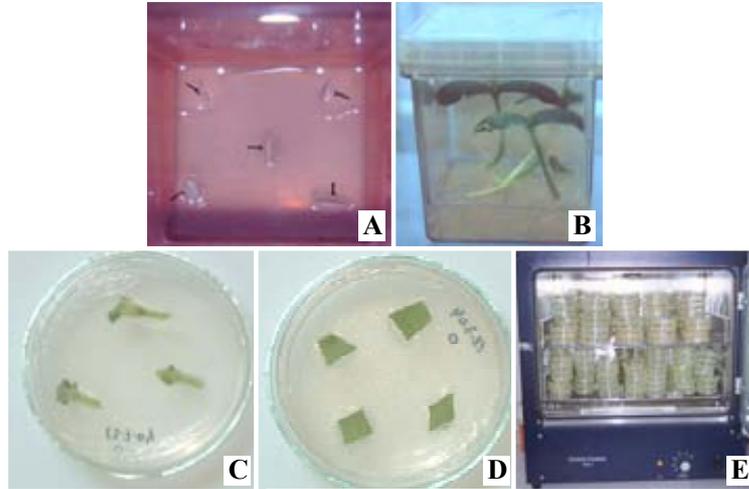
Species and cultivar	Pathogen	Result	Reference
Melon ( <i>Cucumis melo</i> cv. ‘Yuva’)	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i>	Ongoing study	
Cucumber ( <i>C. sativus</i> cv. ‘Borszczagowski’ and Gy-3)	<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	7% of regenerated plants were resistant	Malepszy and El-Kazzaz (1990)
Melon ( <i>C. melo</i> )	<i>F. oxysporum</i> f. sp. <i>melonis</i>	Correlation between filtrate density and explant growth established	Megnégneau and Branchard (1991)
Cucumber ( <i>C. sativus</i> cv. ‘Borszczagowski’ and Gy-3)	<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	Selected plants showed various modes of resistance expression when exposed to 100% culture filtrate	El-Kazzaz and Malepszy (1992)



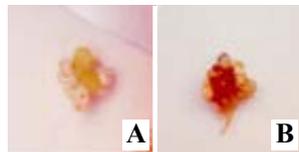
**Figure 14.1.** Plant breeding scheme by induced mutations and somaclonal variations (modified from Van Harten, 1998).



**Figure 14.2.** The steps of filtrate preparation. (A) Fungal cultures were filtered through filter paper to remove mycelium; (B) Fungal cultures were sterilised with a 0.22  $\mu\text{m}$  Sartorius filter unit with a vacuum for complete removal of fungal cells; (C) After sterilisation; (D) Fungal culture filtrate, which was added to autoclaved basal medium.



**Figure 14.3.** The steps for culture initiation. (A) *In vitro* seed culture. Seeds were cultured after removal of the seed cover; (B) *In vitro* plantlet production; (C) Hypocotyls with leaf and cotyledon explants; (D) Cotyledon explants; (E) Incubation period.



**Figure 14.4.** Selection for resistant melon calli with fungal culture filtrate. (A) Live callus (resistant); (B) Dead callus (susceptible).



## Chapter 15

### Screening for resistance to lettuce downy mildew (*Bremia lactucae*)

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#### Abstract

Screening methods of *Lactuca* spp. for resistance to *Bremia lactucae* (lettuce downy mildew) are described in this chapter. Procedures of isolation, cultivation and maintenance of *B. lactucae* isolates are summarised. Resistance screening can be carried out either on lettuce seedlings, detached cotyledon leaves, leaf disks or parts of true leaves from adult plants. Methodology of inoculum preparation, inoculation and incubation conditions is described. For the assessment infection degree, a qualitative or quantitative approach is used. A basic differential set of *Lactuca* spp. used for testing *B. lactucae* isolates and virulence phenotypes of the most important *B. lactucae* races was established, including the denomination of races by the sextet code. The methods described have a broad application both in practice and research. They are useful for resistance screening of new breeding material, for searching for new sources of resistance in *Lactuca* spp. germplasm, for investigating plant resistance mechanisms and also for the study of virulence variation in *B. lactucae* isolates and populations.

#### INTRODUCTION

Lettuce is one of the most important vegetable crops and has a long history of germplasm research and resistance breeding (Lebeda *et al.*, 2007). Lettuce downy mildew, caused by the peronosporaceous fungus *B. lactucae* Regel (Figure 15.1A, B), is a serious disease of cultivated lettuce (*Lactuca sativa* L.) distributed worldwide wherever the lettuce crop is grown (Marlatt, 1974). However, it can also attack more than 200 other species from about 40 genera of the family Compositae (Crute and Dixon, 1981). The most common wild host species of this pathogen is *Lactuca serriola* (prickly lettuce) and it can also be frequently found on some *Sonchus* species (Petrželová and Lebeda, 2004). However, it has been proven that *B. lactucae* is highly specific and mostly limited to the same genus of plants (Crute and Dixon, 1981; Lebeda and Syrovátko, 1988; Lebeda *et al.*, 2002). Thus, except for some *Lactuca* species, weedy growing Compositae cannot serve as a source of inoculum for cultivated lettuce (Lebeda and Syrovátko, 1988).

Several different mechanisms of resistance to *B. lactucae* have been identified in cultivated lettuce (Lebeda *et al.*, 2001). Most of the resistance is considered to be race-specific (Lebeda *et al.*, 2002). Race specificity is also common in some wild *Lactuca* species and some closely related genera (Lebeda and Boukema, 1991; Lebeda, 1998; Lebeda and Petrželová, 2001, 2004b). The interaction between *L. sativa*, *L. serriola* and *B. lactucae* conforms to the gene-for-gene relationship (Illot *et al.*, 1989; Crute, 1992a), in which resistance is determined by dominant *Dm* resistance genes (R-factors) in the hosts, and matched by dominant factors for avirulence (Avr) in the pathogens (Hammond-Kosack

and Jones, 1997). More than 40 resistance *Dm* genes or R-factors have been identified in *L. sativa* and *L. serriola* so far (Reinink, 1999; Sicard *et al.*, 1999; Lebeda *et al.*, 2001, 2002; Michelmore *et al.*, 2002), and the same number of complementary avirulence (Avr) genes or factors are thought to exist in *B. lactucae*. The existence of many additional resistance genes in populations of *L. serriola* is also expected (Lebeda, 1986a; Farrara and Michelmore, 1987; Bonnier *et al.*, 1994; Lebeda and Petrželová, 2004b, 2004c). Cross inoculation experiments with *B. lactucae* originating from *L. sativa* and wild *L. serriola* may contribute to unveiling novel sources of resistance in lettuce. However, so far there is only limited information available on the distribution of race-specific resistance in natural populations of *L. serriola* (Lebeda and Petrželová, 2004b, 2004c). More detailed research in this field is needed.

Only a limited number of race-specific resistance genes have been utilised in commercial lettuce breeding programmes. In Europe, the most frequently used resistance genes in lettuce are *Dm2*, *Dm3*, *Dm4*, *Dm5/8*, *Dm6*, *Dm7*, *Dm11*, *Dm16*, R18 and their combinations (Crute, 1992b; Lebeda and Zinkernagel, 1999, 2003a).

Race-specific resistance has a big disadvantage as it does not provide durable protection against lettuce downy mildew, and the introduction of new resistant cultivars is mostly followed by the appearance of new virulent races of the pathogen (Lebeda and Zinkernagel, 1999, 2003b). During the last few decades, lettuce resistance breeding has been focused on searching for and utilisation of novel sources of resistance to *B. lactucae* from wild *Lactuca* spp. and closely related species, especially *L. serriola*, *L. saligna* and *L. virosa* (Norwood *et al.*, 1981; Bonnier *et al.*, 1992; Lebeda, 1998; Maisonneuve *et al.*, 1999; Jeuken and Lindhout, 2002; Lebeda *et al.*, 2007). The transfer of genes for resistance from wild species to lettuce may, however, make the latter species vulnerable to attack by races more usually associated with original, wild host species (Lebeda, 1984).

Reliable methods for screening for resistance/susceptibility in lettuce species are, therefore, extremely important for the success of breeding programmes (Lebeda *et al.*, 2007). Ideally, these methods should allow a large number of plants to be screened simultaneously. This chapter describes the use of seedlings and leaf disks inoculated with live isolates of downy mildew.

## **MATERIAL AND METHODS**

### **Pathogen isolation, cultivation and maintenance**

One or two leaves with symptoms of lettuce downy mildew (i.e., chlorotic lesions surrounded by veins and covered with pathogen conidiophores) are usually taken from infected lettuce plants. The leaf samples should be placed in small, closed plastic boxes lined with moistened cellulose cotton-wool, and they must be transported to the laboratory as soon as possible. If the samples are sporulating, the spores may be used directly for pathogen isolation. However, leaves with chlorotic spots must most often be incubated for 1-3 days at 10-15°C and with a 12 hour photoperiod until profuse sporulation occurs (Lebeda, 1986a, 1986b; Lebeda and Petrželová, 2004a).

Isolates of *B. lactucae* can be obtained either from localised leaf spots or monosporic isolates, i.e., inoculum is prepared by isolation and cultivation of one conidium. Nevertheless, isolates that originate from one lesion are not necessarily genetically homogeneous. For detailed genetic studies (e.g., genetics of virulence, hybridisation between virulence phenotypes etc.), it is better to use monosporic isolates (Lebeda, 1986b).

For isolation of spores from localised spots, the spots are cut out of infected leaves with sterile scissors. There are two ways of isolating the pathogen from these leaf pieces. The first one is based on washing off spores from the leaf surface with tweezers into a small amount (~5 ml) of distilled water in a beaker. Prepared conidial suspension is sprayed onto seedlings of universally susceptible cultivars/accessions, e.g., *L. sativa* cvs. 'Cobham Green' (Lebeda and Blok, 1991) or 'Hilde' (Lebeda, 1997), or *L. serriola* line 'LSE/57/15' (Lebeda and Petrželová, 2004a) by a glass chromatography

sprayer. The second and more efficient method is based on direct contact of cut leaf pieces covered with conidiophores with a pre-moistened surface of the seedlings. This way, one can avoid dilution of inoculum and the probability that conidia will reach the leaf surface of seedlings is much higher.

Isolates are maintained and multiplied on seedlings of susceptible plants. Re-inoculation is needed every 10-12 days. Between individual experiments, isolates can be stored frozen at  $-20^{\circ}\text{C}$  on infected seedlings. However, the vitality of conidia decreases rapidly at this temperature after 2-3 months of storage. For longer storage periods, isolates can be safely maintained up to 6-7 months in a  $-80^{\circ}\text{C}$  freezer (Lebeda, 2002). Inoculum from frozen conidia is then prepared by re-suspending them in distilled water.

### **Preparation of plant material for screening**

The test must be carried out in containers or dishes where high air humidity and optimal conditions for incubation can be maintained throughout the experiment. The best way to carry out extensive resistance tests with lettuce is to use plastic photographic boxes ( $320 \times 265 \times 60$  mm) lined with three layers of moistened cellulose cotton-wool and one layer of filter paper, and closed with a glass cover (Figure 15.2). These boxes are suitable for tests performed with seedlings or leaf parts (Figures 15.2 and 15.4). In the case of lettuce seeds, the seeds to be tested are sown into lines with a spacing of  $\sim 2.5$ -3 cm, and the boxes should be kept in a cultivation room under a night/day temperature regime of  $10/15^{\circ}\text{C}$  and a 12 hour photoperiod until the seedlings have fully expanded cotyledonary leaves ( $\sim 5$ -7 days after sowing). Before applying the inoculum, it is necessary to remove the seed coats from the seedlings (Lebeda, 1986b).

In addition to intact seedlings, the tests can be performed on detached cotyledon leaves that are placed with their axial side downwards on moistened filter paper. It is also possible to use parts of true leaves from adult plants (2-3 months after transplantation). Leaf disks (diameter: 10 mm, Figure 15.4) or segments of approximately the same size are cut out of leaves by the use of a cork borer and treated the same way as detached cotyledon leaves (Lebeda, 1986a, 1986b). Seedlings and parts of leaves can also be grown in Petri or Drigalski dishes (90, 150 or 200 mm), or in small plastic boxes (Lebeda and Pink, 1998) lined with moistened cellulose cotton wool and filter paper under the conditions described above. They are good for testing smaller numbers of samples and should be prepared a short time before inoculation.

### **Inoculum preparation, inoculation and incubation**

The inoculum is prepared by washing 1-2 day old spores ( $\sim 9$ -12 days after inoculation) from infected seedlings of susceptible plants with distilled water. The optimal concentration of a conidial suspension is approximately  $10^5$  spores/ml (Lebeda, 1979, 1986b). Once the spores are placed in the solution, the inoculum must be applied to seedlings or leaf disks as soon as possible.

The inoculation may be performed either by spraying or soaking. Conidial suspension is usually sprayed onto tested plants with a glass chromatography sprayer (Lebeda, 1984). For one plastic photographic box, approximately 5-6 ml of inoculum is needed as the surface of the leaves must be completely wet. This method is not well applicable, for instance, to some wild *Lactuca* species due to the wax layer on the leaf surface causing the sprayed inoculum to agglomerate into small droplets. Crute and Dickinson (1976) described a method of soaking seedlings or leaf disks in prepared inoculum; however, the spraying method is still more efficient (Lebeda, 1986b).

*B. lactucae* requires specific conditions for its growth. The optimal temperature for conidial germination and penetration ranges from  $10$ - $15^{\circ}\text{C}$ . For successful infection, high relative humidity (90-100%) and a dark period during penetration are also needed (Lebeda, 1986b). Boxes or dishes with inoculated seedlings or leaf disks (or parts of leaves) are incubated in a cultivation room at a night/day temperature of  $10/15^{\circ}\text{C}$ . Temperatures  $>20^{\circ}\text{C}$  inhibit the development of the pathogen. For

the first 12-24 hours after inoculation, the boxes must be covered with black foil. After that, they are kept under a 12 hour photoperiod. Under optimal conditions, sporulation usually occurs 7-9 days after inoculation. The final assessment of infection is usually carried out on day 13 or 14 after inoculation. In the case of incompletely compatible responses, the assessment of infection may be performed even later, at approximately 17-21 days after inoculation (Lebeda, 1986b).

### **Disease assessment**

There are two methods (qualitative and quantitative) for assessing resistance/susceptibility of lettuce to lettuce downy mildew (Lebeda, 1986b). The qualitative assessment is good for the screening of breeding material, whereas the quantitative method is usually used for research purposes.

#### *Qualitative assessment*

The following scale is mostly used for the assessment of infected seedlings, but it can also be used for leaf disks (Lebeda, 1986a, 1986b; Lebeda and Pink, 1998):

- + susceptible response: macroscopically visible sporulation on 80-100% of seedlings;
- resistant response: no visible sporulation on seedlings;
- (-) incompletely resistant response: limited sporulation often followed by macroscopically visible necrotic response or chlorosis (Lebeda *et al.*, 2001);
- (+) heterogeneous response: mixture of completely susceptible and some resistant plants in tested sample; it may be caused by a segregation or impurity of used seed, or by a heterogeneity of an isolate (Crute and Dickinson, 1976).

#### *Quantitative assessment*

The main criterion for the quantitative or semi-quantitative assessment is the intensity of pathogen sporulation (Figures 15.3 and 15.4). The degree of infection is usually assessed at two day intervals 6-14 days after inoculation.

For the assessment of sporulation intensity on cotyledon or true leaves, a scale ranging from 0-3 is used (Figure 15.3) as described by Dickinson and Crute (1974):

- 0 = no visible conidiophores on leaves;
- 1 = limited sporulation, sporadic conidiophores present;
- 2 = < 50% of cotyledon area covered with conidiophores;
- 3 = > 50% of cotyledon area covered with conidiophores.

For the assessment of sporulation intensity on leaf disks (Figures 15.2 and 15.4), a scale ranging from 0-4 is used (Lebeda, 1983, 1986a):

- 0 = no visible conidiophores;
- 1 = ≤ 25% of leaf disk surface is covered with conidiophores;
- 2 = > 25 - ≤ 50% of leaf disk surface is covered with conidiophores;
- 3 = > 50 - ≤ 75% of leaf disk surface is covered with conidiophores;
- 4 = > 75 % of leaf disk surface is covered with conidiophores.

For both scales, the final value of sporulation intensity (degree of infection, DI) is expressed as the percentage of the maximum score according to Townsend and Heuberger (1943):

$$P = \frac{\sum(n \times v)}{x \times N} \times 100$$

Where: P = the total degree of infection (DI); n = number of plants in each assessed category (infection degree); v = infection degree (for leaf disks 0-4); x = scale range (for leaf disks = 4) and N = total number of assessed plants or leaf disks.

Each test usually comprises 20-25 seedlings per tested sample in one replication. In the case of a variable reaction, the test must be repeated. When testing adult plants, five leaf disks or segments originating from one plant are included in one test, and a total of five plants are tested (or more depending on the uniformity of the material used).

### **Determination of pathogenic variability**

Virulence of isolates is examined by screening the differential set of *Lactuca* accessions with well-characterised race-specific *Dm* genes and/or R-factors. The basic differential set of *Lactuca* spp. genotypes used for *B. lactucae* is listed in Table 15.1. Isolates' virulence and the sextet codes of the most important races in the recent past are summarised in Table 15.2.

## **CONCLUSIONS**

The methods described in this chapter have broad applications, not only for research purposes but also in lettuce resistance breeding programmes (Lebeda *et al.*, 2007). They are used for the routine resistance screening of new breeding Material and for searching for new sources of resistance among *Lactuca* spp. germplasm but also for screening of virulence of pathogen populations occurring on cultivated lettuce (Lebeda and Zinkernagel, 2003a, 2003b). The method using lettuce seedlings especially has a big advantage as it allows testing of huge numbers of genotypes simultaneously.

### **Acknowledgements**

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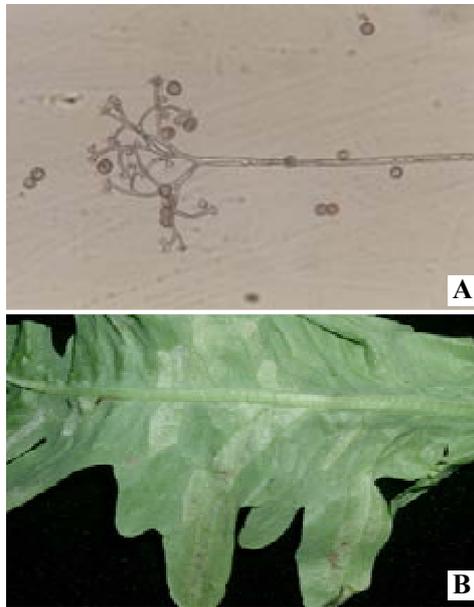
**Table 15.1.** Basic differential set of *Lactuca* spp. used for testing *B. lactucae* isolates\*

Sextet number (see Table 15.2.)	Differential genotype	<i>Dm</i> gene/R-factor (see Table 15.2.)
	Cobham Green	<i>Dm</i> 0/R?
1	Lednický	<i>Dm</i> 1
2	UCDM2	<i>Dm</i> 2
3	Dandie	<i>Dm</i> 3
4	R4T57D	<i>Dm</i> 4
5	Valmaine	<i>Dm</i> 5/8
6	Sabine	<i>Dm</i> 6
7	<i>L. serriola</i> (LSE/57/15)	<i>Dm</i> 7+R?
8	UCDM10	<i>Dm</i> 10
9	Capitan	<i>Dm</i> 11
9	Hilde × <i>L. serriola</i> (H×B)	<i>Dm</i> 11
10	British Hilde	R12
11	Pennlake	<i>Dm</i> 13
12	UCDM14	<i>Dm</i> 14
13	<i>L. serriola</i> (PIVT 1309)	<i>Dm</i> 15
14	<i>L. serriola</i> (LSE/18)	<i>Dm</i> 16
15	<i>L. serriola</i> (LS-102)	R17
16	Colorado	R18
17	Ninja	R36
18	Discovery	R37
19	Argeles	R38

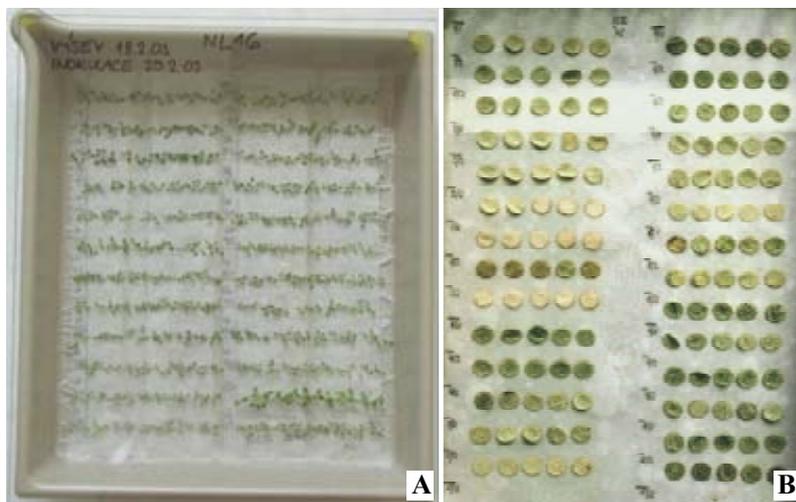
R?: Resistance factor (R-factor) not known or not well characterised.

\*Based on Van Ettehoven and Van der Arend, (1999).

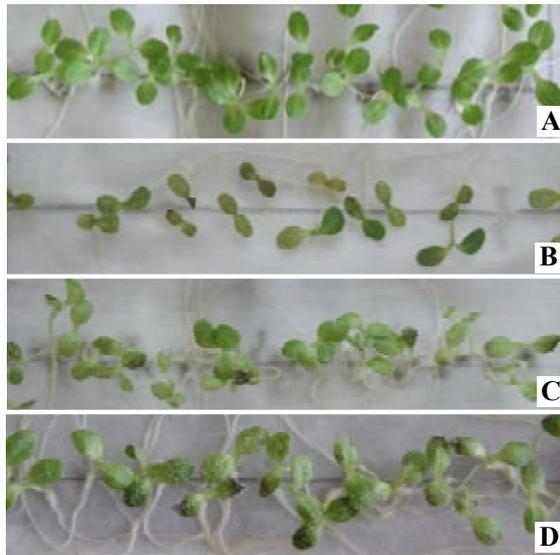




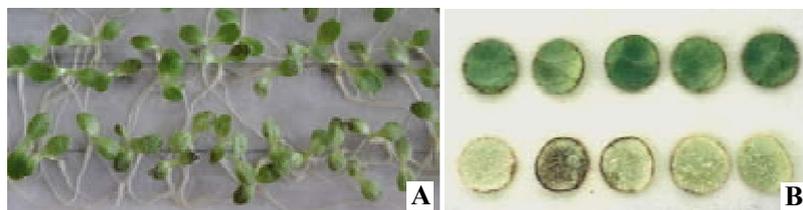
**Figure 15.1.** (A) Microphotograph of *Bremia lactucae* conidiophore and conidia. (B) Abaxial leaf surface of *Lactuca sativa* with sporulating *Bremia lactucae*.



**Figure 15.2.** Boxes with inoculated seedlings (A) and leaf disks (B) of *Lactuca* spp.



**Figure 15.3.** Detail of seedlings with different degrees of infection (DI) 12 days after inoculation by *Bremia lactucae*. (A) DI = 0; (B) DI = 1; (C) DI = 2; and (D) DI = 3.



**Figure 15.4.** Comparison of resistant and susceptible responses in seedlings (A) and leaf disks (B) of *Lactuca sativa* 14 days after inoculation with *Bremia lactucae*.

## Chapter 16

### Screening for resistance to tomato powdery mildew (*Oidium neolycopersici*)

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#### Abstract

This paper provides basic knowledge about a devastating disease of greenhouse tomato plants, namely tomato powdery mildew (*Oidium neolycopersici*), including information about its host range, sources of resistance to this pathogen within wild *Lycopersicon* spp., its pathogenic variability (a preliminary differential set is presented), the genetic background of resistance and mechanisms of resistance. The basic methods of pathogen isolation, cultivation and maintenance, and methods of preparation of experimental plants are briefly described. Two methods of resistance screening are described, leaf disk method (as modified from Mieslerová *et al.*, 2000) and the whole plant method (as modified from Bai *et al.*, 2003), including a description of the scales used for assessment of the degree of infection (DI).

#### INTRODUCTION

Tomato powdery mildew, caused by *Oidium neolycopersici* (Kiss *et al.*, 2001), is a relatively new disease, which occurs predominantly in greenhouse raised tomato crops. Since the mid 1980s, when epidemics first started in Western Europe, the pathogen has rapidly spread throughout Europe and also to the New World (Mieslerová and Lebeda, 1999). Only the anamorph of this fungus has been found until now (Figures 16.1. and 16.2).

The symptoms of tomato powdery mildew infection are white circular pustules, which appear primarily on the upper sides of leaves, and can also affect stems and petioles. The colonies of *O. neolycopersici* are initially small, 3-10 mm in diameter, then enlarge and can cover the whole leaf surface. In heavily infected plants, powdery mildew also develops on the lower side of leaves, but never on the fruit. *O. neolycopersici* can be morphologically easily distinguished from *Leveillula taurica* (Lév.) Arnaud/*Oidiopsis sicula* Scalia, syn. *O. taurica* (Lév.) Salmon, which causes heavy infections of tomatoes and red pepper in tropical and semi-arid areas (Palti, 1988).

Until now, experimental studies have been concentrated mainly on the morphological and molecular characterisation of this pathogen (Whipps *et al.*, 1998; Lebeda and Mieslerová, 1999; Jones *et al.*, 2000; Kiss *et al.*, 2001; Mieslerová *et al.*, 2002), its host range (Whipps *et al.*, 1998; Lebeda and Mieslerová, 1999; Lemaire *et al.*, 1999; Huang *et al.*, 2000a) and on searching for resistance sources within the genus *Lycopersicon* (Lindhout *et al.*, 1994a; Kumar *et al.*, 1995; Ignatova *et al.*, 1997; Milotay and Dormanns-Simon, 1997; Ciccarese *et al.*, 1998; Mieslerová *et al.*, 2000).

Based on a comprehensive study including light microscopy, scanning electron microscopy (SEM) analysis and internal transcribed spacer (ITS) sequence analysis, Jones *et al.* (2000) placed the fungus to *Erysiphe* sect. *Erysiphe* very close (nearly identical) to *E. aquilegiae* var. *ranunculi* and clearly distinct from *E. orontii* and *E. cichoracearum*. Kiss *et al.* (2001), in their detailed studies, identified and described the powdery mildew on tomatoes from Australia (*O. lycopersicum*) as a species distinct from the tomato powdery mildew (*O. neolycopersici*) widespread in Europe, Africa, North and South America, and Asia.

A detailed study of the host range of *O. neolycopersici* revealed that this fungus mainly infects some species of the families Solanaceae and Cucurbitaceae although the experiments gave different results, mainly concerning representatives of the Cucurbitaceae family (Fletcher *et al.*, 1988; Lebeda and Mieslerová, 1999; Lemaire *et al.*, 1999; Huang *et al.*, 2000a). No susceptible taxa have been found in such families as Brassicaceae, Compositae, Leguminosae and Poaceae, while some susceptible species have been reported from the families Apocynaceae, Campanulaceae, Crassulaceae, Cistaceae, Linaceae, Malvaceae, Papaveraceae, Pedaliaceae, Scrophulariaceae, Valerianaceae and Violaceae (Whipps *et al.*, 1998).

The most valuable donors of resistance were detected in accessions of *Lycopersicon hirsutum*, *L. parviflorum*, *L. peruvianum* and *L. pennellii* (Lindhout *et al.*, 1994a; Ignatova *et al.*, 1997; Milotay and Dormanns-Simon, 1997; Ciccicarese *et al.*, 1998; Mieslerová *et al.*, 2000), while only a few resistant genotypes were found in *L. esculentum* and *L. pimpinellifolium* (Kumar *et al.*, 1995; Ciccicarese *et al.*, 1998; Mieslerová *et al.*, 2000), which are considered to be the closest relatives of tomato. Macroscopically, resistance to *O. neolycopersici* in wild tomato species is characterised by strongly restricted mycelial growth and lack of sporulation (Lindhout *et al.*, 1994a). Microscopically, the hypersensitive reaction (HR) is the major mechanism of resistance to *O. neolycopersici* in *Lycopersicon* species (Huang *et al.*, 1998; Mieslerová *et al.*, 2004). A biochemical study of the generation of reactive oxygen species and peroxidase activity during *O. neolycoersici* infection of *Lycopersicon* species has been performed (Mlíčková *et al.*, 2004; Tománková *et al.*, 2006).

However, limited information is available on the pathogenic variability of *O. neolycopersici*. Host range experiments have revealed considerable differences, mainly in the ability of different *O. neolycopersici* isolates to infect representatives of the Solanaceae family, which suggests the existence of different pathotypes (*formae speciales*) (Huang *et al.*, 1998; Mieslerová and Lebeda, 1999). Because nearly all recent commercial tomato cultivars are considered to be highly susceptible (Kozik, 1993; Lindhout *et al.*, 1994a; Mieslerová *et al.*, 2000), these are not useful for the differentiation of *O. neolycopersici* isolates. Based on pathogenicity studies of isolates of *O. neolycopersici* originating from different European countries, Lebeda and Mieslerová (2002) proposed a preliminary differential set of genotypes (Table 16.1).

Intensive study of the genetic basis of resistance, mainly in wild *Lycopersicon* species (*L. hirsutum*, *L. peruvianum*), has revealed the existence of six genes responsible for resistance to *O. neolycopersici* (*Ol-1-6*) (Lindhout *et al.*, 1994b; Ciccicarese *et al.*, 1998; Huang *et al.*, 2000b; Bai *et al.*, 2004, 2005). According to these results, the near-isogenic lines were produced from crosses which contained the different dominant *Ol* genes in an *L. esculentum* genetic background. The disease tests with local *O. neolycopersici* isolates demonstrated that the resistance conferred by different *Ol* genes was isolate-dependent and may be race-specific (Table 16.2).

## **MATERIAL AND METHODS**

### **Pathogen isolation, cultivation and maintenance**

Leaves of tomato highly infected by *O. neolycopersici* are used as the source of inoculum. An important feature for the successful isolation of the pathogen is the quality of the inoculum. The leaves with pathogen should not be too old (i.e., they should not exhibit seriously necrotised leaf tissue), and should not be collected in wet weather, because this causes the conidia not to be released from the conidiophores. The isolates of *O. neolycopersici* are maintained on plants of highly susceptible cultivars of tomato (e.g., cv. 'Amateur') that are about two months old. The upper side of two or three leaves of a susceptible tomato plant are inoculated by surface contact (dusting/tapping) using infected plants (Lebeda and Mieslerová, 1999).

These plants are kept under plastic covers in a growth chamber at a temperature of 20-25°C with a 12 hour photoperiod. In two- or three-week intervals, new tomato plants are re-inoculated with the isolate.

## Preparation of plant material for screening

Seeds of tested plants are treated with 6% chloramine for 20 minutes and then sown into plastic boxes, 8 × 11 × 2.5 cm, containing perlite. Seedlings with fully developed cotyledons are transferred to 7 cm plastic pots containing garden soil/peat (2:1 [v/v]). Plants are grown in a growth chamber at 20°C with a 12 hour photoperiod, and are used for experiments at the three to five true leaf stage (about eight weeks old) (Lebeda and Mieslerová, 1999).

## Inoculum preparation, inoculation, incubation and disease assessment

### *Leaf disk method (modified from Mieslerová et al., 2000)*

The leaf disks (diameter: 14 mm) are cut using a cork borer from true leaves and placed with the adaxial side up on water agar (15 g/l, Agar ST Imuna, Imuna Pharm Ltd., Šarišské Michalany, Slovakia) in Petri dishes. The upper side of each leaf disk is inoculated by surface contact (dusting/tapping) using leaves of tomato cv. 'Amateur' that have 80-100% of the surface covered with fresh sporulating mycelium of *O. neolycopersici*. The average number of powdery mildew conidia delivered to leaf disks is  $65 \pm 15/\text{mm}^2$ . After inoculation, the Petri dishes are placed in a growth chamber and incubated at 18/20°C (night/day) with a 12 hour photoperiod.

The method of spraying leaf disks with a suspension of powdery mildew conidia, which is frequently used in experiments with downy mildews (of the order Peronosporales), was also tested. However, poor results were achieved. Although the very high humidity inside the Petri dishes usually supports the germination of conidia, the water drops on the leaf surface inhibited the development of tomato powdery mildew mycelium, resulting in weak sporulation even in the positive control accession.

The degree of susceptibility of the tested accessions to the *O. neolycopersici* isolates was evaluated macroscopically 5-14 days after inoculation at two-day intervals. For assessing the DI, a 0-4 scale was used (Figure 16.3): 0 = without symptoms of pathogen development; 1 = mild development of mycelium without sporulation; 2 = well-developed mycelium with mild sporulation; 3 = intensive sporulation and well-developed mycelium covering <50% of the leaf disk area; 4 = intensive sporulation and well-developed mycelium covering 50-100% of the leaf disk area. For each accession, the mean and standard deviation (SD) of the P (percentage of the total degree of infection) was calculated according to the formula of Townsend and Heuberger (1943) as follows:

$$P = \frac{\sum(n \times v)}{x \times N} \times 100$$

Where: P = the total degree of infection (DI); n = number of plants in each assessed category (infection degree); v = infection degree (0-4); x = scale range (in this case = 4) and N = total number of assessed plants.

Data recorded on the 14th days after inoculation were used to calculate the DI. Plants were considered to be resistant when the % max DI was between 0 and 30, as susceptible when the % max DI was between 60 and 100, and as incompletely resistant when the % max DI was between 30 and 60. The presence or absence of an HR was macroscopically assessed.

### *Whole plants method (modified according to Bai et al., 2003)*

The 6-8 week old plants are sprayed with a suspension of  $2 \times 10^4$  conidia/ml. The inoculum is prepared by washing conidial spores from freshly sporulating leaves of heavily infected tomato plants in tap water and is used immediately. Inoculated plants are placed under plastic covers at 18/20°C (night/day) with a 12 hour photoperiod. At least five plants per accession are used.

The inoculated plants are evaluated according to the following DI scale (Figure 16.4): 0 = no visible sporulation; 1 = very few fungal spots (weak sporulation); 2 = a moderate number of fungal spots

(heavy sporulation); 3 = a very high number of fungal spots (heavy sporulation). The plants are first evaluated five days after inoculation and for the last time on the 18th day following inoculation. The last evaluation (if plants are not totally destroyed) is used for determining the susceptibility/resistance of the tested accession. Plants are considered to be resistant when the  $DI \leq 1$ , as susceptible when the  $DI > 2$  and as incompletely resistant when  $1 < DI \leq 2$ . In addition, the presence of necrotisation in infected leaf tissue is macroscopically assessed.

## CONCLUSIONS

Tomato powdery mildew (*O. neolycopersici*) has started to cause serious infections in tomato crops since the late 1980s. Vast amounts of information have been obtained about this pathogen, including its interaction with the host *Lycopersicon* spp. Studies on the host range and sources of resistance have produced a considerable amount of data that can be applied to the breeding of resistant tomato cultivars and disease management. However, there is still only limited data available about the pathogenic variability of this fungus. It is assumed that the interactions of *Lycopersicon* spp. and *O. neolycopersici* are based on race-specificity. However, only detailed research on these interactions will elucidate the exact mechanisms of resistance and their genetic background. The methods described in this paper have a broad potential for application both in screening *Lycopersicon* spp. germplasm and in subsequent tomato breeding programmes. These methods can also be applied to the research of pathogenic variability of *O. neolycopersici*. The methodology described is useful for basic research of host-pathogen interactions, pre-breeding and selection mass screening, as well as for characterisation of resistance of commercial tomato cultivars.

## Acknowledgements

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**Table 16.1.** List of *Lycopersicon* spp. accessions recommended as a basis for a preliminary differential set of genotypes and differentiation of pathogen races

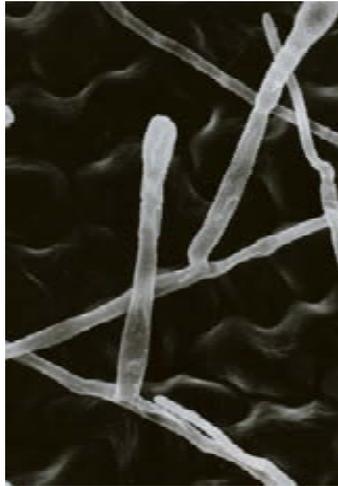
<i>Lycopersicon</i> spp.	Accession	<i>Oidium neolycopersici</i> race/response		
		OL 1	OL 2	OL 3
<i>L. esculentum</i>	cv. 'Amateur'	S <sup>1</sup>	S	S
<i>L. hirsutum</i>	LA 94	R	S	M
<i>L. hirsutum</i>	LA 1738	R	R	S
<i>L. hirsutum</i>	LA 1731	R	R	M
<i>L. hirsutum</i> f. sp. <i>glabratum</i>	LA 2128	R	R	R

<sup>1</sup>Reaction (DI = degree of infection): R = resistant (DI = 0-30%); M = moderately susceptible (DI = 30-60%); S = susceptible (DI = 60-100%). Source: Lebeda and Mieslerová (2002).

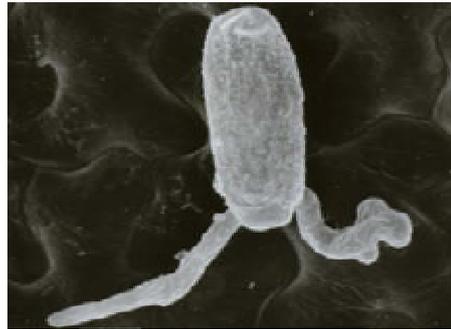
**Table 16.2.** List of *Lycopersicon* spp. accessions and their reactions with local isolates of *Oidium neolycopersici*

<i>Lycopersicon</i> spp.	Accession	On-Ne	<i>Oidium neolycopersici</i> isolate/response				
			On-Hu	On-Cz	On-It	On-Fr	On-Fl
<i>L. esculentum</i>	'Moneymaker'	S <sup>1</sup>	S	S	S	S	S
Crosses based on <i>L. esculentum</i>	NIL <i>Ol-1</i>	R	MR	R	R	R	R
	NIL F3- <i>Ol-2</i>	R	R	R	R (MR)	R	R
	NIL <i>Ol-3</i>	R	R	R	R	R	R
	NIL <i>Ol-4</i>	R	R	S	R	R	R
	NIL <i>Ol-5</i>	R	MR	R	R	R	R
	NIL <i>Ol-6</i>	R	R	S	R	R	R

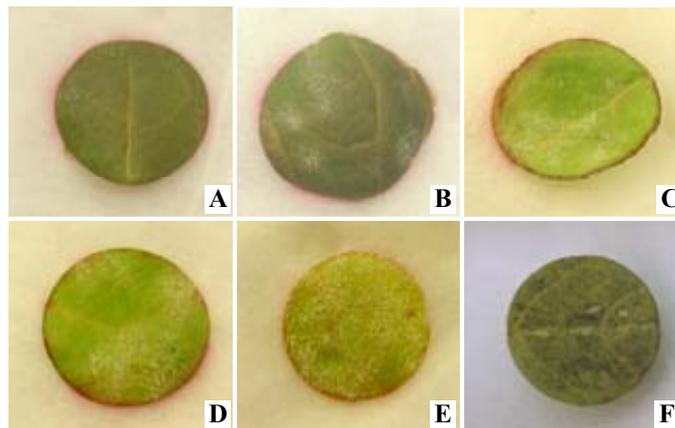
<sup>1</sup>Reaction: R = resistant (DI ≤ 1); MR = moderately resistant (1 < DI ≤ 2); S = susceptible (DI > 2) (see Figure 16.4). Source: Bai *et al.* (2005).



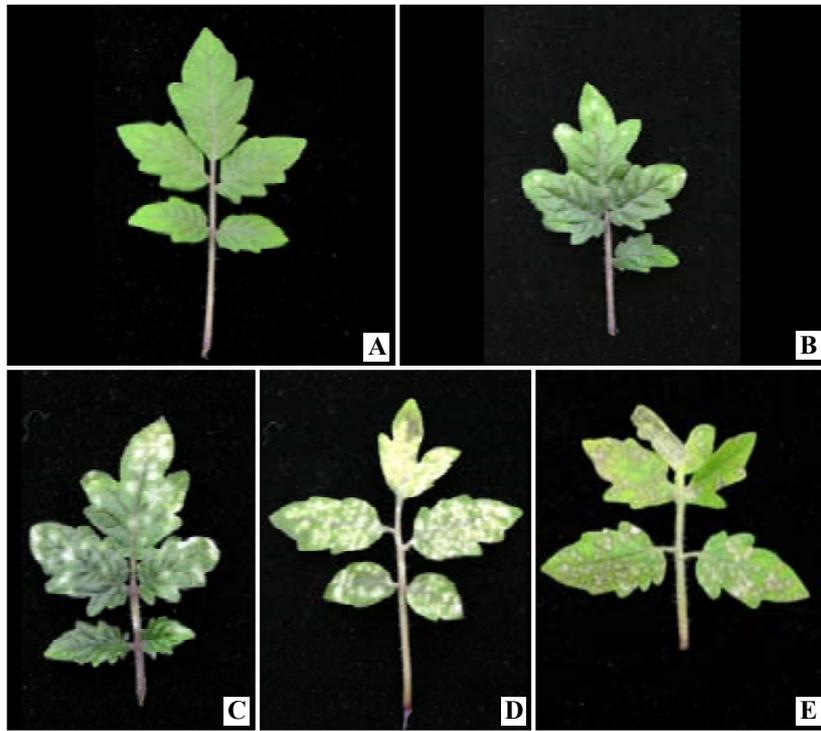
**Figure 16.1.** Electron micrograph showing conidiophores of *Oidium neolyopersici*.



**Figure 16.2.** Electron micrograph showing a germinating conidium and appressorium formation of *Oidium neolyopersici*.



**Figure 16.3.** Assessment of the degree of infection (DI) of tomato powdery mildew on leaf disks. (A) DI = 0: without symptoms of pathogen development; (B) DI = 1: mild development of mycelium without sporulation; (C) DI = 2: well-developed mycelium with mild sporulation; (D) DI = 3: intensive sporulation and well-developed mycelium covering <50% of the leaf disk area; (E) DI = 4: intensive sporulation and well-developed mycelium covering 50-100% of the leaf disk area; (F) HR: Hypersensitive response (dark spots at the site of fungal penetration).



**Figure 16.4.** Assessment of the degree of infection (DI) on leaves of the whole plants. (A) DI = 0: no visible infection; (B) DI = 1: very few fungal spots (weak sporulation); (C) DI = 2: a moderate number of fungal spots (sporulation); (D) DI = 3: a very high number of fungal spots (heavy sporulation). (E) Pustules of sporulating *Oidium neolycopersici* accompanied by necrotic surrounding cells.



## Chapter 17

### ***In vitro* and *in vivo* selection of black pepper (*Piper nigrum*) mutants tolerant to Fusariosis**

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#### **Abstract**

The cultivation of black pepper plants in Brazil is severely limited by Fusariosis, a disease caused by *Fusarium solani* f. sp. *piperis* that leads to plant death. Considering the fact that the black pepper plants in Brazil are derived from a few plants introduced from Singapore, and that introduction of other germplasm has been extremely difficult, the genetic variability found within the populations is very low. Conventional breeding methods have, therefore, been highly ineffective for obtaining resistance or tolerance to Fusariosis disease. In order to generate genetic variability and selection of plants resistant to Fusariosis, the objective of the present study was to establish useful methodology using gamma irradiation in association with *in vitro* and/or *in vivo* techniques. For this purpose, it is suggested that: (1) a dose of 20 Gy of gamma-rays be applied for *in vitro* mutation induction of buds from young plants; (2) generation advancement be done at least three times by subculturing (micropropagation) in order to eliminate chimerism; (3) *in vitro* selection be carried out with a filtrate of *F. solani* f. sp. *piperis*; (4) *in vivo* selection be performed through the application of a suspension of fungal spores onto the plants; and (5) agronomic evaluation of selected plants be carried out in an area with a high incidence of *F. solani* f. sp. *piperis*.

#### **INTRODUCTION**

Black pepper (*Piper nigrum*) originates from India and belongs to the Piperaceae family, whose members have different numbers of chromosomes, 36, 48, 52, 60, 104 and 128 (Sharma and Bhattacharyya, 1959; Dasgupta and Datta, 1976). The plant is considered autogamic, the inflorescence is a pendulous spike 5-20 cm long, and there are female, male and hermaphrodite plants. Natural pollination is performed by geitonogamy through the dispersion of pollen by water, dew or rain droplets; fruit formation occurs six months after pollination (Nambiar *et al.*, 1978; Poltronieri *et al.*, 1999).

The crop thrives in hot and moist weather, requiring an average rainfall of > 2500 mm/year, moisture of > 80%, average temperatures of ~ 23-28°C, and soils of good drainage (Albuquerque *et al.*, 1989). The main producing countries are India, Indonesia, Brazil and Malaysia. Seeds as well as vegetative cuttings are used for propagation. Propagation with seeds is used mostly in breeding programs, while propagation with cuttings is more commonly used for commercial cultivations (Nambiar *et al.*, 1978; Albuquerque *et al.*, 1989).

Given the narrow genetic base of the Brazilian cultivars, black pepper cultivation has been severely attacked and limited by a disease known as Fusariosis, which is caused by the fungus *Nectria haematococca* f. sp. *piperis* (*F. solani* f. sp. *piperis*). The symptoms of Fusariosis include root decay and drying of the branches. The disease spreads quickly all over the planting regions, destroying large areas of cultivation in a short period of time (Albuquerque and Duarte, 1977; Duarte and Albuquerque, 1999).

Tissue culture techniques can be used to create genetic variation through a process called somaclonal variation, which causes changes similar to those induced by physical and chemical mutagenic agents, and which can be incorporated into genetic breeding programmes. *In vitro* selection allows selection of mutants tolerant to abiotic factors and resistant to diseases in a short period of time, and can be used as an auxiliary to field selection. The use of molecular markers is ideal for distinguishing genetic somaclonal from epigenetic changes (Jain, 2001).

*In vitro* culture techniques were first applied for the formation of calluses and differentiation of multiple sprouts and roots of black pepper plantlets. More recently, regeneration of black pepper plants via somatic embryogenesis has been achieved (Mathews and Rao, 1984; Khoon and Talib, 1985, Philip *et al.*, 1992; Joseph *et al.*, 1996).

Conventional and non-conventional breeding programmes produce genetic variability in the population followed by selection of the desired genotypes (Wenzel, 1985). The use of mutation induction techniques is an alternative because it allows the generation of genetic variability and has sped up success in breeding cultivated species (Maluszynski *et al.*, 1995; Donini and Sonnino, 1998). Regarding black pepper cultivation in Brazil, conventional methods for obtaining genotypes with resistance or tolerance to the Fusariosis disease have brought little progress, for no tolerance to the disease has been observed within the species (Poltronieri *et al.*, 1999). Elsewhere, the best results through conventional breeding were obtained with the hybrid Panniyur-I (Nambiar *et al.*, 1978).

For plants with an unknown genetic source of a desirable feature or even for apomictic or sterile plants in which species hybridisation is not an option, the induction of mutations is an alternative method of plant breeding (Tulmann Neto *et al.*, 1998). The selection of variants is an important step in plant breeding by mutation and, for the most part, is harder than the induction itself (IAEA Technical Reports, 1977). According to Ahloowalia and Maluszynski (2001), more than 1800 cultivars were obtained directly or indirectly through mutation induction in plants with seed propagation, such as wheat, rice, barley, peanuts and beans. For vegetatively propagated plants, 465 mutants were obtained, most of them for ornamental plants (chrysanthemum, rose, begonia, azalea, dahlia, etc.) and a few for fruit-bearing plants (apple, Japanese pear, grapefruit, banana, pineapple, date).

In contrast to conventional *in vivo* cultivation, *in vitro* cultivation results in a quick dissolution of chimeras and the recovery of genetically stable mutants (Tulmann Neto *et al.*, 1998). For *in vitro* selection, phytotoxic effects of fungal filtrate have been proven in the selection process for resistance to the disease caused by *Fusarium* in pineapple (Hidalgo *et al.*, 1998, 1999; Borrás *et al.*, 2001), soybeans (Jin *et al.*, 1996) and banana plants (Matsumoto *et al.*, 1999a,b). Variations in the level of tolerance to the toxins produced by *Fusarium in vitro* are mostly due to the plant variety or genotype, which is similar to the responses observed following pathogen attacks under field conditions. Such variations reflect the different resistance mechanisms of the host that operate at various levels, from the prevention of fungal invasion and expansion to tolerance or degradation of the fungal toxin (McLean, 1996).

## MATERIAL AND METHODS

In the present study, we conducted: (1) tests for radiosensitivity of *in vitro* buds to gamma radiation; (2) cultivation of *F. solani* f. sp. *piperis* fungus; (3) tests using the culture filtrate as a selective agent; (4) *in vitro* and *in vivo* selection of variants; and (5) agronomic evaluation of V<sub>5</sub> plants in areas of Fusariosis occurrence.

### **Radiosensitivity of *in vitro* buds to gamma radiation**

Doses ranging from 0 to 80 Gy were tested, and an entirely randomised experimental design was used with treatment doses of 0, 20, 30, 40 and 50 Gy, with seven replications, each of which consisted of 20 buds per Petri plastic sterile plate (diameter: 8.5 cm), totalling 700 buds. Each plate contained agar water (0.7% w/v) and was individually irradiated at a dose rate of 1297 kGy/hour with a gamma-cell at CENA/USP, Piracicaba, São Paulo. After irradiation, buds were cultivated in Murashige and Skoog (MS) semi-solid basal medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/l 6-benzylaminopurine (BAP) and 0.2 mg/l indole-3-acetic acid (IAA). After 45 days of cultivation at 25 ± 3°C with 16 hours of daylight, with a light intensity of ~3000 lux, the survival rate, number of buds per explant and explant weight (g) were evaluated. The mean values were subjected to linear regression analysis using the software Microsoft Excel to determine the dose of gamma radiation necessary to reduce the survival rate, number of buds and explant weight to 30-50% (Figure 17.1).

### ***In vitro* phytotoxicity of the culture filtrate of *F. solani* f. sp. *piperis***

The influence of the Czapek-Dox medium components in the multiplication culture – basic MS culture medium supplemented with 0.5 mg/l BAP and 0.2 mg/l IAA – was evaluated by examining the performance of *in vitro* buds in a range of Czapek-Dox concentrations (0, 20, 30, 40 and 50% [v/v]) in the bud multiplication medium. The test was performed in an entirely randomised experimental design with three replications, each replication represented by a flask with five buds. The data on explant survival and buds per explant after 45 days of cultivation were subjected to variance analysis and Tukey tests for a comparison of the averages.

On the other hand, isolates of *F. solani* f. sp. *piperis* of infected plants were cultivated on potato sucrose agar (PSA) culture medium. Two weeks later, 1 cm disks containing fungal mycelium were cultivated for 35 days on Czapek-Dox medium, and an evaluation of the number of micro- and macroconidia per ml was carried out on the 8th, 11th, 14th, 17th, 23rd, 29th and 35th days after inoculation. At the end of the period, hyphae were dried in the greenhouse at 40°C for 48 hours and weighed. The data was used to generate a growth curve using the software Microsoft Excel.

In order to test the filtrate phytotoxicity, an experiment was conducted with a filtrate from a 28 day old fungal culture at different concentrations (0, 20, 30, 40 and 50% [v/v]) in basal MS medium supplemented with 0.5 mg/l BAP and 0.2 mg/l IAA – bud multiplication medium – and two forms of filtrate sterilisation. The first form of sterilisation was filter sterilisation (FS) using a sterile filter with a pore size of 0.22 µm coupled to a vacuum pump, and adding the filtrate to the autoclaved bud multiplication medium in an aseptic flux chamber. In the second sterilisation method, the fungal culture filtrate was autoclaved for 20 minutes, and added to the bud multiplication medium at the desired concentration, and then autoclaved for a second time (AII). The evaluation was done by calculating the percentage of bud lethality through variance analysis and a Tukey test for comparison of the averages, and linear regression analysis.

### ***In vitro* selection of variants that survived the treatment with fungal culture filtrate**

The buds that were subjected to gamma irradiation and surviving buds underwent several multiplication cycles in order to eliminate chimerism and to obtain homogeneous mutants. They were subjected to *in vitro* selection on a culture medium supplemented with a selection agent: fungal culture filtrate at concentrations of 50 and 55% (v/v) of the total filtrate obtained after 28 days of fungal cultivation in Czapek-Dox medium. A total of 4117 buds were subjected to *in vitro* selection, of which 2332, 532, 160 and 1093 buds originated from doses of 10, 20, 30 and 40 Gy, respectively. Survival was evaluated and converted into percentages of selection, and the selection index for each of the selection agent conditions applied was determined using chi-square tests.

## Preparation of spores and plant inoculation

The fungus *F. solani* f. sp. *piperis* was cultivated on PSA medium for two weeks, and spores were collected by adding sterile water and filtering through four layers of gauze. The spores were then counted in a Neubauer chamber, and adjusted to the desired concentration with sterile water. Susceptible black pepper plants cultivated in black polyethylene bags in the greenhouse were inoculated with different fungal concentrations ( $0$ ,  $2 \times 10^2$ ,  $2 \times 10^4$  and  $2 \times 10^6$  spores/ml) either applied to the soil or to aerial parts of the plant. 5 ml of the inoculum for each concentration, and five plants per treatment were used.

Seedlings regenerated from the buds selected *in vitro* were planted in soil, acclimatised for six months, and used for inoculations with the fungal spores. The inoculation was performed by spraying the aerial plant parts and watering the soil. Evaluations of seedling mortality were performed visually starting two months after inoculation. Re-isolation of the pathogen was done for confirmation based on Koch's postulate that "the microorganism must be re-isolated from the diseased organism and correspond to the original microorganism in pure culture".

## Field selection and evaluation of plants in areas of Fusariosis incidence

Evaluated and selected black pepper lineages were obtained from an experiment started in 1978 (Ando *et al.*, 1984). 428 cuttings, originating from the cultivar 'Singapore', were subjected to gamma irradiation with doses of 20 and 25 Gy, and planted in a recently deforested area in the municipality of Tomé-Açu, State of Pará, Amazonia, Brazil, where irradiated and surviving plants were grown to produce young plants  $V_1$ .  $V_1$  plants were pruned and a total of 500 cuttings with 2-3 internodes were prepared from which  $V_2$  plants were grown individually in 5 l plastic buckets containing a soil substrate compound. In the third month after planting, the soil of the  $V_2$  plants was inoculated with a spore suspension ( $5 \times 10^4$  spores per ml; 10 ml per 5 l bucket) of *F. solani* f. sp. *piperis* grown in PSA for 15-20 days. Surviving  $V_2$  plants (43 individuals) were transplanted to an area of high levels of Fusariosis occurrence in order to undergo natural selection. After three years of cultivation, three plants had survived from which  $V_3$  plants were produced and preliminary characterisation of the materials was undertaken.  $V_4$  plants were produced from the  $V_3$  plants with the best performances regarding the occurrence of the disease, growth vigour, shape and productivity of fruits in the fourth year of cultivation. The  $V_4$  plants with the best field performances based on evaluations by technicians after five years of cultivation were used to produce lineages of  $V_5$  plants.

## Agronomic evaluation of the $V_5$ plants

The experiment was started in March 1994 in an area of natural occurrence of Fusariosis. Lineages originating from both 16  $V_5$  cuttings and one non-irradiated cutting (cultivar 'Singapore') were used. The evaluation was conducted in the field, each consisting of a repetition of eight plants, varying from one to seven repetitions per lineage.

All  $V_5$  lineages were evaluated for the occurrence of Fusariosis and survival, length and weight of the spike, number of seeds per spike, wet and dry weight of 100 fruits, and individual plant production. In addition, the blooming period and the number of orthotropic and plagiotropic branches were also evaluated.

Averages and variation of the variables were obtained. A comparison made using the Tukey test was conducted based on three years of data collected in the field, as well as an analysis of genetic divergence through main components and canonical variables.

## RESULTS AND DISCUSSION

### Radiosensitivity of *in vitro* buds to gamma radiation

Buds subjected to different doses of gamma radiation (0 to 80 Gy) showed different responses. It was observed that the higher the dose, the greater the physiological effects, both in terms of survival rates of explants and in terms of differentiation of buds and explant weights. For doses > 40 Gy (60 and 80 Gy) the survival rates were 0%; and for the 10 Gy dose, the irradiation effects were not visible as the *in vitro* responses were similar to those of non-irradiated buds.

Determination coefficients ( $R^2$ ) were > 0.91, representing a > 95% correlation, and the resulting equations allowed us to calculate the gamma radiation doses necessary to achieve a 30-50% reduction in the parameters evaluated (survival rate, number of buds and explant weight) in comparison to non-irradiated material. The optimal doses varied from 14.17 to 18.47 Gy for a 30% reduction and 23.62 to 30.78 Gy for a 50% reduction (Table 17.1). The data revealed a direct relationship and functional response between the applied dose and the results obtained, which are shown in Figures 17.2. It should be noted that the most accurate correlation was observed for explant weight, followed by survival rate and number of different buds per explant.

Based on the physiological effects of gamma irradiation (Figure 17.3) and on suggestion by Predieri (2001), a dose of 20 Gy was chosen, as this dose would cause a determined physiological effect of > 30% and < 50% reduction of the evaluated parameters. The same dose was established by Ando *et al.* (1984), from which three surviving plants were selected after artificial and natural selection against Fusariosis. Likewise, higher doses caused more DNA alterations, which are mostly prejudicial and may compromise other desirable features of the original cultivar (Przybyla, 1994).

After selecting the dose, 1204 buds were subjected to 20 Gy of gamma radiation, and 741 buds survived, which represents 72.36% of the total number irradiated, close to the 72.29% determined before by the equation. As to the number of differentiated buds per explant, an average of 1.64 against the expected mean of 1.56 was obtained. Therefore, it can be said that the results obtained with a dose of 20 Gy were very similar to those expected (Ahloowalia and Maluszynski, 2001).

### *In vitro* toxicity of the culture filtrate of *F. solani* f. sp. *piperis*

Buds cultivated in a multiplication culture medium at different Czapek-Dox concentrations (0 to 50%) presented a similar response, for both bud proliferation and survival, after 45 days of cultivation. The average number of buds per explant varied from 2.67 to 3.20 and the survival rate from 69.79 to 100%, respectively at concentrations of 50% and 0% Czapek-Dox medium (Table 17.2). It should be emphasised that buds considered as non-survivors presented green tissue even though there was no differentiation of new buds. It is believed that the constituent compounds in the Czapek-Dox medium had no noticeable negative effect on the *in vitro* responses of cultures. This was a key condition for the use of tests with the fungal culture filtrate in the multiplication medium of buds to determine its concentration as a selection agent, in order to avoid the selection of other culture medium constituents different from the fungal culture filtrate, as suggested by Daub (1986).

In fungal cultures grown to produce the filtrate to be used as a selection agent, the average conidium production from three flasks followed the fungus growth curve equivalent to the estimated number of conidia per ml. On the 8th day of cultivation, conidium production was about  $8 \times 10^4$ , and  $3 \times 10^5$  on the 29th day, and decreased thereafter. Likewise, the production of hyphae varied from 1070 to 1153 mg dry weight after 35 days of cultivation, reaching an average of 1098 mg for every 200 ml of culture medium.

The resulting growth curve indicated that the most intense growth phase happened between 0 and 20 days after the beginning of cultivation, and that a stationary phase started from the 23rd day, with a decline occurring after the 29th day (Figure 17.4). This allowed us to infer that the tendency of more

intensive production of secondary metabolites and consequent phytotoxins might take place between the 23rd and 29th day of cultivation. *F. solani* f. sp. *piperis* culture filtrate was also produced by Duarte (1993) between 20 and 25 days of cultivation.

To assess the possibility of using the culture filtrate of *F. solani* f. sp. *piperis* as a selection agent, tests were performed to determine the toxicity of the culture filtrates to *in vitro* buds. For this purpose, two types of sterilisation of the culture filtrates were tested: FS and AII (Table 17.3). After FS, no bud differentiation was observed at concentrations > 30% FS, while 40% FS was estimated to be lethal to buds of plants susceptible to Fusariosis (Figures 17.5A and 17.6A, B). When the culture filtrate was sterilised by AII, no evidence of bud differentiation was found for 50% AII. 80, 60 and 20% of bud differentiation was observed in 20, 30 and 40% AII, respectively. Regression analysis allowed us to estimate that 52.1% AII concentration, as a selection agent, would cause 100% mortality of susceptible buds (Figures 17.5B and 17.6C, D). Matsumoto *et al.* (1995) and Hidalgo *et al.* (1999) established that the optimal concentrations of culture filtrates to be used as selection agents for *in vitro* selection of banana and pineapple were 15% (*F. oxysporum* f. sp. *cubense*) and 20% (*F. subglutinans*) (see Chapters 6 and 10).

### ***In vitro* selection of variants which survived the fungal culture filtrate**

The concentration of the culture filtrate of *F. solani* f. sp. *piperis* played an important role in the survival rate of buds, considering that lower survival rates of buds were observed when a 55% filtrate concentration was used as a culture medium for all buds, independent of the gamma radiation dose applied to them previously. In buds that originated from irradiation with 30 Gy, no survival was observed with a 55% concentration. Only three buds were selected with a 50% concentration among 160 buds initially transferred to the selection medium. On the other hand, a higher percentage of selected buds on a 50% filtrate concentration was obtained from buds subjected to gamma irradiation with a 10 Gy dose (11.35%), followed by 20 Gy (9.13%) and 40 Gy (4.22%) as shown in Table 17.4.

It is important to point out that a 50% concentration of the selection agent was not very effective in the selection process, considering that > 40% of buds survived, except for those originating from a 30 Gy dose (4.76%). Finally, fungal culture filtrate at a 55% concentration proved significantly more effective as a selection agent and could be used for screening materials for potential resistance to Fusariosis (Figure 17.7).

### **Determination of spore concentration and method of inoculation**

The form of fungal infection that proved to be the most efficient in the process of selection was that of soil inoculation where a concentration of  $2 \times 10^6$  spores/ml caused 100% of plant death after six months (Figure 17.8). Inoculation by spraying caused plant death, but survivors could still be found. In the case of soil inoculation, symptoms of the disease initially appeared within one to two months of planting, with leaves showing chlorosis, a yellowish colour and consequent necrosis. The leaves and stem dried, and plant death occurred about six months after planting, under the weather conditions at Piracicaba, State of São Paulo, in greenhouse cultivation. A similar artificial selection procedure was used by Ando *et al.* (1984) to select plants resistant to Fusariosis using soil inoculation with a suspension of  $4 \times 10^5$  spores/ml of the fungus.

### **Agronomic evaluation of plants originating from gamma-irradiated cuttings**

The selection of three surviving  $V_2$  plants from 428 irradiated  $V_1$  plants in 1978 allowed six generations to be propagated in the past 20 years. During the whole process, plants had been cultivated in areas of Fusariosis incidence. Through continuous selection of plants of good architecture, normal morphology as well as vigorous and favourable production features, propagation was attained through cuttings. From the fourth generation on ( $V_4$  plants), cuttings were planted in lineages. Such lineages planted in areas of Fusariosis incidence were evaluated for their agronomic traits, principally based on production characteristics, and lethality/survival after contact with the disease (Figure 17.9).

## Agronomic evaluation of the V<sub>5</sub> plant lineage

Based on evaluations conducted up to the year 2000, from the lineages consisting of three or more repetitions, C25, C45, C132 and C170 stood out by presenting lower death rates caused by Fusariosis. Nevertheless, other climatic or physiological factors such as drought and an excess of water in the soil affected the survival of lineages originating from the V<sub>1</sub> plants more significantly than the control (Table 17.5). It should be emphasised that death by Fusariosis incidence was less pronounced (19.3%) in lineages originating from irradiated cuttings than from the control (47.4%). In principle, the effect of gamma irradiation and selection for resistance or tolerance to Fusariosis favoured higher survival rates of plants in areas of fungal occurrence.

Plant flowering varied greatly, both in the V<sub>5</sub> plants of all lineages and in the control plants, beginning in mid-November when the rain season had just started, until March. Fructification also varied, beginning six months after the first flowering, verified from late May through September, with its peak in August, when most spikes presented fruits with a colour ranging from yellowish to reddish, a common situation for black pepper plants in the region according to Poltronieri *et al.* (1999).

Production characteristics evaluated in the year 2000 presented variation with regard to spike characteristics. Spike length varied from 6.60 to 9.28 cm (C163 and C26, respectively), with an average of 7.91 cm; spike weight between 3053 and 5870 g (C163 and C70, respectively) with an average of 4589 g; and a number of fruits from 27.3 to 49.6 per spike (C163 and C70, respectively) with an average of 38.1.

As for the weight per 100 fruits, variation between 8.66 g (N108) and 14.84 g (C137) was found, with an average of 11.61 g. Regarding average production per plant, lineage C45 presented the smallest production (1540 g) while the highest production (8900 g) was observed in lineage N135 (Table 17.6). The range of variation in spike length, spike weight and weight per 100 fruits was small, whereas the variation range for the number of fruits per spike was more prominent. The general production average per plant in the mutant lineages was 3912 g, while in the control plants (CT), originating from the traditional cultivar 'Singapore', the average per plant reached 2700 g.

Better performance was found for the V<sub>5</sub> plants than for the control, which provides evidence that the V<sub>5</sub> plants cultivated in an area of Fusariosis incidence show a more favourable response when subjected to unfavourable cultivation conditions.

Mutagenesis through gamma irradiation aiming at genetic breeding of black pepper plant against Fusariosis disease seems to be a more viable alternative when associated with *in vitro* technology such as *in vitro* selection and generation advancement through micropropagation and with genetic/molecular evaluation for detection of new allelic forms as well as adequate methods of artificial and natural selection against the fungus. All these integrated factors enable the manipulation of large populations under mutagenic treatment and reduce the time for stabilisation of chimeras, allowing *in vitro* screening of potential materials for resistance or tolerance to the disease, whereas genetic variation of selected plants can be detected through genetic and molecular analysis, confirming the expression of the character by appropriate methods of selection during greenhouse cultivation as well as in the field. Therefore, once a mutant possessing disease resistance or tolerance and potential productivity is obtained, it can be cloned *in vitro* in a shorter period of time and in a larger quantity. A new cultivar can be launched and/or incorporated into genetic breeding programmes of black pepper plants. Technologies thus generated and integrated will be powerful tools, especially regarding time and cost reduction, in the development of new cultivars.

## CONCLUSIONS

The use of mutation induction through gamma *in vitro* technology is a viable alternative for genetic breeding of black pepper plant that is aimed at obtaining genotypes resistant and/or tolerant to Fusariosis, since no source of genetic resistance has yet been found within the species. Procedures of

micropropagation, mutagenesis and *in vitro* selection of variants tolerant to metabolites of the fungus should be coupled with adequate strategies of artificial selection with fungal spore suspension and natural selection in areas of incidence of the disease as well as with evaluations of the agronomic traits of the selected material with regard to the production features.

This study has led to the following conclusions: (1) A dose of 20 Gy of gamma radiation is appropriate for mutagenesis of *in vitro* cultivated buds; (2) *In vitro* selection of variants against Fusariosis is possible with a 55% concentration of *F. solani* f. sp. *piperis* culture filtrate cultivated for 28 days in Czapek-Dox medium; (3) A concentration of  $2 \times 10^6$  spores/ml of fungus in suspension applied to the soil is adequate for artificial selection against Fusariosis under greenhouse conditions; and (4) V<sub>5</sub> plants selected from gamma-irradiated cuttings show higher longevity and productivity in areas of Fusariosis incidence.

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**Table 17.1.** Effect of radiation dose on survival, number of buds and weight per explant, linear equation, determination coefficient and adequate dose for a reduction of 30 and 50% of the evaluated parameters

Dosage (Gy)	Average		
	Survival	Buds/Explant	Weight/Explant
0	100.0	2.96	1.18
20	87.86	1.47	0.93
30	51.57	0.51	0.59
40	32.83	0.34	0.31
50	19.60	0.22	0.20
Equation	$y = -1.7395x + 107.08$	$y = -0.0572x + 2.7024$	$y = -0.01198x + 1.1847$
R <sup>2</sup>	0.9322	0.9152	0.9501
30% <sup>1</sup>	74.956 = 18.47 Gy	1.8917 = 14.17 Gy	0.8293 = 17.95 Gy
50% <sup>2</sup>	53.54 = 30.78 Gy	1.3512 = 23.62 Gy	0.5924 = 29.91 Gy

<sup>1</sup>30% reduction for each evaluated parameter according to the linear equation. <sup>2</sup>50% reduction for each evaluated parameter according to the linear equation.

**Table 17.2.** Effect of different concentrations of Czapek-Dox medium in the bud multiplication medium on the survival and proliferation of buds per explant

Coefficient of variation	Degree of freedom	Mean square		F test		Czapek-Dox medium (%)	Survival (%) <sup>1</sup>	No. of buds per explant <sup>2</sup>
		Survival	Buds/Explant	Survival	Buds/Explant			
Treatments	4	0.0065	0.1441	2.06 <sup>NS</sup>	1.32 <sup>NS</sup>	0	100.0a <sup>3</sup>	3.20a
						20	93.19a	3.00ab
						30	89.83a	3.15ab
Residue	14	0.0032	0.1094			40	79.48a	2.78ab
						50	69.79a	2.67b

<sup>1</sup>General survival average = 87.62%; standard deviation = 4.197; CV = 4.79%. <sup>2</sup>General survival average = 2.99; standard deviation = 0.331; CV = 11.06%. <sup>3</sup>Values within a column followed by the same letters are not significantly different at < 0.05. <sup>NS</sup> = Not significant.

**Table 17.3.** Effect of the filtrate added to bud multiplication medium on bud differentiation per explant after 45 days of cultivation

Coefficient of variation	Degree of freedom	Mean square	F test	Filtrate (%)	Non-differentiation (%) <sup>1</sup>			
					FS	AII		
Sterilisation (A)	1	0.142	19.961**	0	22.47b <sup>2</sup>	A	9.58c	A
				20	89.83a	A	19.02c	B
Filtrate (%) (B)	4	0.118	16.639**	30	100.00a	A	40.00bc	B
				40	100.00a	A	78.30ab	A
Factor A × B	4	0.024	3.395*	50	100.00a	A	100.00a	A
Treatments	9	0.079		Equation	$y = 1.526x + 39.728$		$y = 1.892x - 2.973$	
				R <sup>2</sup>	0.7474		0.8829	
Residue	13	0.007		100% mortality <sup>3</sup>	40.0% FS		52.1% AII	

<sup>1</sup>General non-differentiation average = 62.74%; standard deviation = 4.98; CV = 7.94%. <sup>2</sup>Values followed by the same letter do not differ between each other. Lower case letters: comparison within the column; Capital case: comparison. <sup>3</sup>Filtrate concentration that should cause 100% mortality according to the linear equation. \*, \*\* Significant at  $p < 0.05$  and  $p < 0.01$ , respectively.

**Table 17.4.** *In vitro* selection of buds surviving in culture medium containing two concentrations of fungal filtrate as a selection agent after six weeks of cultivation

Dosage (Gy)	Filtrate 55%				Filtrate 50%				General		
	Live	Dead	Total	%	Live	Dead	Total	%	Live	Dead	%
10	123	961	1084	11.35	550	698	1248	44.07	673	2332	28.86
20	24	239	263	9.13	119	150	269	44.24	143	532	26.88
30	0	97	97	0.00	3	60	63	4.76	3	160	1.88
40	43	977	1020	4.22	54	19	73	73.97	97	1093	11.27
<b>Total</b>	<b>190</b>	<b>2274</b>	<b>2464</b>	<b>7.71</b>	<b>726</b>	<b>927</b>	<b>1653</b>	<b>43.92</b>	<b>916</b>	<b>4117</b>	<b>22.49</b>

**Table 17.5.** Fusariosis incidence and plant survival during 1999 and 2000

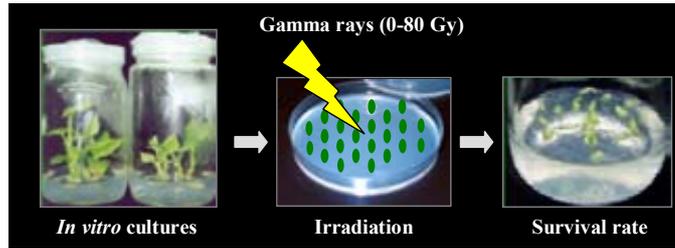
Lineage <sup>1</sup>	Total No. of plants	Replication	Surviving plants (%)		Plants killed by Fusariosis (%)	
			1999	2000	1999	2000
C25	22	3	7 (31.8)	7 (31.8)	1 (6.7)	1 (6.7)
C26	40	5	18 (45.0)	12 (30.0)	2 (9.1)	8 (28.6)
C45	24	3	7 (29.2)	6 (25.0)	1 (5.9)	1 (11.1)
C70	56	7	29 (51.8)	19 (33.9)	0 (0.0)	9 (24.3)
C123	40	5	19 (47.5)	15 (37.5)	1 (4.8)	4 (16.0)
C132	24	3	9 (37.5)	8 (33.3)	0 (0.0)	0 (0.0)
C137	24	3	13 (54.2)	10 (41.7)	1 (9.1)	3 (21.4)
C163	56	7	23 (41.1)	12 (21.4)	3 (9.1)	11 (25.0)
C166	56	7	19 (33.9)	11 (19.6)	4 (10.8)	11 (24.4)
C170	48	6	18 (37.5)	15 (31.3)	0 (0.0)	3 (9.1)
N27	8	1	4 (50.0)	4 (50.0)	1 (25.0)	1 (25.0)
N108	16	2	7 (43.8)	5 (31.3)	1 (11.1)	3 (27.3)
N121	8	1	5 (62.5)	3 (37.5)	0 (0.0)	2 (66.7)
N135	8	1	4 (50.0)	3 (37.5)	0 (0.0)	1 (25.0)
N148	8	1	3 (37.5)	3 (37.5)	0 (0.0)	0 (0.0)
<b>Total</b>	<b>438</b>		<b>185 (42.2)</b>	<b>133 (30.4)</b>	<b>15 (5.9)</b>	<b>59 (19.3)</b>
<b>Control</b>	<b>24</b>	<b>3</b>	<b>13 (54.2)</b>	<b>5 (20.8)</b>	<b>2 (18.2)</b>	<b>9 (47.4)</b>

<sup>1</sup>Lineages selected from irradiated cuttings and plants from non-irradiated cuttings (control) in Tomé-Açu, Brazil.

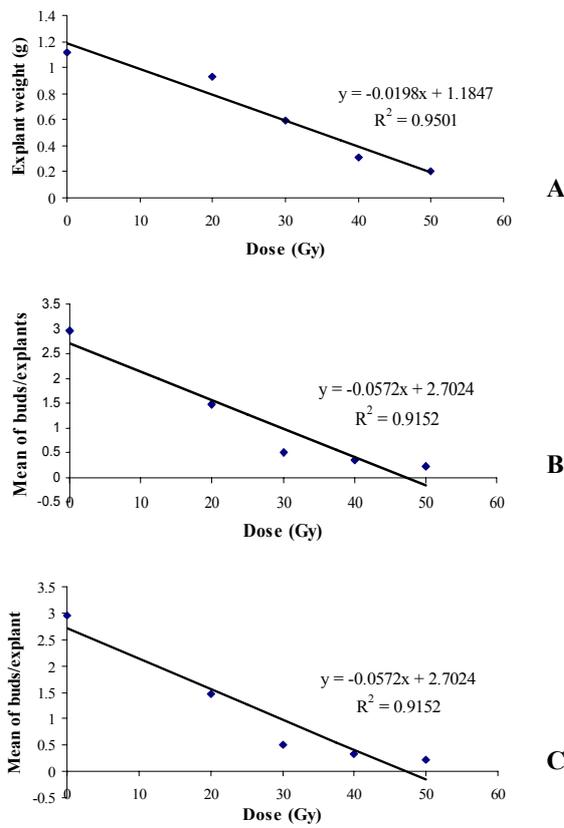
**Table 17.6.** Spike features, weight per 100 fruits and average production per green pepper plant of V<sub>5</sub> lineages after six years of cultivation

Lineage	Spike <sup>1</sup>		Fruits	Weight/100 fruits (g)	Production (g) average/plant
	Length (cm)	Weight (g)			
C26	9.28	4.197	43.0	10.68	4661.11
C132	7.85	4.842	32.6	12.36	3650.00
C166	8.03	3.874	32.5	12.45	4521.43
N135	8.42	5.054	43.6	10.79	8900.00
C70	8.79	5.870	49.6	12.07	4258.89
N148	7.33	4.686	42.8	8.80	2925.00
C25	7.94	4.581	35.5	10.52	3000.00
C45	6.90	4.624	33.7	14.40	1540.00
N1A	7.47	4.897	39.3	10.55	5400.00
C163	6.60	3.053	27.3	12.52	3072.22
C123	7.70	4.759	41.8	10.47	4629.17
N121	9.07	5.614	45.4	12.44	3950.00
C170	8.72	4.758	31.8	13.90	2722.22
C137	7.36	4.087	34.8	14.84	2566.67
N27	7.68	4.321	36.4	10.33	3203.33
N108	7.49	4.206	39.3	8.66	3600.00
<b>Average</b>	<b>7.91</b>	<b>4.589</b>	<b>38.1</b>	<b>11.61</b>	<b>3912.50</b>

<sup>1</sup>Average of 10 spikes.



**Figure 17.1.** Radiosensitivity test of *in vitro* buds irradiated with various doses of gamma-rays ranging from 0 to 80 Gy and cultivation in a bud multiplication medium.



**Figure 17.2.** Effect of gamma radiation on explant weight (A), survival (B) and number of buds per explant (C).

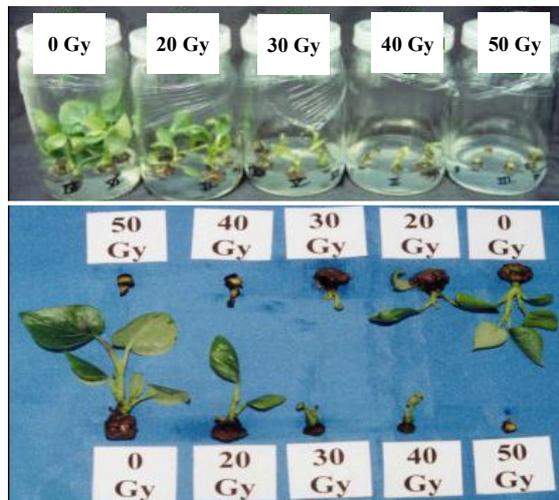


Figure 17.3. Effect of gamma radiation, doses ranging from 0 to 50 Gy, on *in vitro* buds.

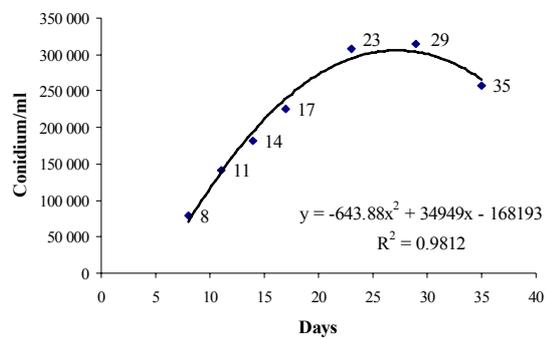
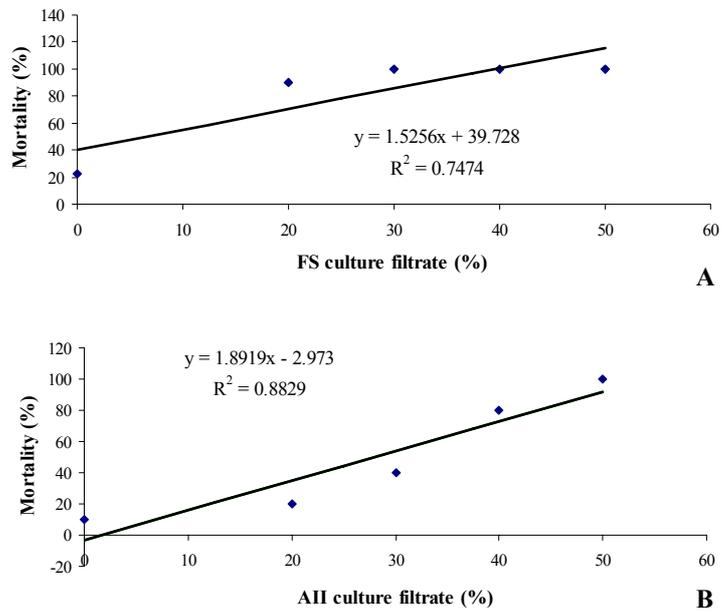
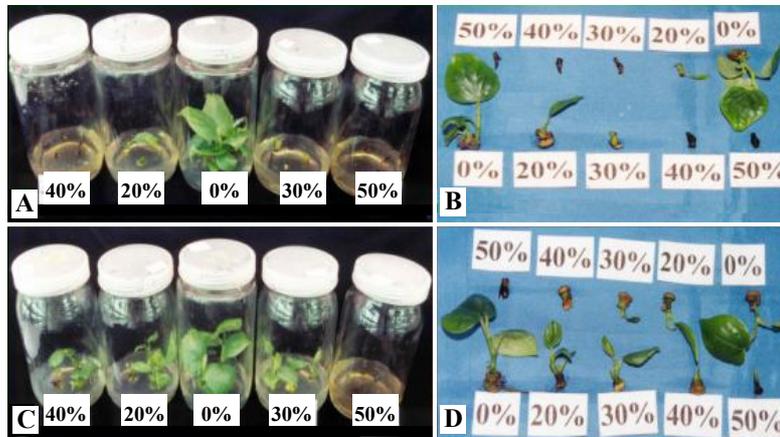


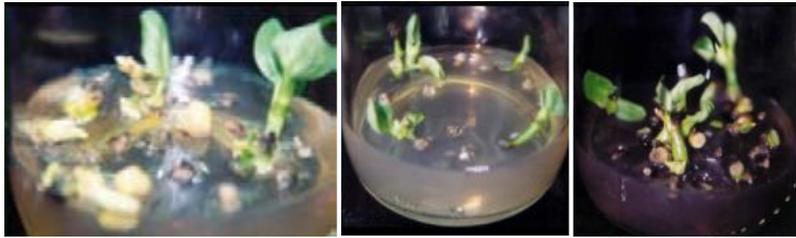
Figure 17.4. Growth curve of *Fusarium solani* f. sp. *piperis* cultivated on Czapek-Dox medium for 35 days.



**Figure 17.5.** Effect of two different forms of culture filtrate sterilisation on bud differentiation. (A) filter sterilisation (FS); (B) Double autoclaving (AII).



**Figure 17.6.** Effect of different filtrate concentrations (0, 20, 30, 40 and 50%) as a selective agent in bud multiplication medium. (A-B) Filter sterilisation (FS) after 45 days of cultivation; (C-D) Double autoclaving (AII) after 45 days of cultivation.



**Figure 17.7.** *In vitro* selection of variants originating from gamma irradiation of buds which survived the culture filtrate of *Fusarium solani* f. sp. *piperis* cultured for six weeks.



**Figure 17.8.** Plants subjected to inoculation with fungal spores (0 to  $2 \times 10^5$ ,  $\times 10^4$  and  $\times 10^6$  spores/ml). (A) One month after inoculation; (B) Six months after inoculation: Left (E) via spraying and right (D) via soil inoculation.



**Figure 17.9.** Mortality and survival of  $V_5$  plants in an area of high incidence of Fusariosis. (A-C) death by *Fusarium solani* f. sp. *piperis* attack; (D-E) surviving  $V_5$  plants; (F) control plant originated from non-irradiated cuttings.

## Chapter 18

### Screening for resistance to cucurbit downy mildew (*Pseudoperonospora cubensis*)

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#### Abstract

Basic methods of screening for resistance to *Pseudoperonospora cubensis*, causing cucurbit downy mildew, are described in this chapter. Procedures of pathogen isolation, cultivation and maintenance in *in vitro* conditions are summarised. The preparation of plant material suitable for such tests is also given. The methodology of inoculum preparation, inoculation and incubation is described. Both qualitative and semi-quantitative methods for disease assessment exemplified by using leaf disks are described. A well characterised set of differential germplasm for determination of *P. cubensis* pathotypes (Lebeda and Widrechner, 2003), including 12 cucurbitaceous genotypes from six genera, is presented. The denomination of pathotypes using unique (tetrad) numerical codes and following the differential set is proposed. Methods described in this chapter have broad applications in research and practical breeding.

#### INTRODUCTION

The Cucurbitaceae is a remarkable plant family, deserving of our attention because of its economic, aesthetic, cultural, medicinal and botanical significance. In the Old and New Worlds, cucurbits have been associated with human nutrition and culture for more than 12000 years. Thus, the Cucurbitaceae, along with the Brassicaceae and Asteraceae, can be considered to be families of extraordinary importance to humans, and follow after cereals and legumes in their economic significance for the human economy (Lebeda *et al.*, 2007).

Cucurbit downy mildew, caused by *P. cubensis* (Berk. and Curt.) Rostov (Figures 18.1 and 18.2) is one of the most important diseases affecting field and glasshouse cucumbers, and other cucurbits around the world (Lebeda and Widrechner, 2003). Although cucurbit downy mildew most often occurs in tropical, subtropical and warm, temperate areas of the world (Lebeda and Widrechner, 2003), it is also known to occur in cooler regions, such as Sweden (Forsberg, 1986) and Finland (Tahvonen, 1985), to where inoculum is transported by air flows. Overwintering as oospores is known and was observed in natural conditions in Japan (Hiura and Kawada, 1933), China (Chen *et al.*, 1959; Zhang *et al.*, 2006), India (Bains *et al.*, 1977), Italy (D'Ercole, 1975) and Austria (Bedlan, 1989). *P. cubensis* attacks a broad spectrum of cucurbitaceous plants, especially cucumbers (*Cucumis sativus*), muskmelons (*Cucumis melo*), watermelons (*Citrullus lanatus*) and cucurbits (*Cucurbita pepo*, *C. maxima*). Palti and Cohen (1980) reported about 40 host species; however, more recently at least 60 species have been reported to be affected (Lebeda 1992a, 1999; Lebeda and Widrechner, 2003, 2004).

Interactions and host-parasite specificity between Cucurbitaceae and *P. cubensis* are very heterogeneous and complex. The existence of *P. cubensis* pathogenicity variation on the level of pathotypes and races has been confirmed in different countries around the world (Lebeda and Widrechner, 2003; Lebeda *et al.*, 2006). The differentiation of pathotypes is described in the final part of this chapter (Tables 18.1-3).

Race-specificity was observed in interactions between *C. melo* and *P. cubensis*, and between *Cucurbita* spp. and *P. cubensis* (Lebeda, 1991, 1999; Lebeda and Gadasová, 2002; Lebeda and Widrlechner, 2003, 2004); however, until now no differential set for race determination has been developed (Lebeda *et al.*, 2006). Recently, some previously unknown and highly virulent isolates were described from Israel (Cohen *et al.*, 2003) and the Czech Republic (Lebeda and Urban, 2004a, 2004b).

Disease control through the growing of resistant cucumber (*C. sativus* L.) cultivars has not, as yet, been effective (Lebeda, 1992b; Lebeda and Prášil, 1994; Lebeda and Widrlechner, 2003; Lebeda and Urban, 2004a, 2004b). Despite research progress in this field (Lebeda, 1999; Lebeda and Doležal, 1995; Lebeda *et al.*, 1996), no effective source of resistance of *C. sativus* has been found. Sitterly (1972) only reported field resistance of some cultivars to *P. cubensis*. On the contrary, effective sources of resistance based on race specificity are known in the case of *C. melo* and *Cucurbita* spp. (Lebeda, 1991; Lebeda and Křístková, 1993, 2000; Lebeda and Widrlechner, 2004). However, according to the terminology of McDonald and Linde (2002), *P. cubensis* belongs to the group of “the highest risk pathogens” with high evolutionary potential (Lebeda and Urban, 2004a; Lebeda *et al.*, 2006; Urban and Lebeda, 2006) and, thus, the use of resistance genes has to be combined with other practices of an integrated management system (antifungal compounds and plant defence inducers, balanced fertiliser input, forecasting systems and diagnostics etc.) to minimize the risk of the pathogen attack.

## MATERIAL AND METHODS

### Pathogen isolation, cultivation and maintenance

In order to isolate the pathogen, leaf samples infected by *P. cubensis* are placed in plastic pots (110 × 85 × 45 mm) on wet filter paper. Depending on the degree of infection and destruction of the plant tissue, it is possible to maintain these samples for 2-3 days at 10-15°C. Leaves are placed with the adaxial surface down as conidiosporangiophores are especially produced on the abaxial surface. The highest level of sporulation is usually achieved in fresh chlorotic lesions.

The primary inoculum is prepared from a lesion, surrounded by intact tissue, with well-developed conidiosporangiophores with spores (Figure 18.1). The lesion is cut out with sterile scissors and shaken by tweezers in a beaker (25 ml) with distilled water (approximately 5 ml); sterilised water is more suitable. The plant tissue is then removed from the water, and the inoculum is atomised over the abaxial surface of a leaf of a highly susceptible genotype (e.g., *C. sativus* ‘Marketer 430’ or *C. sativus* ‘Stela F1’). The leaf is then placed with the adaxial surface down on a moistened filter paper in a Petri dish (Lebeda, 1986).

For genetic studies, it is more appropriate to use monospore isolates, which are prepared by isolation and cultivation of a conidiospore as follows: A conidiospore suspension is prepared from a primary isolate and diluted in distilled water (in a ratio of approximately 1:5 to 1:10). A suspension drop is then placed on a slide with water agar (2-3 mm thick) and isolation is carried out using a monospore insulator (Fassatiová, 1979). The agar column with a conidiospore is then transferred onto a leaf disk (20 mm in diameter), placed with the adaxial surface down on a moistened filter paper in a Petri dish. A modification of this method was described by Michelmore and Ingram (1982). It is also possible to isolate a monospore with the help of a micropipette (Koch and Blok, 1985).

Pure cultures (isolates) are cultivated on leaf disks or whole leaves of a susceptible genotype placed in Petri dishes. After ~ 10-14 days (optimum 10 days), re-inoculation onto fresh plant material must be carried out. Longer time periods are not recommended because of destruction of plant tissues, loss of isolate vitality or possible infection by saprophytic microflora (Lebeda, 1986). *P. cubensis* isolates can be stored in Petri dishes packed in aluminium foil at – 80°C. The spores are vital for about six months; after this period, it is necessary to renew the cultures through fresh inoculations (Lebeda and Urban, 2004a).

## Preparation of plant material for screening

In our experience, true leaves (6-8 week old plants, 3-6 true leaf stage) are the most suitable for assessing resistance. Plants should be grown in a glasshouse under optimal conditions (25°C/15°C day/night, daily watering and weekly fertilisation), and should not be treated with chemicals (fungicides, etc.) as these could significantly influence the infection of the pathogen isolates. Leaves used in the tests have to be fully turgescient and without any signs of infections or pests. On the other hand, it is also possible to use a primary intact leaf for the infection tests (Thomas, 1982).

## Inoculum preparation, inoculation and incubation

The inoculum is multiplied on leaf disks (20 mm in diameter) or whole leaves of a highly susceptible cultivar (depending on the original host of the *P. cubensis* isolate), which are placed with the adaxial surface down on moistened filter paper in Petri dishes. The optimal conditions for inoculum incubation are as follows: Usually, 7-9 days after inoculation, the abaxial surfaces of the leaves/disks are covered with conidiosporangiophores and spores of the pathogen (Figure 18.2). The inoculum is prepared by shaking disks/leaf pieces with well-developed spores in distilled water. The remaining plant tissue should then be removed from the water, and the spore suspension diluted to  $10^5$ - $10^6$  spores/ml. This concentration is optimal for maximum infection pressure (Lebeda, 1986). However, lower concentrations ( $5 \times 10^3$ ) are also possible (Thomas, 1982).

The inoculum is applied onto leaf disks that were placed with the adaxial surface down on the moistened filter paper in Petri dishes. Alternatively, water agar can be used instead of the moistened filter paper. The inoculum is atomised over the surface of the disks with a glass sprayer (approximately 2-3 ml per Petri dish). The leaf surface should be uniformly covered by the spore suspension and the whole surface of the disks should touch the filter paper, otherwise only limited infection could occur (Lebeda, 1986).

Infection by *P. cubensis* is strongly influenced by environmental conditions. For that reason, it is necessary to ensure stable microclimate conditions during *in vitro* incubation. In field conditions, incubation of *P. cubensis* takes 4-12 days, and the shortest incubation occurs at 25°C (day) and 15°C (night). The production of spores requires high air humidity and a dark period of approximately six hours, and is inhibited by water drops on the surface of the leaves (Cohen, 1981). In *in vitro* conditions, inoculated disks are incubated in the growth chamber with stable conditions, i.e., a 12 hour photoperiod, 18°C/15°C during light/dark cycles. During the first 16-24 hours after inoculation, it is necessary to keep the inoculated leaves in the dark by covering the Petri dishes with black plastic foil. The pathogen usually produces conidiosporangiophores with spores 7-9 days after *in vitro* inoculation. The final evaluation is carried out 14 days after inoculation (Lebeda, 1986).

## Disease assessment

The assessment of resistance/tolerance is made between 6 and 14 days after inoculation at two day intervals. The level of tolerance of a particular host plant with known genotype and accession to a pathogen isolate can be quantified both qualitatively and semi-quantitatively.

For a qualitative evaluation, three degrees of infection are used to determine the level of tolerance according to Lebeda and Widrlechner (2003) (Figure 18.3):

- resistance: no visible symptoms of sporulation or very sparse sporulation ( $\leq 10\%$  of leaf disks covered by sporangiophores with spores);
- ± incomplete resistance: 10.1-35% of leaf disks covered by sporangiophores with spores;
- + susceptibility:  $> 35.1\%$  of leaf disks covered by sporangiophores with spores.

For a semi-quantitative evaluation, a 0-4 scale is used (Lebeda, 1986; Lebeda and Widrlechner, 2003) (Figure 18.4):

- 0 without sporulation;
- 1 ≤ 25% of the disk area covered by sporangiophores with spores;
- 2 25.1-50% of the disk area covered by sporangiophores with spores;
- 3 50.1-75% of the disk area covered by sporangiophores with spores;
- 4 > 75.1% of the disk area covered by sporangiophores with spores.

In both scales, the intensity of sporulation (i.e., the total degree of infection, DI) is expressed as a percentage of the maximum scores according to Townsend and Heuberger (1943):

$$P = \frac{\sum(n \times v)}{x \times N} \times 100$$

Where: P = the total degree of infection (DI); n = number of disks in every category of infection; v = the category of infection (0-4); x = the range of the scale (in this case = 4); N = the total number of evaluated disks. At least five disks in three replicates (i.e., 3 plants/accession) are tested in the case of homogeneous plant material. Highly susceptible cultivars such as *C. sativus* 'Marketer 430' or *C. sativus* 'Stela F1' are used as a control in the case of *P. cubensis* isolates from *C. sativus*.

### Determination of pathogenic variability

The first differential set of cucurbitaceous plants showing varying degrees of resistance to *P. cubensis* pathotypes was published in 1987 (Thomas *et al.*, 1987). This set included six differential genotypes from three cucurbitaceous genera (*Cucumis*, *Cucurbita*, *Citrullus*); however, they were not defined either at the species (*Cucurbita* spp.) or genotype (*Cucumis*, *Citrullus*) levels. For this reason, a new differential set was developed, and includes 12 cucurbitaceous genotypes from six genera, which are sufficiently characterised at the levels of species, subspecies and genotypes/accessions (Table 18.1) (Lebeda and Widrechner, 2003). *P. cubensis* pathotypes (Table 18.2) are described by using unique (tetrad) numerical codes following the proposed differential set of Cucurbitaceae (Table 18.1) according to Lebeda and Widrechner (2003) as shown in Table 18.3.

## CONCLUSIONS

The methods described in this chapter have broad applications in basic research, screening of plant germplasm and resistance breeding programmes of cucurbits (Lebeda *et al.*, 2007). The method using leaf disks is especially advantageous as it allows screening of resistance of a huge amount of plant material in a relatively limited space and in precisely defined conditions, as well as the characterisation of a large number of *P. cubensis* isolates from the viewpoint of pathotypes and races (Lebeda *et al.*, 2006). Data at the population level can contribute to elucidating temporal and spatial pathogen variation and dynamics, as well as to clarifying host-pathogen interactions. It is also important to consider the practical application of these data in resistance breeding, studies of fungicide effectiveness and disease management (Urban and Lebeda, 2006).

### Acknowledgements

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**Table 18.1.** Differential set of cucurbit taxa for determination of pathogenic variability in *Pseudoperonospora cubensis*\*

No.	Taxon	Accession number		Cultivar name	Country of origin
		Donor	EVIGEZ <sup>1</sup>		
1	<i>Cucumis sativus</i>		H39-0121	‘Marketer 430’	USA
2	<i>C. melo</i> subsp. <i>melo</i>	PI 292008	H40-1117	‘Ananas’ ‘Yoqne’am’	Israel
3	<i>C. melo</i> subsp. <i>agrestis</i> var. <i>conomon</i>	CUM 238/1974	H40-0625	‘Baj-Gua’	Japan
4	<i>C. melo</i> subsp. <i>agrestis</i> var. <i>acidulus</i>	PI 200819	H40-0611		Myanmar
5	<i>Cucurbita pepo</i> subsp. <i>pepo</i> <sup>2</sup>	PI 171622	H42-0117	‘Dolmalik’	Turkey
6	<i>C. pepo</i> subsp. <i>ovifera</i> var. <i>texana</i>	PI 614687	H42-0130		USA
7	<i>C. pepo</i> var. <i>fraterna</i> <sup>3</sup>	PI 532355	H42-0136		Mexico
8	<i>Cucurbita maxima</i>		H42-0137	‘Goliáš’	Czechoslovakia
9	<i>Citrullus lanatus</i>		H37-0008	‘Malali’	Israel
10	<i>Benincasa hispida</i>	BEN 485	H15-0001		USA
11	<i>Luffa cylindrica</i>		H63-0010		N/A
12	<i>Lagenaria siceraria</i>		H63-0009		N/A

<sup>1</sup> EVIGEZ: Czech genebank number; <sup>2</sup> Taxonomy of *Cucurbita* species is adjusted according to the recent correspondence with J. H. Wiersema (ARC, Beltsville, USA); <sup>3</sup> Originally described as *Cucurbita fraterna* (Lebeda and Gadasová, 2002). \* Adapted from Lebeda and Widrlechner (2003).

**Table 18.2.** Examples of reaction patterns of some *Pseudoperonospora cubensis* isolates originating in Europe and their degree of pathogenicity\*

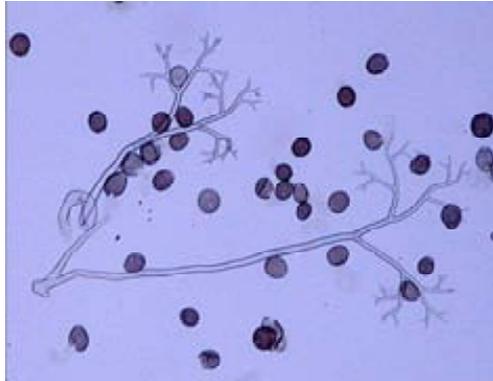
No.	Differential genotype	<i>P. cubensis</i> /isolate (PC) <sup>a</sup>							
		3/00 <sup>b</sup>	13/00	1/88	3/98	1/98	4/00	12/00	1/97
1	<i>Cucumis sativus</i>	+ <sup>c</sup>	+	+	+	+	+	+	+
2	<i>C. melo</i> subsp. <i>melo</i>	+	- <sup>d</sup>	+	-	+	-	+	+
3	<i>C. melo</i> subsp. <i>agrestis</i> var. <i>conomon</i>	-	-	-	-	-	+	-	+
4	<i>C. melo</i> subsp. <i>agrestis</i> var. <i>acidulus</i>	-	+	-	-	-	-	+	+
5	<i>Cucurbita pepo</i> subsp. <i>pepo</i>	-	-	-	-	-	-	-	-
6	<i>C. pepo</i> subsp. <i>ovifera</i> var. <i>texana</i>	-	+	+	+	+	+	+	+
7	<i>C. pepo</i> var. <i>fraterna</i>	-	-	-	-	-	+	-	-
8	<i>C. maxima</i>	-	+	-	+	+	+	+	+
9	<i>Citrullus lanatus</i>	-	-	-	-	-	-	-	+
10	<i>Benincasa hispida</i>	-	-	+	+	+	+	+	+
11	<i>Luffa cylindrica</i>	-	-	-	-	-	-	+	-
12	<i>Lagenaria siceraria</i>	-	-	+	+	+	+	+	+

<sup>a</sup> PC codes represent the isolate number/year of collection; <sup>b</sup> 3/00 isolated in France, all others in the Czech Republic; <sup>c</sup> resistant response (with no visible symptoms of sporulation or very sparse sporulation converging < 10% of infected area; <sup>d</sup> susceptible response with intensity of sporulation > 35% of infected area (Lebeda and Křístková, 1993). \* Modified according to Lebeda and Gadasová (2002).

**Table 18.3.** Unique (tetrad) numerical codes for *Pseudoperonospora cubensis* pathotypes (Table 18.2) following the proposed differential set of Cucurbitaceae (Table 18.1)\*

<b>Genotype</b>		<b>Cucumis spp.</b>				<b>Cucurbita spp.</b>				<b>Other cucurbits</b>				<b>Identifier</b>
<b>Differential number</b>		<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>	
<b>Isolate</b>	<b>Scoring value</b>	<b>1</b>	<b>2</b>	<b>4</b>	<b>8</b>	<b>1</b>	<b>2</b>	<b>4</b>	<b>8</b>	<b>1</b>	<b>2</b>	<b>4</b>	<b>8</b>	
PC 3/00		1	2	0	0	0	0	0	0	0	0	0	0	3.0.0
PC 13/00		1	0	0	8	0	2	0	8	0	0	0	0	9.10.0
PC 1/88		1	2	0	0	0	2	0	0	0	2	0	8	3.2.10
PC 3/98		1	0	0	0	0	2	0	8	0	2	0	8	1.10.10
PC 1/98		1	2	0	0	0	2	0	8	0	2	0	8	3.10.10
PC 4/00		1	0	4	0	0	2	4	8	0	2	0	8	5.14.10
PC 12/00		1	2	0	8	0	2	0	8	0	2	4	8	11.10.14
PC 1/97		1	2	4	8	0	2	0	8	1	2	0	8	15.10.11

\* According to Lebeda and Widrechner (2003)



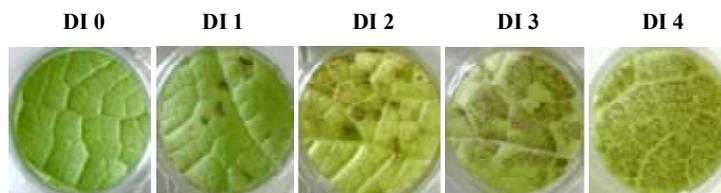
**Figure 18.1.** Conidiosporangiophores and spores of *Pseudoperonospora cubensis*.



**Figure 18.2.** Abaxial leaf surface of *Cucumis sativus* cv. 'Marketer 430' with sporulating *Pseudoperonospora cubensis*



**Figure 18.3.** Different responses in leaf disks to inoculation by *Pseudoperonospora cubensis* (nine days after inoculation). (A) Resistant response of *Cucurbita pepo* subsp. *pepo* cv. 'Dolmalik'; (B) Susceptible response of *C. pepo* subsp. *texana* (reduced sporulation of *P. cubensis*); (C) Susceptible response of *Cucumis sativus* cv. 'Marketer 430' (profuse sporulation of *P. cubensis*).



**Figure 18.4.** Detail of leaf disks with different degree of infection (DI 0-4) nine days after inoculation with *Pseudoperonospora cubensis*.

## Chapter 19

### Screening for resistance to cucurbit powdery mildews (*Golovinomyces cichoracearum*, *Podosphaera xanthii*)

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#### Abstract

In this chapter is summarized the basic knowledge about cucurbit powdery mildew (CPM) which is the major cause of losses in cucurbit production worldwide. The disease is caused by two obligate biotrophic ectoparasites, *Golovinomyces cichoracearum* s.l. (*Gc*) (syn. *Erysiphe cichoracearum* s.l.) and *Podosphaera xanthii* (*Px*) (syn. *Sphaerotheca fuliginea*). They can be distinguished easily under light microscopy according to the morphology of conidia and their germ tubes. Powdery mildew species differ in host range, ecological requirements, and geographic distribution. Broad pathogenic variation is represented by the existence of different pathotypes and races. Fungicide resistance of CPMs is a serious problem worldwide, therefore suitable sources of resistance and resistance breeding are considered as very important for all cucurbit crops. There are briefly described the basic methods of pathogen determination, isolation, cultivation and maintenance, including preparation of plant material for screening. The methods of adult leaf disks inoculation, disease assessment and calculation of degree of infection (DI) are described, as well as determination of pathogenic variability (pathotypes and races) by both species of cucurbit powdery mildew.

#### INTRODUCTION

Cucurbit powdery mildews (CPMs) cause a foliar fungal disease and are among the most intensively studied powdery mildews because of their economic impact (Cohen *et al.*, 2004; McCreight, 2006), not only in Europe but also worldwide (Jahn *et al.*, 2002). CPMs occur in all areas where cucurbits are cultivated, both in field and greenhouse crops. In field conditions, CPMs are less problematic than in the greenhouse. CPMs primarily infect leaf blades that can be totally destroyed during heavy infection. Reduction or destruction of assimilatory leaf surface can markedly influence yield and the quality of fruits (Skalický, 1961; Sitterly, 1978; Jahn *et al.*, 2002).

During recent years, the taxonomical division of the order Erysiphales has been revised (Braun *et al.*, 2002) and two ascomycete fungi are considered to be the causal agents of CPM infection: *Golovinomyces cichoracearum* (DC.) V. P. Gelyuta (*Gc*) (formerly *E. cichoracearum* resp. *E. orontii*) (Figure 19.1) and *Podosphaera xanthii* (Castag.) U. Braun and N. Shish. (*Px*) (formerly *Sphaerotheca fuliginea* or *S. fusca*) (Shishkoff, 2000) (Figure 19.2). The two species differ in their host range (Braun, 1995), ecological requirements (Sitterly, 1978; Lebeda, 1983), response to certain fungicides (McGrath, 2001; Sedláková and Lebeda, 2008) and pathogenicity (Bardin *et al.*, 1997, 1999; Lebeda *et al.*, 2007a). The identification of both species is relatively easy by microscopic examination of the morphological characters of conidia (Lebeda, 1983) (Figures 19.1 and 19.2). The occurrence of cleistothecia (chasmothecia) is recorded quite rarely (Figures 19.3 and 19.4).

In general, *Px* occurs more frequently in warmer regions and on protected crops (greenhouses, plastic tunnels). *Gc* is more common in temperate and cooler regions than in warmer areas. This fact was confirmed in the 1970s and 1980s during studies on the distribution of CPMs in the former

Czechoslovakia (Lebeda, 1983) and specified by Zlochová (1990) for the territory of Slovakia. It was also verified in recent observations (Lebeda, 1983; Křístková *et al.*, 2002; Lebeda and Sedláková, 2004), and a potential tendency of *Px* spread to northern areas of the Czech Republic has been indicated (Křístková *et al.*, 2002; Lebeda and Sedláková, 2004). It has been shown that the CPM species spectrum in the Czech Republic is markedly different from that in some western and southern European countries, and many other parts of the world (Křístková *et al.*, 2009), *Px* being the common or even the predominant CPM species (Bertrand *et al.*, 1992; McGrath, 1994; Vakalounakis *et al.*, 1994; Cohen *et al.*, 2004). The genetic background of heterothalism of both CPM species was found to be monogenic (McGrath *et al.*, 1996). The low frequency of occurrence of sexual stages (teleomorphs, Figure 19.4) is probably due to unequal distribution of sexually compatible mycelia (Bertrand, 1991; McGrath *et al.*, 1996).

The pathogenicity variation among *Px* and *Gc* has been described at the level of pathotypes and races (Bardin *et al.*, 1997, 1999). Pathotypes basically express pathogenicity variation at the host range level (Table 19.1); on the other hand, races represent the level of virulence on a set of selected genotypes of one host species (recently *Cucumis melo*, Table 19.2) with different resistance factors. Both species are characterised by broad pathogenic variation represented by the existence of different pathotypes and races (Bertrand *et al.*, 1992; Vakalounakis and Klironomou, 1995; Jahn *et al.*, 2002). 12 races of *Px* (Cohen *et al.*, 2004) and two races of *Gc* have been identified on melons, but recent results suggest that even more pathotypes and races exist (Lebeda and Sedláková, 2004, 2006; Lebeda *et al.*, 2004; McCreight, 2006; Lebeda *et al.*, 2008).

Jahn *et al.* (2002) published a comprehensive review of sources and genetic control of resistance to powdery mildew in cucurbits. It is clear that only relatively little progress has been made towards breeding *Cucumis sativus* resistant to CPM. The main reason for this is the absence of good resistance resources (Lebeda *et al.*, 2007). As opposed to this, in watermelon (*C. melo*) there are many sources of race-specific resistance available (Lebeda, 1991), including commercially cultivated cultivars (Lebeda *et al.*, 2007b). Broad variation of resistance against powdery mildews is known in gourds (*Cucurbita* spp.). We found important differences in field resistance of *C. pepo* and in some other species (Lebeda and Křístková, 1994, 1996; Lebeda *et al.*, 1999; Jahn *et al.*, 2002).

The application of fungicides is the principal tool for managing powdery mildews (Hollomon and Wheeler, 2002). Fungicide resistance of CPMs is a serious problem worldwide, as many reports show that strains are becoming resistant to eight groups of fungicides (e.g., fenarimol, benomyl, triforine etc.) (Epinat *et al.*, 1993; McGrath, 1996, 2001; and McGrath and Shishkoff, 2001, 2003). Resistance to some fungicides (e.g., triadimefon) can be expected to develop rapidly (McGrath and Shishkoff, 2001). The occurrence of fungicide resistance of CPM on cucurbit crops was also recently reported in the Czech Republic (Sedláková and Lebeda, 2003, 2004a, 2004b, 2008). Further research should focus on obtaining more comprehensive data about the geographical distribution, spatial and temporal variability, and shift of fungicide resistance, including the relationship with pathogenicity variation of CPM (Křístková *et al.*, 2004; Lebeda and Sedláková, 2006; Lebeda *et al.*, 2007a).

## **MATERIAL AND METHODS**

### **Pathogen isolation, cultivation and maintenance**

Fresh and heavily infected leaves of host plants (e.g., *Cucurbita pepo* L., *C. maxima* Duchesne, *Cucumis sativus* L. and *C. melo* L.) must be collected in the field or from under cover. Before isolation, it is necessary to carry out microscopic examination of the sample to identify the pathogen. Mixed cultures are not suitable for this work. The identification of *Px* and *Gc* is carried out by microscopic examination of the morphological characters of fresh or dry conidia in a 3% KOH solution (Lebeda, 1983). Conidia of pure cultures are transferred by tapping on the primary leaves of the highly susceptible cucumber (*C. sativus*) cultivar 'Stela F<sub>1</sub>' or other susceptible host genotype/species. By doing this, it is possible to obtain a pathogen culture (population of conidia) that is considered to be homogeneous.

An exact description for obtaining monosporic isolates of *Gc* is given in Schnathorst (1958). Leaves with pathogen cultures are softly atomised with distilled water with a glass sprayer. Conidia are then transferred by tapping them onto the surface of water agar in Petri dishes. Single conidia are isolated (under the microscope) and transferred with a glass needle in a drop of water to the surface of the leaf, which is placed on a piece of filter paper in a Petri dish. Incubation is at room temperature.

Because powdery mildew cannot be cultivated on synthetic media, it is necessary to maintain the culture on the primary leaves of susceptible host seedlings. Isolates are cultured in plastic boxes (24°C /18°C day/night with a 12 hour photoperiod) and transferred at approximately two week intervals (Lebeda, 1986). Sitterly (1978) also mentioned that it is possible to store CPM on dry leaves in paper bags for longer periods of time (up to one year) at room temperature. Based on the research of Bardin and Nicot (1999) and our own preliminary experiments (unpublished data), it appears that conidia of *Px* can withstand long-term storage in a deep freezer (– 40°C) after thorough drying. Pérez-García *et al.* (2006) recommend cold storage of *Px* conidia desiccated with silica gel at – 80°C. This is probably possible due to oil droplets present in the conidia.

### **Preparation of plant material for screening**

Resistance screening of cucurbits against powdery mildews is mostly performed with adult plants. Plants at the seedling stage (cotyledons) are not recommended for screening because expression of resistance mostly appears in later developmental stages (Angelov and Petkova, 1979; Lebeda, 1984). Plants at the stage of 3-6 true leaves are the most suitable for screening (Lebeda, 1986) and should be cultivated in greenhouses or growth chambers at 22-26 °C. It is recommended that plants have buttress (wire, string) for climbing. During the collection of leaves for resistance screening, the plants must be fully turgescient and not treated with chemicals (fungicide application can considerably influence the final response to powdery mildew). Throughout cultivation, it is necessary to keep plants free of spontaneous infection with powdery mildews, which can frequently occur during the summer months. From this viewpoint, the winter months are the most suitable time to perform these experiments as the risk of a spontaneous inoculum transfer and infection from outside the greenhouse or growth chamber is very low at this time.

### **Inoculum preparation, inoculation and incubation**

The fungal inoculum is prepared and multiplied on fully developed cotyledons or the first primary leaves of a highly susceptible cucumber cultivar (e.g., ‘Stella F<sub>1</sub>’) or another host species. Plants are grown in plastic pots filled with Perlite with two or three seedlings per pot (Figures 19.3 and 19.5). After the inoculation of cotyledons or primary leaves, the pots are put in transparent plastic boxes with a detachable cover (Figure 19.5). The inside of the box must be moderately ventilated to prevent the formation of water drops on the walls of the box and/or on the leaf surface. High air humidity may reduce sporulation and cause limited dispersion of conidia during inoculation. A sufficient amount of fresh conidia appears on the leaves 9-12 days after inoculation (Figure 19.6).

As mentioned above, it is best to use leaves from adult plants at the 3-6 leaf stage for inoculation. The disks (diameter: 15 mm; Figure 19.7) are cut with a cork borer from the leaves, and placed with their abaxial side on moistened filter paper in the Petri dishes (Lebeda, 1984, 1986). Another method has been proposed in which the leaf disks are put onto polystyrene pills floating in distilled water in the Petri dishes (Nagy, 1972). In this case, the Petri dishes must be opened every two days following inoculation so that the evaporated water can be gradually added. Leaf disks may also be cultivated on water agar supplemented with benzimidazole (40 mg/l) (Király *et al.*, 1974). Inoculation of leaf disks is performed by mechanical transfer of conidia (tapping, dusting). After inoculation, the surface of the leaf disks should be covered with a white coat of conidia (Lebeda, 1986). Sitterly (1978) proposed that the inoculum be prepared as a water suspension of conidia. However, we do not recommend this method because the conidia germinate badly in water drops, and mycelium does not develop well on the moist leaf surface.

Opinions about the optimal conditions for powdery mildew infection differ (Nagy, 1976; Sitterly, 1978; Bashi and Aust, 1980; Zlochová, 1990). However, in our experience, the most suitable temperature for incubation is 18-22°C with a photoperiod of 12 hours (Lebeda, 1986). During the incubation, it is necessary to keep the temperature constant, because it is known that, in cucumbers, the expression of resistance versus susceptibility may depend on the temperature (Munger, 1979). For optimal infection and good sporulation, the humidity inside the Petri dishes must be low (~ 60 - 70%).

### Methods of disease assessment

The assessment of infection intensity versus resistance is first carried out on the basis of the degree of coverage of disks with mycelium. Eventually, it is possible to combine this criterion with sporulation intensity. Disks are evaluated 14 days after inoculation using the following scale (Lebeda, 1984, 1986) (Figure 19.7):

- 0 = without symptoms of infection;
- 1 = ≤ 25% of disk surface covered with mycelium;
- 2 = > 25 - ≤ 50% of disk surface covered with mycelium;
- 3 = > 50 - ≤ 75% of disk surface covered with mycelium;
- 4 = > 75% of disk surface covered with mycelium.

Each plant genotype is represented by five-leaf disks (diameter: 15 mm) in at least three replicates (one replicate per plant) totalling at least 15 disks per genotype.

The intensity of sporulation, also called the degree of infection (DI), is expressed as a percentage of the maximum scores according to Townsend and Heuberger (1943):

$$P = \frac{\sum(n \times v)}{x \times N} \times 100$$

Where: P = the total degree of infection (DI); n = the number of disks in every category of infection; v = the category of infection (0-4); x = the range of the scale (in this case = 4) and N = the total number of evaluated disks.

Genotypes with low or no sporulation (i.e., DI = 0 - 1) are considered to be resistant (R); genotypes with a DI of 2 - 4 are scored as susceptible (S).

### Determination of pathogenic variability

To determine pathogenic variability (pathotypes and races), isolates are also screened using a leaf-disk method (Lebeda, 1986; Bertrand *et al.*, 1992; see above). In this case, a set of six differential cucurbit taxa (*C. melo* Védraçais and PMR 45, *C. sativus* Marketer, *C. pepo* Diamant F<sub>1</sub>, *C. maxima* Goliáš [Czech origin] and *Citrullus lanatus* Sugar Baby) (Bertrand *et al.*, 1992) is used for determination of pathotypes as shown in Table 19.1. The races are identified by using a set of 11 differential genotypes of *C. melo* (Iran H, Védraçais, Solartur, PMR 45, WMR 29, Edisto 47, PI 414723, PMR 5, PI 124112, MR-1, Nantais Oblong) as shown in Table 19.2 (Bardin *et al.*, 1999). Recently is in progress the development of a new system of determination and denomination of pathotypes and races of both cucurbit powdery mildews species (Lebeda *et al.*, 2008).

## CONCLUSIONS

Cucurbit powdery mildew is one of the most devastating diseases of cucurbits and is caused by two species (*Golovinomyces cichoracearum* and *Podosphaera xanthii*) which are distributed worldwide. However, till now there is limited success in resistance breeding of the most cucurbit crops because of enormous pathogenic variation of both CPM species and rather limited sources of efficient and durable resistance. Detailed and broad international research of CPMs pathogenic variability and sources of resistance, including their genetic background, can yield new knowledge in this area. For this purpose

are required unified and efficient methods of resistance screening by using well defined pathotypes and races (Lebeda *et al.*, 2008). The methods described in this chapter have a broad potential for applications both in screening of Cucurbitaceae germplasm and in cucurbits breeding programmes (Lebeda *et al.*, 2007b). The methodology is also useful for basic research of host-pathogen interactions, pre-breeding and mass-screening selection. The described methods can be also applied to the research of pathogenic variability (determination of pathotypes and races) of both powdery mildew species, as well as in their population studies (Lebeda *et al.*, 2008).

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**Table 19.1.** Response of different genotypes of Cucurbitaceae to pathotypes of *Golovinomyces cichoracearum* and *Podosphaera xanthii*

Differential genotype/abbreviation	France						Czech Republic					
	A <sup>2</sup>		Gc (4) <sup>1</sup>		Px (3)		Gc (10)		Px (6)			
	AB1B2	AC	ACD	A	AB1C	AB1B2C	AB1B2CC	AB1B2CCm	AB1CC	AB1CCm	D	D
<i>Cucumis sativus</i> 'Markete 430'	S	S	S	S	S	S	S	S	S	S	S	S
<i>Cucumis melo</i> 'Védrantais'	R	R	R	R	S	S	S	S	S	S	S	S
<i>Cucumis melo</i> 'PMR 45'	R	R	R	R	R	S	S	S	R	R	R	R
<i>Cucurbita pepo</i> 'Diamant F <sub>1</sub> '	R	R	S	R	S	S	S	S	S	S	S	S
<i>Cucurbita maxima</i> 'Goliáš'*	-	-	-	-	-	-	-	S	S	S	S	S
<i>Citrullus lanatus</i> 'Sugar Baby'	R	R	S	R	R	R	R	R	R	R	R	S

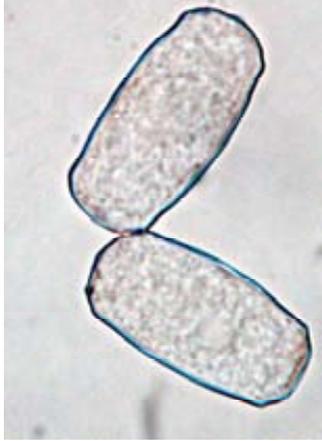
<sup>1</sup>Pathogen species (No. of identified pathotypes): Gc = *Golovinomyces cichoracearum*, Px = *Podosphaera xanthii*. <sup>2</sup>Designation of pathotypes based on the host response reaction: R = resistant, S = susceptible, - not tested; \*proposed by Lebeda and Sedláková (2004). Sources: for France: Bertrand (1991), Bertrand *et al.* (1992); for the Czech Republic: Lebeda and Sedláková (2004, 2006), Lebeda *et al.* (2007a).

Table 19.2. Examples of reaction patterns of *Golovinomyces cichoracearum* and *Podosphaera xanthii* for determining of pathogen races

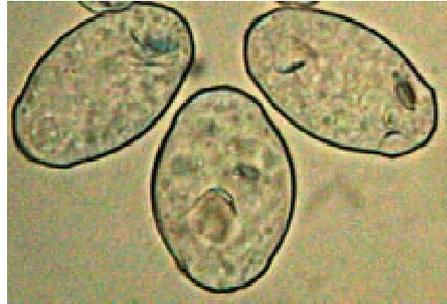
Race	Cucumis melo genotypes*												
	IrH	Véd	P45	W29	E47	PI41	P5	PI12	MR1	TpM	Ans	Nobl	
<i>Golovinomyces cichoracearum</i>													
0	S	R	R	R	R	R	R	R	R	-	-	R	Pitrat <i>et al.</i> (1998)
1	S	S	S	S	S	R	R	R	R	-	-	R	
f	R	S	S	R	R	R	R	R	R	-	-	S	Lebeda and Sedláková (2004, 2006), Lebeda <i>et al.</i> (2007a)
k	S	S	S	R	R	S	S	R	R	-	-	R	
S	S	S	S	S	S	S	S	S	S	-	-	S	
<i>Podosphaera xanthii</i>													
0	S	R	R	R	R	R	R	R	R	R	R	R	Pitrat <i>et al.</i> (1998)
1	S	S	R	R	R	R	R	R	R	S	S	S	
2US	S	S	S	het	S	S	R	R	R	S	S	S	
2F	S	S	S	R	R	R	R	R	R	S	S	S	
2S	S	S	S	het	R	S	R	R	R	S	-	-	McCreight (2006)
2Z	S	-	S	R	R	R	R	R	-	-	-	-	Alvarez <i>et al.</i> (2000)
3	-	S	S	-	R	R	S	R	R	S	S	S	Pitrat <i>et al.</i> (1998)
4	S	S	S	S	R	R	R	R	R	S	S	S	
5	S	S	S	S	S	R	R	R	R	S	S	S	
6	-	S	S	S	S	R	R	R	-	-	-	-	Bertrand (2002)
N1	-	-	R	R	R	S	R	-	-	-	-	-	Hosoya <i>et al.</i> (2000)
N2	-	-	S	R	R	S	R	-	-	-	-	-	
N3	-	-	R	R	S	S	R	-	-	-	-	-	
N4	-	-	R	R	S	R	R	-	-	-	-	-	
R1	R	S	S	R	R	S	S	R	R	-	-	S	Lebeda and Sedláková (2004, 2006), Lebeda <i>et al.</i> (2007a)
G	S	S	S	S	S	S	S	R	R	-	-	S	
H	S	S	S	R	S	S	S	S	S	-	-	S	
F	S	S	S	S	S	S	S	S	S	-	-	S	

Reaction: R = resistant, S = susceptible, het = heterogeneous, - = not tested.

\**C. melo* genotypes: IrH ('Iran H'), Véd ('Védrañtais'), P45 (PMR 45), W29 (WMR 29), E47 ('Edisto 47'), PI41 (PI 414 723), P5 (PMR 5), PI12 (PI 124 112), MR1 (MR-1), TpM ('Top Mark'), Ans ('Ananas'), Nobl ('Nantais Oblong'). Sources: Pitrat *et al.* (1998), Bardin *et al.* (1999), Jahn *et al.* (2002), McCreight (2006), Lebeda *et al.* (2008).



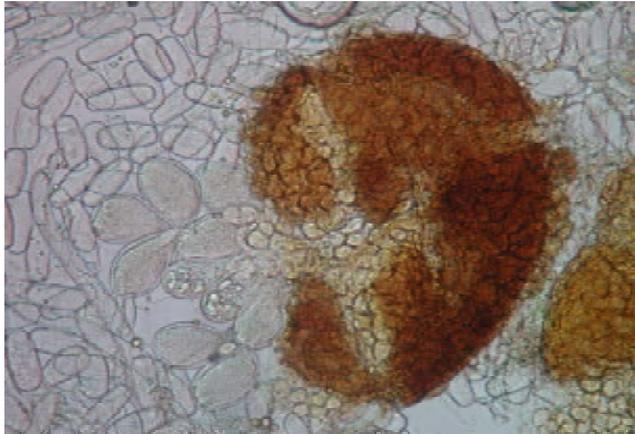
**Figure 19.1.** Conidia of *Golovinomyces cichoracearum*.



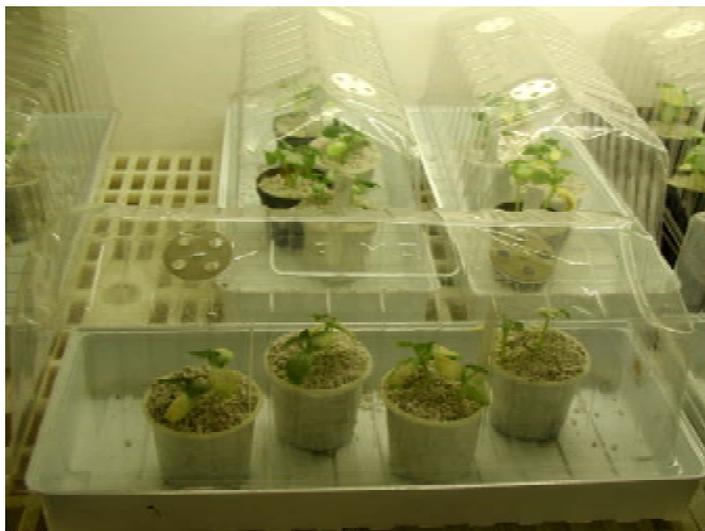
**Figure 19.2.** Conidia of *Podosphaera xanthii*.



**Figure 19.3.** Sporulation of cucurbit powdery mildew on cotyledons of *Cucumis sativus*, susceptible cv. 'Stela F<sub>1</sub>' (formation of cleistothecia on the necrotic tissue).



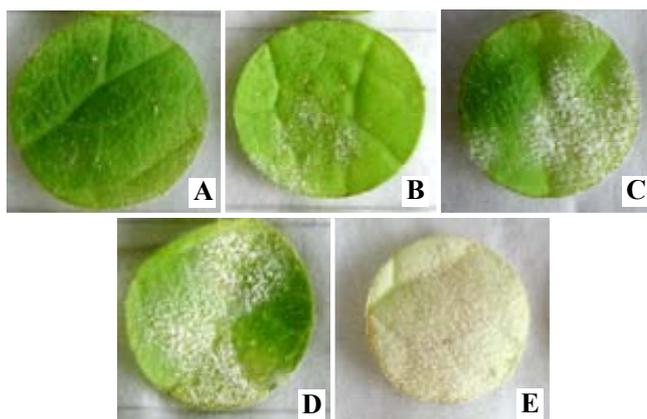
**Figure 19.4.** Cleistothecium with asci of *Golovinomyces cichoracearum*.



**Figure 19.5.** Maintenance of cucurbit powdery mildew isolates.



**Figure 19.6.** Sporulation of cucurbit powdery mildew (*Golovinomyces cichoracearum*) on cotyledons of susceptible *Cucumis sativus* cv. 'Stela F<sub>1</sub>'.



**Figure 19.7.** Detail of leaf disks with different degrees of infection (DI) 14 days after inoculation with *Golovinomyces cichoracearum*. (A) DI = 0: no symptoms of pathogen development; (B) DI = 1: mild development of mycelium without sporulation; (C) DI = 2: well-developed mycelium with mild sporulation; (D) DI = 3: intensive sporulation and well-developed mycelium covering <50% of the leaf disk area; (E) DI = 4: intensive sporulation and well-developed mycelium covering 50-100% of the leaf disk area. *Source:* Lebeda (1983).



## Chapter 20

### Screening onions and related species for resistance to Anthracnose (*Colletotrichum gloeosporioides*)

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#### Abstract

Anthracnose (*Colletotrichum gloeosporioides*) is an airborne disease which causes significant yield losses in onion (*Allium cepa* var. *cepa*) and shallot (*A. cepa* var. *ascalonicum*) in tropical regions. There is scant variation in the response to the pathogen within *A. cepa*, but high resistance was found in accessions of *A. fistulosum*, *A. galanthum*, *A. roylei* and other onion-related species. Their introgression recently became feasible, and their resistances to temperate diseases are currently being exploited. Screening for resistance to anthracnose is facilitated by *in vitro* multiplication of the plant material and the use of massive experimental inoculation under conditions highly conducive to the disease (27°C, 95% relative humidity) in a growing chamber. These elements provide economic, quick, repeatable and reliable screening. Protocols for the *in vitro* introduction of *Allium* plant material, for its multiplication and its acclimatisation are provided. On average, the number of explants doubles every three to four weeks. An aggressive strain should be selected, and the inoculum produced as a suspension of conidia, which is mass sprayed on the populations to be screened. High relative humidity (100%) should be ensured by covering the plants with plastic bags during the incubation period (48 to 96 hours after inoculation). The disease is scored after two weeks. A high level of resistance was found in accessions of *A. galanthum* and *A. fistulosum*, and partial resistance in *A. roylei*. A genetic analysis of the resistance in *A. roylei* to a Brazilian isolate, revealed that it is dominantly inherited and most probably determined by more than one gene.

#### INTRODUCTION

Anthracnose (*C. gloeosporioides* Penz; teleomorph: *Glomerella cingulata* Spould & Schrenk) is an airborne disease which causes significant yield losses in onion (*A. cepa* var. *cepa*) and shallot (*A. cepa* var. *ascalonicum*) in tropical regions. It is widespread in Indonesia, India, Brazil and other countries, and in extreme cases can affect 100% of the crop (Chawda and Rajasab, 1992; Wietsma *et al.*, 1998; Pedrosa *et al.*, 2004). Anthracnose causes abnormal twisting and elongation of the pseudostem, slender and distorted leaves, and soft rotting of the bulbs. Leaf lesions, scattered with minute, slightly raised acervuli (fruiting bodies) carrying pink masses of conidia, develop (Chawda and Rajasab, 1992; Boff, 1996; Weeraratne, 2002).

Temperature and wetness period are major factors contributing to infection and disease development. The optimum temperature for spore germination is 26°C, while maximum mycelium growth occurs at 23-27°C, and spore production is optimal at 27°C (Suhardi, 1994; Carneiro and Amorim, 1999). A leaf wetness period of two hours can be enough to cause infection (Carneiro and Amorim, 1999), although Suhardi (1991) found that the longer the wetness period, the higher the disease severity. The control of onion and shallot anthracnose is based on chemicals, and cultural practices that tend to reduce the

levels of initial inoculum and to cause unfavourable conditions for disease development (Suhardi, 1993; Haddad *et al.*, 2003; Pedrosa *et al.*, 2004).

Breeding resistant cultivars may be an environmentally sound method of disease control; however, there is very limited variation in the response within *A. cepa*. The shallot cultivar 'Sumenep' showed reduced disease severity in Indonesia (Suhardi, 1993), but being sterile, it is not useful for breeding.

In Brazil, variation in the reaction of onion cultivars to anthracnose was found. The local cultivar 'Barreiro' was identified first, and its derivative 'Pira Ouro' was selected for resistance (Melo and Costa, 1983). Recently, after screening 15 isolates under controlled conditions, cultivars developed locally such as 'Vale Ouro IPA 11' and 'Roxinha de Belem' differed from the susceptible cultivars 'Texas EG 502' and 'Brownsville' (Assunção *et al.*, 1999). In the same way, Pedrosa *et al.* (2004) screened a set of Brazilian onion cultivars and Uruguayan germplasm. Local germplasm was more resistant than introduced susceptible cultivars, and differed in the frequency of initial infection (4.85 to 3.64 lesions/leaf) and in the rate of disease development (Gompertz  $r_g$ : 0.13 to 0.20), but not in the maximum severity nine days after inoculation, nor in the latent period (13.3 to 14.5 days). This scant variation in the local germplasm, in a highly conducive screening test, was also found for other onion diseases (Galván *et al.*, 2004), and is an important tool for small household farming, and therefore a valuable source of resistance.

Higher levels of resistance to anthracnose were found in accessions of *A. fistulosum*, *A. galanthum*, *A. roylei* and other onion-related species (Galván *et al.*, 1997). Introgression of resistances and other useful characters from onion-related species has been viable since the late 1980s, with successful crosses with *A. roylei* and *A. galanthum*. The generation of tri-hybrid populations by the bridge cross *A. cepa* × (*A. roylei* × *A. fistulosum*) allows the introduction of *A. fistulosum* features into the genetic basis of the crop (Kik, 2002).

The exploitation of resistances to temperate diseases from *A. cepa*-allied species is ongoing. *A. roylei* resistance to *Peronospora destructor* was found to be controlled by one gene, located in the distal portion of the linkage group assigned to chromosome 2 (Kofoet *et al.*, 1990; Kik, 2002). Resistance to *Botrytis squamosa* was found in *A. roylei* (de Vries *et al.*, 1992) and *A. fistulosum* (Currah and Maude, 1984), the former being exploited by backcrossing (Alan *et al.*, 2003). Similar introgression schemes could be implemented to breed for high resistance to *C. gloeosporioides*.

The development and availability of accurate screening to identify the response of specific genotypes become an important issue for the use of molecular techniques, such as the development of molecular markers via bulk segregant analysis, or via linkage disequilibrium. Indeed, the screening stage forms a bottle-neck for many pathosystems and, therefore, deserves attention. As a general goal, the adopted screening method should be economical, quick and repeatable.

*In vitro* multiplication of *Allium* species is a useful technique when screening inter-specific populations and other valuable plant material (de Melo *et al.*, 2003). This technique allows: (1) the maintenance of specific genotypes and populations over long periods, and (2) the availability of a large enough number of replications for the proper characterisation of specific genotypes.

Screening for resistance to anthracnose is facilitated by *in vitro* multiplication of the plant material and the use of mass experimental inoculation under conditions highly conducive to disease development in a growing chamber. These elements provide a reliable screening procedure.

## MATERIAL AND METHODS

### Inoculum production

*C. gloeosporioides* isolates differ in their virulence, when comparing isolates from different onion or shallot cultivation areas (Suhardi, 1994; Assunção *et al.*, 1999; Pedrosa *et al.*, 2004) and continents (Galván *et al.*, 1997). There is no indication of isolate-specific responses, but rather differences in the presence of pathogenic factors among isolates, thus leading to differences in virulence. Suhardi (1991) also found differences between two isolates with regard to their temperature requirements for *in vitro* spore germination and mycelium growth, suggesting ecological adaptation to local conditions.

An aggressive strain, isolated from the target cropping area, should be selected for screening. So far, no advantage has been gained by mixing isolates in the resistance tests.

The pathogen is *in vitro* multiplied on potato dextrose agar (PDA) at 27°C in darkness. The inoculum is produced as a suspension of conidia by gently rubbing 10 day old colonies (usually fully sporulated) and filtering with a cheese-cloth. The suspension is then adjusted to  $1.0-1.2 \times 10^6$  spores/ml and a surfactant such as Tween-80 is added (10 drops/l). The inoculation is performed with a hand atomizer.

### Plant material

Onion plants are produced from seeds in pots in a greenhouse, and shallot cultivars are vegetatively propagated as usually done for commercial purposes. The test is carried out with 60 to 90 day old plants (three to four true expanded leaves). In this chapter, the response of *A. fistulosum*, *A. roylei* and *A. galanthum* to *C. gloeosporioides* are presented and discussed, including results on genetic analysis of an F<sub>2</sub> *A. cepa* × *A. roylei* segregant population screened at Wageningen University and Research Centre (The Netherlands) and results previously reported in Galván *et al.* (1997). In addition, the response of tri-hybrid *A. cepa* × (*A. roylei* × *A. fistulosum*) clones is reported and discussed (Galván, 1996). The parental species were propagated from seeds, and the F<sub>1</sub> and F<sub>2</sub> genotypes were vegetatively cloned in a greenhouse.

When testing the response of genetically valuable populations (e.g., inter-specific progenies), *in vitro* propagation allows the maintenance of the genotypes, and increases the number of replications (de Melo *et al.*, 2003). The introduction is performed by direct plant regeneration, using pieces of the basal stem as initial explants (Kahane *et al.*, 1992). Plants in vegetative growth would be preferable for obtaining explants; however, non-dormant bulbs could be used as an alternative (dormant bulbs and flowering stems give a lower rate of success).

The *in vitro* culture protocols presented here are, in essence, the same as those described by de Melo *et al.* (2003). First, the plants (or bulbs) are washed with tap water. The outer sheaths, the roots and senescent parts of the basal plate are removed, and the false stem is cut to 15-25 mm in length from the basal plate. This piece is surface disinfected under aseptic conditions (flux chamber). This involves: (1) 20 minutes of immersion in 96% ethanol, followed by (2) 90 minutes of immersion in 15 g/l sodium hypochlorite (pH6) plus 10 drops/l of Tween-80 or a similar surfactant, under frequent or permanent shaking; and (3) rinsing with sterile water three times to remove the hypochlorite.

Next, the outer sheaths of the disinfected basal stems are again removed, and the stem or bulb is cut shorter just above the basal plate containing the sprouting points (5-10 mm). This basal plate is divided in a radial pattern to obtain the final explants measuring 3 to 8 mm in all dimensions, each one potentially containing sprouting points. Vegetatively growing plants yield two to six explants (dependent on the initial diameter of the basal plate), whereas bulbs may yield 10 to 20 explants (Kahane *et al.*, 1992).

Initial explants are *in vitro* established using MS growing medium (Murashige and Skoog, 1962), including vitamins, 40 g/l of sucrose, 2 mg/l of benzyl-amino-purine (BAP) and 0.2 mg/l of naphthalene acetic acid (NAA), adjusted to pH5.7. 125 mg/l of cefotaxime is added as an antibiotic, during cooling down. Both the initial establishment and the sub-culture are incubated at 20-25°C and a photoperiod of 16 hours using fluorescent light (70  $\mu\text{mol}/\text{m}^2/\text{s}$ ). During sub-culturing, the leaves and the roots of the plants are trimmed. For sub-culture and multiplication, the growing medium is half-concentrated MS with vitamins and 40 g/l of sucrose adjusted to pH5.7. On average, the number of plantlets doubles every three to four weeks, by naturally generated lateral stems or by splitting, although the rate depends on the genetic background of the material.

Plantlets with well developed roots are chosen for acclimatisation. The temperature should be in the range of 15-25°C. The relative humidity should be kept close to 100% during the first days to prevent desiccation by covering the pots with a plastic film and, if necessary, a shadowing net to prevent excessive temperature and sun burning. After a few days, the plastic is progressively removed, allowing the plants to gradually adapt to the conditions of the greenhouse.

At least three to four replications per genotype should be included in the screening, up to a maximum of 10 whenever possible. All genotypes and/or accessions should also be included in a non-inoculated control treatment, sprayed with pure water.

### **Mass screening test**

The screening is carried out under controlled conditions in a growing chamber or phytotron. The temperature is set to 26°C, relative humidity is 95-100%, and the plants are exposed to a photoperiod of 12 hours (78  $\mu\text{mol}/\text{m}^2/\text{s}^1$ ) (Figure 20.1). The pots are transferred to the phytotron a few days before inoculation.

The suspension of conidia is mass sprayed onto the accessions being screened. Pedrosa *et al.* (2004) reported that spraying was faster, simpler, and provided higher infection and lower variability than inoculation by deposition of mycelial disks on the leaves. Figures 20.2A and B show the distribution of *C. gloeosporioides* conidia on the leaves, 24 hours after atomization, aligned along the edges of the dried drops. In order to obtain uniform distribution of conidia, the atomizer should be adjusted to produce very tiny drops. Excessive spraying should be avoided as this rinses the inoculum.

Immediately after inoculation and during the first two days (incubation period), high relative humidity (100%) should be ensured by covering the plants with plastic bags (Figure 20.1A). Afterwards, high relative humidity (95-100%) should be achieved by including a fog producer, spraying water on the plants and keeping the floor wet. The effect of different durations of the “incubation” treatment studied in Galván (1996) is presented and discussed in this chapter.

The disease is scored one and/or two weeks after inoculation by considering the characteristic anthracnose spots, and avoiding other reactions of the plants by comparison with the control treatment. A simple, ordinal scale is preferred to record the observed genetic variation. Working with *A. cepa*-related species, a logarithmic-based scale was appropriated (Galván *et al.*, 1997): 0: no symptoms; 1: < 5% of the leaf area affected, one or a few small spots; 2: 5-20% of the leaf area affected; 3: > 20% of the leaf area affected.

## **RESULTS AND DISCUSSION**

### **Screening procedure**

Three different “incubation” periods were tested, in which plants remained covered with plastic bags for one, two and four days after inoculation (Galván, 1996). A minimum duration of 48 hours was necessary to cause maximum disease severity (Figure 20.3).

This result is in agreement with Suhardi (1994), who reported that disease severity increases with the duration of the wetness period in the range of 6 to 45 hours, but not with Carneiro and Amorim (1999), who found that disease severity was not correlated with wetness duration. Although a few hours wetness period could be enough to produce infections, at least 48 hours is suggested in order to ensure highest disease expression.

Germination of *Colletotrichum* conidia comprises the development of a germ tube, followed by the formation of appressoria (Bailey *et al.*, 1992). Subsequently, the cuticle is penetrated. Preliminary microscopic observations, 24 hours after inoculation (Figures 20.2C and D), showed the germinated conidia and appressoria, but no clear fungal growth through the epidermis yet. These observations suggest that, if the incubation period to increase leaf wetness is too short, the efficiency of detecting susceptible genotypes may be affected.

To discriminate between susceptible and resistant responses, scoring disease expression 14 days after inoculation was better than 7 days after inoculation (Figure 20.3). Pedrosa *et al.* (2004) found that scoring 9 days after inoculation gave the best results when distinguishing the reaction among Brazilian onion cultivars. However, for testing *Allium* species related to onion, the largest differences between susceptible and resistant germplasm were found two weeks after inoculation, when the susceptible controls were significantly diseased.

## Resistance

A high level of resistance was found in accessions of *A. galanthum* and *A. fistulosum* (Figure 20.3). These species were highly resistant to a Brazilian isolate from Santa Catarina, and partially resistant to an Indonesian and a Nigerian isolate (Galván *et al.*, 1997). Partial resistance in these species is characterised by a much lower number of infections, which mainly occur in older leaves, than in a susceptible control. In partially resistant material, the infections lead to spots of limited size with suppressed or delayed sporulation (Figure 20.4). Although *A. cepa*-type infections were observed, the typical reaction was tiny white spots (Galván *et al.*, 1997).

*A. roylei* was resistant to the Brazilian isolate, but was susceptible to the Indonesian and Nigerian isolates. The pattern of reaction was similar to that of *A. galanthum* and *A. fistulosum*, but with a lower level of resistance (Galván *et al.*, 1997).

After infection with *Colletotrichum* diseases, two phases are recognised: (1) a short hemi-biotrophic phase, followed by (2) a visible and destructive necrotrophic phase (Bailey *et al.*, 1992). Considering the first phase, two types of *Colletotrichum* pathosystems are distinguished: (1) intracellular hemi-biotrophic species with formation of haustoria, and (2) the subcuticular intramural species. In both systems, there is contact between the pathogen and the host living cells, which makes it possible for resistance responses to be triggered before the collapse of the host tissue. These phases have not been described for *A. cepa* anthracnose.

The mode of resistance found in *A. fistulosum*, *A. galanthum*, *A. roylei* and other *Allium* species possibly involves mechanisms operating during the early infection process, which might be combined to other mechanisms at a later infection stage delaying the progress of the disease (Galván *et al.*, 1997).

The screening of an F<sub>2</sub> inter-specific population of *A. cepa* × *A. roylei* with the Brazilian isolate was carried out in order to analyse the genetic basis of the resistance in *A. roylei* (Figure 20.5). The experiment was repeated twice and gave rise to different results, probably as a result of the screening conditions. Seven days after inoculation, *A. cepa* was already very affected, while most of the *A. roylei* replications, F<sub>1</sub> and F<sub>2</sub> clones had few or no symptoms. 14 days after inoculation, disease expression increased overall, but the difference between the parents remained significant. The F<sub>1</sub> clones and most of the F<sub>2</sub> clones were partially resistant, a response similar to that in *A. roylei* parent, revealing a

dominant inheritance pattern. The occurrence of significant differences between F<sub>1</sub> plants, suggests the presence of more than one gene controlling *A. roylei* resistance (Galván *et al.*, 1997).

Tri-hybrid clones *A. cepa* × (*A. roylei* × *A. fistulosum*) (known as CC × RF, Wageningen Accession No. 89446) were screened in the aforementioned experiments (Galván, 1996). Out of 10 clones, seven and eight in each experiment fell into the resistant or partially resistant classes. Clones from the backcross of *A. cepa* (CC × [CC × RF], No. 91074) were also screened, choosing parents with no previous selection for resistance. In each experiment, eight out of 15 and one out of six clones, were still characterised as resistant or partially resistant. These results suggest a combined effect of *A. fistulosum* and *A. roylei* resistances, and the potential of the try-hybrid approach for the introgression of *A. fistulosum*, as suggested by Kik (2002). Further research, combining efficient screening with molecular tools, would provide insight into the genetic control of these resistance sources.

## CONCLUSIONS

Successful and efficient screening for resistance to onion and shallot anthracnose can be achieved under conditions that are highly conducive for disease development. The screening can be facilitated by the use of *in vitro* propagation of *Allium* plant material, allowing clonal replication of genetically valuable populations.

Under these conditions, the establishment of a reliable screening test, playing a key role in speeding up the selection procedures, and applying molecular tools oriented to understanding the genetic basis of the resistance, is attainable.

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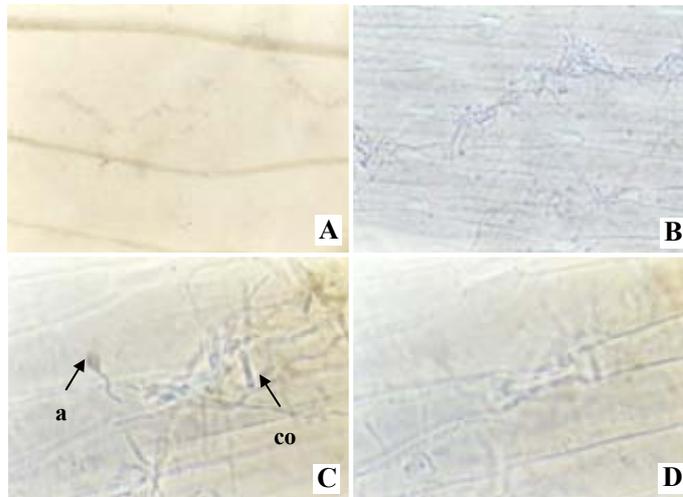
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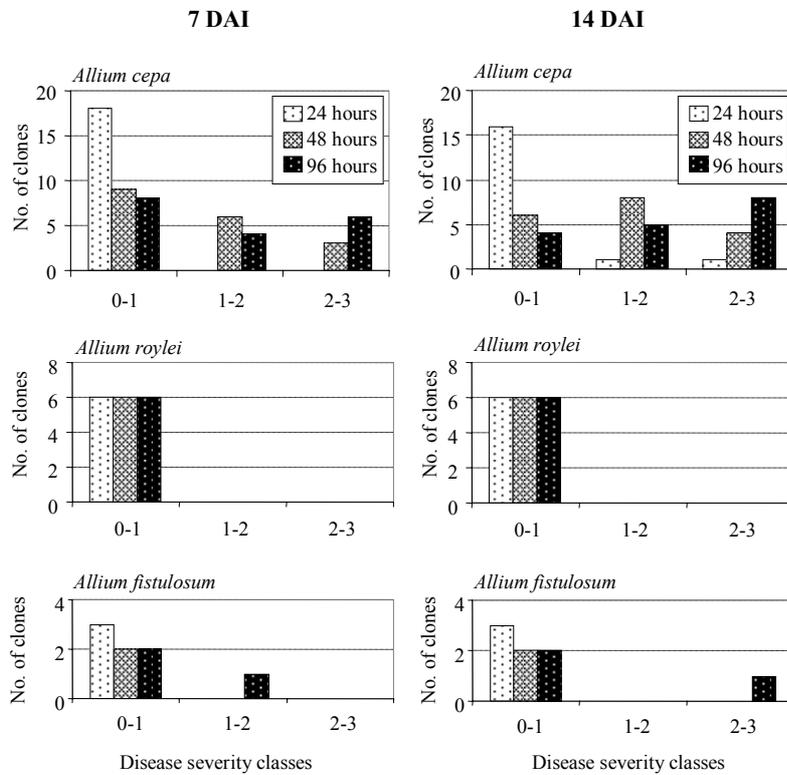
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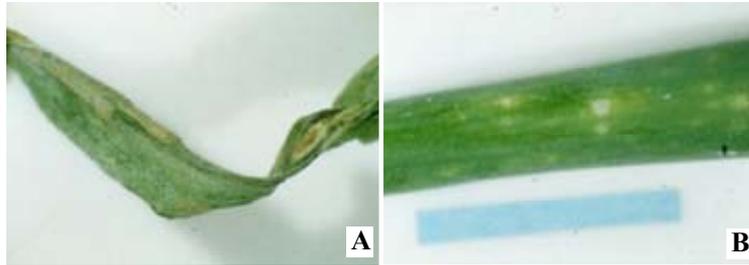
**Figure 20.1.** Overview of the screening tests in *Allium* spp. under controlled conditions. (A) Incubation period, in which the plants are covered with a plastic bag to ensure 100% relative humidity. (B) Thereafter, fog is produced with a humidifier.



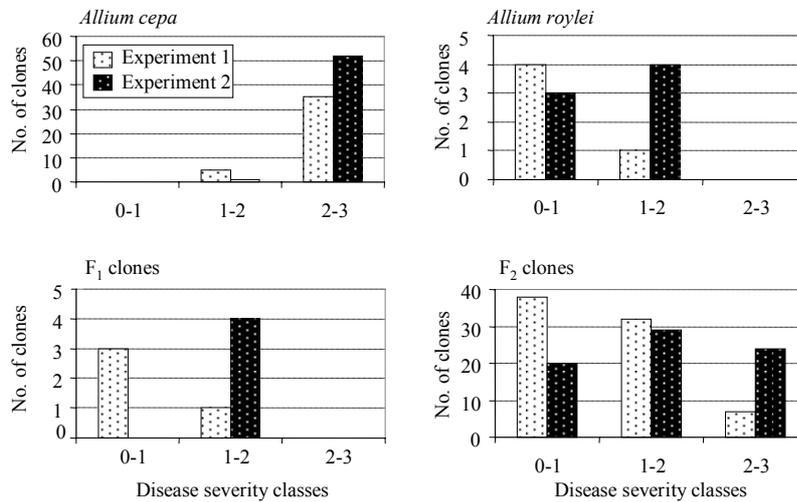
**Figure 20.2.** Microscopic observations of onion leaf pieces 24 hours after spray inoculation with a suspension of *Colletotrichum gloeosporioides* conidia. (A) 200 $\times$  magnification; (B) 300 $\times$ ; (C) 400 $\times$ , above the epidermis; (D) 400 $\times$ , epidermal layer. Arrows point at an appressorium (a) and a conidium (co).



**Figure 20.3.** Distribution over disease severity classes of genotypes of *A. cepa* (cvs. ‘Albeno’ and ‘Marathon’), *A. roylei* (Wageningen Accession No. 95001) and *A. fistulosum* (No. 84036), after 24, 48 and 96 hours of “incubation” treatment, 7 and 14 days after inoculation (DAI). A Brazilian isolate of *Colletotrichum gloeosporioides* from Santa Catarina State was used. The disease severity classes in the x-axis comprise the lower limit, but not the upper one (Galván *et al.*, 1997).



**Figure 20.4.** (A) Reaction to anthracnose caused by *Colletotrichum gloeosporioides* on a susceptible onion (*Allium cepa*). (B) Partial resistance of *A. fistulosum*, with small to tiny white spots developing and little or no sporulation.



**Figure 20.5.** Distribution over disease severity classes of an individual observation of *A. cepa* plants (cvs. ‘Albeno’ and ‘Marathon’), and clonal medians of *A. roylei* (Wageningen Accession No. 84037), F<sub>1</sub> *A. cepa* × *A. roylei* clones (No. 86282), and F<sub>2</sub> *A. cepa* × *A. roylei* clones (No. 89163), in two experiments represented by light and dark bars, respectively, 14 days after inoculation. A Brazilian isolate of *Colletotrichum gloeosporioides* from Santa Catarina State was used. The disease severity classes in the x-axis comprise the lower limit, but not the upper one (Galván *et al.*, 1997).



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