IAEA-TECDOC-1426

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Proceedings of a final Research Coordination Meeting
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GENETIC IMPROVEMENT OF UNDER-UTILIZED AND NEGLECTED CROPS IN LOW INCOME FOOD DEFICIT COUNTRIES (LIFDCS) THROUGH IRRADIATION AND RELATED TECHNIQUES

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FOREWORD

The majority of the world's food is produced from only a few crops, and yet many neglected and under-utilized crops are extremely important for food production in low income food deficit countries (LIFDCs). As the human population grows at an alarming rate in LIFDCs, food availability has declined and is also affected due to environmental factors, lack of improvement of local crop species, erosion of genetic diversity and dependence on a few crop species for food supply. Neglected crops are traditionally grown by farmers in their centres of origin or centres of diversity, where they are still important for the subsistence of local communities, and maintained by socio-cultural preferences and traditional uses. These crops remain inadequately characterised and, until very recently, have been largely ignored by research and conservation. Farmers are losing these crops because they are less competitive with improved major crop species.

Radiation-induced mutation techniques have successfully been used that benefitted the most genetic improvement of "major crops" and their know-how have a great potential for enhancing the use of under-utilized and neglected species and speeding up their domestication and crop improvement. The FAO/IAEA efforts on genetic improvement of under-utilized and neglected species play a strategic role in complementing the work that is being carried out worldwide in their promotion.

This CRP entitled Genetic Improvement of Under-utilized and Neglected Crops in LIFDCs through Irradiation and Related Techniques was initiated in 1998 with an overall objective to improve food security, enhance nutritional balance, and promote sustainable agriculture in LIFDCs. Specific objectives addressed major constraints to productivity of neglected and under-utilized crops by genetic improvement with radiation-induced mutations and biotechnology in order to enhance economic viability and sustain crop species diversity, and in future to benefit small farmers.

This TECDOC describes the successful results obtained by the use of radiation-induced mutations and biotechnology since the start of this CRP in 1998. Additionally, a total of 25 research papers were published in conference proceedings and international refereed journals (see Annex 1). K. Nichterlein, formally of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, initiated this CRP. Special acknowledgement goes to S. Ochatt (France) for help in the preparation of this publication. The IAEA officer responsible for this TECDOC is S.M. Jain, of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

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SUMMARY

1. INTRODUCTION

Only a limited number of crops produce the bulk of food consumed in the world. Many neglected and under-utilized species are extremely important for food production in low-income food deficit countries (LIFDCs). They are locally well adapted to marginal lands and constitute an important part of the local diet, providing valuable nutritional elements (e.g. protein, vitamins, minerals) and spices, often lacking in staple crops. Their role in traditional medicine is also well known. Neglected and under-utilized crops also represent an important source of revenue for local economies and are part of the rich cultural and traditional heritage of communities around the world. In addition, these crops are important sources of resistance genes for biotic and abiotic stress breeding that can be utilized also for the genetic improvement of commodity crops. As compared to the major crops, they require relatively low inputs and, therefore, contribute to sustainable agricultural production. So far, little attention has been paid to these species in terms of research and development due to lack of government funding and interest of the private sector. These traditional crops are often low yielding and cannot compete with major crops, even though many of them have the potential to become economically viable. Lack of genetic improvement and often narrow genetic diversity for important agronomic traits hamper the development of these crops. Other constraints are the lack of knowledge on the taxonomy, reproductive biology, and the genetics of agronomic and quality traits.

Genetic improvement of neglected and under-utilized crops can be achieved by various techniques such as classical breeding, mutagenesis, and biotechnological approaches. Mutagenesis has a distinct advantage in that small genetic changes, which affect critical agronomic traits, can be induced and transferred to well adapted and culturally acceptable local cultivars. In addition, it is a simple and cost effective technology. The summation of these techniques, which are commonplace in developed countries, are either unavailable or are in their infancy in LIFDCs.

Improved crop cultivars play a key role in enhancing crop production and security. During the FAO Fourth International Technical Conference on Plant Genetic Resources for Food and Agriculture (PGRFA) held in 1996 in Leipzig, Germany, a Global Plan of Action (GPA) was adopted by more than 150 FAO member states which included recommendations on specific priority activities for the utilization of plant genetic resources. Activity 12 of the GPA specifically called for efforts to promote the development and commercialisation of under-utilized crops and species.

This Coordinated Research Project (CRP) on Genetic improvement of neglected and under-utilized crops in LIFDCs through irradiation and related techniques was initiated in December 1998 with a view to:

- identifying and prioritising major constraints for the development of locally adapted and neglected crops;
- establishing a network of research cooperation and germplasm conservation and use;
- overcoming the constraints of neglected and under-utilized species using genetic improvement techniques including selection and cross breeding, mutation techniques and appropriate biotechnological approaches;
- developing germplasm for a number of selected neglected and under-utilized crops;
- developing protocols for genetic improvement techniques of individual under-utilized crops; and

- transferring technology and disseminating information through publications and participation at both national and international conferences.

To achieve these objectives, 15 research projects were funded to support both seed and vegetatively propagated crops in LIFDCs, and this volume will consider these two groups of crops separately, describing results obtained by the different participating teams for each individual crop, and ending with matters relating to international policy towards these crops.

1.1. Seed propagated crops

Amaranth (RSA)

Amaranth is a multipurpose crop, used as vegetable, grain crop, medicinal, forage, and for ornamental purposes. The crop is particularly suitable for marginal areas and is known to be resistant to both drought and salinity. In most LIFDCs the crop is not grown for commercial purposes, but rather as part of a local sustainable agriculture traditional system. Amaranth is very rich in vitamins (A, B), minerals (Ca, Fe, P, Mg), proteins (particularly lysine and methionine), fibres (5–25%) and lipids (4–10%). A greater drought tolerance trait is among those desirable characteristics needed to enhance the use of this crop in RSA, along with day-light insensitive genotypes that would allow its cultivation in winter time, when food insecurity is more severe in poor rural areas. The ultimate objective of this project was to broaden the agro-biodiversity food basket and strengthen livelihood options for both nutrition and income generation of local communities.

Amaranth (Slovakia)

Amaranth in Europe is used as a nutritionally rich food particularly for its high content in vitamins and proteins as well as other biologically pro-health compounds such as the antioxidant squalene. It is also used as an alternative source of energy and in the decontamination of heavy metal-affected soils. In Slovakia, grain yield is low (2 to 3 tons/ha) and on average unstable. Cultivation constraints include also post-harvest losses and presence of anti-nutritional factors in the seed. The project was aimed at improving selected grain cultivars adapted to local conditions, to increase both quantity and quality of seed production and to develop suitable varieties for mountainous marginal areas of Central Slovakia for income generation.

Bambara groundnut (Ghana)

Bambara groundnut is a major source of inexpensive protein in sub-Saharan Africa. The crop has high lysine and methionine content. Humans, pigs, consume seeds and poultry while the haulm is used as fodder for livestock. The crop also features prominently in cropping systems in Ghana and makes a significant contribution to soil fertility through symbiotic nitrogen fixation. The project in Ghana was aimed at collecting and evaluating bambara groundnut germplasm for desirable agronomic traits and select superior ones lacking specific traits for improvement through mutagenesis.

Bambara groundnut (France)

In spite of the importance of bambara, there has been little research on the crop. Its long life cycle and floral biology do not facilitate improvement of the crop. A shortening of the generation cycles would allow rapid progress in breeding. In addition, knowledge of the *in*

vitro requirements for regeneration would help fasten mutagenesis and biotechnology-based generation of genetic novelties in this species. The French effort focused on developing *in vitro* techniques for plant regeneration and shortening of the generation cycle.

Grass pea (India)

The grass pea, with its large eco-physiological plasticity and very low production costs, is a cheap source of dietary protein available for subsistence farmers in LIFDCs where vast surfaces of this crop are grown. However, there are some limits to grass pea consumption deriving from the sometimes relatively high (up to 0.76%) seed content of β,-N-oxalyl-α,β diaminopropionic acid (ODAP), the toxin responsible for neurolathyrism. It is therefore needed to develop cultivars with decreased levels of this toxin. In India, this project was aimed at mutagenesis, and *in vitro* regeneration techniques to introduce transgenes blocking the biosynthesis of oxalic acid.

Grass pea (France)

The grass pea (*Lathyrus sativus*) is a seed legume that tolerates extreme environmental conditions and has high protein content and a significant resistance to anthracnose. Grass peas would also be a useful genetic resource for resistance breeding of peas (*Pisum sativum* L). Unfortunately, grass peas have a long generation cycle, are cross-incompatible with most other cultivated seed legumes and insufficient research input has been devoted to them in the past, so that little is known on their floral biology, cytogenetics and breeding in general. In France, *in vitro* selection, a shortening of generation cycles and somatic hybridization were developed and a series of fundamental, cytological, genetic and biophysical studies were performed.

Naranjilla (Ecuador)

This is a traditional high altitude culture in Ecuador that is also found in other South American countries but also in New Zealand and the USA. However, the species is very much in danger of genetic erosion due to its floral biology, and the main problem for cultivation is diseases and pest, especially nematodes. In the absence of adequate phytochemical treatments to avoid these, farmers are contaminating the soil and the fruit and causing a negative ecological impact. There is therefore an urgent need to obtain novel, nematode resistant genotypes of Naranjilla, and this project is aimed at the development and use of mutation induction through gamma rays to induce nematode resistant plants from the landrace Baeza, which is characterized by its valuable organoleptic traits and high market demand, but also by its high susceptibility to nematodes.

Quinoa (Mexico)

Quinoa is one of the pseudocereals which are widely recognised nowadays as having great potential to contribute to solve the malnourishment problems affecting great sectors of various populations in marginal rural zones, as this species is also able to cope with low soil fertility, drought and frost. However, the main problem in this crop is the high saponin content and one goal of this project was the application of radio-induced mutagenesis techniques to the locally cultivated variety Barandales of quinoa, to obtain low-saponin, high yielding lines. In addition, the exploration of valuable germplasm of *Chenopodium berlandieri ssp muttaliae* native landraces was performed and some genotypes were recollected to be incorporated as progenitor in future breeding programmes of quinoa. A prerequisite to this was the detailed

cytological characterization of such materials and this was therefore another main goal in this project.

1.2. Vegetatively propagated crops

Bitter potato (Bolivia)

Potato and other Andean tubers are the most important crops in Bolivia. Bitter potatoes are cultivated in high lands (4,000 m above sea level) and are among the few species that tolerate extreme eco-physiological conditions, including freezing. A major challenge to enhance the use of bitter potatoes is their high glycoalkaloid content. Another problem of this crop is the gradual loss of many local, native varieties, which are being replaced by sweeter genotypes. The objectives of this project were to collect such valuable genetic material, and also to develop improved varieties with low solanine and solasodine content through the application of mutations induced by irradiation of *in vitro* cultures.

Cocoyam (Ghana)

Cocoyams (*Xanthosoma sagittifolium* and *Colocasia esculenta*) are important starchy staples in Ghana providing people with carbohydrates, proteins and vitamins. The corms of *Colocasia* and the cormels of *Xanthosoma* are used in various food preparations whilst the leaves of *Xanthosoma* are consumed as a vegetable. Despite their importance, they have received little attention by R&D. The major constraints of these crops are low yields and diseases (e.g. root rot and leaf blight). The project was aimed at characterizing the genetic resources of cocoyams and selecting superior genotypes for mutagenesis to develop varieties resistant to the root rot and leaf blight diseases.

Cocoyam (Costa Rica)

Cocoyam (*Xanthosoma sagittifolium*) is an important source of carbohydrates in Costa Rica. It is a neglected crop and its cultivation is hampered by root rot disease caused mainly by *Fusarium* spp. Cultural practices and chemical treatments have failed to control the disease and no resistant varieties have been developed so far. The project was aimed at characterizing available germplasm and generating genetic variability to develop mutant varieties resistant to the root rot disease, by mutagenesis induced through radiation of shoot bud apices followed by their culture *in vitro*.

Okra (Thailand)

Okra (*Abelmoschus* spp.) is a multipurpose vegetable crop in Thailand, whose fruits are an important source of Vitamins (A, C) and minerals (particularly Ca). The plant is also used for many other purposes, including medicinal (in treating peptic ulcers). The total area under okra cultivation in Thailand increased to 1,600 ha in 1996. However, due to severe disease attack, both area and production decreased dramatically in 1997 (up to 50%). Infections by okra yellow vein mosaic virus are among the most damaging ones, causing stunting in the plant and reduced number of fruits. The tobacco white fly transmits this disease. To address this problem, a breeding program on okra was established in 1996 in Thailand and activities were undertaken using resistant commercial varieties originated in India. Induced mutation using gamma radiation to develop resistant varieties was selected as a viable breeding option; in view that okra has always received modest research investment, particularly in Thailand.

Taro (Indonesia)

Taro (*Colocasia esculenta*) is an important staple crop in several regions of Indonesia. The corms and leaves are major sources of carbohydrates, proteins, minerals and vitamins. However, there are production constraints including the leaf blight disease caused by *Phytophthora colocasiae*, low yields and acridity of varieties. The limited genetic diversity is a major constraint in crop improvement. The aim of this project was to obtain high yield and early maturing genotypes, with leaf blight resistance and improved taste through mutagenesis using gamma irradiation and tissue culture.

Yam (Costa Rica)

Yam (*Dioscorea* spp.) is an important starchy staple food crop in Costa Rica. It is also used as animal feed as well as in pharmacology. Exports have increased recently due to high demand in U.S.A. Cultivation is constrained by fungal diseases (e.g. Anthrachnose) and lack of suitable morpho-agronomic types. The goals of this project were to induce genetic variability through *in vitro* mutagenesis, to obtain resistant varieties with desirable plant architecture.

2. SPECIFIC ACHIEVEMENTS AND RECOMMENDATIONS FROM THE DIFFERENT PROJECTS

2.1. Seed propagated crops

2.1.1. Project: Development and evaluation of drought tolerant mutant germplasm of Amaranthus sp. K. de Ronde (South Africa), team

2.1.1.1. Achievements

- 15 selected M5 drought tolerant leafy mutants (*A. tricolor*) were selected in the field for their comparatively higher performance in artificially created drought conditions.
- Halogen lamps were successfully used to grow leafy amaranth in off-season in the greenhouse.
- Five day-insensitive mutant lines were identified.

2.1.1.2. Recommendations

- The putatively drought resistant mutants must be further analysed using molecular markers.
- The remaining mutated lines must be screened for drought tolerance.
- The promising day-insensitive selections would also require further investigation to confirm the findings.
- Evaluation for nutritional properties of the mutants is needed.

2.1.2. Project: Improvement of selected Amaranthus cultivars by means of mutation techniques and biotechnological approaches. A. Gajdosova (Slovak Republic), team

2.1.2.1. Achievements

- A successful selection was carried out in two different grain amaranth genotypes up to the M5 generation.
- 48 *A. cruentus* selected mutants and 18 mutants of K-433 hybrid were obtained, that bore various useful traits, i.e. determinate growth, uniformity in flowering and seed maturity, leaf-less inflorescences, and an increased seed size.

2.1.2.2. Recommendations

- Selected mutants need further molecular studies.
- Studies on the nutritional composition of the mutants are recommended.

2.1.3. Project: Improving bambara groundnut productivity through mutation breeding and in vitro techniques. H.K. Adu-Dapaah (Ghana), team

2.1.3.1. Achievements

- Involvement of farmers in participatory varietal breeding (PVB), to identify production constraints and set research objectives to solve specific problems using mutagenesis (Adu Dapaah et al., 2003).
- Assembly, evaluation and selection of superior bambara groundnut landraces for breeding.
- Determination of LD_{50} at 178 Gy and 0.78% EMS.
- Identification of mutants resistant to *Cercospora* leaf spot with desirable agronomic traits.

2.1.3.2. Recommendations

- Characterization of mutants using molecular markers.
- Evaluation of mutants for nutritional properties.
- Validation of the strategy to shorten the generation cycles.
- Evaluation of mutants across various agro-ecological zones.

2.1.4. Project: Biotechnological approaches for genetic improvement of bambara groundnut: in vitro plant regeneration and shortening of generations using in vitro and in vivo systems. R.S. Sangwan (France), team

2.1.4.1. Achievements

- A new technique has been developed for the shorten of the generation cycle of bambara groundnut using a combination of *in vitro* and *in vivo* methods (Ochatt et al, 2002b).
- An efficient *in vitro* shoot regeneration system has been developed in bambara groundnut by combining phytohormones and using embryonic explants (Lacroix et al 2003).
- True-to-typeness of regenerants was established (Lacroix et al 2003).

2.1.4.2. Recommendations

2.1.5. Project: Suppression of neurotoxic amino acid in seed storage protein of Lathyrus sativus L. via mutation techniques and gene transfer. Mohapatra, U. (India), team

2.1.5.1. Achievements

- Plants were regenerated from cotyledonary explants and explant-derived callus.
- Gene transfer with *Agrobacterium tumefaciens* + GUS gene was successful as regenerated transgenic plants expressed GUS activity inherited in the progeny in a Mendelian fashion.
- The optimum doses for gamma irradiation and EMS treatment were determined.

2.1.5.2. Recommendations

- Further gene transfer experiments should include the gene blocking oxalic acid biosynthesis in order to eliminate ODAP from the seed storage proteins.
- Mutation breeding remains infant, and research in this domain should be completed.

2.1.6. Project: Development of biotechnological approaches for breeding of grass pea (Lathyrus sativus L.). Ochatt, S. (France) team

2.1.6.1. Achievements

- Fertile plants were regenerated from various explants, with or without callusing (Ochatt et al, 2001).
- The genetic mechanism underlying hyperhydricity was identified (flow cell cytometry) (Ochatt et al, 2002a).
- Two tetraploids and one mixoploid were obtained and several more variants were identified and characterized (isozymes, RAPDs, flow cytometry).
- The cross-incompatibility between pea and grass pea was confirmed (Ochatt et al, 2003a).
- Leaf protoplast-derived calluses were obtained and, after fusion of grass pea and pea protoplasts, somatic hybrid calli were produced (Durieu & Ochatt 2000).
- Cytogenetic tools were developed for the characterization of *Lathyrus* metaphase chromosomes using fluorescent *in situ* hybridisation.
- A novel strategy for shortening of generation cycles *in vitro* gave 7 and > 4 generation cycles/year for pea and grass pea, respectively (Ochatt et al 2002b).
- Early predictors (cytological and bio-physical) of somatic embryogenesis competence have been identified (Ochatt et al 2003b).
- The shortening of generation cycles developed permits a faster breeding and ensures an absolute self-pollination of plants, useful for single seed descent.

2.1.6.2. Recommendations

- The fundamental studies engaged with the various genotypes must be pursued..
- The analysis of seed protein quality has to be performed on *in vitro* regenerants compared to the mother plants, especially for those with different ploidy levels.
- Somatic hybrid plants should be regenerated after fusion of pea and grass pea protoplasts for an assessment of their agronomic value.

2.1.7. Project: Nematode Resistance through Mutation Induction in a Local Variety of Naranjilla (Solanum quitoense Lam) in Ecuador. Muñoz L.(Ecuador), team

2.1.7.1. Achievements

- Mutations were induced by gamma ray irradiation of true seeds and axillary buds of the traditional Naranjilla variety Baeza.
- Seed-derived mutants were observed in the greenhouse and 35 nematode resistant genotypes were kept for further analyses.
- Tissue culture-derived mutants were also evaluated in the greenhouse and plants are available for future field evaluation.

2.1.7.2. Recommendations

- *In vitro* propagated material should be used in future, as it appeared to be more amenable for mutation work than true seed-derived material.
- Evaluation and characterization of further generations should be performed before stable, nematode-resistant material of Naranjilla can be proposed to farmers.

2.1.8. Project: Characterization of Chenopodium germplasm, selection of putative mutants, and its cytogenetic study. A. Rubluo (Mexico), team

2.1.8.1. Achievements

- A large number of *Chenopodium quinoa*, including radio-induced mutants, and two accessions of *C. berlandieri* ssp. *Muttaliae* was characterized in terms of morphology, productivity and saponin cotent.
- Several groups of quinoa germplasm were distinguished on the basis of plant architecture, stem diameter and yield.
- Three mutant lines exhibiting high yield and low saponin content in the M_7 generation were obtained.
- Molecular markers (RAPDs) for the identification of low- and high-saponin content quinoas were preliminary developed.
- Cytological studies with *C. quinoa* var. Barandales permitted to determine its chromosome complement, as well as details on the chromosome length, total chromatin, nuclear DNA content and genome size.

2.1.8.2. Recommendations

- Work on the development and exploitation of molecular markers should be continued and extended to other chenopod genotypes.

- Characterization of the mutants and lines collected should be completed.

2.2. Vegetatively propagated crops

2.2.1. Project: Breeding of bitter potato (Solanum juzepczukii) through mutation induction and tissue culture techniques. Murillo, R.A. (Bolivia), team

2.2.1.1. Achievements

- A genetic diversity bank was established.
- Conditions for *in vitro* culture and virus cleaning for several genotypes were optimised (Murillo, 1998).
- Dosimetry studies were carried out and optimum doses were 22 Gy for Bola Luck'y and 28 Gy for Luck'y Kheto (Murillo, 2002).
- Chimaeras were dissociated by multiplication through to M_1V_4.
- Mutants were acclimated in the greenhouse for further characterization.
- An efficient method for the determination of the total glycoalkaloids was adapted to bitter potatoes.
- A total of 14 potentially useful mutants were kept for further field experiments.

2.2.1.2. Recommendations

- Molecular characterization of the mutants produced must be performed.
- The mutants should be tested in their natural habitat.
- The release to the market of novel agronomically useful mutants as new varieties should be carried out.

2.2.2. Project: Improvement of cocoyams (Xanthosoma sagittifolium) and (Colocasia esculenta) using gamma irradiation, tissue culture and molecular markers. E.Y. Danquah (Ghana), team

2.2.2.1. Achievements

- The genetic relationships amongst 70 accessions has been established by DNA fingerprinting (Danquah et al 2003).

- Genetically distant accessions have been identified and will provide very useful information to plant breeders and germplasm conservationists (Danquah et al 2001, 2003).
- The LD_{30} of both species was established at 10 Gy and two mutant populations have been generated.
- *Fusarium* spp., *Penicillium* spp., *Botryodiplodia* spp., *Pythium* spp. and *Rhizoctonia* spp. were isolated from infected samples of *X. sagittifolium* collected from farmer fields from four different geographic locations in Ghana.

2.2.2.2. Recommendations
- Further characterization of germplasm using more efficient molecular markers is needed.
- Molecular characterization of the mutant populations is recommended.
- Screening of the mutant populations for root rot and leaf blight should be completed before new varieties can be proposed to farmers.

2.2.3. *Project: Induction of genetic variation in Xanthosoma spp. Saborio-Pozuelo, F (Costa Rica) team*

2.2.3.1. Achievements
- A mutation induction methodology for cocoyam using gamma rays was achieved: recollection of plant material, virus cleaning, *in vitro* cultures, determination of the LD30, dissociation of chimaeras.
- Establishment of a greenhouse screening methodology for *Fusarium* spp.
- 17 putative tolerant mutants were obtained after irradiation and screening.
- Development of a DNA extraction method and standardization of RAPDs (Saborio et al, 2002a, b).
- Molecular and greenhouse phenotypic characterization of collected and mutated material.
- Establishment of a greenhouse germplasm bank of cocoyam.

2.2.3.2. Recommendations
- The mutant clones should be characterized morphologically, using the cocoyam descriptors guide developed by IPGRI to complement the results from molecular data.
- Some of the promising agronomic traits observed are still to be confirmed in the field
- A finer molecular characterization is required.
- The nutritional value of mutants has to be ascertained.

2.2.4. Project: Induced mutation for resistance to yellow vein mosaic virus in okra. V. Phadvibulya (Thailand), team

2.2.4.1. Achievements
- The optimum dose for gamma irradiation technique (LD50 400–600 Gy) in okra was identified (Phadvibulya et al 2001).
- The white fly transmission technique was developed in greenhouse conditions in order to reduce environmental interferences to a minimum.
- 22 mutant lines with resistance to OYVMD in greenhouse and field conditions were obtained, 12 of these gave satisfactory yields but fruits had an undesirable shape, the remaining 10 mutant lines produced fruits of a desirable shape but the yield is still to be determined.

2.2.4.2. Recommendations
- The resistance to OYVMD in mutant lines has to be confirmed.

- Further selection for more attractive fruit characteristics (in particular better shape, colour, texture and size) has to be undertaken.
- Confirmation of induced mutation would be best pursued using molecular markers.

2.2.5. Project: Development of early maturing and leaf blight resistant taro (Colocasia esculenta) with improved taste. L. A. Sukamto (Indonesia), team.

2.2.5.1. Achievements
- Protocols for *in vitro* propagation of taro have been developed.
- The LD_{30} of *Colocasia esculenta*, cultivar Bentul was established as 10 Gy.
- Plants were multiplied through M_1V_4 and transplanted to the greenhouse and in the field.
- Three mutants tolerating leaf blight and more palatable have been identified and screened for early maturity and improved yield.
- Three variants (B43, B63 and B133) had the desired characters: high tolerance to leaf blight disease, early maturing, heavy corms and good taste.

2.2.5.2. Recommendations
- Screening of mutants for root rot disease resistance is needed.
- DNA fingerprinting of mutants is necessary.
- Multi-site evaluation of mutants is needed to assess their adaptability before release to the farmers.

2.2.6. Project: Application of biotechnology and mutation techniques for anthracnose resistance and compact plants in Dioscorea spp. Argüello, J (Costa Rica) team

2.2.6.1. Achievements
- A reliable strategy for plant regeneration from *in vitro* cultured nodal explants has been developed.
- The LD50 was determined to be between 10 and 20 Gy, and plants were irradiated at 15 Gy.
- Plants were multiplied to the M_1V_6 with different substrate and acclimatization conditions (relative humidity, temperature) tested.
- The causal agent of anthracnose was isolated, multiplied and characterized.
- All putative mutants were inoculated with *Colletotrichum gloesporioides.*
- Reliable techniques for the micropropagation and mutagenesis were developed.
- Dwarfs plants were selected and are presently micropropagated.
- So far, pathogen tolerance was not detected among the mutants.

2.2.6.2. Recommendations
- Optimum conditions for inoculation with the fungus should be precisely determined.
- The inoculation of at least 5000 plants for subsequent selection should be envisaged.
- Molecular characterization of selected mutants is needed; once analysed and characterized, mutants will have to be tested in the field.

2.2.7. Project: Enhancing the use of under-utilized plant species: strategies, approaches and experiences at IPGRI. Padulosi, S. (Syria) team

Political and sociological issues related to research and increased utilisation of neglected crops around the world, and the role of international organisations in this area are underlined. The experience of IPGRI in this respect is reported through a series of examples, in different

areas of the world (particularly in LIFDCs) and on different neglected and under-utilized crops. The activities of IPGRI towards improving cultivation of this group of species around the world, as well as the role it can play *viz à viz* other organisations to help fund their development through a better dissemination of information and the creation of a greater public awareness and policy is stressed.

3. GENERAL RECOMMENDATIONS

The continuation of the research projects in this CRP should be facilitated, as they have generated very useful results, to ensure continuity and realization of the goals of the projects.

Research on the evaluation of the mutants generated from this CRP for nutritional quality and acceptability by consumers is of the utmost importance. Mechanisms for the release of the improved varieties that might result from these efforts should also be established so as to allow farmers to benefit quickly from them.

This will require the improvement of facilities in participating countries and the training of personnel in the use of mutagenesis, tissue culture and other biotechnologies to improve neglected and under-utilized crops.

Human resources development activities are highly recommended to allow the dissemination of know-how from this CRP, to benefit researchers from various LIFDCs. If supported, short-term workshops/training programmes in developing countries for technology transfer should accomplish this.

A greater cooperation with other existing projects addressing neglected and under-utilized species, e.g. the IPGRI-IFAD Project on Andean grains, Bamnet, Tarogen, International Centre for Under-utilized Crops, Global Facilitation Unit of Global Forum for Agricultural Research, and FAO, will be essential to create important synergies and experience sharing among partners.

Sharing of germplasm among participating countries is also highly desirable. The assistance of IPGRI in developing Material Transfer Agreements would be appreciated. The recently approved FAO International Treaty for PGRFA offers opportunities in this respect.

DEVELOPMENT AND EVALUATION OF MUTANT GERMPLASM OF *Amaranthus*

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Abstract

Seeds of *Amaranthus tricolor* were gamma irradiated and M_1 seedlings planted in the field or in wooden boxes in greenhouses to screen for early drought tolerance. Drought tolerance was confirmed in similar subsequent screening steps, and seeds were collected from selected drought tolerant M_2, M_3, M_4 and M_5 plants. The mutation induction and selection procedure, constraints and characteristics of the mutated plants are described, with emphasis on selection for early drought tolerance. *A. tricolor* putative mutants showed strong drought avoidance and drought tolerance characteristics during severe moisture stress, and recovery after rewatering was within a few hours, with re-growth within a few days. This study is part of a project to develop tolerant genotypes of neglected vegetable crops that could contribute to food production in rural areas in Africa and the rest of the world.

1. INTRODUCTION

In South Africa, where drought is a severe problem, tolerance to drought stress of economically important crops is of great value. Since most plants can only survive a limited period of drought, development of plants with improved drought tolerance, growth, and yield is essential for food production in marginal areas.

Arid lands, where the precipitation is insufficient to support conventional agriculture, are rapidly increasing throughout the world because of several factors including, notably, deforestation and the related global warming trend known as the "greenhouse effect" [1]. There is also a tremendous growth in the world population, putting further pressure on the food sources. Of the world's total land surfaces, 35 percent are already considered to be intensely arid to semi-arid [2], which means that increases in the population will mean greater need for use of this land. Data on global warming suggests that developing crops tolerant to water deficit, while maintaining or increasing productivity, will be even more critical in the future [3]. It is estimated that the world's population will soar from 5.4 billion people in 1992 and 6.3 billion by the year 2000, to 8.5 billion in 2025 [4]. The population in the third world alone is almost 5 billion at present, nearly equal to the entire population 10 years ago and will increase to 7 billion by 2025. The global growth rate was 1.7% in 1993 but according to recent figures Africa has expanded at a rate of 3% a year in the 1990's, by far the highest in the world. According to the author about 95% of the future population growth will occur in less-developed countries in Asia, Africa and Latin America, the countries least capable of being able to deal with this, while the industrialised world will grow with less than 5% [4]. All indications are that this tendency will continue for the next decade.

Amaranth is a multi-purpose plant utilized by millions of people as grain, leafy vegetable, animal feed and also has medicinal uses. Vegetable amaranth or "potherbs" are herbaceous annuals [5] that are utilised as a staple food or delicacy by both rural and urban populations,

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where they play an important role in family self-subsistence [6]. The leaves of amaranth present an excellent protein, vitamin and fibre source to many tropical, subtropical and temperate region populations at a very low cost. Vegetable amaranth shows a wide diversity in growth habit, leaf shape, colour and size, plant size and inflorescence characteristics, but typically have broad succulent leaves, tender stalks, and small inflorescences with a low seed production (200–500 kg/ha) [7].

Vegetable amaranth has all the characteristics to be improved for better yield, drought tolerance and improved nutritional qualities to be of worldwide value. Unfortunately, good quality agricultural areas are limited by available water, where drought is the most common and most severe limitation to plant productivity [8]. *Amaranthus* is especially grown in semi-arid areas with no modernised irrigation equipment, and harvest productivity and quality completely depends on the environmental conditions, where rainfall is most critical. Although most of the species are tolerant to high temperatures, and flourish at temperatures of 22–30°C, harvest quality and quantity is severely influenced by drought, and vegetable amaranth is known to be more droughts sensitive. *Amaranthus* are cultivated in both dry and wet seasons, but multiple irrigation is usually needed for a better harvest, depending on the growth stage and water hold capacity of the soil [9].

The aim of this project was to develop new genotypes of vegetable amaranth (*A. tricolor*) with improved drought tolerance, improved leaf yield and photoperiod insensitivity through mutation induction techniques. Developing vegetable *Amaranthus* crops for successful cultivation in marginal areas could further contribute to family subsistence and food security.

2. MATERIALS AND METHODS

2.1. Establishing dosimetry

The first dosimetry test was conducted at 0, 100, 200, 300, 400 and 500 Gy (n = 100 for each treatment) and seeds were germinated *in vitro* and in soil seedling trays. From results obtained from this experiment, we further tested different irradiation doses of 0, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 Gy (n = 100 for each treatment). Seeds were either germinated under aseptic conditions on Murashige & Skoog (MS) medium [10] or in soil in seedling trays in a greenhouse. For *in vitro* germination, seeds were surface sterilised after irradiation using 1% NaOCl, by placing them in cheesecloth bags (the seeds are very small and difficult to handle) and slightly shaking them on a shaker for 25 minutes. Thereafter seeds were rinsed 3X with sterile distilled water and were germinated on MS medium [10] in test tubes (n = 30 for each treatment) to determine the LD-50. Seeds were incubated at a photoperiod of 16 h at low light of a photosynthetic photon flux (PPF) of 10 μmol m^{-2} s^{-1} GEC Alsthom cool white light. At 6–8 weeks after germination, growth and survival calculations were made for the different treatments (Tables I and II). The LD-50 was determined by observing germination rate, growth and survival after 6–8 weeks of growth.

For greenhouse treatments, seeds were sown (n = 50 for each treatment) in seedling trays in a 2:1:1 (peat:sand:vermiculite) soil mixture. The seeds were kept moist at all times, and Multifeed™ was applied weekly. The LD-50 was determined after 6–8 weeks of growth as above (Table I and II).

2.2. Selection procedures

2.2.1. Screening for drought tolerance

48,000 *A. tricolor* seeds were vacuum packed in plastic bags and send to the Atomic Energy Corporation, Pelindaba, to be gamma-irradiated at 180 Gy. A Part of the M_1 seedlings (12 400 seedlings) were germinated and grown outside under shade cloth in seedling trays in mist beds during 2000/2001, where after they were transplanted to the field after four weeks. The rest of the M_1 seedlings (35,800) were grown and kept in seedling trays in a greenhouse. For both treatments the plants were allowed to grow for four months or until seed was formed, and seed was harvested for the M_2.

The M_2-M_5 seed was then tested for early drought tolerance and confirmation of early drought tolerance in wooden boxes or in rainout shelters over a period from 2000–2003. Selection criteria were mainly based on survival after re-watering, but growth vigour, disease appearance, shoot length and branching were also noted.

2.2.1.1. Wooden boxes

The wooden boxes are 1,830 mm X 915 mm X 180 mm, according to the technique developed by [11], in a 2:1:1 (peat:sand:vermiculite) soil mixture. Seeds were sown in rows, 10 cm apart, allowing 28 progenies per box (Figure 1a), of which two control progenies per box were included. Seedlings were thinned out after germination, to leave rows of 10 plants per progeny (Figure 1a). After approximately 3 weeks of growth, watering was completely stopped (Figure 1b). Boxes were re-watered after approximately 80% of the plants died (Figure 1c). Seed was collected from surviving plants (Figure 1d) after being replanted into 25 cm pots with a similar soil mixture.

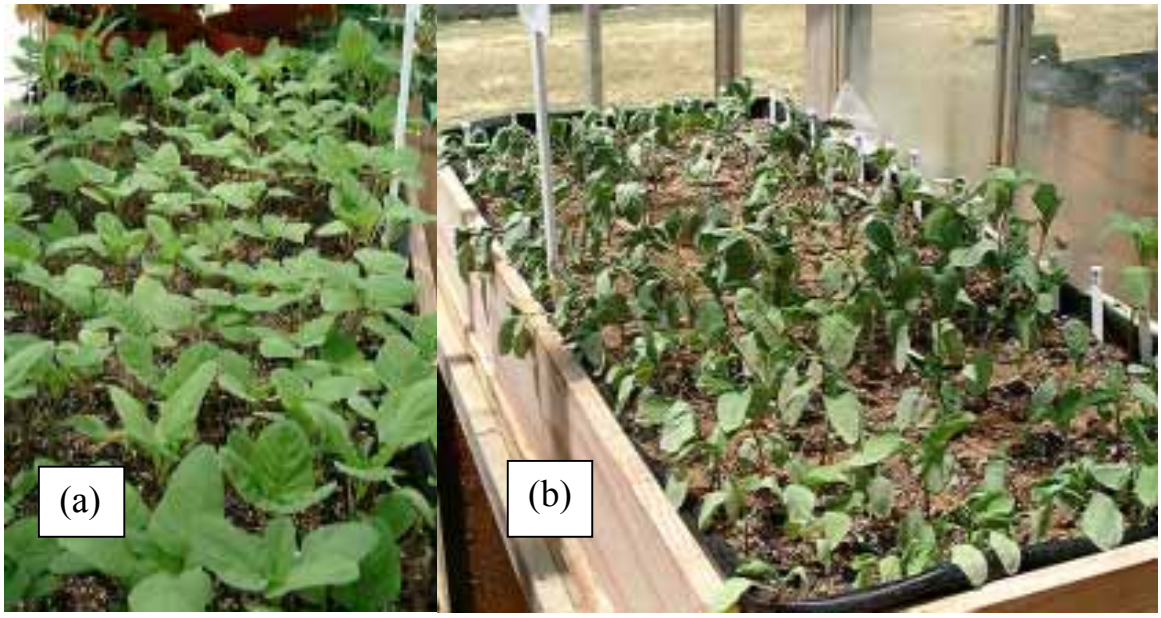

Fig. 1. A. tricolor M_2 progenies screened for early drought tolerance in a wooden box: (a) seedling growth, (b) drought stress two weeks after watering was stopped.

Fig. 1. A. tricolor M_2 progenies screened for early drought tolerance in a wooden box (Cont.): (c) drought stress six weeks after watering was stopped (d) rewatering and re-growth after 4–8 weeks for seed selection.

2.2.1.2. Rainout-shelters

651 M_2 progenies were germinated in seedling trays in a greenhouse. After two weeks, seedlings were thinned out and transplanted into prepared soil in a rainout shelter, 12 seedlings per row, rows 30 cm apart and 50 cm between rows. Two rows of barrier plants (control) were planted on the outside of the plot, as well as three control rows inside the plot. The plot was watered twice a week, and 10 days after transplant of the seedlings watering was completely stopped.

3. RESULTS AND DISCUSSION

3.1. Establishing dosimetry

A.tricolor irradiated seeds should be evaluated for germination as well as survival in order be able to determine the LD-50. Although all the seed germinated, even at high dosages of irradiation (300–500 Gy), not all treatments survived the irradiation after 6–8 weeks of growth.

Most of the seedlings from seeds treated at 300 Gy and higher died *in vitro* after eight weeks (Table I). Vigorous plantlets developed from the Control and 100 Gy treatments. Very poor growth was observed in the glasshouse from seeds treated at 300 Gy and higher (Table I), and the plantlets remained short and stunted. Germination rate was high for all treatments.

TABLE I. RESULTS OF GAMMA IRRADIATION (CO_{60}) ON GERMINATION, SURVIVAL RATE AND SEEDLING HEIGHT OF *Amaranthus tricolor* AFTER 8 WEEKS OF GROWTH *IN VITRO* (N=30) AND IN SEEDLING TRAYS (N=50)

Treatment (Gy)	Germination (%)		Survival (%)		Seedling height (mm)	
	In vitro	Greenhouse	*In vitro*	Greenhouse	*In vitro*	Greenhouse
Control	96	85	100	100	33	51
100	95	85	87	86	32	55
200	95	75	45	52	25	49
300	96	95	26	35	22	30
400	96	95	2	10	22	20
500	95	80	0	0	20	20

It was clear from the first irradiation treatment at 100–500 Gy (Table I) that the dosimetry of 300–500 Gy was too high, and all treatments except the100–200 Gy eventually died off after eight weeks of growth.

In the second treatment at 20–200 Gy the germination rate, survival and length of the plantlets was not that much influenced by the different irradiation dosages after six weeks of growth in the greenhouse or *in vitro*, but the general plant vigour was influenced in the greenhouse (Table II). At 140–180 Gy, approximately 50% (LD–50) of the plantlets developed into vigorously growing seedlings (Figure 2). The bulk *A. tricolor* seeds were then irradiated at 180 Gy for this trial.

TABLE II. RESULTS OF GAMMA IRRADIATION (CO_{60}) ON GERMINATION, SURVIVAL RATE, CHLOROPHYLL PRODUCTION AND SEEDLING HEIGHT OF *Amaranthus tricolor* AFTER 6 WEEKS OF GROWTH *IN VITRO* (N=30) AND IN SEEDLING TRAYS (N=50)

Treatment (Gy)	Germination & survival (%)		Lack of chlorophyll production (%)	Seedling height (mm)	
	In vitro	Greenhouse	*In vitro*	*In vitro*	Greenhouse
Control	93	96	0	25	41
20	100	87	2	25	40
40	100	83	6	24	42
60	97	96	6	25	40
80	93	96	13	26	40
100	100	92	20	25	39
120	100	92	51	23	36
140	93	96	60	26	41
160	100	96	70	25	33
180	97	96	73	26	32
200	97	92	73	24	32

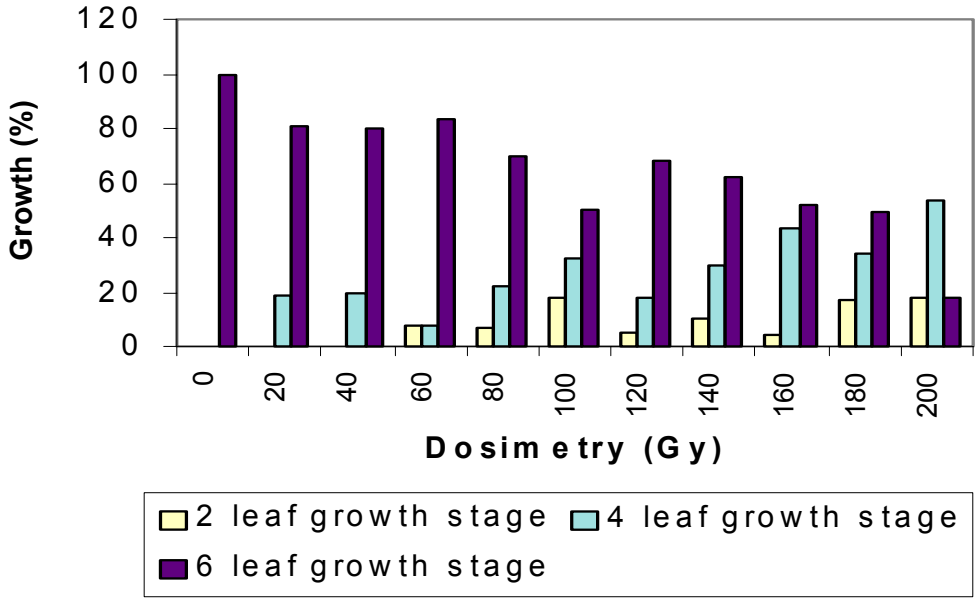

Fig. 2. The effect of different gamma irradiation (Co_{60}) doses on the vigour of Amaranthus tricolor plantlets after 6 weeks of growth in the greenhouse.

3.2. Selection procedures

3.2.1. Screening for early drought tolerance

3.2.1.1. M_1 progenies

A large number of the M_1 seeds did not germinate, and some were lost due to insect pests. Only 5,124 M_2 progenies (approximately 10%) survived. Phenotypic differences in leaf size, shape, colour and branching were noticed in M_1 (Figure 3) and in M_2 (Figure 4) plants.

Fig. 3. Phenotypic differences in growth vigour, leaf size, height, branching and leaf colour in M_1 plants.

Fig. 4. Phenotypic differences in growth vigour, leaf size, height, branching and leaf colour were noticed in M_2 plants.

3.2.1.2. Wooden boxes

After rewatering, plants were allowed to recover, but only a small number of plants survived the drought treatment. Single plant survival (approximately 60%) (Figure 5a) as well as two or more survivors per progeny (approximately 40%) (Figure 5b) were noted.

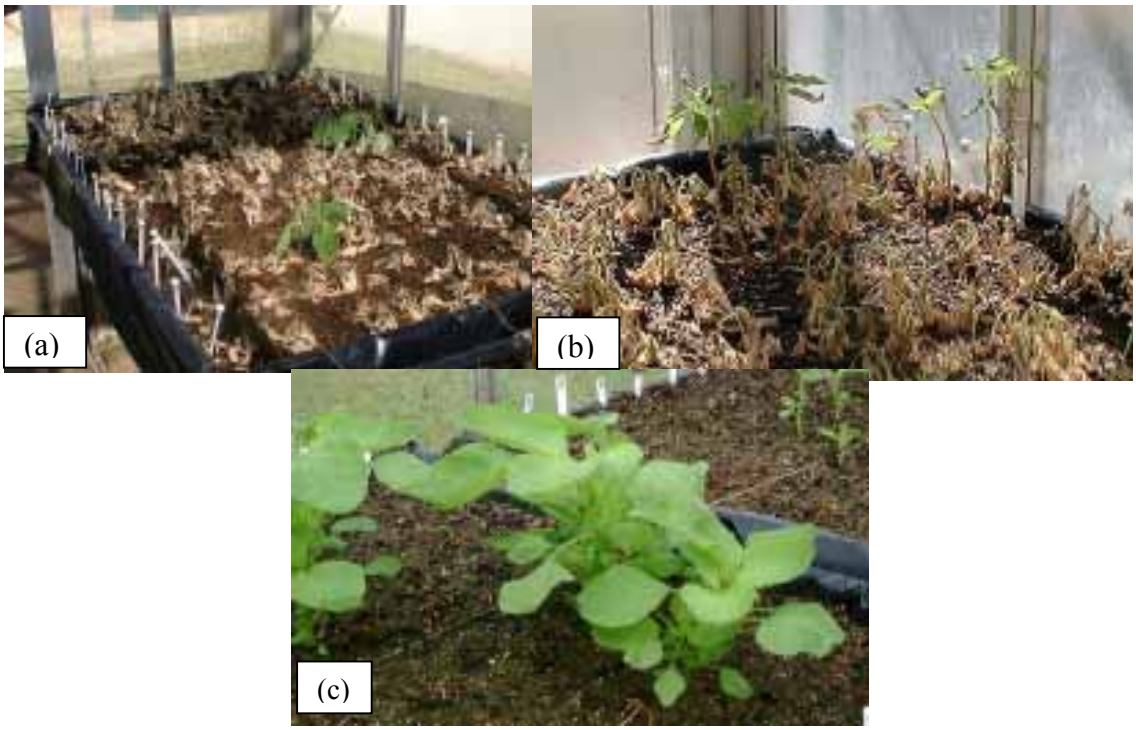

Fig. 5. A. tricolor M_3 progenies screened for early drought tolerance in a wooden box: (a) single plant survival per progeny (b) multiple plant survival per progeny (c) "budding" of shoots to survive.

It was also noticed that about 30% of the stressed plants were completely wilted, but recovered to full turgor after rewatering, and survived drought as a whole plant without leaf loss. This seems to be an indication of metabolic drought tolerance. Metabolic mechanisms

leading to this tolerance will be very useful for selection purposes under conditions of intermittent water-deficit stress [12)] These amaranth plants tolerate dehydration by having a high desiccation tolerance, which is the ability to survive low tissue water status.

The other 70% of the M_2–M_5 plants tested so far shed their leaves and the tip of the branches died down, and they started to shoot again 6 days after rewatering (Figure 5c). This could be an indication of drought avoidance mechanisms at this early developmental stage, where the oldest leaves dry out to reduce the evaporating leaf surface area by 80–100% in order to promote survival of the plant. A small percentage (2%) of the latter also showed the formation of anthocyanin in either their leaves, stems, or both (Figure 5d). Anthocyanin has been shown to improve drought tolerance in some plants. About 1% of selected surviving plants had a decrease in leaf size, probably decreasing the possible transpiration area. Another 1% showed a general increase in plant vigour. It was also noticed that one M_3 progeny was the only row with woolly aphid infestation, indicating a possible increased susceptibility to aphid infection. This selection was however not early drought tolerant.

Fig. 5. A. tricolor M_3 progenies screened for early drought tolerance: (d) formation of anthocyanin (e, f) selection in a rainout shelter.

Rehydration upon rewatering is fast, which means that even small amounts of rain will be utilised sufficiently to ensure survival of amaranth in the field. The initial water uptake after rewatering can take place via existing roots, followed by new roots formed after the drought treatment [13]. According to the latter author it is possible for plants to shed no surface roots during prolonged periods of drought. This factor could also have contributed to the fast recovery of amaranth seedlings after severe moisture stress periods, although it was not studied during this trial.

3.2.1.3. Rainout-shelter

Plants grown in the rainout-shelter proved to be more difficult to screen for early drought tolerance. Insects were a problem to control, and beetles, aphids and cutworms posed a serious problem that had to be controlled via insecticides. It was also realized that the watering of the plants should be done superficially, since amaranth seedlings have a very good ability to sense the onset of drought, whereby the roots react by growing very fast and deep into the soil within a few days. This has the effect that the plants are able to reach very deep into the soil for available soil water, and moisture stress then only starts after 4 months, when the plants are mature and have seeded already. Selection at this age is not only very difficult, but the criteria also differ from that of the wooden boxes (Figure 5e, f).

3.2.1.4. Photoperiod insensitivity

Single seed descendant M_2 seed of 5124 progenies were planted in wooden boxes, but results were not yet available at the time of writing this report.

3.2.2. Constraints

During this trial various constraints were encountered using either the wooden box or rainout-shelters. These included the limitation of the number of possible progenies to be screened each year in wooden boxes. Some screening trials had to be repeated, because it was not always easy to determine the exact correct stage to start rewatering: if too early too many plants survived (Figure 6a), if too late all plants died (Figure 6b), uneven survival of plants due to the box not being exactly horizontal or when last watering was done uneven (Figure 6c).

Other constraints included mice that ate the seedlings during winter months and accidental watering of boxes by personnel during drought stress. As amaranth is a short day plant, greenhouses also had to be equipped with halogen lamps in order to be able to screen in winter months, which allowed for larger number of mutant to be screened every year. Another constraint was human error, where it was neglected to close the rainout shelter, and the plants received 64 mm of rain. Due to this accident, the plants continued to grow for another 3–4 weeks, and at that stage became too big to be stressed again and results compared poorly with other trials.

The M_2 progenies were replanted in wooden boxes in a greenhouse with additional lighting source during January 2002.

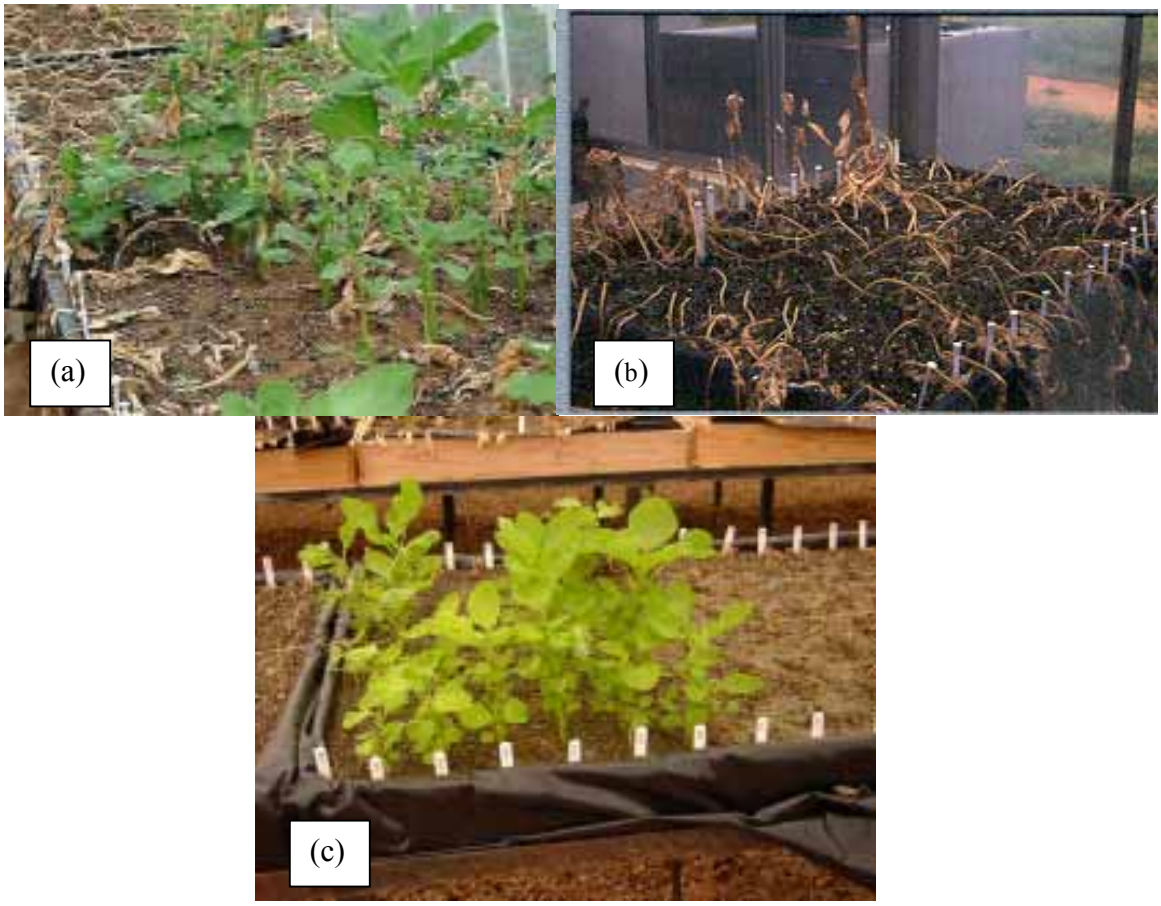

Fig. 6. Rewatering of plantlets: (a) too early - too many plants survived, (b) too late -all plants dead, (c) unevenly watered - "box effect" of survival.

4. SUMMARY

TABLE III. SUMMARY OF RESULTS OBTAINED FROM DECEMBER 1999 TO JUNE 2003 FOR THE IMPROVEMENT OF *A. Tricolor* THROUGH MUTATION BREEDING

Total number of *A. tricolor* seed irradiated	M_1 seedling loss due to irradiation, insects	Total number progenies selected	Number screened in wooden boxes	Number screened in rainout-shelters	Total number screened	Remaining number to be screened
48,000	42,876	M_2= 5,124	2,621	651	3,272	1,852
		M_3= 724	191		191	533
		M_4= 56	50		50	6
		M_5= 12				12

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IMPROVEMENT OF SELECTED *Amaranthus* CULTIVARS BY MEANS OF MUTATION TECHNIQUES AND BIOTECHNOLOGICAL APPROACHES

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Abstract

Pseudocereals like amaranth (*Amaranthus ssp.*), with high numbers of species exhibiting a high degree of variability, may enhance biodiversity within cereal food supply and deliver essential ingredients as grain and vegetable crops suitable for variable climatic conditions and also for people with allergies. Goals in improving cultivars of grain amaranth are similar to those in other grain crops – improvement and stabilization of the yield, increasing pest resistance, and improving harvestibility. The aim of this work was to combine radiation mutagenesis with biotechnology approaches to improve selected *Amaranthus* cultivars. For the experiments, two genotypes of *Amaranthus sp.* have been selected – *Amaranthus cruentus* "Ficha" and hybrid "K–433" which are characterized by a good seed quality and quantity, suitable for food production. The seeds were treated with 175 Gy. During the period of the project duration (10 December 1998–19 May 2003) the M_1 - M_5 generations were established. The phenological observations were performed during all vegetation periods and selection on desired traits was done. The negative plants were removed from the field. The weight of seeds per plant and weight of 1000 seeds (WTS) was recorded and statistically evaluated. Finally, as seed progeny of M_4 generation, 48 samples of *A. cruentus* (irradiated) with WTS > 0.87g and 18 samples of K–433 (irradiated) with WTS > 0.75g were selected and used for establishment of M_5 generation. In several samples of *A. cruentus*, the WTS reached 0.9–1.0g and in K–433 0.8–0.9g with an obvious tendency to stabilization of this trait when comparing them with the mother plants of the previous generation.

1. INTRODUCTION

In substance, all basic components of human nutrition, e.g. proteins, carbohydrates and lipids can be of a plant origin. At present, from more than 250 thousand well-known plant species only approximately 150 are used for food production. From them, only 1/5 cover 95% of energy need, protein, carbohydrate and lipid consumption. From those, approximately 80% are wheat, rice, rye, barley, oat, maize and millet.

Pseudocereals like amaranth (*Amaranthus ssp.*), with high numbers of species exhibiting a high degree of variability, may enhance biodiversity within cereal food supply and deliver essential ingredients as a grain and vegetable crop suitable for wide climatic conditions and also for people with allergies [1].

The extensive cultivation of amaranth is mainly in American, African and Asian countries [2]. In Europe, preliminary research trials of amaranth cultivation have been realized in a few countries (e.g. Czech and Slovak Republics, Poland, Austria, Hungary and Germany). Yields of amaranth biomass are in the range of 10–50 tones of dry matter per hectare annually, depending on chosen plant species, location, as well as cultivation conditions (e.g. soil, climate). Expectable grain yields in Europe will range between 2 and 3 t/ha but yields up to 6 t/ha under conventional agricultural practices have been reported.

Breeding work on grain amaranth is just beginning and shows the necessity of further research for drought resistance; yield improvement and maturation of grain [3,4,5]. The breeding of new varieties has just begun in Europe in the UK, The Netherlands, Germany, Austria, the Czech Republic and Poland. New breeding lines with potential for high grain and biomass yield should be investigated for performance under different climatic conditions.

The potential impact of amaranth cultivation and consumption on human population is based on its very promising nutritional and economic characteristics, as it is a non-allergenic source of basic nutrients, as well as a food source for patients suffering from food intolerance (gluten enteropathy). Amaranth grain has high protein content, with a well-balanced amino acid composition, with high lysine and methionine content. Lipid fractions from amaranth seeds contain high levels of unsaturated fatty acids and possess a high antioxidative activity. Amaranth oil contains also a unique squalene component, an intermediate of steroid synthesis, which is discussed as immuno modulator and is proposed to play a role in the rate of cholesterol synthesis (prevention of cardiovascular diseases). The most promising use of amaranth starch, which possesses high solubility and digestibility, is based on the uniquely small starch grain size of amaranth, which is about one-tenth the size of cornstarch and therefore offers new possibilities for food processing, pharmacology and cosmetics [6]. Furthermore, amaranth-originated products may reduce dietary fibre insufficiency, vitamin deficiency, as well as deficiency of bioactive compounds (antioxidants, folic acid).

Cultivated amaranth varieties are used for food grain, leafy vegetables, forage, ornamental gardening, and energy production by burning the straw material and others.

2. DETAILED RESEARCH OBJECTIVES

The basis for any plant breeding is the natural existing variability within species, which is used for controlled hybridization and selection. The availability of simple, efficient and rapid techniques for increasing genetic variation is an essential component of plant breeding programmes. Conventional plant breeding is based on the use of genetic variation and selection of the desired genotypes. This requires the screening of relatively large populations. Mutation induction and selection for desired traits in combination with *in vitro* techniques offer several advantages over conventional methods [8, 9].

The aim of this work was to improve selected *Amaranthus* cultivars by combining radiation mutagenesis with biotechnology approaches.

At the same time, by collaboration with small farmers in our field experiments, to promote cultivation, commercialization and crop diversity and sustainability in agriculture production in Slovakia.

2.1. Breeding objectives in grain amaranth

Among the problems to be solved in amaranth breeding are the high degree of heterozygosity, low heritability of some traits and susceptibility to some diseases, especially in *A. caudatus* [7]. Numerous major genes have been identified that may be useful for mutation breeding in amaranth, such as genes coding flower, embryo and seed pigmentation, leaf characters, type of starch in perisperm, early/late flowering, inflorescence architecture and vegetative architecture.

Goals in improving cultivars of grain amaranth are similar to those in other grain crops – improvement and stabilization of the yield, increasing pest resistance, and improving harvestibility [3].

There are several desired traits which were recorded: vigorous seedling growth, determination of the plant growth, timing and uniformity of flowering and seed maturation within plants, synchronous drying of plant and seeds, reduction of leafiness in the inflorescence area, reduction in seed retention, increasing size of seeds, pale seed pigmentation, enhanced food quality traits (increasing seed proteins).

3. MATERIAL AND METHODS

3.1. Plant material

For these experiments two genotypes of *Amaranthus sp.* were selected – *Amaranthus cruentus* "Ficha" and hybrid "K–433" which are characterized by a good seed quality and quantity, suitable for food production. Both seed samples were obtained from the collection of Gene Bank of the Research Institute of Plant Production Praha-Ruzyne, Czech Republic.

Amaranthus cruentus "Ficha" is a medium early cultivar with big seeds (WTS 0.85g) light in colour (Fig.1).

Amaranthus hybrid "K–433" is a plant breeding material, product of interspecific hybridization originated from Rodalo Research Center in Pennsylvania, USA. It is medium early with light- coloured seeds (WTS 0.73g) (Fig. 2).

Fig. 1. Amaranthus cruentus "Ficha.".

Fig. 2. Amaranthus hybrid "K–433".

3.2. Research methods carried out

3.2.1. Radiosensitivity test

Before the final irradiation treatment, a radiosensitivity test was performed in collaboration with Joint FAO/IAEA Programme Agency's Laboratories in Seibersdorf, Austria. Samples of 100 seeds were treated with 5 different doses: 500, 750, 900, 1200 and 1500 Gy.

After treatment the seeds were grown under greenhouse conditions. For each dose and non-irradiated control, 15 seeds were planted per tray in three replicated trays with random arrangement of different doses to eliminate border effect. The germination percentage, seedling survival and height of seedlings were measured and evaluated. The seedling height measurement was done once the first leaves were fully expanded and did not continue during elongation. The length from cotyledons to the tip of the first leaves was measured. The hypocotyl itself did not show any radiation effect. The optimal dose for the final treatment was selected after this testing.

3.2.2. Establishment of field experiments

During the period between 10 December 1998 and 19 May 2003 the M_1 - M_5 generations were established, following the scheme as detailed below.

Scheme of field experiments	Vegetative stage	Seed progeny
1198	-	Irradiation of seeds – M_1
1999	M_1	M_2
2000	M_2	M_3
2001	M_3	M_4
2002	M_4	M_5
2003	M_5	M_6

M_1 generation: The irradiated seeds and untreated control were sown in the field of Experimental Station "Dolna Malanta" – University of Agriculture, Nitra on 29 April 1999 in four randomly arranged replicates containing at least 1,000 plants per sample. The harvest of M_1 generation was done in the second half of September 1999.

M_2 generation: was established at the beginning of May 2000. Samples from each plant were sown individually into lines distant 375 mm with 100 mm between plants. Three repetitions in random arrangement were done containing together at least 10000 plants per sample. Isolation belts with *Helianthus tuberosum* were planted between individual samples. The M_2 generation was harvested at the end of September 2000.

For M_3 generation the seeds were sown in territory of Nitra town, on very fertile soil near Nitra River at the beginning of May 2001. Approximately after two weeks the field experiment was completely destroyed by a storm. A second sowing was performed during the first week of June. The seeds from individual plants were sown into separated three m long lines, 375 mm apart and with 100 mm between plants. The number of samples was: 47–control and 264–irradiated for *A. cruentus* and 44–control and 363–irradiated for K–433. The M_3 generation was harvested by mid November 2001.
M_4 generation: was established during the first week of May 2002 on two different sites in Slovakia. The first field experiment was located on the territory of Nitra town in Western

Slovakia, on very fertile soil near Nitra River, an area typical for production of maize, while the second field experiment was established on Research Breeding Station Maly Saris in Eastern Slovakia, an area typical for potato production. For establishment of M_4 generation on each locality, 319 samples of *A. cruentus* (irradiated) and 184 samples of K–433 (irradiated) were used plus control samples of both genotypes. The seeds from individual samples were sown into separated three m long lines, 375 mm apart and at 100 mm between plants. After field selection 155 plants were collected in *A. cruentus* and 122 plants in K–433 during September, 2002 from the experimental field in Nitra and similar numbers from Maly Saris, which were again selected on the basis of weight of 1,000 seeds (WTS).

M_5 generation: was sown [48 samples of *A. cruentus* (irradiated) and 18 samples of K–433 (irradiated)] during the first week of May 2003 on both sites above.

The phenological observations were performed throughout all vegetative periods and selection on desired traits, as mentioned above, was started in M_2 generation. The negative plants were removed from the field.

For harvest, the individual collection of single inflorescences was done. The samples were dried under room conditions, the seeds were isolated from the inflorescence, cleaned and kept in paper bags.

3.2.3. Seed evaluation

The weight of seeds per plant and weight of 1,000 seeds was recorded and statistically evaluated.

3.2.4. Testing of oxalic acid contents

One of the breeding objectives is the reduction of anti-nutritional factors like oxalic acid, which is a precursor of the neurotoxin ODAP/BOAA (beta -A- oxalyl-L-alfa, beta-diaminopropionic acid). For oxalic acid contents in green leaves of amaranth, capillary iso-tachophoretic analysis was tested in collaboration with the State Veterinary Institute in Nitra.

3.2.5. Molecular characterization of selected samples

For molecular characterization of selected irradiated samples of *A. cruentus*, restriction analysis of chloroplast DNA based on PCR-RFLP analysis was done. The young leaves from M_4 plants were collected during July 2002 and used for chloroplast DNA analysis.

3.2.6. In vitro cultivation

The aim was to develop efficient regeneration/multiplication system for each cultivar, which enables multiplication of the selected genotypes under *in vitro* conditions.

In vitro regeneration was tested using different culture media and growth regulators. The seeds were surface-sterilized by dipping them in 70% ethanol for 1 min, then in 0.1% $HgCl_2$ for six min, followed by washing with sterile distilled water. The seeds were germinated in Erlenmeyer flasks on a filter paper. For *in vitro* cultivation, the different parts of 10 days old seedlings were used as explants: epicotyls with several true leaves, hypocotyl segments and root segments.

For *in vitro* cultivation, the following basal culture media were tested:

- MS modified medium with 1/2 concentration of macroelements, 1/4 nitrogen compounds and full concentration of micro-elements and vitamins, solified by 0.8% Plant agar and with 20g.l^{-1} sucrose
- White basal medium, solidified by 0.8% Plant agar and with 20g.l^{-1} sucrose
- DCR basal medium, solidified by 0.8% Plant agar and with 20g.l^{-1} sucrose

The combinations of growth regulators (mg.l^{-1}) tested for adventitious bud induction and shoot regeneration

Combination	BAP	NAA	KIN
1	5.0	0.01	
2	5.0	0.1	
3		0.1	5.0
4	1.5		1.5

The combinations of growth regulators (mg.l^{-1}) tested for callus induction

Combination	BAP	NAA	2,4-D	IAA	Dicamba
1	0.01	6.0			
2	0.1		6.0		
3	0.1			6.0	
4	0.1				6.0
5	0.5		2.0		

4. RESULTS AND DISCUSSION

4.1. Radiosensitivity test

The radiosensitivity test showed that a dose of 500 Gy induced a growth reduction of 67% in *A. cruentus* and of 55% in hybrid "K–433". The growth reduction with higher doses reached 100 % (no first true leaves were established, the seedlings developed only to the cotyledonary stage). From these data it was assumed that a 50% growth reduction (GR50) in seedling height was about 400 Gy. In other crops, with GR50 at 300–400 Gy, useful doses for mutation induction are thought to be in the range of 100–300 Gy. As a result of these measurements, the seeds were finally treated with 175 Gy.

4.2. Evaluation of M_1 generation

The harvest of M_1 generation was done in the second half of September 1999.

The phenological observations during the vegetative period have shown unequal germination of seeds but with no significant differences between treated and untreated samples. On 25 May 1999 the plants had sprouted being partly in the cotyledonary stage or at the first true leaves stage. On 21 June, the first inflorescences started to be visible. All plants in the field formed inflorescences and seeds (100%). The average height of the plants of each sample was as follows (cm):

	21 June 1999	23 July 1999
A. cruentus – treated	35.00	88.75
A. cruentus – control	38.75	89.50
K–433 – treated	28.75	81.00
K–433 – control	33.75	82.25

It is obvious from these data that no significant influence on growth reduction was observed after irradiation treatment. The differences between both cultivars were visible in growth dynamics, where *A. cruentus* showed a more intense growth and formed a denser cover. On 27 July 1999 the formation of seeds began. By 25 August 1999 the seeds were in the stage of wax ripeness and they reached full maturity around half of September.

The weight of seeds per plant and weight of 1,000 seeds have been recorded and statistically evaluated. There were no significant differences in weight of seeds per plant among tested samples. For the weight of 1,000 seeds in samples of M_2 seeds in hybrid K–433 there were differences between non-treated and radiated samples in favour of radiated samples (Table I). When we compared control samples, M_1 and M_2 seeds there were also differences in WTS (Table II). We suppose that these differences are rather the result of hybrid origin (segregation) than radiation. In *Amaranthus cruentus* there were no differences between radiated and control samples, and no differences were confirmed either between control samples from M_1 and M_2 generation**.**

TABLE I. MULTIPLE RANGE ANALYSIS FOR WEIGHT OF 1,000 SEEDS IN M_2 SEEDS

Level	Count	LS mean (g)	Homogenous groups		
Hybrid K–433 control	5	0.672	X		
Hybrid K–433 γ	5	0.742		X	
A. cruentus control	5	0.854			X
A. cruentus γ	5	0.838			X

Method: 95% LSD

TABLE II. MULTIPLE RANGE ANALYSIS FOR WEIGHT OF 1,000 SEEDS OF CONTROLS $M_1 – M_2$

Level	Count	LS mean (g)	Homogenous groups		
Hybrid K–433 control – M_2	5	0.672	X		
Hybrid K–433 control – M_1	5	0.703		X	
A. cruentus control – M_2	5	0.854			X
A. cruentus control – M_1	5	0.856			X

Method: 95% LSD

4.3. Evaluation of M_2 generation

In spite of the fact that from the beginning of May the weather in Slovakia was very dry, the seeds started to germinate 10 days after sowing, and 14 days after sowing the plants were fully sprouted. There were no significant differences between treated and untreated samples on 19 June 2000. The plants were approximately 20 cm high. By the end of June, first

inflorescences started to be visible. Later on, the flowering was synchronous and all plants formed inflorescences and seeds. The individual harvest of M_2 plants was realized at the beginning of October. Only plants with positive traits were collected.

Number of collected plants (samples) of M_2 generation for evaluation and for establishment of M_3 generation was as follows:

A. cruentus (control) - 47 samples
K–433 (control) - 44 samples
A. cruentus (irradiated) - 264 samples
K–433 (irradiated) - 363 samples

Evaluation of weight of seeds per plant and weight of 1,000 seeds are shown in Tables III to VII.

In Table III, we can see that there is significant difference in weight of 1,000 seeds between control and M_3 seeds in hybrid K–433, but no significant differences in *A. cruentus*.

In Tables IV and V, there is a comparison of weight of 1,000 seeds in M_1, M_2 and M_3 seed progeny in both *A. cruentus* and K–433. Again, we can see that there is a larger variability in K–433 than in *A. cruentus*, and this is probably influenced by hybrid genotype of K–433.

Unfortunately, there was no stable tendency in M_3 seed generation to increase size of seeds (weight of 1,000 seeds). The individual evaluation of single seed progeny has to be done as the basis for positive selection for seed size, when minimal values for WTS should be considered the values of original seed samples used for radiation (WTS 0.85g in *Amaranthus cruentus* "Ficha" and WTS 0.73g in K–433). We suppose that this trait could be stabilized by a severe positive selection in the next generations.

In Tables VI and VII, the results for weight of seeds per plant are mentioned. There is significantly higher variability in M_3 generation of *A. cruentus* so that a higher percentage of plants formed smaller inflorescences and had to be removed from the population.

TABLE III. SUMMARY TWO-SAMPLE ANALYSIS RESULTS OF 1,000 SEEDS WEIGHT OF AMARANTH OF M_3 SEEDS

Genotypes of amaranth	Count	LS mean (g)	Homogeneous groups		
Hybrid K–433 – control	5	0.79	X		
Hybrid K–433 – γ	363	0.71		X	
A. cruentus L. – control	5	0.85			X
A. cruentus L. – γ	264	0.82			X

Hypothesis test for H0 (alpha = 0.05)

TABLE IV. SUMMARY TWO-SAMPLE ANALYSIS RESULTS OF 1,000 SEEDS WEIGHT OF *Amaranthus cruentus* L. IN M_1, M_2 AND M_3 GENERATION

Generation	Count	LS mean (g)	Homogeneous groups
M_1 control	5	0.85	X
M_2 control	5	0.85	X
M_3 control	5	0.85	X

Hypothesis test for H0 (alpha = 0.05)

TABLE V. SUMMARY TWO-SAMPLE ANALYSIS RESULTS OF 1,000 SEEDS WEIGHT OF AMARANTH HYBRID K–433 IN M_1, M_2 AND M_3 GENERATION

Generation	Count	LS mean (g)	Homogeneous groups		
M_1 control	5	0.70	X		
M_2 control	5	0.67		X	
M_3 control	5	0.79			X

Hypothesis test for H0 (alpha = 0.05)

TABLE VI. SUMMARY STATISTICS OF SEED WEIGHT PER PLANT OF AMARANTH

Statistics	*A. cruentus* L. control	Hybrid K–433 control	*A. cruentus* L. γ	Hybrid K–433 γ
Sample size	47.00	44.00	264.00	363.00
Average (g)	30.97	20.09	21.46	20.23
Median	26.8	15.48	18.71	18.1
Mode	25.24	15.28	13.69	15.09
Variance	297.28	123.12	129.04	123.52
Standard deviation	17.24	11.09	11.35	11.11
Standard error	2.51	1.67	0.69	0.58
Minimum	6.88	7.01	2.07	1.24
Maximum	71.13	63.38	56.21	58.5
Range	64.25	56.37	54.14	57.26
Coeff.of variation	55.67	55.24	52.94	54.93
Sum	1,455.6	883.84	5,665.05	7,343.83

TABLE VII. SUMMARY TWO-SAMPLE ANALYSIS RESULTS OF SEED WEIGHT PER PLANT OF AMARANTH

Genotypes of amaranth	Count	LS mean (g)	Homogenous groups	
Hybrid K–433 – control	44	20.09	X	
Hybrid K–433 – γ	363	20.23	X	
A. cruentus L. – control	47	30.97		X
A. cruentus L. – γ	264	21.46	X	

Hypothesis test for H0 (alpha = 0.05)

4.4.Evaluation of M_3 generation

Because of the later sowing (the first field experiment was destroyed by a storm), the vegetation was delayed and the plants were shorter. However, the inflorescences were established on 100% of plants and seeds reached the complete ripe stage at the end of October.

The number of plants collected from M_3 generation was 576 in *A. cruentus* (irradiated) and 440 in K–433 (irradiated). Seed progenies of these plants were evaluated for the weight of seeds per plant and weight of 1,000 seeds. The average weight of 1,000 seeds (WTS) was the lowest in this generation – 0.80g in *A. cruentus* and 0.63g in K–433 (with no significant differences between irradiated and control samples) in comparison with other generations.

The selection of samples was done on the basis of WTS. The selected samples had to reach at least the same average WTS as the original irradiated samples (0.85g in *A. cruentus* and 0.73g in K–433). This criterion was, in to our opinion, strict enough considering the high reduction in seed size in M_3 generation which was caused by later sowing and delay of the vegetation period. From selected plants of M_3 generation of *A. cruentus*, in 69 plants the weight of 1,000 seeds was the same as in control (non significant differences), in 36 plants WTS was significantly higher (at level 0.05), and in 50 plants the differences in increase of WTS were statistically highly significant (at level 0.01).

In hybrid K–433 the majority of selected M_3 plants had significantly higher WTS in comparison with control. Among selected plants, 90 plants had a significantly higher WTS at 0.01 level, in 11 plants WTS was higher at a 0.05 level, and in 21 plants the differences were non significant in comparison with the control.

The differences in WTS among progenies of a same plant (for example C17 / 1, 2, 3, 4, 5 where five M_3 plants were selected in 2001 as progeny of one M_2 plant) were in some cases high. For 8 plants, progenies were found in which WTS was non significantly different from the control and also progenies with significant differences. In some cases, however, all selected progenies of one plant had the same tendency and their WTS was significantly higher.

In general, differences in WTS among M_3 plants were not too high, as confirmed by the low variation coefficient. On the other hand the weight of seeds/plant exhibited very high variation coefficient.

The variability and/or stability of weight of 1,000 seeds have to be assessed in the next generations (M_4, M_5) and, together with positive selection; it should be the basis for seed size increase.

4.5. Evaluation of M_4 generation

After field selection during September 2002, 155 plants of *A. cruentus* and 22 plants of K–433 1 were collected from the experimental field in Nitra, and a similar number from Maly Saris. From the collected samples the subsequent selection was performed based on significant differences in size of seeds. In *A. cruentus* (irradiated), 48 samples were selected with WTS > 0.87g (WTS of control–0.85g, Nitra), and in K–433 (irradiated) 18 samples were selected with WTS > 0.75g (WTS of control–0.68g, Nitra). These were used for the establishment of M_5 generation. In several samples of *A. cruentus* the WTS reached 0.9–1.01 g and in K–433 0.8–0.9g with an obvious tendency towards the stabilization of this trait when comparing this samples with the previous generation.

4.6. Determination of oxalic acid content

Capillary isotachophoretic analysis proved to be the suitable method for oxalic acid content determination in green leaves of amaranth. Hereafter, all selected samples will be tested by this method on oxalic acid content.

4.7. Molecular characterization of selected samples

Restriction analysis of chloroplast DNA based on PCR–RFLP analysis of the flanking regions between the genes *trn*S [tRNA–Ser (UGA)] and *psb*C (psII 44kDa protein) revealed no

differences in *Eco* RI and *Hae* III restriction patterns of the control plant and irradiated M_3 plants. The *Eco* RI restriction profiles of a control plant obtained from non-irradiated seeds and the 23 plants of M_3 generation grown from irradiated seeds consisted of 1 restriction fragment of an approximate size of 2000 bp. On the contrary, the *Hae* III patterns consisted of 5 restriction fragments of 1300, 500, 210, 100, and 70 bp, respectively, uniformly occurring in all the samples compared.

Additional experiments are planned oriented towards the RAPD–PCR analysis of some other genes of both genomic and chloroplast DNA.

4.8. *In vitro* cultivation

4.8.1. Adventitious shoot regeneration

The best shoot regeneration was achieved when the upper parts of seedlings with epicotyl and several true leaves were used as primary explants.

From the culture media tested, MS medium was the most favourable for shoot regeneration and multiplication. The best shoot regeneration and multiplication rates were achieved on modified MS with 5 $mg.l^{-1}$ BAP and 0.01 $mg.l^{-1}$ NAA. A high cytokinin:auxin ratio favours *Amaranthus* shoot regeneration. Strong cytokinins, such as BAP, seem to be the most effective agents for shoot regeneration [10].

No significant differences were apparent in regeneration ability between both cultivars.

4.8.2. Callus induction

Callus formation occurred in all tested combinations of growth regulators, confirming the great capacity of *Amaranthus* explants to form callus. The most effective combination for callus growth in *A. cruentus* seams to be 6 $mg.l^{-1}$ NAA + 0.1 $mg.l^{-1}$ BAP, while in hybrid K–433 a combination 2 $mg.l^{-1}$ 2,4–D + 0.5 $mg.l^{-1}$ BAP showed a stronger effect on callus growth. Similar results can be found in the literature, where NAA or 2,4–D in combination with BAP were successfully used for callus induction [11].

Further experiments are necessary for the optimisation of shoot multiplication, elongation and rooting, and for callus induction and regeneration with respect to interacting factors such as genotype, age and type of explants and cultural parameters.

5. CONCLUSIONS AND RECOMMENDATIONS

Amaranth is a crop known for its tolerance to drought and salinity, resistance to diseases, insects and weeds. Also, it does not require strong chemical inputs polluting the environment. Introducing amaranths as a C4 plant into agriculture could contribute to mitigate CO_2 concentration in the atmosphere, the major factor provoking greenhouse effects. It can be used also as a renewable energy source. Introducing amaranth to agriculture will also be coherent with sustainable agriculture because of its low factor input, high yield potential and possibility of cultivation on marginal lands and contaminated soils.

For these reasons it is desirable to continue with amaranth breeding, to determine the most suitable varieties of *Amaranthus sp.* for introduction into European agriculture, taking into account an assessment of agronomic practices, processing and economical costs.

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BAMBARA GROUNDNUT IMPROVEMENT THROUGH MUTATION BREEDING IN GHANA

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Abstract

A study was initiated to improve bambara groundnut productivity using mutation breeding. Two landraces of varieties Tom and Nav were treated with gamma rays ranging from 50 to 400 Gy and with EMS at concentrations from 0.2 to 1.2%. Pooled mean LD_{50} for gamma irradiation were 178.5 Gy and 0.78% for EMS treatment respectively. Gamma irradiation gave effective dosages for mutation frequency at 150 Gy for Tom and 200 Gy for Nav, while EMS treatment gave 0.6% for both Tom and Nav. Genetic variation of up to four times the control was estimated for both gamma irradiation and EMS treatment compared to the untreated controls. Selected mutants outperformed the untreated control plants in most of the agronomic traits and reaction to diseases like *Cercospora* leaf spot, anthracnose and viruses. The results indicate that mutation breeding could be used to improve bambara groundnut productivity.

1. INTRODUCTION

Bambara groundnut (*Vigna subterranea* (L. verde) is an important source of protein, especially for rural and urban dwellers who cannot afford the high cost of animal protein in sub-saharan Africa [1]. The major producers of bambara groundnut are Nigeria, Niger, Ghana and Ivory Coast. The crop is widely grown in Southern Africa [2]. About half of the world's production occurs in West Africa [3]. It is grown mainly, for its edible protein, which has high lysine and methionine content [4]. Humans, pigs and poultry, consume the seeds while the haulm is used as fodder to feed livestock [5]. Bambara has an interesting content of protein (18–24%) Carbohydrate (51–70%), energy (367–414 kcal per 100 g), iron (4.9–48 mg/100g) [4]. Bambara features prominently in the cropping system in Ghana and makes a significant contribution to soil fertility through symbiotic nitrogen fixation [6]. Production constraints include inherently low yield potential, susceptibility to diseases such as *Cercospora,* mosaic virus, pod discoloration, susceptibility to pests like bruchids, lack of improved varieties, low soil fertility, lack of improved management practices. The most cost-effective and environmentally friendly solution to insect and disease infestation is through the use of resistant varieties.

At a workshop involving stakeholders in bambara groundnut production and utilization, the need to improve bambara productivity came to the fore. For selection to be effective in a genetic enhancement programme, genetic variation must exist. Radiation and other chemical mutagens may be used to increase variability. Induced mutation supplements plant breeding and confers specific improvement without significantly altering acceptable phenotypic traits [7]. The value of induced mutation in bambara improvement has not been fully determined and utilized. Literature on the use of mutagens and *in vitro* techniques to improve resistance

to diseases and pests in bambara is scanty. A systematic programme was initiated to improve bambara groundnut productivity.

The objectives of the study are:

- To conduct baseline survey/needs assessment to identify farmers needs.
- To conduct radiosensitivity tests to determine the LD_{50} for chemically treated and gamma irradiated seeds.
- To screen local and exotic bambara groundnut germplasm, radiation and chemically induced mutants for resistance to *Cercospora*, anthracnose and bruchid in two locations.
- To investigate the effects of gamma irradiation and chemical mutagens as a means of increasing genetic variability.

2. MATERIALS AND METHODS

2.1. Informal survey/needs assessment

The objective of the informal survey was to obtain baseline information on bambara groundnut production constraints to prioritise the constraints and to identify those that could be addressed by breeding through the use of induced mutation and *in vitro* techniques. Informal surveys were conducted in the major bambara groundnut growing areas in Ghana. The country was divided into three zones viz: (a) northern zone (b) middle zone and (c) southern zone. Each zone had a team comprising of (i) breeder (ii) agronomist, (iii) rural sociologist/social-economist, and (iv) pathologist/entomologist. The Participatory Rural Appraisal method was used. This includes farmer discussion groups; interviewing key informants in some villages, farm visits to meet with farmers as well as discussions with personnel of the Ministry of Food and Agriculture. Some of the questions asked include the cropping system, varieties planted, source of seed for planting, agronomic practices, diseases and pests, harvesting and storage, seed selection, marketing and utilization as well as resource allocation. Production constraints were prioritised. The role of gender in bambara production was also discussed.

2.2. Radiosensitivity test

(a) The radiosensitivity test was conducted on local accessions that were found to be high yielding but susceptible to *Cercospora* and Mosaic virus. Seeds of uniform size with initial germination of 100% were selected. Four batches of two hundred seeds each were irradiated by a ^{60}Co gamma ray 220 unit cell at the Ghana atomic Energy Commission at the following doses: 0, 50, 10, 150, 200, 250. 300, 350 and 400 Gy. The irradiated seeds were planted on flat ridges at a spacing of 20 cm x 30 cm. Recommended agronomic practices were carried out.

(b) Seeds of uniform size of varieties Tom and Nav with initial germination of 98% were selected. A set of 200 seeds per treatment was treated with ethylmethane sulphonate (EMS) at a concentration of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 or 1.2%. A stock solution of EMS was prepared and diluted to the required concentration. The seeds were placed in a beaker, the aqueous solution of EMS was poured and the beaker was placed on a shaker for 6 hours. The treated seeds were washed several times in running tap water and planted immediately on the field at a spacing of 20 cm × 30 cm.

Data were collected on the following parameters:

- Emergence count on alternate days until complete germination.
- Number of seedlings growing after germination.
- Seedling height (cm) at weekly intervals (a-sectors) was noted.
- Aberrations on leaf surface (a-sectors) were noted.

Following the estimation of LD_{50}, two landraces of bambara groundnut cvs Tom and Nav were selected from evaluation of germplasm collected from farmers. Two thousand lots of dry seeds of each of the two varieties were irradiated by a ^{60}Co gamma ray 220-unit cell at the Ghana Atomic Energy Commission with dosages of 150 Gy and 200 Gy. Similarly, 2000 lots of dry seeds of the two varieties were treated with 0.6% and 0.8% EMS. The irradiated and EMS treated seeds together with their respective controls were planted in field plots at the Crops Research Institute, Kumasi ($6^{o}43'$N, $1^{o}36'$W). The soil series has been classified as Ferric Acrisol (FAO/UNESCO Legend) and Paleustult (U.S. Dept. of Agriculture Soil Taxonomy). A randomised complete block design with three replications was used. Plants were spaced 0.20 m × 0.5 m. A basal dressing of 30 kg/ha of P_2O_5 was applied. Spraying Karate at 2.5g a.i./l as and when necessary controlled insects. Twenty seeds from each surviving plant were planted to raise the M_2 generation. Plants selected in M_2 were planted for M_3 generation. In each generation selection was made for desirable agronomic characters and the reaction of the plants to *Cercospora* leaf spot, anthracnose and mosaic virus. In the M_3 half of the seeds were reserved and used to screen for resistance to bruchid, a devastating storage pest.

Mutation frequency was estimated by pooling chlorophyll, a-sectors and male sterile mutants as a percentage of segregating progenies as outlined by [8]. Mutagenic effectiveness was assessed according to [9] as outlined by [10]. Data collected in the M_1 and M_2 generations include (i) days to emergence, (ii) seedling survival 30 days after planting (DAP), (iii) number of pods per plant, (iv) 100- seed weight, (v) seed yield per plant and (vi) shelling percentage. Four of these traits were further evaluated using genetic parameters (i) genetic variance, (ii) heritability; and (iii) genetic gain, according to [11] as outlined by [12]. Ten percent selection pressure was applied in each case.

2.3. Screening for bruchid resistance

Bambara groundnut is grown widely in Ghana but the efforts of farmers to increase production are hampered by damage to the seeds by the bruchid, *Callosobruchus maculatus* (F.) (*Coleoptera: Bruchidae*) in storage. However, the loss in storage can drastically be reduced by the use of resistant varieties. The objective of the study was to screen different varieties of bambara groundnut for resistance against attack by *C. maculatus*.

Seeds of 88 accessions of bambara groundnut obtained from the legume breeder, Crops Research Institute (CRI), were fumigated with a Phostoxin tablet in the entomology laboratory at Kwadaso, Kumasi, Ghana for 72 hours to kill the existing insects from the field. The materials were aerated and conditioned to the laboratory environment for 14 days. Ten seeds of each variety were put in separate rearing boxes in four replicates and arranged on shelves in the laboratory. Two pairs of 0–1–day old *C. maculatus* adults were introduced into each box, allowed to oviposit on the seeds and removed after 24 hours. The eggs laid were counted seven days afterwards and the insects emerging were counted daily until emergence ceased. The numbers of insects emerging were used to compute the index of susceptibility (IS) [13], defined as follows:

$$IS = \frac{\log_{10} \text{ per cent } F_1 \text{ adult emergence}}{\text{Developmental period (Dp)}}$$

Dp = time from the mid-oviposition to time of 50% F_1 adult emergence

2.4. Disease score

Forty-four lines previously irradiated or chemically treated with EMS and nine local accessions of bambara groundnut planted in trials at Fumesua and Wenchi, respectively, were screened for their resistance to *Cercospara* leaf spots, Anthracnose and virus diseases. Severity ratings were based on a 1–5 scale, where 1 represents apparently healthy symptomless plants and 5 represents very severely infected plants.

2.5. Multilocation variety testing

Ten entries comprising six mutant lines and four landraces were evaluated in two locations Fumesua and Wenchi. The Randomised Complete Block Design with three replications was used on a 5.0 m long plot of four rows. Spacing was 20 m × 0.50 m. Recommended agronomic practices were followed. Data were collected on number of days to 50% flowering and maturity, number of pods per plant, 100 seed weight, pod yield (t/ha) and shelling percentage.

3. RESULTS AND DISCUSSION

3.1. Informal survey/needs assessment

Results from the informal survey revealed that bambara groundnut is mostly cultivated by women. Production constraints enumerated by farmers include low yields (0.5–0.8 t/ha) compared to 4.0 t/ha obtained on-station, lack of improved varieties, susceptibility to diseases such as *Cercospora* leaf spot, anthracnose, mosaic virus among others; susceptibility to storage pests, bruchid, caterpillars and grass hoppers (sporadic), low soil fertility, lack of improved crop management practices. The constraints were prioritised and those that could be addressed through mutation breeding included developing insect and disease resistant varieties as well as seed quality improvement and availability.

3.2.Radiosensitivity test

Results indicated an LD_{50} 150–200 Gy with a mean of 175 Gy for Tom and 182 Gy for Nav respectively with a pooled mean of 178.5 Gy for the gamma irradiated treatments for emergence count, number of growing after germination and seedling height. Reduction in plant vigour increased with increasing dosage (Table 1). For the EMS mutagenic treatment, an LD_{50} of 0.75% and 0.82%, respectively for varieties Tom and Nav with a pooled mean on 0.79%.

In general, the establishment of the gamma-irradiated plants was better than the EMS treated plants in the M_2 and M_3 generations. Results of the studies are presented in Tables I–IV. The effect of mutagenic effectiveness and frequency LD_{50} and some agronomic traits in M_1 are presented in Table V.

The survival percentage of the irradiated population did not vary appreciably from the non-irradiated population at the lower dosages of 50 Gy and 100 Gy, but a remarkable decrease in

survival rate was observed for 150 Gy–300 Gy for the two varieties. A non-linear decrease with increase in dosage was observed (Table I). On the basis of 30 day-old surviving seedlings, the mean LD_{50} of 175 Gy and 180 Gy were obtained for cultivars Torn and Nav, respectively, with a pooled mean of 178.5 Gy. The effectiveness of the mutagen calculated as the mutation frequency per unit dose established that the most effective dosages for the two cultivars were 150 Gy for Torn and 200 Gy for Nav. These differences might be due to the genetic backgrounds of the two varieties. Mensah and Eruotor [14] made similar observations in lima beans, which corroborate the findings of this study.

TABLE I. EFFECT OF GAMMA IRRADIATION ON MUTAGENIC FREQUENCY AND EFFECTIVENESS, LD_{50} AND SOME AGRONOMIC TRAITS IN BAMBARA GROUNDNUT IN M_1 GENERATION

Variety	Treatment Gy	Mf	ME	De	Sr	LD_{50}
Tom	-	-	-	9.0	93.1	
	50	0.98	0.15	9.5	88.0	
	100	2.50	0.25	10.1	69.0	
	150	3.50	0.29	12.0	59.8	
	200	4.00	0.20	13.0	50.4	175 Gy
	250	4.70	0.19	14.0	40.0	
	300	5.50	0.17	14.6	24.1	
Nav	-	-	-	10.1	90.1	
	50	0.80	0.11	11.2	85.2	
	100	1.80	0.20	12.4	68.0	
	150	2.79	0.21	13.7	55.2	182 Gy
	200	3.61	0.25	14.6	49.6	
	250	4.10	0.18	14.2	36.1	
	300	5.13	0.17	15.1	22.0	

Mf, mutation frequency; ME - mutation effectiveness; De - No. of days to emergence; Sr - survival rate 30 DAP; LD_{50} lethal dose for 50% survival

For the EMS treated population, the survival percentage did not vary much from the untreated control at the lower dosages of 0.2% and 0.4%. A remarkable dose-dependent reduction was however observed for 0.6–1.2% indicating a non-linear decrease with increase in EMS dosage (Table II). Mean LD_{50} of 0.7% and 0.82% were obtained respectively for Torn and Nav. with a pooled mean of 0.79% on the basis of 30–day–old seedling survivals. The effectiveness of the mutation revealed that the most effective dosage for the two cultivars was 0.6%, which is at variance with the results obtained from the irradiated treatment.

A stimulatory effect on number of days to emergence, plant height, number of days to maturity, number of pods per plant and seed yield per plant was recorded at lower dosages of 50 and 100 Gy and 0.2 and 0.4% respectively for the irradiated and EMS treated populations. However, a reduction in the same parameters at higher dosages of 150 to 300 Gy and 0.6 to 1.2% was observed for the two cultivars. In the case of shelling percentage there was little or no effect of irradiation or EMS treatment at lower dosages of 50 and 100 Gy and 0.2 and 0.4% respectively and a progressive decrease with increasing dosage. The ability of irradiation and EMS treatments to stimulate or reduce productivity at certain dosages, as observed in this study, has been reported earlier [12, 15, 14, 16].

TABLE II. EFFECT OF ETHYLMETHANE SULPHONATE (EMS) ON MUTAGENIC EFFECTIVENESS, LD_{50} AND SOME AGRONOMIC TRAITS IN BAMBARA GROUNDNUT IN THE M_1 GENERATION

Variety	Treatment	Mf	ME	De	Sr	LD_{50}
Tom	-	-	-	8.7	94.0	
	0.2%	0.90	0.11	8.9	90.2	
	0.4%	2.10	0.23	9.1	71.1	
	0.6%	2.95	0.29	10.0	69.1	0.75%
	0.8%	3.81	0.21	11.5	60.7	
	1.0%	4.15	0.18	11.9	40.9	
	1.2%	4.93	0.17	12.0	38.1	
Nav	-	-	-	8.3	05.3	
	0.2%	0.83	0.10	8.7	93.1	
	0.4%	1.79	0.20	8.1	92.3	
	0.6%	2.35	0.27	9.1	80.1	0.82%
	0.8%	2.93	0.23	9.6	71.6	
	1.0%	3.10	0.20	10.5	68.1	
	1.2%	4.41	0.19	11.2	52.0	

Mf, mutation frequency; ME: mutation effectiveness; De: No. of days to emergence; Sr: seedling survival rate 30 DAP; LD_{50} lethal dose for 50% survival

Genetic variance was increased in all the traits under study. High increases from two to four times greater than their respective non-irradiated and non-EMS treated populations were found in days to maturity, number of pods per plant, seed size and seed yield for the two varieties. Similar observations were noted for the EMS treated populations compared to non-EMS treated control populations (Tables III and IV). The increase in genetic variance observed in this study corroborates previous findings in [17], [18] and [14] that genetic variability in peanut, soybean and lima beans could be increased four times following x-ray and gamma ray irradiations in the second generation respectively. Based on selected plants from the M_4 generation, the genetic studies revealed that in bambara groundnut, the number of pods per plant offered the highest opportunity for selection in M_2 followed by seed size and seed yield per plant in the M_3 generation following gamma ray and EMS treatments.

TABLE III. POPULATION MEAN, GENETIC VARIANCE, HERITABILITY AND PREDICTED GENETIC GAIN FOR SOME AGRONOMIC TRAITS IN M_3 PROGENIES OF THE TWO BAMBARA GROUNDNUT VARIETIES GROWN FROM SEEDS TREATED WITH GAMMA RAYS GR AND UNTREATED UT

Trait	Population Mean		Genetic Variance		Heritability		Genetic Gain	
	UT	GR	UT	GR	UT	GR	UT	GR
Pp	66.80±2.5	79.8±3.1	1.50	3.50	89.6	90.2	14.1	15.7
Ss	58.10±1.7	61.2±2.3	1.20	5.71	73.8	85.1	12.1	13.9
Gy	83.40±3.1	97.1±2.7	3.78	9.00	65.0	70.5	11.0	12.0
Sp	54.00±1.1	58.6±1.8	2.70	3.35	59.2	62.0	7.8	8.7
Pp	62.1±1.5	70.8±2.3	4.10	9.70	85.10	86.31	9.7	11.9
Ss	51.0±2.4	62.1±3.1	6.10	9.30	84.60	85.21	13.6	14.7
Gy	76.2±1.9	94.5±2.7	3.20	7.00	82.40	86.71	10.2	11.8
Sp	55.3±1.1	58.2±1.0	5.10	10.20	27.10	28.10	8.3	9.2

Pp: No. of pods per plant Ss: Seed size (100 seed weight); Gy: grain yield; Sp: shelling percent

TABLE IV. POPULATION MEAN, GENETIC VARIANCE, HERITABILITY, AND PREDICTED GENETIC GAIN FOR SOME AGRONOMIC TRAITS IN THE M_3 PROGENIES OF TWO BAMBARA GROUNDNUT VARIETIES GROWN FROM SEEDS TREATED WITH ETHYLMETHANE SULPHONATE (EMS) AND UNTREATED CONTROL

	Population Mean		Genetic Variance		Heritability		Genetic Gain	
Trait	UT	EMS_T	UT	EMS_T	UT	EMS_T	UT	UMS_T
Tom								
Pp	69.3±2.5	64.1±1.7	1.57	4.11	82.4	90.1	14.5	15.9
Ss	57.9±1.7	54.6±2.4	1.32	5.31	80.1	87.5	12.7	13.1
Gy	85.2±3.5	86.7±2.9	4.11	9.71	70.2	77.3	11.3	12.8
Sp	55.1±0.9	57.0±1.0	3.10	4.93	54.8	61.2	7.1	9.3
Nav								
Pp	69.2±1.9	50.3±2.2	1.49	4.15	81.2	93.1	13.9	15.2
Ss	52.1±2.0	50.2±1.7	1.28	4.49	78.4	89.3	12.0	13.2
Gy	79.3±3.1	89.9±2.8	3.99	8.81	69.4	78.1	11.0	12.6
Sp	56.7±1.2	59.1±0.9	3.01	4.97	52.6	62.4	6.9	9.1

Pp: No. of pods per plant Ss: Seed size (100 seed weight); Gy: grain yield; Sp: shelling percent

Results from the present investigation indicated that there is an appreciable contribution of heredity to the traits under study. Furthermore, irradiation and chemical mutagenesis (EMS) induced a high degree of variability within the gamma-irradiated and EMS treated populations. It could therefore be inferred that gamma irradiation and EMS treatments could serve as a means of improving bambara groundnut.

Evaluation of M_2–M_4 progenies. The number of progenies selected, seeds planted and number established are presented in Table V.

TABLE V. NUMBER OF PROGENIES SELECTED IN M_2, SEEDS PLANTED AND NUMBER ESTABLISHED FOLLOWING GAMMA IRRADIATION AND EMS TREATED

	Irradiation				EMS		
Treatment	No. of progenies selected	No. of seeds planted	No. of plants established	Treatment	No. of progenies selected	No. of seeds planted	No. of plants established
M_2 0	-	1,000	700	0	-	1,000	800
150 Gy	100	7,000	4,300	0.6%	50	4,040	2,010
200 Gy	60	6,250	3,690	0.8%	39	3,210	1,920
Total	160	14,250	8,690	Total	89	8,250	4,730
M_3 0	-	1,000	741	0	-	1,000	793
150 Gy	54	4,000	2,630	0.6%	26	3,516	2,110
200 Gy	34	2,731	1,421	0.8%	18	1,342	1,010
Total	88	7,731	4,792	Total	44	5,858	3,913
M_4 0	-	500	420	0	-	1,000	650
150 Gy	10	1,250	1,730	0.6%	5	593	421
200 Gy	8	680	540	0.8%	5	621	462
Total	18	2,430	2,690	Total	10	2,214	1,633

3.3. Screening for bruchid resistance

The developmental periods and the indexes of susceptibility of the insect on the various accessions ranged from 23.8 to 26.5 days and 0.071–0.082 respectively. An index, which is close to 0.1, indicates high susceptibility of the material attacked by the insect. Thus, none of the materials were resistant to attack by the insect. This implies that more work has to be done with the introduction of more landraces and exotic accessions from other parts of Africa for further screening.

3.4. Disease assessment

Based on the amount of disease of the whole plant, disease severity of foliar symptoms was assessed on a five-point scoring system where mean scores of 1.0–1.4 were considered as highly resistant, 1.5–2.5 as moderately resistant, 2.6–3.5 as moderately susceptible and 3.6–5.0 as highly susceptible. Mean scores of disease incidence indicate percentage of plants infected. Figs. 1 and 2 show mutants resulting from mutagenesis showing resistance to *Cercospora* leaf spot and the susceptible untreated controls.

TABLE VI. MEAN INCIDENCE AND SEVERITY SCORES FOR VIRUS INFECTION, *Anthracnose* AND *Cercospora* LEAF SPOTS ON BAMBARA GROUNDNUT AT FUMESUA

Entries	Virus		Anthracnose		*Cercospora* leaf spots	
	Sev.	Inc. (%)	Sev.	Inc. (%)	Sev.	Inc. (%)
Control	2.5	80	2.0	100	1.5	40
150 Gy–M_3	1.6	75	1.4	27	1.9	49
150 Gy–M_4	1.8	60	1.1	5	1.2	20
200 Gy–M_4	2.0	80	1.5	20	1.2	10
250 Gy–M_3	1.8	50	2.0	70	1.3	25
EMS	1.6	57	1.4	27	1.2	12
Tom	2.3	80	1.4	23	1.5	40
Ada	2.3	80	1.1	7	1.2	10
Nav	1.9	57	1.2	14	1.3	19

Generally, all the radiated and the EMS treated lines as well as the local land races performed better than the control at Fumesua (Table VI). With regard to the virus disease, severity of infection was less in 150 Gy–M_3 and EMS treated lines than in the other lines. Incidence of infection was however higher ranging from 50 to 80 percent. In the case of the fungal diseases, anthracnose, caused mainly by *Colletotrichum dematium* was more prevalent than the *Cercospora* leaf spot (caused by *Cercospora canescens*) in the radiated and EMS lines.

The percentage incidences were also higher ranging from 0–100, compared to 0–40% for *Cercospora* leaf spot. There was not much difference in the infection severity of *Colletotrichum* and *Cercospora* among the radiated and EMS lines. However, 150 Gy–M_4 radiated lines appeared the best in terms of both severity and incidence of infection. The local land races had higher resistance to anthracnose than to *Cercospora* leaf spot.

At Wenchi, where only the local land races, Nav and Tom were planted, the Nav lines performed better than the Tom, both in severity and incidence in all the diseases (Table VII). There was virtually no incidence of anthracnose in the Nav lines indicating that the

production of local races is more suitable at the Savanna-forest transitional zone than in the Savanna zone.

Besides *Colletotrichum dematium* and *Cercospora canescens* other fungal disease organisms recorded on Bambara groundnut include *Colletotrichum gloeosporioides*, *Phomopsis sp*., *Corynespora cassiicola*, *Cladosporium herbarum*, *Phoma sp*., *Fusarium solani*, *Bipolaris papendorfii* and *Botryodiplodia theobromae*. *Macrophomina phaseolina*, a well-known pathogen on leguminous plants has also been recorded in Ghana as a seed-borne of Bambara Groundnut [19]. *Cercospora* leaf spot and virus infection have also been reported in Zimbabwe (Rothwell, 1983 cited by [20]).

TABLE VII. MEAN INCIDENCE AND SEVERITY SCORES FOR VIRUS INFECTION, *Anthracnose* AND *Cercospora* LEAF SPOTS ON BAMBARA GROUNDNUT AT WENCHI

Entries	Virus		Anthracnose		*Cercospora* leaf spots	
	Sev.	Inc. (%)	Sev.	Inc. (%)	Sev.	Inc. (%)
Nav	1.4	26	1.0	0	1.3	16
Tom	2.0	50	1.5	20	2.0	40

Figures 1 and 2 show the effect of mutagenesis in inducing resistance to *Cercospora* leaf spot.

Fig. 1. A mutant resulting from EMS treatment showing resistance to Cercospora leaf spot (a) and an untreated control showing susceptibility to Cercospora leaf spot (b).

Fig. 2. M_4 Gamma irradiated mutant showing resistance to Cercospora leaf spot (a), and a non-irradiated susceptible control (b).

3.5. Mutilocational testing

There were no significant differences in days to 50% flowering and days to maturity (Table VIII). There were however, significant differences ($p<0.05$) in pods per plant, 100 pod weight and pod yield/ha among the lines. Pods/plant ranged from 36 to 87, with a mean of 52. EMS M_4–22 produced the highest number of pods/plant but had lowest seed weight. Hundred seed weight ranged from 48.1 to 81.9 g, with a mean of 59.3 g. Pod weight ranged from 3112 to 6,012 kg/ha. 150 Gy–14–1 had the highest pod yield. The mutants produced higher pod yields than the landraces. In a related study Karikari [21] observed variation between local and exotic bambara groundnut. He also observed significant differences in grain yield and other yield components, which are in consonance with observations made in this study.

4.CONCLUSIONS

From the study it can be concluded that: (i) Bambara groundnut is mostly cultivated by women and is a food security crop in Ghana. (ii) A pooled mean LD_{50} of 178.56 Gy and 0.87 respectively were obtained for gamma irradiation and EMS treatments. (iii) Mutagenesis induced genetic variation of up to four times than the untreated control in bambara groundnut. (iv) Mutagenesis could be used to improve bambara groundnut productivity since the selected mutants out-performed their untreated control plants in most of the agronomic traits and reaction to *Cercospora* leaf spot, anthracnose and viruses.

TABLE VIII. MEAN PERFORMANCE OF 10 BG MUTANT AND LANDRACES PLANTED IN TWO LOCATIONS IN 2002.

Accessions	DF	DM	Pd^{-1}	100 seed weight (g)	Pod yield t/ha	Shelling %
1.Nav 7	33	92	51	44.8	4,033	61
2.150 Gy–M_4–3	35	96	39	74.8	5,038	52
3.Nav 2	33	91	45	61.2	3,112	58
4.Nav 3	36	96	49	49.1	3,272	50
5.Tom	36	96	36	52.8	3,592	55
6.150 Gy–M_4–5	35	93	42	68.2	4,060	67
7.200 Gy–M_4–91	34	95	40	64.1	4,781	59
8.EMS–M_4–22	34	95	87	49.1	4,033	65
9.EMS–M_4–3	35	97	54	81.9	5,403	58
10.150 Gy–14–1	38	100	80	51.3	6,012	48
Mean	34.9	95.1	52.3	59.7	4,333.6	57.3
CV (%)	0	0.4	28.8	22.9	24.6	7
LSD (0.05)	0	0.7	37.1	30.1	2,110	12

Df- days to flowering DM- Days to maturity Pd-1- Pods/plant

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BREEDING OF SHORTENING OF GENERATION CYCLES FOR A FASTER BAMBARA GROUNDNUT

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Abstract

Manipulation of generation cycles for a faster breeding of food legumes through *in vitro* culture systems is an attractive but technically challenging goal. We report here, for the first time, that generation cycles were drastically shortened in the food legume Bambara groundnut (*Vigna subterranea*) to be of value in breeding programmes when *in vitro* and *in vivo* strategies were used. Thus, we used a greenhouse strategy as the control system and an *in vitro* plus *in vivo* strategy with various Bambara landraces. Using *in vitro* plus *in vivo* system and embryo axis explants, we obtained more than four generations per year as compared to 1 or <2 generations in the control field/greenhouse system. The landraces of Bambara groundnut tested slightly differed in their generation cycle responses, but plants obtained through *in vitro* plus *in vivo* systems were morphologically normal and fertile, as their progeny. Our results demonstrate that it is possible to use *in vitro* and *in vivo* strategies to significantly reduce the duration of generation cycles in Bambara groundnut, thus offering novel and viable strategies for breeding of this important crop.

1. INTRODUCTION

Within the context of plant breeding and the creation of genetic novelties, it is most interesting to dispose of strategies permitting the acceleration of generations, via a shortening of each generation cycle [1]. It is also useful to induce flowering and seed set under the controlled (nearly risk-less) *in vitro* conditions, particularly for rare and valuable genotypes where initial seeds are in very short supply or non existent [2]. These strategies would thus favour a faster fixation of any new and useful genetic traits. The protein legumes are a good example in this respect, being frequently regarded as genotypes recalcitrant to most *in vitro* approaches [3, 4, 5, and references therein]. In addition, they are also a material particularly adapted for comparisons of different methods aimed at reducing the duration of each generation cycle, as a minimum of 10–12 generations are needed before the registration of a novel genotype as a new variety and, at best, two generations per year only are feasible in the field [1].

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Indeed, the increasing needs of plant proteins as animal feed on account of BSE disease, and for human consumption in low- income food-deficit countries (LIFDC) have led over the past few years to the development of protein-rich sources other than soybean, in an effort to warrant a diversity of sources adaptable to varying applications. Thus, some seed legumes have gained (or are gaining) importance, and particularly those with a relatively high (20 to 40%) protein content, including pea (*Pisum sativum* L.), presently the major protein legume in Europe, and two grain legumes lesser utilized in Europe but quite important elsewhere, such as Bambara groundnut (*Vigna subterranea* L.) [6], widely cultivated in Africa. Despite being grown by subsistence farmers across semi-arid Africa, Bambara groundnut production has been sustained largely through tradition and has been accorded very little research effort. Recently, we have developed several efficient *in vitro* tissue culture systems for mutation-cum biotechnological applications in Bambara groundnut [7].

Modern genetics finds its foundations on the work by Mendel on peas, and controlled breeding in pea is very old as compared to Bambara groundnut. In turn, Bambara groundnut requires breeding for an improvement of yield and resistance to diseases before its efficient exploitation. Against this background, the studies described in this article were aimed at reducing the length of the generation cycles *a*) in the greenhouse, and *b*) with an intermediate methodology involving *in vivo* plus *in vitro* stages. Moreover, to ensure the validity of the approaches developed, they were applied to four landraces of Bambara groundnut (*Vigna subterranea* L.). This system may be used in single seed descent (SSD) selection as an alternative to pedigree selection, when it is not feasible for a breeder to handle large pedigree populations.

2. MATERIALS AND METHODS

2.1. Greenhouse strategy

We have tested the acceleration of generation cycles for four landraces of Bambara Groundnut (BG): two landraces from Ghana referred to as GB1 and GB2 and two land races from Mali referred to as MB1 and MB2, were grown in the greenhouse with a thermoperiod of 27 ± 1 C (day) 25 ± 1 C (night), under a short day condition of 10h light with a light intensity of about 5000 lux. For certain experiments, we have also used long day conditions (16h light, 8h dark cycle), for plant growth in the greenhouse. These landraces were provided by Dr. H. K. Adu-Dapaah (Crop Research Institute, Kumasi, Ghana) and Prof. A. Bretaudeau (IPR, Katibougu, Mali). Seeds were sown in 51 cm × 31 cm multi-welled trays, placed on top of plain trays for recovery of percolating liquid to avoid surface greening during watering. Soil or Perlite was used as the substrate and plants were watered and nourished by capillarity with a nutrient solution. The rapid maturation of plants was obtained by stopping all watering and nourishing of plants at the stage when pods were whitish, corresponding to a dry matter content between 50 and 60% in the seeds. In this respect, the use of Perlite, a neutral substrate with very little retention capacity, favored the dehydration of plants. Harvest was made by hand at full maturity so as to preserve a maximum germination power, and seeds were re-sown immediately following the same procedure.

2.2. *In vitro* plus *in vivo* strategy

This *in vitro* plus *in vivo* strategy is modified from that reported by Stafford and Davies [8] as detailed by Ochatt *et al* [9] and is briefly described below.

Sterilization: The seeds of Bambara groundnut (50–100 seeds) were imbibed overnight in 50 ml conical tubes containing 30 ml of distilled water at room temperature. They were rinsed 3–4 times with distilled water, and were surface sterilised by dipping them successively into ethanol 70% (1 min) and 5% calcium hypochlorite (30 minutes). They were then rinsed 3–4 times with sterilised distilled water. Pods with mature non-dry seeds were detached from plants of each landrace, and entire pods were surface sterilised as detailed above.

Culture Media: The culture medium contained Murashige and Skoog [10] macroelements, microelements and vitamins of Nitsch and Nitsch [11], 2% sucrose and 0.6% Difco-Bacto agar. This medium hereafter referred to as BM medium, contained different types and concentration of growth regulators. For seed germination only half-strength hormone free BM was used. For a better root growth 0.5–1 mg/l naphthalene acetic acid (NAA) was added to the BM. Poured in disposable sterile containers, these media could be stored at 4°C for many months without any damage.

Culture conditions: For *in vitro* plus *in vivo* shortening of generation, three different types of *in vitro* cultural protocols were used: Protocol 1. Entire seeds-unpeeled control seeds, Protocol 2. Seeds without seed coats-peeled seeds, and Protocol 3. Embryonic axes isolated from the unpeeled seeds. Seed coats and embryonic axes were isolated under a binocular microscope from surface sterilised seeds/germinating seedlings. Seeds were cut with the scalpel (3 incisions in a triangle were made in order to isolate the embryo axis) around the radicle without injuring the embryonic axis. The seeds and the embryonic explants were cultured on half-strength BM in plastic Petri dishes (20 ml of medium per dish) and or culture tubes, kept in a culture room under a short day regime (5000lux, for 10 h light) at 27 ± 1°C (day) and 25 ± 1°C (night). Plantlets (3–4 cm height) were transferred to the greenhouse for seed set.

Pods containing mature embryos but non-dry seeds were detached from the greenhouse grown plants of each landrace, with entire pods surface sterilised by dipping them successively into ethanol 70% (1 min), calcium hypochlorite solution 50 g/l (30 min) and sterile distilled water (20 min) as above. Pods were opened aseptically and the seeds of each pod were chosen at random for the next sowing. The wet integument/seed coat was carefully opened and discarded with a scalpel avoiding to damage the cotyledons and embryo axes, and paying particular attention not to break the root tip with the cap (two conditions to favour a rapid germination).

3. RESULTS AND DISCUSSION

3.1. Greenhouse strategy

In order to obtain a very short generation cycle with Bambara groundnut, it is essential to control the development of plants whose growth is naturally of an indeterminate type. In this context, for a maximum shortening of the production cycle there is the need to produce plants with a reduced vegetative development and producing few seeds. Hence, the final goal being to integrate this technique into a single seed descent (SSD) selection scheme, one or two seeds per plant suffice. Therefore, we have tested both the short day and long day conditions for plant growth in our greenhouse. There were no significant differences in terms of plant height between the two photoperiodic (long/short day) conditions tested (data not shown).

The main goal of these experiments in the greenhouse was to be able to produce one or two generation cycles per calendar year, and this was achieved for all the landraces/ genotypes tested. The duration of the greenhouse production cycle was similar to that observed in the fields in Mali, where the normal duration of each generation cycle is of 170 to 200 days.

3.2. *In vitro* plus *in vivo* strategy

In preliminary experiments carried out *ex vitro* and *in vitro*, a number of facts were clearly observed:

First, the presence of the integument always delayed the root growth/germination by several days and these had to be removed as a mechanical obstacle to obtain the fastest germination.

Secondly, the best rates of germination (90 to 100%) were always obtained under the sterile *in vitro* conditions, by avoiding fungi and desiccation, as compared with non-sterile conditions. Such experiments justified the use of bare embryos with *in vitro* conditions in a first period of the plant development.

The third fact was that a composition of the nutrient solution as simple, cheap and efficient as possible, such as the BM medium described in Materials and Methods, was the best.

TABLE I. FREQUENCY OF SEED GERMINATION AND PLANTLET DEVELOPMENT ON HALF-STRENGTH BM MEDIUM, FOR BAMBARA GROUNDNUT LANDRACE MB2, AFTER FOUR WEEKS OF CULTURE. FOR EACH TREATMENT ABOUT 70 TO 80 SEEDS AND OR EMBRYO EXPLANTS WERE USED. RESULTS ARE EXPRESSED AS MEANS ± STANDARD ERROR

Days	Unpeeled Seeds (%)	Peeled Seeds (%)	Embryo-axis (%)
7	0	15.3±1.8	38.2±4.2
14	14.8±1.6	35.8±2.6	62.5±3.6
21	49.3±3.4	50.8±3.6	95.2±2.6

TABLE II. FREQUENCY OF PLANTLET DEVELOPMENT ON HALF-STRENGTH BM MEDIUM, FOR BAMBARA GROUNDNUT LANDRACE MB2, AFTER FOUR WEEKS OF CULTURE. FOR EACH TREATMENT ABOUT 70 TO 80 SEEDS AND OR EMBRYO EXPLANTS WERE USED. RESULTS ARE EXPRESSED AS MEANS ± STANDARD ERROR

Explant Type	% germination	Plant Size	Root Length	Stem Height	No. of stem Branches
Unpeeled Seeds	48.2±4.2	6.8±0.8	17.4±2.1	1.5±0.6	12±2
Peeled Seeds	51.4±3.6	7.1±1.2	18.2±1.8	2.2±0.8	13±2
Embryo-axis	94.6±4.8	1.9±0.6	3.6±0.8	2.1±0.6	4±1

The responses of the unpeeled control, peeled and embryo explants following a three-week incubation period are summarized in Tables I and II. There were three main types of response:

(1) Germination is faster for the peeled seeds i.e. peeled seeds start germination seven days after culturing while unpeeled control germinate about 14 days after culture. However, after 21 days of culture, the percentage of germination and plant morphology were nearly comparable between peeled and unpeeled seeds.

(2) On BM medium normal root growth and plant development was observed. However, with auxin such as NAA (0.5 to 1 mg/l), root growth was better than on BM medium without growth regulators (data not shown).

(3) As shown in Table 2, there was a large difference in development (including plantlet size) between plantlets derived from embryo axis explants and those from peeled or unpeeled seeds, which were nearly four times larger than those obtained from the embryo axis, after 21 days of culture. This was probably due to the presence of reserves stored in the cotyledons of the peeled/unpeeled seeds, while embryonic axis explants have little stored reserves. On the other hand, the embryo axis explants germinated much faster, i.e. 4–5 days of culture as compared to seven days for the peeled and 14 days for the unpeeled seeds. The difference in plantlet size, however, played none or little role in the duration of flowering or seed set.

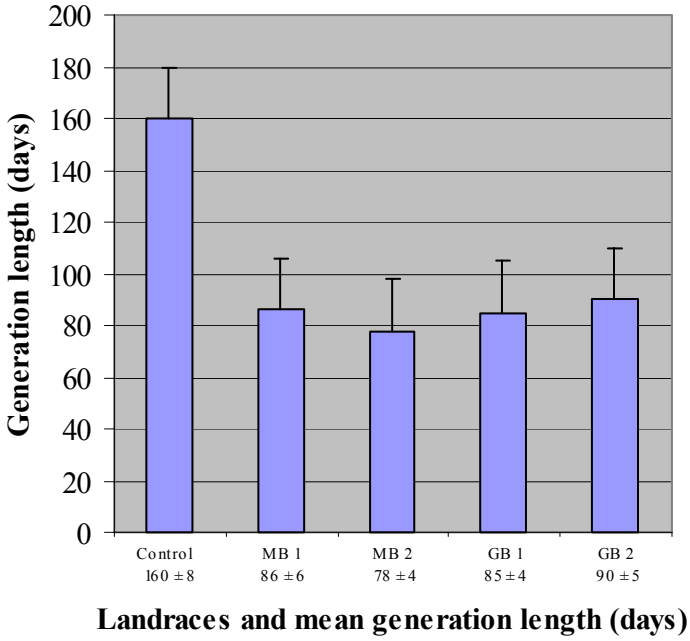
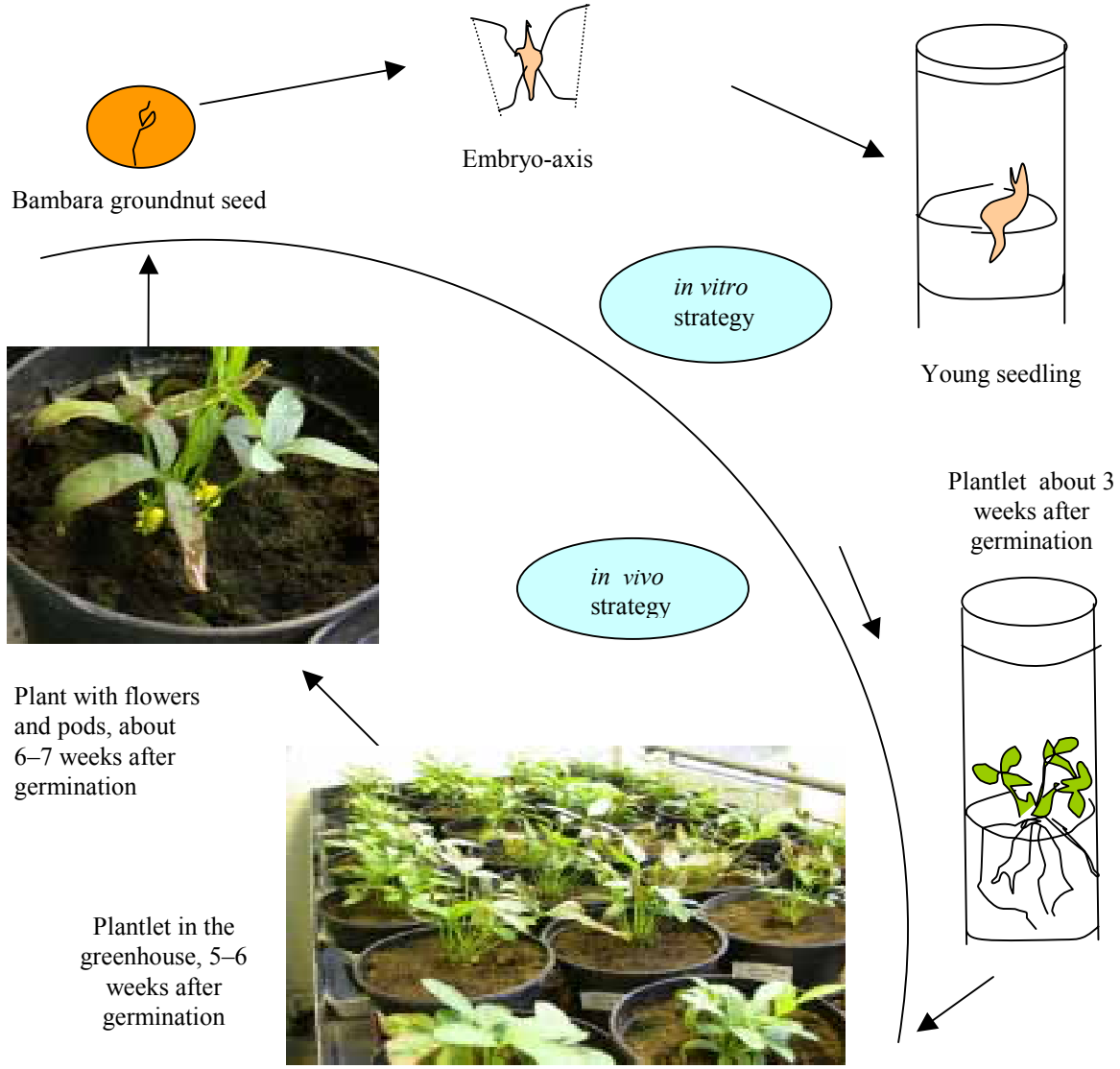


Fig. 1. Mean generation length (in days) of Bambara groundnut with the in vitro plus in vivo strategy.

Rooted Bambara groundnut plantlets were grown in a greenhouse under a short day condition for seed set. Preliminary results indicate that all four landraces produced small pod yields under our greenhouse conditions. There were small differences in landraces in terms of mean leaf number per plant, leaf canopy, pod dry weight etc. Similar to pea [9], in Bambara groundnut, better results were obtained through successive generations from seed to seed, by alternating a first step *in vitro* for germination and a second step *ex vitro* for full development. In such conditions, the mean time span for one generation was of 86.6 ± 6.2 days in MB1, 78.1 ± 4.3 days in MB2 and 85.4 ± 3.9 days in GB1 (Fig. 1**).** As an example, Bambara groundnut landrace MB2 has a mean cycle length of about 170 days in the field (unpublished data from Prof. Bretaudeau, personal communication), which allows for two generations per

year at best. When looking at the duration of each generation cycle over a two-year period in artificial conditions, some fluctuations could be observed, in accordance with the seasons (data not shown).

Preliminary experiments were carried out to determine the duration of generation cycle, in the greenhouse conditions. For example, the duration of seed to seed cycle for MB2 was 160 ± 8 days, similar to that observed in the field in Mali (unpublished data, personal communication from Prof. Bretaudeau). Moreover, we also compared the generation cycle between unpeeled control seeds and the peeled seeds, which were found to be 125 ± 5 days and 110 ± 6 days, respectively. This indicates that simply removing the seed coat/integuments accelerates the germination and reduces the duration of the cycle. In addition, by combining the *in vitro* and *in vivo* cycles, there was a drastic decrease in the duration of the cycle. A schematic representation of the *in vitro* and *in vivo* cycles is shown in Figure 2. For example, for landrace MB2, the duration of the cycle was 78 ± 4 days. This indicates clearly that the duration of seed to seed cycle in Bambara groundnut can be highly reduced, and thus can be manipulated for application in breeding programmes, e.g. when using a SSD selection system. Plants obtained through *in vitro* plus *in vivo* systems were morphologically normal and fertile, as was their progeny. Therefore, the system developed for pea [9] is also applicable to this important seed crop.



Today where more plant proteins are needed, not only for human consumption but also as a feed for cattle and poultry, an improvement in the quality of seeds is necessary. The application of Single-Seed-Descent (SSD) breeding programmes is most interesting in this context, and methods to accelerate generations in Bambara groundnut should help plant breeding programmes to meet this goal.

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SUPPRESSION OF THE NEUROTOXIC AMINO ACID IN SEED STORAGE PROTEIN OF *Lathyrus sativus* L. VIA MUTATION TECHNIQUES AND GENE TRANSFER

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Abstract

Plant regeneration was achieved in grasspea (*Lathyrus sativus* L.) by *in vitro* shoot proliferation in cotyledonary nodes from axenically grown seedlings, *de novo* shoot organogenesis in callus cultures or adventitious shoot formation directly from explants. Factors influencing *Agrobacterium*-mediated genetic transformation were optimized using a binary vector with T-DNA cassette carrying the selectable marker *npt*II and the reporter gene *gus*-intron. The detection of GUS activity in glasshouse-grown primary transformants substantiated a stable integration and expression of the *gus*-intron gene. Mutagenesis was induced using γ-irradiation as well as two chemical mutagens viz. ethyl methane sulphonate (EMS) and N-methyl-N-nitro-nitrosoguanidine (NG). The implications of these investigations are discussed in the context of producing somaclonal variants, genetic transformants or mutants with a reduced level of the neurotoxin ODAP.

1. INTRODUCTION

Lathyrus sativus L. (grasspea) is grown in many parts of India, Bangladesh, Nepal, China, Iran, Southern Europe, Africa and South America [1], where it has a proven potential owing to its inherent tolerance to abiotic stress and disease resistance combined with high protein content.

In spite of these merits of this crop, a serious pathological condition, neurolathyrism, results from excessive and prolonged consumption of the seeds or young shoots of the plant. This is due to the presence of a neurotoxic amino acid β-N–oxalyl–L-α,β-diaminopropionic acid (ODAP/BOAA) in seed storage protein [2]. The neurotoxin ODAP is known to interfere with the metabolism of glutamic acid, hence with the transmission of nerve impulses to the brain. Thus, in extreme cases, this condition causes paralysis of the lower limbs in human, even leading sometimes to death. The neurotoxin ODAP is present in all plant parts. However, the maximum concentration has been reported in leaves during the vegetative phase and in the embryo during the reproductive phase [3]. As a result, despite its rich protein content, *Lathyrus sativus* is not recommended for human consumption and the crop remains as an under–utilized genetic resource.

The current project was undertaken aiming at rendering *Lathyrus sativus* suitable for human consumption, by substantially reducing ODAP content in this crop via mutation techniques and gene transfer.

The main concern of the project was to explore the possibility of transferring the oxalate decarboxylase gene, known to degrade oxalic acid, which is an essential substrate for the synthesis of the neurotoxin ODAP. A necessary prelude to this activity was to examine the suitability of *Lathyrus sativus* as a potential recipient of various transgenes in the form of a dominant selectable marker *neomycin phosphotransferase* II (*npt*II) and reporter gene *gus*-intron, through *Agrobacterium*-mediated transformation. Uppermost of this pre-requisite was to develop regenerable systems using different explants of *Lathyrus sativus*.

The second objective of this project was to induce mutants through irradiation and chemical mutagens and a further selection of improved cell lines.

2. MATERIALS AND METHODS

2.1. Tissue culture

In the present study five genotypes of *Lathyrus sativus* L. were used. Seeds of four improved genotypes namely IC-120451, IC-120453, IC-120478, IC-120487 were obtained from the National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India and a local genotype (Nayagarh local) was collected from the Pulses Research Station, Nayagarh, Orissa, India.

The seeds were surface-sterilized with 0.1% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) for 5 min, and were inoculated in MS [4], B_5 [5] or BM [6] basal media gelled with 0.8% (w/v) agar, and with pH adjusted to 5.8. The seeds were allowed to germinate at 25 ± 1°C with 35 μmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes. Seven days-old axenic seedlings served as the source of explants. After removal of the radicle and the primary shoot, the cotyledonary node of all the genotypes were inoculated on MS medium supplemented with 2.0 mgl^{-1} BAP. Cotyledonary nodes of the different forms (explant without cotyledons with intact embryonic axis, explant with a single whole cotyledon with embryonic axis, explant with two proximal halves of cotyledon and explant with two whole cotyledons) were inoculated on MS medium supplemented with 0.5–5.0 mgl^{-1} BAP or 0.5–5.0 mgl^{-1} kinetin (Kn) or 0.01–2.0 mgl^{-1} thidiazuron (TDZ) (pH 5.8). The original cotyledonary node explants were repeatedly sub-cultured on shoot multiplication medium [MS + 2.0 mgl^{-1} BAP] after each harvest of shoots. Shoots obtained from each harvest were cut into single node pieces (0.8–1.2 cm) and cultured on MS medium containing 2.0 mgl^{-1} BAP. At the same time different explants (cotyledon, hypocotyl, epicotyl, internode and leaf) were used for callus mediated plant regeneration and for direct adventitious plant regeneration. For these, different growth regulators alone or in combination were tested.

2.2. *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens strain LBA 4404 [7] harbouring the disarmed virulence plasmid pAL4404 [8] and a pBIN19 [9]- derived pMOG18 [10] based binary vector was used for transformation. The T-DNA cassette contained the selectable marker *npt*II and the reporter gene *gus*-intron.

Epicotyls (without meristematic zone of the cotyledonary node) from 7–10–day-old *in vitro* seedlings were excised and wounded by a sterile scalpel blade. For *Agrobacterium* inoculation, explants were floated (10 min) with their wounded surfaces in contact with an overnight bacterial culture (OD_{600} = 0.6) diluted with liquid MS medium supplemented with 2.0 mgl^{-1} NAA and 4.0 mgl^{-1} BAP. The inoculated and control explants were then transferred to semi-solid MS medium supplemented with 2.0 mgl^{-1} NAA and 4.0 mgl^{-1} BAP and incubated for 48 h After this co-cultivation period, the inoculated explants were transferred to the medium as above with 100 μgml^{-1} kananycin sulphate. The shoots regenerated in kanamycin sulphate supplemented medium were considered as putatively transgenic and thought to have arisen from the transformed cells in which *npt*II gene of the T-DNA cassette had been incorporated and expressed thus detoxifying the kanamycin effect. GUS histochemical analysis of randomly selected inoculated explants was carried out 8 days after

inoculation as described [11]. Shoots regenerated from inoculated and control explants were subsequently transferred to half-strength MS medium for rooting. The rooted shoots were then transferred to the glasshouse.

Seeds collected from putative transgenic plants, which showed GUS activity in primary transformants, were germinated on MS medium supplemented with 100 μgml^{-1} kanamycin sulphate and kanamycin resistance was studied 1 month after germination.

2.3. Induced Mutagenesis

Three mutagens were used, out of which one was a physical mutagen i.e.γ-rays (GR) and two others were chemical mutagens i.e. ethyl methane sulphonate (EMS) and N-methyl-N-nitro-nitrosoguanidine (NG). The source of γ-rays was a 1,930 curie ^{60}Co gamma cell installed in the Department of Chemistry, Utkal University. EMS and NG were obtained from Sigma Chemicals (USA).

2.3.1. Treatment methodology

2.3.1.1. GR treatment

Healthy and uniform seeds of *Lathyrus sativus* L. cv. 'Nayagarh Local' were irradiated with GR (gamma radiation) at 10, 20, 40, 60, 80, 100 and 120 KR from a ^{60}Co source available in the Department of Chemistry, Utkal University, Bhubaneswar, India. For each treatment, approximately 100 seeds were taken.

For physical mutagenesis, well-filled and matured seeds were irradiated with 10 different doses of γ-rays (100, 200, 300, 400, 500, 600, 700, 800, 1,000 and 1,200 Gy).

2.3.1.2. Chemical mutagen treatment

Seeds were treated with EMS at different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1.0%). For each treatment, twenty seeds were used. Seeds were soaked in double distilled water for 10 h prior to chemical treatment for easy penetration of mutagens into the seeds. After pre-soaking, water was drained off completely and excess moisture was blotted out from seeds in order to prevent any dilution of mutagens. The volume of the mutagenic solution was large enough to completely submerge the seeds. The control seeds were kept in double distilled water. The containers were kept throughout the entire treatment period of three hours on a mechanical shaker at 28 ± 1°C, to swirl the contents for maintenance of uniform concentration and for uniform penetration of mutagens into seeds. Thereafter, seeds were washed in running tap water for 1.5 h to remove excess mutagen, which would otherwise damage the germinating embryos.

For chemical mutagenesis, seeds were pre-soaked in double distilled water for 12, 18 and 24 h followed by chemical treatment with 10 concentrations of EMS (in %, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0) and NG (in %, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, 0.4 and 0.5) for 2, 3 and 4 h at room temperature (25°C –30°C) with intermittent shaking.

For each treatment 300 seeds were used. After the treatment with chemical mutagens the seeds were thoroughly washed in running tap water to remove traces of mutagens adsorbed to the seed coat, and the seeds were then dried with autoclaved tissue papers. Thus, in total, 190 mutagenic treatments for each cultivar were effected (10 treatments of γ-rays, 90 treatments of EMS and 90 treatments of NG).

2.3.2. Laboratory experiments

Twenty seeds from each treatment and of the untreated (control) seeds were germinated in Petri dishes on moist filter paper, with experiments repeated 10 times. Seven days later, the competence of seeds for measuring the percentage of germination and the length of roots and shoots assessed germination produced. Any abnormal feature, such as root tip bulging or bent root tips was scored by visual observation.

2.3.3. Field experiments

Untreated and treated seeds from each treatment (100 from each) were sown in the field with a spacing of 30 cm row-to-row and 10 cm plant to plant. The percentage of plant survival, the number of pods per plant, the number of seeds per pod and the number of seeds per plant were taken at maturity. Plants that exhibited chimera and seedling abnormalities with respect to leaflet number, shape of leaflets, leaflet injuries, etc. including albinos were scored.

For pollen sterility studies, three mature flowers (before anthesis) were collected from the first inflorescence of each of ten random plants in each treatment. Fully mature anthers were squashed in 15% acetocarmine. The deeply stained pollen grains of standard size were considered as fertile. The smaller sized, poorly stained or non-stainable pollen grains were considered as sterile.

2.3.4. M_1 Generation

2.3.4.1. Laboratory experiment

One hundred and fifty seeds from each treatment along with the control were sown on the filter/tissue paper in Petri dishes and replicated three times for germination in the laboratory condition. Observations were recorded on germination, root length and seedling height.

2.3.4.2. Field experiment

The remaining 150 seeds from each treatment along with control were sown in the field during March 2001, with a spacing of 30 x 10 cm to raise the M_1 generation. The biological effects of different treatments were evaluated with respect to variability (% of germination), lethality (% of survival), sterility (% of reduction in seed set) and injury (% of seedling height reduction). Observations were taken on 30 randomly chosen competitive plants from each treatment for six quantitative traits (plant height, number of branches/plant, number of pods/plant, number of seeds/pod, 100 seed weight and seed yield/plant). Selfed seeds from M_1 generation were harvested to raise the M_2 Generation.

3. RESULTS

3.1. Tissue Culture

3.1.1. Cotyledonary node-based plant regeneration

Of the five genotypes studied, IC-120487 had the highest percentage shoot development (93.3%), maximum shoot numbers (averaging 11.3 shoots/explants) and longest average shoot length (4.9 cm) on MS medium + 2.0 mgl^{-1} BA after 15 days of inoculation of the explants (Table I).

TABLE I. REGENERATION RESPONSE OF COTYLEDONARY NODE EXPLANTS (WITH TWO WHOLE COTYLEDONS) OBTAINED FROM DIFFERENT GENOTYPES OF *Lathyrus sativus* CULTURED ON MS + 2.0 MGL^{-1} BA

Genotype	% Cultures regenerating shoots	Shoots/explant	Shoot length (cm)
IC-120451	70.0	6.3	3.1
IC-120453	80.0	7.4	3.6
IC-120478	86.6	8.7	4.1
IC-120487	93.3	11.3	4.9
Nayagarh Local	88.3	10.6	3.8

The cotyledonary node explants failed to regenerate shoots on MS basal medium. Incorporation of a cytokinin to the medium was essential to induce axillary shoot proliferation. Of the three different cytokinins tested (BAP, Kn and TDZ), BAP at 2.0 mgl^{-1} was the most effective (Table II). Shoot development was increased with increase in the concentration of cytokinin (BAP/Kin/TDZ) up to a certain threshold beyond which the frequency of shoot development was reduced markedly. On TDZ containing media, the formation of a small amount of callus was observed at the base of the explants and so was also the production of stunted shoots (Table II).

The effect of the number and size of cotyledons associated with the explants on shoot regeneration responses was studied by culturing the cotyledonary node explants along with none, a single or both cotyledons, either intact or as the two proximal halves. It was observed that the different forms of cotyledonary node explants affected multiple shoot formation to a great extent in the presence of a cytokinin. Highest shoot numbers were recorded when explants with two whole cotyledons were used (Table III).

TABLE II. EFFECT OF CYTOKININS ON SHOOT DEVELOPMENT FROM COTYLEDONARY NODE EXPLANTS WITH TWO WHOLE COTYLEDONS OF IC-120487

Cytokinin (mgl^{-1})		% Cultures regenerating shoots	Shoots / explant	Shoot length (cm)
Control		-	-	-
BA	0.5	55.0	7.9	3.1
	1.0	80.6	8.7	4.2
	2.0	93.3	11.3	4.9
	3.0	83.3	10.4	4.4
	4.0	71.6	9.3	4.1
	5.0	51.6	7.8	3.0
Kn	0.5	45.0	5.9	2.3
	1.0	51.6	7.5	2.9
	2.0	58.3	8.3	3.6
	3.0	63.3	8.7	4.1
	4.0	50.0	6.7	3.2
	5.0	48.3	6.1	2.7
TDZ	0.01	-	-	-
	0.05	48.6	8.8	1.2
	0.1	51.1	9.2	0.9
	0.5	-	-	-
	1.0	Callus	Callus	Callus
	1.0	Callus	Callus	Callus

TABLE III. EVALUATION OF DIFFERENT FORMS OF COTYLEDONARY NODE EXPLANTS OF IC-120487AFTER 15 DAYS OF INOCULATION ON MS + 2.0 MGL^{-1} BA

Explant type	% Cultures regenerating shoots	Shoots / explant	Shoot length (cm)
Explant without cotyledons with intact embryonic axis	48.3	3.1	2.9
Explant with single whole cotyledon with embryonic axis	68.3	4.2	3.2
Explant with two proximal halves of cotyledons	81.6	8.3	3.6
Explant with two whole cotyledons	93.3	11.3	4.9

Shoot cultures were established by repeatedly sub-culturing the original cotyledonary nodes on fresh multiplication medium [MS + 2.0 mgl^{-1} BAP] after each harvest of newly formed shoots. Each explant was sub-cultured for 3 successive passages and produced an average of 8–9 shoots per cycle. In 30 days, about 30–35 shoots were obtained from a single cotyledonary node. Nodal segments derived from *in vitro* primary shoots of all the five grass pea genotypes cultured on MS containing 2.0 mgl^{-1} BAP failed to regenerate shoots even after 30 days of inoculation.

3.1.2. Callus-mediated plant regeneration

The leaf explants of five genotypes of *Lathyrus sativus* were cultured on MS and B_5 basal media supplemented with different growth regulators to evaluate the efficiency of the two basal media for callus induction and proliferation. Basal media were supplemented with either NAA (2.0 mgl^{-1}) + BAP (0.5 mgl^{-1}) or 2,4–D (2.0 mgl^{-1}) + BAP (0.5 mgl^{-1}). Calli were transferred to fresh medium of similar composition every 2 weeks. Fresh weight of calli was taken at 15 days interval (Table IV).

Taking the fresh weight gain of callus after 45 days of inoculation compared the callusing ability of different explants. When 2,4–D was used the explants produced mostly white to greenish white, soft calli with root like structures. In this study it was found that shoots did not regenerate from such tissues. On the contrary, in cultures containing NAA calli were compact and green from which shoots regenerated. The results showed that, in the presence of NAA addition of any concentration of BAP did not help shoot regeneration. Conversely, replacing NAA by BAP induced shoot regeneration. On media with BAP lower than that used in the callusing medium (i.e. 0.3 mgl^{-1}), shoot buds did not regenerate. When BAP was increased to 1.0 mgl^{-1} shoots started to develop, but beyond 1.0 mgl^{-1} BAP had an adverse effect on shoot regeneration.

TABLE IV. EVALUATION OF MS AND B_5 MEDIA FOR CALLUS INITIATION*

Genotypes	MS+0.5 mg/l BAP + 2.0 mg/lNAA Fresh weight of callus (g)			B_5+0.5 mg/l BAP + 2.0 mg/lNAA Fresh weight of callus (g)		
	15 days	30 days	45 days	15 days	30 days	45 days
Nayagarh Local	0.153	0.285	0.366	0.103	0.176	0.311
IC-120451	0.135	0.241	0.342	0.093	0.158	0.217
IC-120453	0.116	0.218	0.283	0.089	0.123	0.195
IC-120478	0.085	0.115	0.173	0.027	0.095	0.116
IC-120487	0.169	0.314	0.389	0.117	0.198	0.336
Genotypes	MS+0.5 mg/l BAP+ 2.0 mg/l 2,4–D Fresh weight of callus (g)			B_5+0.5 mg/l BAP+ 2.0 mg/l 2,4–D Fresh weight of callus (g)		
	15 days	30 days	45 days	15 days	30 days	45 days
Nayagarh Local	0.118	0.241	0.315	0.104	0.193	0.249
IC-120451	0.102	0.325	0.288	0.091	0.150	0.203
IC-120453	0.092	0.121	0.206	0.078	0.136	0.181
IC-120478	0.068	0.099	0.145	0.019	0.079	0.109
IC-120487	0.144	0.269	0.344	0.128	0.218	0.277

*Mean fresh weight of callus at 15 days interval

TABLE V. GROWTH OF CALLUS FROM DIFFERENT EXPLANTS OF THE FIVE CULTIVARS TESTED**

	MS + NAA (3.0 mgl^{-1}) + BAP (0.3 mgl^{-1})			
Genotype	Epicotyl	Hypocotyl	Leaf	Internode
Nayagarh Local	0.197±0.002	0.104±0.003	0.834±0.002	0.447±0.004
IC-120451	0.194±0.002	0.101±0.004	0.793±0.002	0.403±0.003
IC-120453	0.164±0.003	0.094±0.005	0.646±0.004	0.343±0.004
IC-120478	0.097±0.003	0.083±0.006	0.224±0.004	0.124±0.003
IC-120487	0.204±0.003	0.109±0.004	0.999±0.001	0.553±0.002
	MS + 2,4–D (3.0 mgl^{-1}) + BAP (0.3 mgl^{-1})			
Genotype	Epicotyl	Hypocotyl	Leaf	Internode
Nayagarh Local	0.173±0.015	0.189±0.004	0.931±0.003	0.432±0.015
IC-120451	0.157±0.003	0.179±0.003	0.821±0.004	0.399±0.006
IC-120453	0.129±0.004	0.137±0.004	0.491±0.005	0.323±0.005
IC-120478	0.089±0.003	0.101±0.004	0.317±0.004	0.100±0.004
IC-120487	0.187±0.005	0.203±0.003	0.951±0.004	0.535±0.006

** Data on callus fresh weight (x ± S.D.) pooled from four experiments, each with 3 replicates.

This led to the belief that complete elimination of NAA from the medium coupled with maintenance of an optimal concentration of BAP (i.e. 1.0 mgl^{-1}) was supposedly a requirement for shoot regeneration. In this medium, calli were maintained for 7–10 days during which 4–6 shoots developed from each green compact callus.

3.1.3. Adventitious shoot organogenesis

Explants such as cotyledons, hypocotyls, epicotyls, leaves and internodes from seven-day-old *in vitro* seedlings of all five different grasspea genotypes were cultured on MS and B_5 medium supplemented with different growth regulators e.g. NAA, 2,4–D, BAP, TDZ, either alone or in combinations. Of these, the epicotyl explants responded best in terms of direct shoot regeneration, on MS + 4.0 mgl^{-1} BAP + 2.0 mgl^{-1} NAA (Table VI).

Among the genotypes tested, IC-120487 showed the highest shoot regeneration frequency (80%) coupled with the highest average shoot number (8.2) and the production of the longest shoots (4.1 cm) (Table VII).

TABLE VI. EVALUATION OF DIFFERENT EXPLANTS OF IC-120487 AFTER 15 DAYS OF INOCULATION ON MS + 4.0 MGL^{-1} BAP + 2.0 MGL^{-1} NAA

Explant type	Number of days required for shoot bud initiation	% Cultures regenerating shoots	Shoots / explant	Shoot length (cm)
Cotyledon	No response	-	-	-
Hypocotyl	19	73.3	4.4	3.7
Epicotyl	20	80.0	8.2	4.1
Internode	22	70.0	3.8	2.8
Leaf	26	48.3	2.1	2.0

TABLE VII. REGENERATION RESPONSE OF EPICOTYL EXPLANT OBTAINED FROM DIFFERENT GENOTYPES OF *Lathyrus sativus* CULTURED ON MS + 4.0 MGL^{-1} BAP + 2.0 MGL^{-1} NAA

Genotype	% Cultures regenerating shoots	Shoots/explant	Shoot length (cm)
IC-120451	35.0	2.1	2.2
IC-120453	43.3	3.2	2.8
IC-120478	71.6	4.9	3.5
IC-120487	80.0	8.2	4.1
Nayagarh Local	75.0	5.2	3.8

By sub-culturing every 15 days in the same medium, about 8.2 shoots were regenerated from each epicotyl explants. On media fortified with TDZ at lower concentrations shoot buds were formed but they were fewer than on media with BAP + NAA. At higher concentrations, TDZ gave rise to abundant hyper-hydrated callus formation (macroscopic dead cells full of water) in all explants studied (Table VIII).

3.1.4. Rooting of shoots

Rooting of *in vitro* regenerated shoots was achieved on half-strength MS supplemented with either IAA or IBA or IPA. No root formation was observed when shoots were cultured in a medium devoid of auxins. IAA at 0.5 mgl^{-1} (2.85 μM) induced the highest frequency of

rhizogenesis (81.8%), with the maximum root number (4.8 roots / shoot) and the longest average root length (4.9 cm). Auxin concentrations exceeding 1.0 mgl^{-1} showed a reduction in rooting response. Of the three auxins tested, IAA was the best, as compared to IBA or IPA (Table IX). Nevertheless, roots were able to elongate only after the rooted shoots were transferred to half-strength MS lacking auxin.

TABLE VIII. EFFECT OF DIFFERENT CYTOKININS ON SHOOT DEVELOPMENT FROM EPICOTYL EXPLANT OF IC-120487

Cytokinins (mgl^{-1})	% Cultures regenerating shoots	Shoots / explants	Shoot length (cm)
Control	-	-	-
BA			
0.5	-	-	-
1.0	13.3	1.0	1.7
2.0	21.6	1.2	2.1
3.0	35.0	1.8	2.9
4.0	53.3	3.2	3.0
5.0	46.6	2.7	2.8
BA + NAA			
4.0 + 0.5	65.0	3.7	3.1
4.0 + 1.0	73.3	4.2	3.7
4.0 + 2.0	80.0	8.2	4.1
4.0 + 3.0	51.6	3.8	3.2
TDZ			
0.25	18.3	1.2	0.9
0.5	25.0	1.8	1.1
1.0	Callus	Callus	Callus
2.0	Callus	Callus	Callus

TABLE IX. EFFECT OF DIFFERENT AUXINS ON ROOTING OF *IN VITRO* SHOOTS DERIVED FROM DIFFERENT EXPLANTS OF IC-120487

Auxins (mgl^{-1})		% Rooting	Roots / shoot	Root length (cm)
½ MS (Zero auxin)		9.0	1.0	1.5
IAA	0.25	57.5	1.7	2.4
	0.5	81.8	4.8	4.9
	1.0	69.6	2.8	3.3
	2.0	42.3	2.0	2.1
IBA	0.25	-	-	-
	0.5	24.1	2.4	2.4
	1.0	15.1	1.8	1.4
	2.0	9.0	1.3	1.1
IPA	0.25	-	-	-
	0.5	-	-	-
	1.0	21.1	1.3	1.3
	2.0	9.0	1.0	0.9

3.1.5. Acclimatization of plants

The plantlets regenerated *in vitro* were successfully acclimatized in vermi-compost and eventually in soil. Plantlet survival was 66% following transfer to vermi-compost and 78% of these plants survived after being transferred to soil.

3.2. *Agrobacterium*-mediated transformation

A gradual reduction in the survival of epicotyl explants was associated with an increasing concentration of the phytotoxic antibiotic kanamycin. However, as compared to many plant species whose tissues are naturally sensitive to kanamycin at 25–30 μgml^{-1}, grass pea epicotyls exhibited an inherent tolerance to a much higher antibiotic concentration. All most all explants bleached at 75 μgml^{-1} with a total loss of chlorophyll pigmentation coupled with arrest in shoot proliferation capacity while they were necrotic at the lethal dose of 100 μgml^{-1}. Of the two different types of *Agrobacterium* strains (LBA 4404 & EHA 105) employed, LBA 4404 was found to be more infectious. The transformation efficiency in the case of LBA 4404 type was about 40% while with EHA 105 it was 34%. At a late log phase with OD_{600} = 0.6 (corresponding to 10^9 cells/ml) maximum transformation was achieved as measured by the GUS assay (Fig. 1). An increase or decrease in the OD value resulted in a decrease in transformation efficiency. Values greater than 0.8 were not suitable for transformation and extensive tissue damage occurred at OD values greater than 1.0 due to bacterial over growth. Infection period of about 10 min was suitable and explants co-cultivation with either of the strains (EHA 105 & LBA 4404) for 4 days resulted in the highest transformation frequency (Fig. 2). In the present study, co-cultivation medium with a pH of 5.6 yielded the highest number of transformation events (Fig. 3).

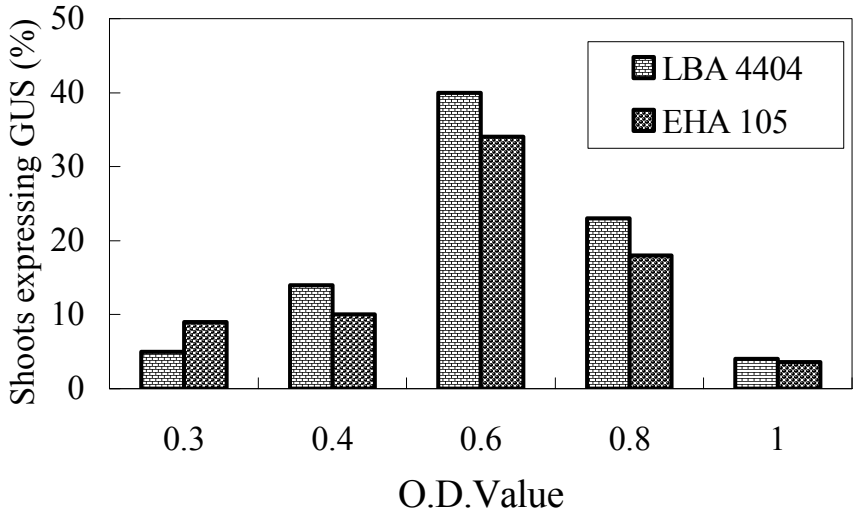

Fig.1. Relationship between stages of growth of Agrobacterium culture and transformation efficiency.

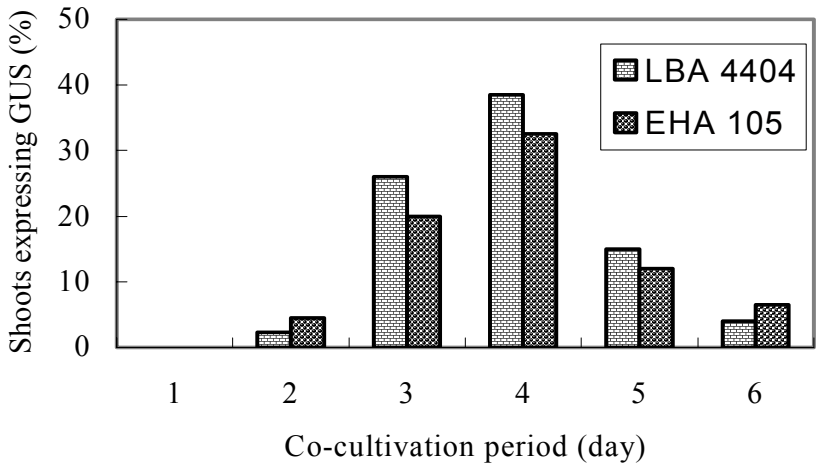

Fig.2. Effect of duration of co-cultivation of epicotyl with Agrobacterium on transformation efficiency.

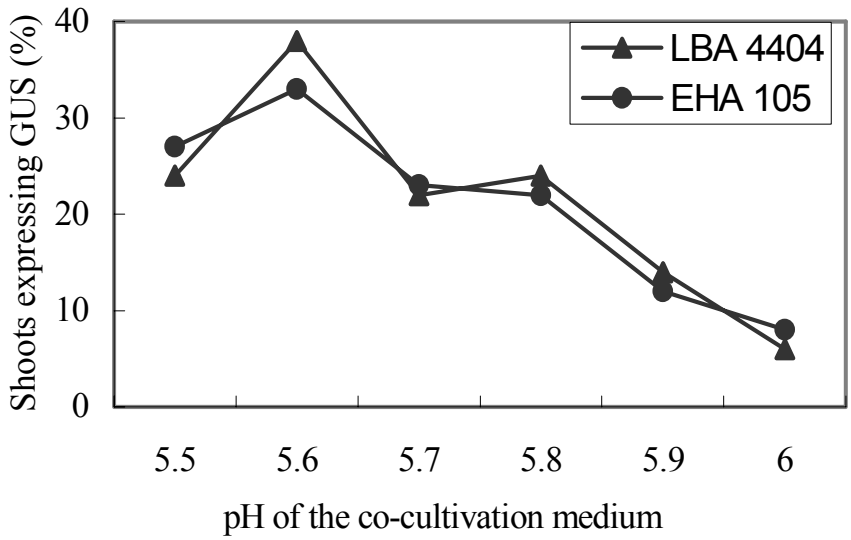

Fig.3. Effect of pH of co-cultivation medium on the transformation.

GUS histochemical analysis of leaf discs collected from glasshouse-grown putative transgenic plants showed the expression of *gus*-intron gene in 5 primary transformants out of 29 putative transgenic plants. The level of GUS activity, as verified by both GUS histochemical and GUS fluorometric analyses [12] showed wide variation amongst different plants.

The seeds collected from the primary transformants were germinated on MS-based medium supplemented with 100 $\mu g ml^{-1}$ kanamycin sulphate, with results as summarized in Table X.

TABLE X. SUMMARY OF RESULTS OF TRANSFORMATION EXPERIMENTS

Sl. No	Code of Primary Transformants (R0)	GUS Histochemical Analysis* A	B	C	GUS Fluorometric Analysis**	Kanamycin resistance of R1 seedlings*** Resistant	Sensitive
1	1 (C) II	-	-	-	35	0	20
2	1 (C) V	-	-	-	41	0	10
3	1 (G) III	-	-	-	47	0	20
4	1 (H) I	-	-	-	39	0	20
5	1 (H) II	-	-	-	50	0	20
6	2 (B) III	-	-	-	38	0	20
7	2 (C) I	++	++	++	4,312	17	3
8	2 (C) IV	+	+	+	2,213	15	5
9	2(C) V	++	+	++	3,075	18	2
10	3 (A) I	-	-	-	40	0	10
11	3 (B) II	-	-	-	45	0	10
12	3 (B) III	-	-	-	43	0	20
13	3 (D) II	-	-	-	45	0	20
14	3 (E) I	-	-	-	47	0	20
15	3 (E) II	-	-	-	38	0	20
16	3 (E) III	-	-	-	39	0	20
17	7 (B) II	-	-	-	49	0	10
18	8 (E) II				43	12	8
19	8 (G) I	-	-	-	45	10	10
20	9 (A) IV	-	-	-	43	0	20
21	12 (B) II	-	-	-	29	0	20
22	14 (C) I	-	-	-	54	0	10
23	15 (G) II	-	-	-	43	0	10
24	15 (G) III	-	-	-	45	0	20
25	15 (H) IV	-	-	-	45	0	20
26	16 (A) II	-	-	-	50	0	10
27	18 (E) III	-	-	-	47	0	10
28	18 (E) IV	-	-	-	42	0	10
29	18 (E) V	-	-	-	34	0	10
30	Control 22 (A) II	-	-	-	45	0	20

*Three leaf discs (A,B and C) of primary transformants were observed after GUS histochemical staining. GUS activity is expressed as ++ = high, + = weak, - = absent.
**GUS fluorometric analysis of leaf extracts expressed as pmol MU mg protein^{-1} min^{-1}
***Sensitivity of seedlings to 100 μgml^{-1} kanamycin sulphate, 1 month after germination.

In this experiment, 8 explants (numbered from A to H) were taken from each of the twenty Petri dishes (Petri dishes were numbered from 1 to 20). From 36 explants, which survived on medium supplemented with kanamycin sulphate, 145 putative transgenic shoots were regenerated. However, on transfer to rooting medium only 52 shoots developed sufficient roots. After the transfer of rooted shoots to the glasshouse only 29 plants survived and reached maturity.

3.3. Induced mutagenesis

In this experiment it was observed that with an increased dose of either GR or EMS, the percentage germination decreased, while the growth of shoots and roots was also inhibited. The results of analysis of GR and EMS treatments are given in Tables XI and XII.

TABLE XI. EFFECT OF DIFFERENT DOSES OF GR ON SEED GERMINATION OF *L. sativus*

Dose of GR KR	Percentage Germination	Average Length of Shoots in cm	Average Length of Roots in cm
10	90±0.78	10.82±0.35	6.63±0.25
20	88±1.17	6.71±0.46	4.72±0.27
40	85±1.23	1.54±1.21	2.40±0.04
60	80±1.49	1.03±0.34	1.89±0.03
80	76±0.82	0.78±0.04	0.94±0.02
100	56±0.52	0.54±0.03	0.85±0.03
120	45±0.63	0.42±0.03	0.83±0.03
Control	95±0.67	12.71±0.26	7.9 ±0.29

Values given as ± indicate standard deviation

TABLE XII. EFFECT OF DIFFERENT DOSES OF EMS ON SEED GERMINATION OF *L. sativus*

Dose of EMS Percent (v/v)	Percentage Germination	Average Length of Shoots in cm	Average Length of Roots in cm
0.1	87±1.25	9.82±0.67	4.89±0.67
0.2	72±2.2	7.51±0.35	3.27±0.34
0.4	67±2.69	3.20±0.4	1.91±0.04
0.6	48±5.25	2.21±0.65	1.71±0.02
0.8	35±7.56	1.82±0.67	1.5 ±0.02
1.0	11±4.35	0.81±0.83	0.62±0.03
Control	95±0.67	12.71±0.26	7.9 ±0.29

Values given as ± indicate standard deviation

With the M_1 generation, at higher doses of γ-rays (700–1200 Gy), EMS (0.8%–1.0%) and NG (0.3%–0.5%) treated plants failed to survive. Pre-soaking with double distilled water for 12 h followed by chemical mutagen treatment for 3 h was found to be most effective on the basis of LD_{50} value. For physical mutagenesis seeds harvested from M_1 generation of γ-rays treatments (100–600 Gy) will be used in of future generations. Similarly, for chemical mutagenesis, seeds obtained from pre-soaking with double distilled water for 12 h followed by treatment with 0.1%–0.7% EMS for 3 h and 0.05%–0.2% NG for 3 h will be utilized in advancement of future generations. Hence seeds harvested from the 20 treatments mentioned above resulted in a reduction of M_1 parameters as compared to the control.

4. CONCLUSION

The present work confirms that it is possible to regenerate large numbers of adventitious shoots from leaf explants of *Lathyrus sativus*. The feasibility of gene transfer through *Agrobacterium*-mediated transformation was also confirmed in this study. Active integration and expression of T-DNA in *Agrobacterium*-inoculated explants allowed them to grow on a kanamycin sulphate-supplemented medium and to exhibit GUS expression. The presence of GUS activity in primary transformants transferred to the glasshouse suggested stable expression of the *gus*-intron gene. Kanamycin resistance of seedlings grown from seeds of putative transgenic plants indicate the expression of the *npt*II gene in the seed derived generation. This result suggests that both transgenes have been integrated in some transgenic lines and the expression of *npt*II gene remains stable in the seed-derived generation. The stable expression of these two transgenes in subsequent generations suggests that the crop is amenable to genetic modification through *Agrobacterium*-mediated transformation. Standardization of parameters for stable expression of these transgenes for several generations is thought to be a prerequisite for transfer of a gene of agronomic significance such as oxalate decarboxylase coding sequence. In future, the transfer of oxalate decarboxylase gene to *Lathyrus sativus* through *Agrobacterium*-mediated transformation will be an important method in significantly reducing ODAP content from the crop to make it suitable for human consumption. Moreover, more attention is required to develop and screen the putative mutants with reduced ODAP content.

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DEVELOPMENT AND EXPLOITATION OF BIOTECHNOLOGICAL APPROACHES FOR BREEDING OF GRASS PEA (*Lathyrus sativus* L.)

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Abstract

The grass pea (*Lathyrus sativus*) is a wild relative of pea, which may be a useful genetic resource for the acquisition of interesting stress resistance traits. However, as grass pea is cross incompatible with pea and its seeds contain a toxic amino acid, hybrids will have to be generated following protoplast fusion, and low-toxin containing plants produced by genetic transformation. It is therefore essential that regenerated plants are fertile, true-to-type and not chimaeric in nature, when they have been obtained in absence of any selection treatment. Various biotechnological approaches have been developed and are being exploited for the breeding of novel grass pea genotypes. The auxin/cytokinin balance of regeneration media affected the rooting ability of regenerants. Conventional and somatic hybridisations with pea were attempted. Flow cytometry permitted to characterise *in vitro* regenerants in terms of nuclear DNA content. Plant regeneration competence was genotype-dependent and strongly correlated with a normal DNA content. Isoenzymes, molecular biology and FISH techniques were also developed and used for the characterization of all regenerants produced.

1. INTRODUCTION

The grass pea (*Lathyrus sativus* L.; 2n=2x=14) is a seed legume capable of growth in semiarid and arid zones in tropical and subtropical areas, due to is remarkable resistance to extreme environmental conditions, including flooding and drought, salinity and low soil fertility. It also possesses a relatively high protein content and a significant resistance to anthracnose (*Mycosphaerella pinodes*). The summation of these traits leads to very low production costs and makes of grass pea the cheapest source of dietary protein available for subsistence farmers in LIFDCs such as those of the Indian subcontinent and Africa but also for dry lands everywhere else.

Despite this, there are some limits to grass pea consumption deriving from the sometimes relatively high (up to 0.76%) seed content of β,-N-oxalyl-α,β diaminopropionic acid (ODAP), the toxin responsible for neurolathyrism, a disease that provokes paralysis of the lower limbs in humans and of all limbs in animals, following a prolonged period of high levels of ODAP consumption in the diet. It would therefore be interesting to develop cultivars with decreased levels of this toxin [1], to ensure a wider and safer use of this environmentally adaptable, yet largely under-utilized crop [2], particularly since recent studies showed that grass pea compares favourably with lupine or pea as feed for sheep and pigs [3].

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Grass peas would also be an interesting genetic resource for resistance breeding of peas (*Pisum sativum* L) [2, 4, 5, 6]. In this context, biotechnology, by *in vitro* selection [7] and gene transfer, would permit to produce novel grass pea genotypes that, while retaining their rusticity, are better adapted for human consumption and, also, to introduce interesting traits from *Lathyrus* into the cross-incompatible common pea, by somatic hybridisation [8].

Few groups have developed *in vitro* approaches with *Lathyrus* species and, as with other legumes, the main bottleneck has been the regeneration of whole plants from cultured tissues. Pecket and Selim [9] have cultured *L. sativus* embryos and Delgado-Montero and Moreno [10] various tissue sources, but both groups obtained callus only. Other authors, using juvenile tissues comprising meristems at the time of explanting, reported on the subsequent regeneration of buds and shoots from callus [7, 11, 12, 13, 14, 15]. Rooted plants were difficult to produce and, often, shoots only grew on the callus surfaces, probably developing from those pre-existing meristems. Only twice was *de novo* regeneration obtained from callus [11, 16], while organogenesis was reported from leaf discs [17] and entire seeds [18]. More recently, fertile plants were prolifically regenerated from meristematic tissues [19] and from hypocotyls segments [20].

With respect to protoplast technology, Razdan et al. [21] produced leaf mesophyll protoplast-derived callus of *L. odoratus*, McCutchan et al. [4] established suspension cultures coupled with the isolation of non-dividing protoplasts for *L. sativus*, while Durieu and Ochatt [8] reported on the sustained division of protoplasts isolated from grass pea leaves and their fusion with pea mesophyll protoplasts, followed by the production of somatic hybrid microcalluses [5].

Hyperhydricity, formerly known as vitrification [22], has been observed *in vitro* in cultures of many species. The morphology of cultures has been described and the influence of various physiological and ecological parameters extensively reviewed [23]. However, information on the fundamental genetic mechanism(s) underlying the occurrence of hyperhydricity is practically non-existent.

In this chapter, we describe the development of reliable *in vitro* strategies for de novo regeneration of fertile, true-to-type plants of *Lathyrus sativus* L. and, also, various other biotechnological breeding approaches that we have developed in our laboratory for grass pea.

2. *IN VITRO* REGENERATION OF GRASS PEA FERTILE PLANTS

In our laboratory, dry seeds of the grass pea genotypes LB, LIII and L12 (Table I) were surface sterilised and water-imbibed overnight (20–22 h), the embryo axes were excised and germinated on hormone-free B5 medium [24] with 10 mM NH_4Cl, 3% sucrose and 0.6% agar (pH 5.6) [20, 25]. Dishes were kept at 22/24°C, under a 16/8 h night/dark photoperiod of 100 $\mu mol.m^{-2}.sec^{-1}$ from warm white fluorescent tubes. Within 5–10 days, 5 mm long hypocotyl segments without any pre-existing meristem were used as explants. Shoot bud regeneration was induced on media based on MS salts [26] with B5 vitamins [24], 3% sucrose, 0.6% agar and 1.0, 3.0 or 5.0 mg/l BAP in combination with 0.0, 0.01, 0.05, 0.1 or 0.5 mg/l NAA, at pH 5.6, as previously used with pea [25]. Two other media used for pea [27] were also tested, with 2.2 mg/l thidiazuron (medium TDZ) or 10 mg/l zeatin and 1.0 mg/l ABA (medium ZABA) added to the basal medium supplemented with 0.1% casein enzymatic hydrolysate and 5% mannitol. Regenerated shoot buds were transferred to the medium used for germination of embryo axes above or to hormone-free MS medium (Mso) for internode

elongation. For rooting, elongated shoots were transferred onto half-strength hormone-free B5 medium [16], or onto half- or full-strength MS medium with 0.0 or 1.0 mg/l NAA, and rooted plants were acclimatised in the greenhouse [20] where they were kept until they flowered and set seed. Experiments were repeated three times or more with at least ten replicates per medium per genotype. Results, expressed as a mean percentage for regeneration and rooting and as the mean number of buds regenerated per explant, were statistically analysed using the Newman-Keuls test (P = 0.05).

Normal, fertile, true-to-type plants were regenerated from the hypocotyl explants of all three genotypes and the whole procedure, from the explant to the grain harvested from regenerants took in average 4 months (for LB) to 5 months (for LIII and L12). The optimum percentage of shoot bud regeneration was obtained on a hormonal combination specific for each genotype studied. The white-seeded LB responded best on NAA-free medium with 5.0 mg/l BAP, while for both coloured-seeded genotypes a medium with 0.01 mg/l NAA was best, in combination with 1.0 mg/l BAP for LIII, but with 3.0 mg/l BAP for L12 (Table II). For all genotypes, whenever half-strength hormone-free B5 medium was used rooting remained erratic. The best rooting responses were obtained on a half-strength hormone-free MS medium, but differences were apparent between regenerated shoots depending on the hormonal balance used for the induction of regeneration from explants.

TABLE I. MAIN PHENOTYPIC TRAITS OF THE THREE *Lathyrus sativus* L. GENOTYPES STUDIED

Genotypes		LIII	L12	LB
Production Area		Asia, India, Nepal Pakistan		Mediterranean Basin
Grains	Size	Small		Large
	Shape or characteristics	Wrinkled, round		Smooth, flat
	Colour	Brown-reddish		White
	TGW (g) [a]	177	236.12	414.14
Flower Colour		Variable (blue, rose, red)		White
Content	ODAP [b]	High		Low
	Antinutritional Factors	High		Low

[a], TGW: weight of one thousand grains; [b], β,-N-oxalyl-α,β diaminopropionic acid

3. IDENTIFICATION OF THE GENETIC MECHANISM UNDERLYING HYPERHYDRICITY OF SOME *IN VITRO* REGENERANTS

The true-to-typeness of regenerants compared with germinated plants from each genotype was assessed in terms of phenotype and also by flow cytometry as described elsewhere [28]. Briefly, the DNA content of regenerants from the different media was examined on nuclei isolated mechanically from chopped leaf tissues [29]. The suspension obtained was filtered (40 μm), the A-T binding specific fluorochrome DAPI (4',6-diamidino-2-phenylindole) was added at 1 μg/ml, and the DNA content was analysed using a Partec PAS-II flow cytometer equipped with a HBO–100 W mercury lamp and a dichroic mirror (TK420). Data were

plotted on a semi-logarithmic scale, whereby the successive peaks obtained (2C, 4C, 8C) are evenly distributed along the abcissa. The integrals of each peak in the histograms were obtained using the built-in software Partec DPAC V2.0. Two independent DNA content analyses were made for each sample from each regenerant.

Dependent on the regeneration medium employed, explants with shoot buds exhibiting severe symptoms of hyperhydricity were observed, particularly in presence of a low NAA content (0.01 or 0.05 mg/l) plus a high (5.0 mg/l) BAP dose, or when TDZ (for LB and L12) or zeatin (for L12) was used as the cytokinin and no auxin was added (Table 2). In turn, such hyperhydric shoots proved incapable of rooting and never succeeded to produce normal, flowering plants. Conversely, non-hyperhydric shoot buds were readily multiplied and rooted *in vitro* (provided the medium adequate for each genotype was used) and gave normal flowering plants, both *in vitro* and in vivo, from which true-to-type grains could be harvested.

Differential responses were also observed between genotypes in terms of the frequency of appearance of hyperhydricity, that was never observed for LIII irrespectively of the hormones employed for regeneration, while regenerants of LB were very prone to hyperhydricity and those of L12 were roughly intermediate, as in terms of regeneration competence (Table II).

TABLE II. PERCENTAGE OF CAULOGENESIS AND FREQUENCY OF HYPERHYDRICITY RELATED TO THE NATURE AND CONCENTRATION IN CYTOKININS OF THE REGENERATION MEDIUM

Genotype	Cytokinin (mg/l)	n[c]	% regeneration[d]	buds/explant[d] (mean number)	% hyperhydric shoots[d]
LB	BAP (1.0)[a]	60	0 f	0 f	0 f
	BAP (3.0)[b]	96	20.8 b	1.9 c	44.8 c
	BAP (5.0)[b]	96	45.8 a	3.2 a	74.8 b
	TDZ (2.2)	96	10.4 c	2.5 b	84 b
	Zeatin (10.0)	96	0 f	0 f	0 f
LIII	BAP (1.0)[a]	76	12.2 c	1.2 d	0 f
	BAP (3.0)[b]	98	6.1 d	2.0 c	0 f
	BAP (5.0)[b]	98	0 f	0 f	0 f
	TDZ (2.2)	98	1.02 e	1.0 e	0 f
	Zeatin (10.0)	98	0 f	0 f	0 f
L12	BAP (1.0)[a]	50	16 bc	1.6 cd	7.7 e
	BAP (3.0)[b]	50	52 a	1.3 d	20 d
	BAP (5.0)[b]	50	14 bc	3.0 a	14.3 d
	TDZ (2.2)	50	2 e	1.0 e	100 a
	Zeatin (10.0)	50	2 e	1.0 e	100 a

[a], mean data for media containing also 0.01 or 0.05 mg/l NAA;
[b], mean data for media containing also 0.01, 0.05, 0.1 or 0.5 mg/l NAA;
[c], n = number of explants;
[d], figures within a column followed by different letters differed significantly at P = 0.05

In order to understand the mechanisms underlying the preferential occurrence of hyperhydricity on some of the regeneration media used, samples from shoots produced on different media were analysed by flow cytometry (Figs. 1, 2). Thus, for over 70 hyperhydric regenerants of LB and L12 analysed, hyperhydricity symptoms were systematically associated with abnormal flow cytometry profiles for such regenerants, that always exhibited three peaks corresponding to the 2C, 4C and 8 C DNA levels (Table III, Fig. 1). Conversely,

all phenotypically normal regenerants obtained for all three genotypes also showed a normal, DNA content, as evidenced by the diploid flow cytometry profiles with the two peaks corresponding to the 2C and 4C DNA levels only, comparable to those of seedling tissues.

We have also analysed the interaction observed between the hormonal composition of the regeneration media and the occurrence of hyperhydricity, and the link of these with a modified DNA level were both confirmed. In this context, auxins had a much more important effect than cytokinins, as an increase in the BAP content of the medium (Fig. 2a,c) had no effect on hyperhydricity or DNA content, whereas the sole addition of NAA to a medium with a hitherto already high BAP level sufficed to induce hyperhydricity of regenerated shoots (Fig. 2c,e). Conversely, the only clear effect of cytokinins was when BAP was compared with TDZ, where the latter were more prone to provoking hyperhydricity.

TABLE III. THE CORRELATION BETWEEN HYPERHYDRICITY AND DNA CONTENT OF REGENERANTS

Genotype	Phenotype of regenerants	Number of regenerants analysed	Flow cytometry profiles with	
			2 peaks (2C, 4 C)	3 peaks (2C, 4C, 8C)
LB	Normal	98	97	1
	Hyperhydric	58	0	58
LIII	Normal	27	27	0
	Hyperhydric	0	Not applicable	
L12	Normal	40	40	0
	Hyperhydric	15	0	15

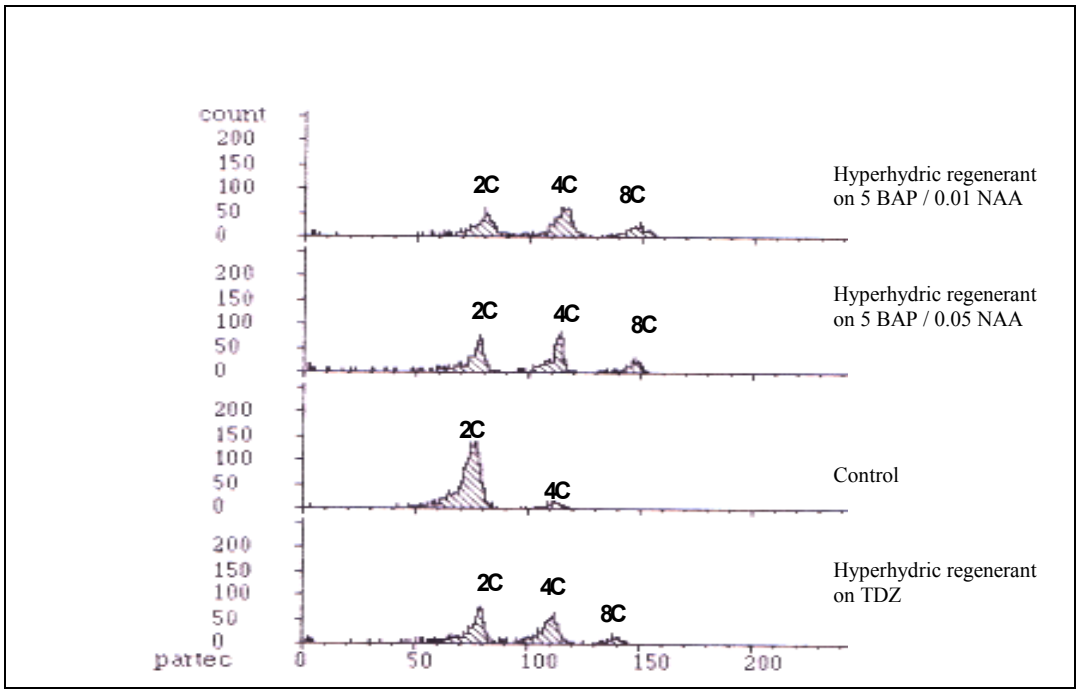
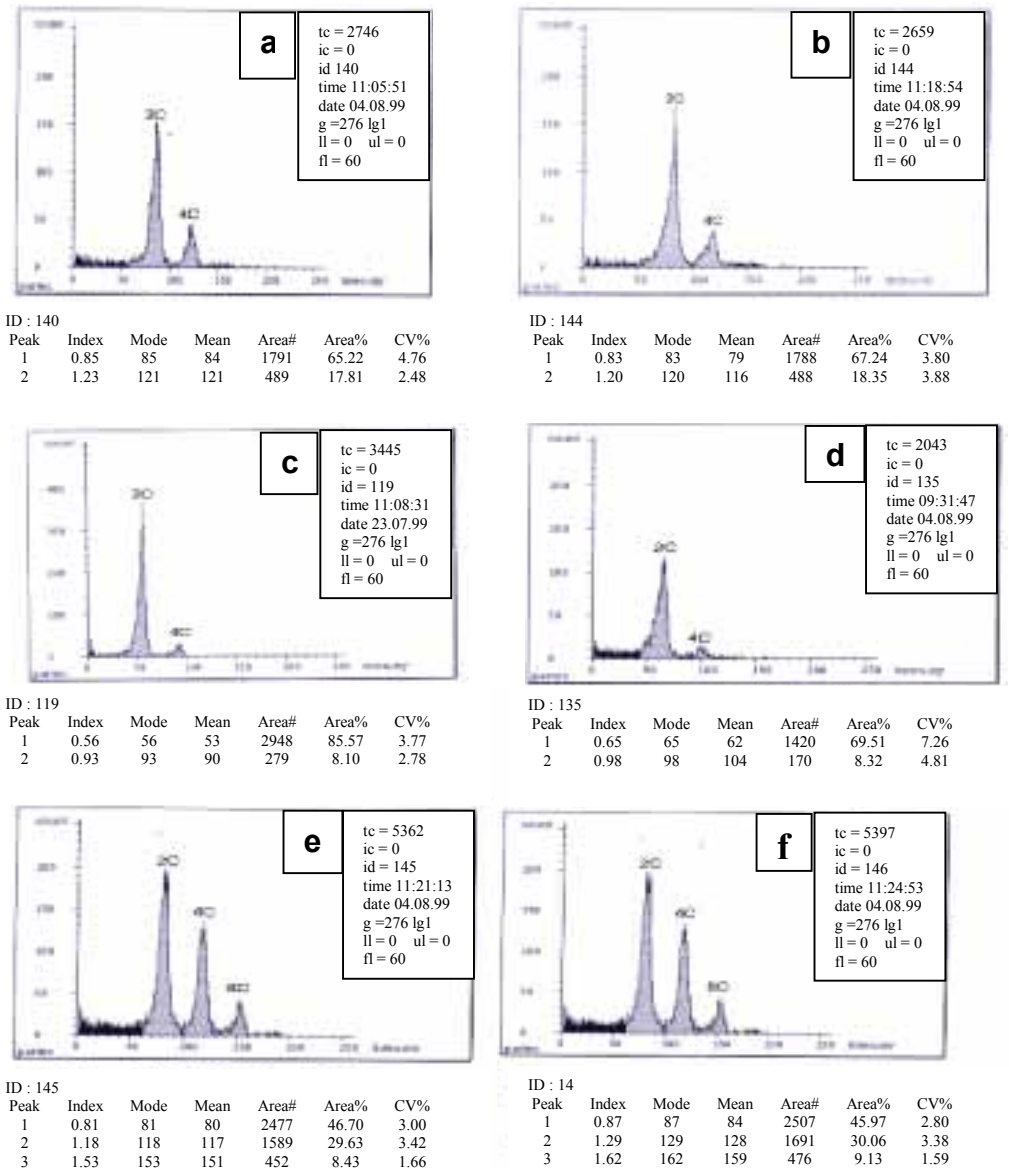


Fig. 1. Flow cytometric profiles of various regenerants of genotype LB on different regeneration media.

On the other hand, tissues taken from shoots regenerated on a same medium, where the only difference was the number of culture passages in vitro on a hormone-free medium at the time of the measurements (Fig. 2). The time in culture did not provoke any modification of DNA content (Fig. 2b,d,f), nor did it have any effect on the occurrence of hyperhydricity, contrasting claims that a prolonged period of culture may result in the occurrence of hyperhydricity [22, 23].

These results, added to those obtained with protoplast-derived tissues of pea [28], open up the way for the utilisation of cell cytometry as an early screening strategy for the avoidance of hyperhydricity in cultured tissues and hence for the optimisation of the whole plant regeneration process in grass pea.



ID : 140

Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.85	85	84	1791	65.22	4.76
2	1.23	121	121	489	17.81	2.48

ID : 144

Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.83	83	79	1788	67.24	3.80
2	1.20	120	116	488	18.35	3.88

ID : 119

Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.56	56	53	2948	85.57	3.77
2	0.93	93	90	279	8.10	2.78

ID : 135

Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.65	65	62	1420	69.51	7.26
2	0.98	98	104	170	8.32	4.81

ID : 145

Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.81	81	80	2477	46.70	3.00
2	1.18	118	117	1589	29.63	3.42
3	1.53	153	151	452	8.43	1.66

ID : 14

Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.87	87	84	2507	45.97	2.80
2	1.29	129	128	1691	30.06	3.38
3	1.62	162	159	476	9.13	1.59

Fig. 2. Cytometric profiles of regenerants of genotype L12 obtained on different regeneration media and maintained on medium Mso for different durations. a: with 3 mg/l BAP and no NAA + one passage on medium Mso; b: with 3 mg/l BAP and no NAA + four passages on medium Mso; c: with 5 mg/l BAP and no NAA + one passage on medium Mso; d: with 5 mg/l BAP and no NAA + four passages on medium Mso; e: with 5 mg/l BAP and 0.01 mg/l NAA + one passage on Mso; f: with 5 mg/l BAP and 0.01 mg/l NAA + four passages on Mso.

4. STUDIES ON THE SEXUAL HYBRIDISATION OF GRASS PEA AND PEA (*Pisum Sativum* L.)

Pisum sativum is reportedly cross incompatible both with *P. fulvum* and *Lathyrus sativus*, but no clear data exist in the literature. Thus, greenhouse-grown plants of a number of genotypes of these species were reciprocally hand-pollinated following emasculation of the flowers. In situ germination of pollen grains was performed, and the pollen germination on the stigmata and the growth of pollen tubes on the styles after staining with aniline blue observed, whereby it was verified that pollen tubes were unable to germinate in inter-specific crosses (Fig. 3). However, three putatively hybrid pods (escapees?) were obtained from one cross between pea and *P. fulvum*, and one between pea cv Térèse and LB grass pea. One grain per pod was *in vitro* germinated, then micropropagated for further flow cytometric and isoenzymatic studies, which both showed that while hybrids had been produced between the two *Pisum* species, the plants obtained from the pea x grass pea crossings were not hybrid [6]. In terms of phenotype, the putative inter-generic hybrid Terese pea x LB grass pea matched closely the pea parent with respect to all characters examined. Of the three isoenzymatic systems examined, esterases (EST), malate dehydrogenase (MDH) and leucine aminopeptidase (LAP), none gave conclusive proof of a hybrid nature, the profiles of the inter-generic putative hybrid was an exact copy of that of Terese pea, both in terms of the number of bands and in their intensity. Results from flow cytometry experiments clearly confirmed the Terese pea x grass pea plants not to be inter-generic hybrids at all, as the profiles of samples from such putative hybrids coincided exactly with that of Terese pea, and were very dissimilar to that of LB grass pea.

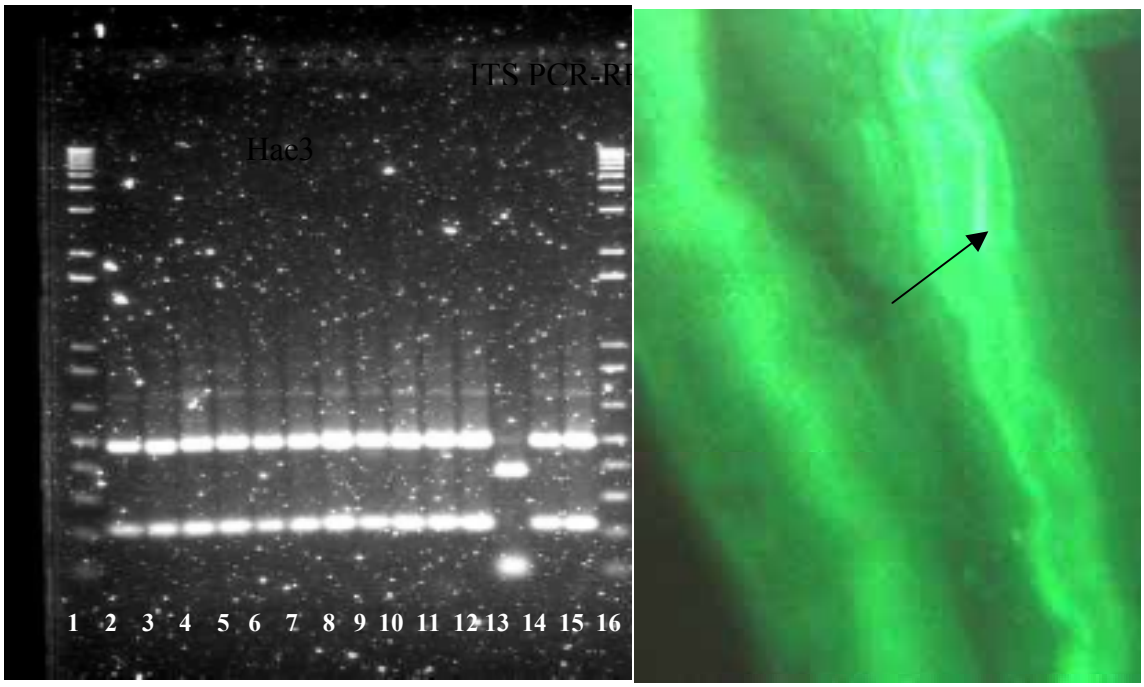


Fig. 3. Right: In situ pollen germination observed after staining with aniline blue under UV light showing that pollen tubes (arrowed) do not reach the ovary. Left: Digestion of ITS PCR products using ITS1 and ITS4 universal primers. Lanes = 1 Kb DNA ladder (BRL) (1 and 16), ITS of P. sativum cv Baccara (2), P. fulvum (3), eight F5 putative hybrids Baccara x P. fulvum (4–11), P. sativum cv Terese (12), Lathyrus sativus cv LB (13) and two F4 putative Terese x L. sativus hybrids (14–15) digested by Hae3.

Further experiments were performed using molecular approaches to characterise the various putative hybrid genotypes produced, compared to the parental genotypes used in the respective crosses. The ITS PCR approach was chosen because of its simplicity, and also because it has proven useful in the past for the analysis of sequence variations in *Pisum* taxa including *P. sativum* and *P. fulvum* [30]. Thus, the digestion by Hae3 and Hinfl of ITS PCR products using ITS1 and ITS4 universal primers was successful to demonstrate unequivocally that the putative inter-generic *P. sativum* X *Lathyrus sativus* hybrids were not hybrid at all, as the profiles obtained for these matched exactly that of Terese pea (Fig. 3).

5. PROTOPLAST ISOLATION, CULTURE, FUSION AND SOMATIC HYBRIDISATION OF GRASS PEA AND PEA

Based on the results above, pea and grass pea protoplasts were, for the first time, efficiently and reproducibly fused. Protoplasts were isolated from leaves of all genotypes and cultured in the media tested for pea [28]. They were labelled with fluorescein diacetate (green), while rhodamine B isothiocyanate (red) was preferred for pea protoplasts [8], and electrofusion (at 750, 1,000, 1,250, 1,500 or 2,000 V/cm) was compared with chemical fusion. The standard macro-method [31] was compared with a micro-method we developed [8], and both were tested with glycine, PEG 6000 and PEG 1540. Heterokaryon viability was assessed and they were cultured in various media. Large numbers of viable mesophyll protoplasts were obtained for all genotypes, and they proliferated to give calluses, but plants have not been regenerated to date. In terms of fusion with pea protoplasts, glycine was the least effective agent (~10% heterokaryons), while PEG was best (>20% heterokaryons). With electrofusion, heterokaryon formation was increased from 750 to 1,500 V/cm, but fell drastically beyond this threshold. In all, electrofusion permitted the largest heterokaryon formation but with a large variability, and PEG 6000 appeared as the most efficient and reproducible fusion agent (Table IV).

TABLE IV. PLATING EFFICIENCY OF *Pisum* (+) *Lathyrus* HETEROKARYONS (MEAN DATA FROM ≥ 200 HETEROKARYONS/TREATMENT AND 3 INDEPENDENT EXPERIMENTS)

Fusing agent	Fusion method	Heterokaryon formation(%)	IPE (%)	FPE (%)
Non-fused LB protoplasts			63.28	2.15
Non-fused LIII protoplasts		Not applicable	37.55	1.44
Non-fused L12 protoplasts			29.22	0.58
Glycine	Micro	11.4	16.12	0.0
	Macro	9.2	19.25	0.0
PEG 1540	Micro	20.6	23.5	2.15
	Macro	19.7	4.65	0.22
PEG 6000	Micro	21.3	28.0	2.75
	Macro	22.5	7.4	0.42
Electrofusion	750	10.1	21.81	1.86
(V/cm)	1,000	13.9	19.46	1.42
	1,250	18.8	22.3	2.85
	1,500	22.1	25.0	3.11

IPE: % heterokaryons dividing once; FPE: % heterokaryons undergoing sustained division.

Heterokaryons fluoresced both green and red under UV light, and divisions could be monitored during several days (up to 4–6 celled colonies; Fig. 4). Several hundreds of somatic hybrid calluses have been obtained and regeneration experiments are under way.

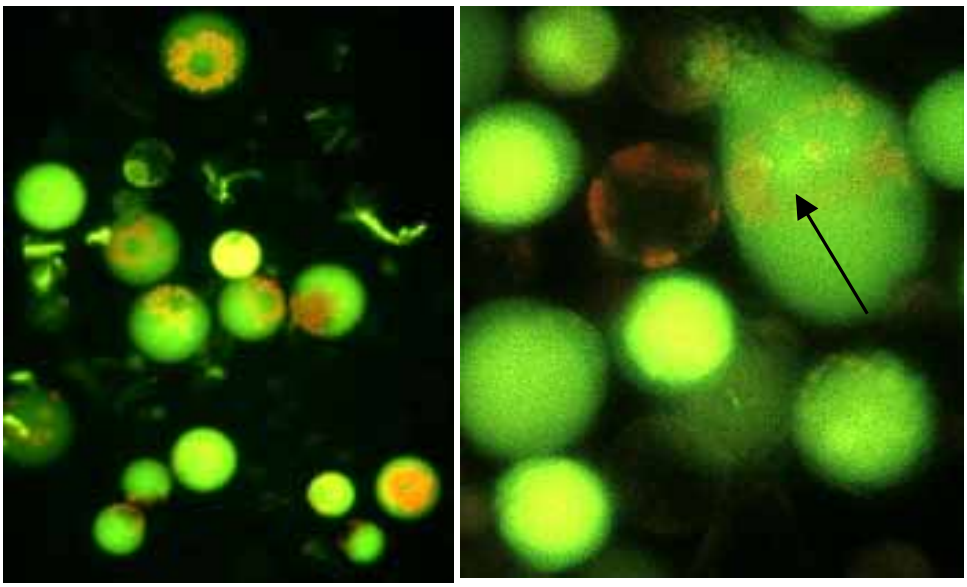


Fig. 4. Fusion of pea and grass pea protoplasts. a. Heterokaryon formation shown by the dual coloration (red from Pisum and green from Lathyrus); b. A dividing heterokaryon-derived cell (arrowed).

6. GENOME ORGANISATIONS AND PHYSICAL MAPPING IN *Lathyrus sativus* L.

The structure, organization and evolution of the rDNA multigene families has been studied in detail in several plant species [32, 33] and, as in all higher eukaryotes, they are separated in two different loci [34]. The 18S–5.8S–25S rRNA genes are organized in tandem arrays within the nucleolar organizer regions (NORs) [35, 36]. The 5S rRNA genes also occur in tandem repeats which map outside the NORs. The two types of ribosomal DNA arrays, associated to heterochromatin, differed in number and position and displayed a high polymorphism in their intergenic sequences [37]. They have been used as a tool for analysing karyotype evolution and behaviour of tandemly repeated gene families. FISH was used to examine the distribution of rDNA and 5S sites in order to provide molecular cytogenetic landmarks that facilitate the ongoing FISH –based karyotype and the development of an integrated map in different legumes. These ribosomal DNA anchoring sites could be also useful to determine the respective homeologous relationships of chromosome bearing the rDNA for comparative mapping.

Figure 5 depicts the results from the fluorescent in situ hybridisation (FISH) studies that are being developed for the precise genomic characterisation of any hybrid material obtained between *Pisum* and *Lathyrus* genotypes. Thus, FISH using PCR-amplified probes has detected a variable number of ribosomal DNA loci. The localisation of the hybridisation sites on four *Lathyrus* and three *Pisum* chromosome pairs represents the first molecular landmarks on chromosomes of these species. The genome of both species can also be distinguished by their repetitive DNA organisation, as revealed by DAPI banding patterns.

Similar results were obtained very recently by Ali et al. [38] when comparing the DNA content, rDNA loci and DAPI bands to assess the phylogenetic distances between various *Lathyrus* species.

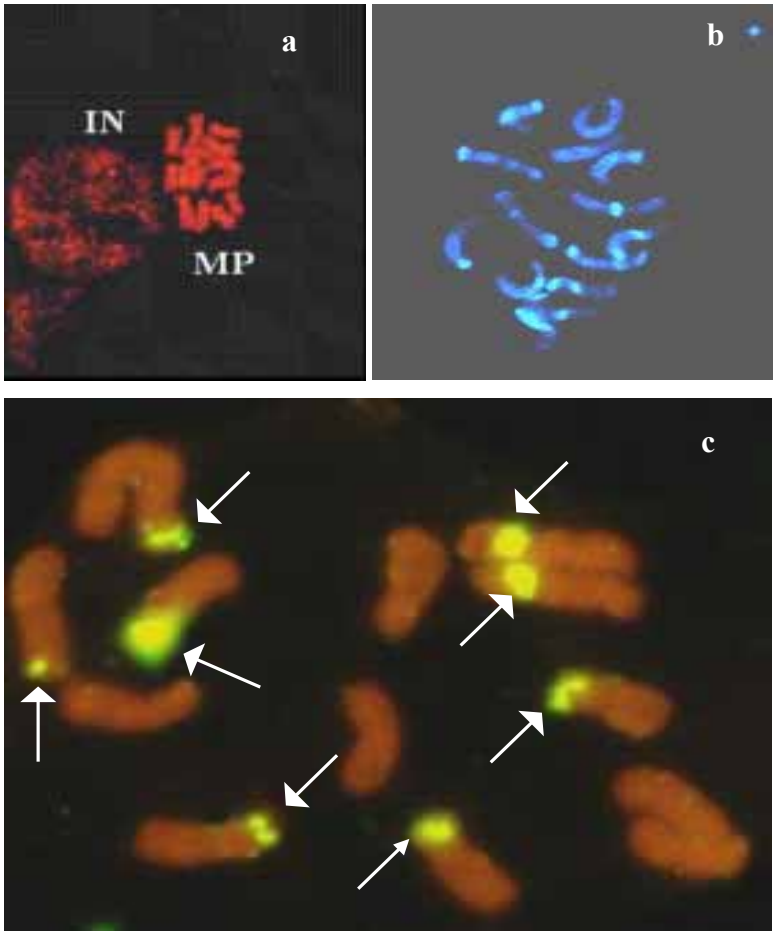


Fig. 5. Genome organisation and physical mapping by FISH (fluorescence in situ hybridisation) in Lathyrus sativus L. a. A fluorescent interphase nucleus (IN) and a metaphase plate (MP) of Lathyrus sativus L. showing 2 n = 14 chromosomes; b. A DAPI stained metaphase plate of Lathyrus sativus L. showing fluorescent bands corresponding to AT-rich sequences; c. The eight 5S rDNA yellow fluorescent sites (arrowed) indicate the molecular landmarks for the identification of four Lathyrus sativus L. chromosome pairs.

7. ACCELERATING GENERATION CYCLES BY THE INDUCTION OF *IN VITRO* FLOWERING AND FRUITING

Generation cycles were drastically shortened in pea (*Pisum sativum*), Bambara groundnut (*Vigna subterranea*), and grass pea (*Lathyrus sativus*) [39]. A greenhouse strategy was used as control, compared to an *in vitro* plus in vivo strategy for pea and bambara groundnut, and to an *in vitro* only strategy for pea and grass pea. Using *in vitro* plus *in vivo* system and embryo axis explants, nearly 6 generations per year for *Pisum* and over 4 generations for *Vigna* were obtained, compared to 2 generations in the field, so that using successive generations from seed to seed in pea, the mean duration for one generation was 67.2±4.6 days in 'Frisson', against a mean of 143 ± 3 days in the field. These results prompted other experiments where all stages from seed to seed were attempted *in vitro* only.

In all cases, the shoots had either been germinated *in vitro* from excised embryo axes on B5 modified medium [25], or regenerated *in vitro* from hypocotyl explants [5, 20]. For all genotypes, shoots of at least 1 cm tall and comprising two internodes were transferred to a simple, hormone-free MS medium where they elongated, flowered and ultimately set seed. Alternatively, shoots were transferred onto half-strength MS medium without hormones or with 1 mg/l NAA [5, 20, 25, 28] for rooting prior to their eventual flowering and fruiting. The number of flowers per shoot, of pods per flower and of seeds per pod was determined for non-rooted and rooted shoots. The number of feasible generations per year was then calculated, based on the number of days elapsed from the transfer of the initial shoot onto the medium up to the harvest of seeds for the first generation (R1), and through the number of days from *in vitro* seed germination up to seed set *in vitro* by the resulting seedlings for subsequent ones (R2 and further). At least 20 (and up to 96) shoots per origin per genotype were tested, and experiments were repeated several (3–5) times throughout a period of 2 years.

Flowering and seed set were consistently obtained *in vitro*, and there was no prerequisite for rooting of shoots preceding it [39]. Moreover, an *in vitro* rooting phase not only lengthened each generation cycle (by about 15–30 days), but it also affected the competence for flowering of shoots. Likewise, the best flowering responses were obtained on a simple, hormone-free medium, whereas the addition of growth regulators systematically resulted in a reduced flowering, and the reduction of salts strength by half provoked a reduced seed set, and was subsequently coupled with a lower germination competence of the seeds produced.

Figure 6 details the results obtained with the different genotypes, over ten successive generations, in terms of mean duration of generation cycles respectively. The end result was that the crop duration, which in the field varies from 150 to 180 days [40], was therefore notably reduced, permitting three generations/year instead of two.

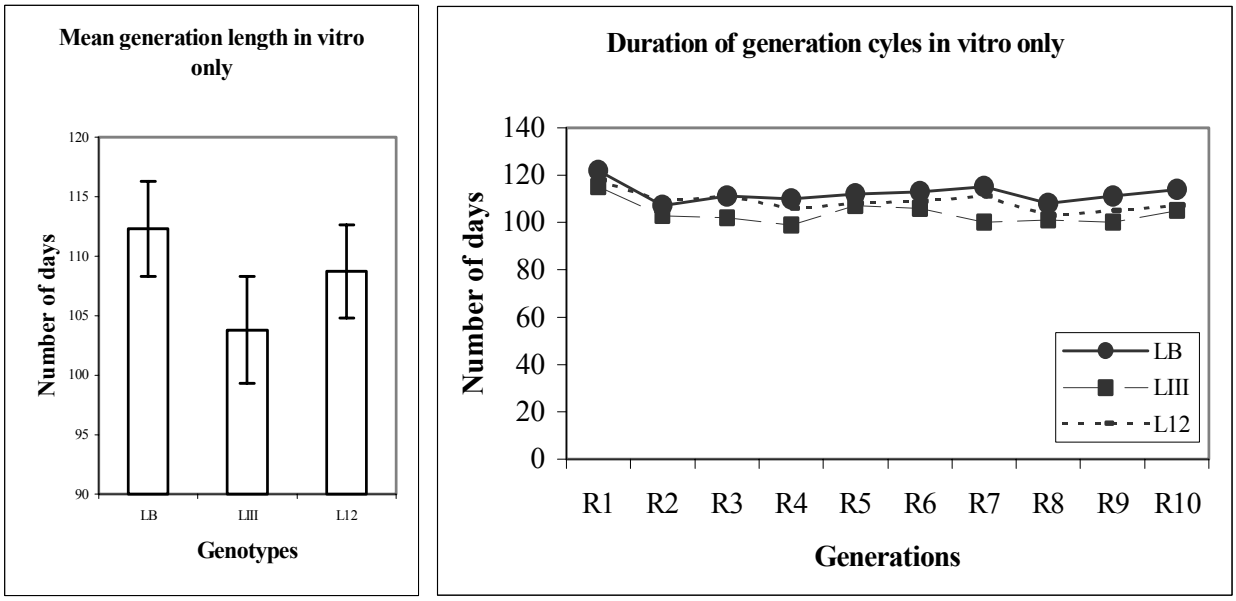


Fig. 6. Shortening of generation cycles in vitro for grass pea.

In vitro flowering had been reported in the past in several species but rarely in legumes, and the plant growth regulator requirements of plants for *in vitro* flowering have been quite variable. Interestingly, in our experiments neither the addition of growth regulators to the shoot medium nor the rooting of *in vitro*-grown shoots were essential for flowering and seed

set, thus reducing the risk of *in vitro*-induced variations derived from the use of hormones [5, 20, 28]. During previous experiments, fertile plants were obtained from explants of grass pea within 17–21 weeks of culture [5, 20]. With these procedures, a more efficient exploitation of those approaches for breeding can be envisaged, as such time span could now be shortened further (by 15 to 30 days), as the rooting step is no longer required with regenerated shoots (generation R1), nor with any subsequent generation.

8. CONCLUDING REMARKS

The availability of reliable regeneration strategies for the production of fertile plants [5, 20] permits to envisage now a better use of biotechnology for the breeding of *Lathyrus sativus* L. itself [1], and also as a protoplast fusion partner for somatic hybridisation with pea [8]. More generally, these results, added to those obtained with protoplast-derived tissues of pea [28], open up the way for the utilization of cell cytometry as an early screening strategy for the avoidance of hyperhydricity in cultured tissues and hence for the optimisation of the whole plant regeneration process in grass pea. Likewise, the possibility to reduce the duration of generation cycles [39] is of the utmost importance when dealing with time gains for breeding of this hitherto neglected species.

The increasing need of plant proteins both as animal feed and for human consumption in low-income food-deficit countries has led over the past few years to the development of protein-rich sources other than soybean, including grass pea (*Lathyrus sativus* L.) [2], a species that has been accorded very little research effort thus far. As stated in the introduction, grass pea is a source of important traits commercially, on account of its low production costs, and from an agronomic standpoint, due to its rusticity. Hence, grass peas require breeding for an improvement of grain quality, specially in terms of a reduction of the content of toxins before they can be efficiently and safely exploited, and biotechnological approaches as those developed in this chapter are likely to help in achieving this aim.

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NEMATODE RESISTANCE THROUGH MUTATION INDUCTION IN A LOCAL VARIETY OF NARANJILLA (*Solanum quitoense* Lam) IN ECUADOR

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Abstract

The main objective of this study was to achieve nematode resistance in *Baeza*, a traditional variety of naranjilla (*Solanum quitoense* Lam.) in Ecuador. Mutations by using gamma ray irradiation (^{60}Co) were performed on true seed and axillary buds of *Baeza*. Radiosensitivity tests determined the most effective doses for naranjilla mutation. Mutated plants from seeds (50 Gy dose) were evaluated until M_2 generation. Nematode-resistant plants from greenhouse evaluation were evaluated *in situ* at the main center of local production of naranjilla (Palora). Nematode resistance was observed for these materials and a severe infestation of *Fusarium oxisporum* and *Pseudomonas solanacearum* provoked their loss except for 35 resistant genotypes that are conserved as seeds in the genebank (-15°C). The evaluation of resistance of these materials in the future may present great interest for breeders. At that moment, our experience determined that the evaluation of materials by using seeds was time and labor consuming, and then we concluded that the vegetative multiplication (tissue culture) was more efficient for naranjilla propagation. By using *in vitro* mutated naranjilla plants we started with M_1V4 material received from the Plant Breeding unit in Seibersdorf, Austria. Materials irradiated with two selected doses (5 Gy and 7 Gy) were evaluated in a greenhouse and plants are ready for field evaluation in Palora. The results achieved so far show that by using mutations clearly nematode-resistant materials can be obtained. However, further evaluation (following generations) must be done, it is clear that naranjilla mutations will contribute, with nematode resistant materials, to overcome the environmental and technical problems that farmers from the center of production of this species experience in the field.

1. INTRODUCTION

The origin of the "Naranjilla" *Solanum quitoense* Lamarck, is not well known because of a lack of archaeological information [1]. However, the first records of Naranjilla come from South America (Ecuador and Colombia) in the middle of the seventeenth century [2]. These countries still constitute the main region of cultivation, principally the valleys of Pastaza and Yungillas in Ecuador and the mountain areas of Cauca and Nariño in Colombia [1; 2]. Fairly recently, cultivation has been undertaken in Panama, Costa Rica and Guatemala [2]. Also, Naranjilla can be found in New Zealand and California [1].

In terms of ancestors, *S. candidum* is suggested as the ancestral species for *S. quitoense*, however, on the basis of crossing results, [2] suggests *S. hirtum* as a progenitor, although the latter hypothesis must be confirmed. Colombia could be the most likely place to find a wild progenitor for this newly domesticated species.

The Naranjilla, from the section *Lasiocarpa*, is an autogamous species that has very little morphological, physiological and organoleptic intra-specific variability [1; 3; 4]. Two varieties of *S. quitoense* Lam. have been recognized: var. *quitoense*, a spineless form, and a form with spines, var. *septentrionales* Schultes and Cuatrecasas [5]. The chromosome number for *S. quitoense* is 2n=24 [6].

This crop in generally grown at an altitude of 1,000 to 1,900 meters above sea level [5]. Naranjilla needs an average temperature between 15 and 22°C with 20°C as the optimum temperature [1; 7]. Also, Naranjilla needs rainfalls ranging from 1200 to 3700 mm [1; 8]. Approximately 6,000 has of Naranjilla are cultivated in Ecuador, with a production of 24,211 tons and a yield average of 4 tons/ha [9].

The common Naranjilla suffers from insect attacks and bacterial and fungal infections [1]. In Ecuador many diseases have been reported such as *Anthracnosis* caused by (*Colletotrichum gloesoporioides*), wilt (*Fusarium* sp), spotted leaves (*Cercospora* sp.), the bacterial wilt caused by *Pseudomonas solanacearum* and pests like fruit worm (*Neoleucinodes elagantalis*) and neck borer (*Faustinus apicalis*) [10; 11]. However, the main problem is the root knot nematode (*Meloydogine* sp.) [1; 3].

The nematode susceptibility of common Naranjilla reduces the plant productive life from five years to one or two years. Symptoms of the nematode attack include reduction of plant growth, foliar chlorosis, withered leaves and root galling. Hence, in Ecuador, once a crop had been infested with nematodes, virgin areas were sought for cultivation (nematode-free soil), and then once an infestation arose in the new territory (usually three years later) the cultivation was moved to new fields [12]. Additionally, farmers used inadequate chemicals in an attempt to eliminate pests and diseases, contaminating the soil and the fruit and causing a negative ecological impact.

The negative impact on the agro-ecosystem as much as the disease susceptibility of Naranjilla, endangered the genetic variability of the species in Ecuador. For this reason, local varieties were supplanted by hybrids that were more resistant to nematode attacks. Then, collecting missions were carried out in Ecuador during 1993 to collect wild relatives as well as landraces from the *Lasiocarpa* Section (Tapia et al., 1993 [10]). Currently, the National Department of Plant Genetic Resources (DENAREF) conserves 46 accessions of *S. quitoense, S. sessiliflorum, S. hirtum, S. arboreum, S. quitoense x S. felinum, S. quitoense x S. sessiliflorum* among others [10; 13].

Due to the extremely low intra-species variation in *S. quitoense*, genes for its improvement most likely will come from inter-species hybridization [14]. In Ecuador, there are two inter-species hybrids between Naranjilla (*Solanum quitoense)* and Cocona (*Solanum sessiliflorum)* called INIAP- Puyo and INIAP- Palora. INIAP-Puyo was obtained by farmer Raúl Viteri, who crossed *S. quitoense* with *S. sessiliflorum*, which is more resistant to pests and diseases. This hybrid is recognizable as it is only half the size of true Naranjilla and contains very few or no seeds [15]. To counteract this reduction in size, farmers began taking advantage of a peculiarity of the hybrid INIAP- Puyo that, after the flowers are sprayed with a very diluted solution of 2,4 D, the fruit increases in size. However, this resulted in concentrations of over 100 to 2,000 ppb of 2,4 D in the fruits as well as other chemicals such as Carbofuran (2,3-dihydro-2,2-Dimetil benzofuran-7,1+l-methylcarbamate, IUPAC) and Aldicarb (2-methyl-2-[methylthio] proprionaldehyde 0-[methylcarbamoyl] oxime), exceeding greatly the permitted range of chemical residue [16]. On the other hand, the hybrid INIAP-Palora does not require any chemical application because it produces larger fruits. This is also an inter-specific hybrid

between *S. quitoense* (var. Baeza Roja) and *S. sessiliflorum* (var. Morona Grande), that is resistant to pests and diseases, but for which resistance to nematodes is not mentioned [11].

At the National Autonomous Institute for Agricultural Research (INIAP), researchers considered the use of mutation-induction through gamma rays to obtain nematode resistant plants from a landrace subject to genetic erosion. This landrace is grown in ever-increasingly small-scale quantities, as its susceptibility to nematodes makes it unfit for usage for commercial production. The local variety Baeza (Fig 1.) was chosen for this purpose, because it was formerly a widely cultivated variety of Naranjilla with valuable organoleptic characteristics and a high market demand, but also with a high susceptibility to nematodes, especially *Meloidogyne* sp.

Fig. 1. Fruits of Baeza, an Ecuadorian landrace of Solanum quitoense Lam.

2. MATERIALS AND METHODS

2.1. Mutation induction of naranjilla by using seeds

2.1.1. Seed collection

Seeds of *S. quitoense* var *Baeza* and *Peluda* were extracted from fruits collected at the *Experimental Farm Palora* (EFP), located in the Amazonic province of Morona Santiago (910 masl, 1° 52´S and 78° 3´W). The fruits were collected at two different time-periods January and March of 1999.

2.1.2. Radiosensitivity test in vivo

Naranjilla seeds (250) from the local varieties Baeza and Peluda were sent to the Plant Breeding Unit in Seibersdorf (Austria), to perform a radiosensitivity test. Seeds were sterilised with 70% alcohol, 30% w/v Clorox (commercial bleach containing 5.2% (w/v) NaOCl) for 10–15 min. Then, they were rinsed three times with sterile water and transferred to MS medium with 0.18% gelrite and 3% sucrose in tubes. The sensitivity test was carried out with 10 seeds per treatment with doses ranging from 100 to 750 Gy including a control.

Simultaneously, 2,000 seeds of Baeza (var.) were sent to the Ecuadorian Commision of Atomic Energy for radiation with a dose of 50 Gy, based on previous studies in Ecuador by [17]. Basic biosafety parameters were applied during the whole process.

2.1.3. Germination of irradiated seeds

Mutated seeds from the Ecuadorian Commission of Atomic Energy were germinated at *the Santa Catalina Experimental Station*, located at 0° 23´S latitude and 78° 31´W longitude, 2480 masl altitude, in the Pichincha province. Seeds were set up in a germination chamber *Seedburo*® with alternate temperatures of 15 and 25ºC every 12 hours, and 80% of relative humidity. Naranjilla seeds were plated in petri dishes of 10 cm diameter with a paper towel imbibed in 75 ppm of GA3. The germination percentage was evaluated after 13, 16, 20 and 26 days of imbibition.

2.1.4. Greenhouse evaluation

Seedlings were transplanted into pots with 500 g of sterile soil (soil, sand and humus in 3:1:1 ratio) and placed in a greenhouse (24°C and 70% of RH). Watering was provided as needed. Variables such as plant length (cm), number of leaves per plant, leaf size (length and width cm), presence or absence of spines were registered after 10, 25, 30 and 42 days in the greenhouse. When plants reached 12 cm (height) and five true leaves (average), they were ready for nematode inoculation.

2.1.5. Nematode inoculation

To establish the nematode-inoculation protocol (previous to the evaluation of 1,500 naranjilla-plants), a preliminary inoculation was carried out on a random sample of five naranjilla plants from irradiated seeds, including the same number of control plants (non irradiated). The inoculation was carried out at the Laboratory of Nematology at Santa Catalina Experimental Station.

Plants of naranjilla were inoculated with 5,000 and 10,000 eggs of *Meloidogyne incognita*. The inoculum was prepared from infected naranjilla-roots collected in a commercial field near Palora**.** The root-galls were washed and cut out into little pieces to isolate nematode eggs according to the sodium hypoclorite technique described in [18] and expressed in eggs per pot. A concentration of 1,000 eggs/ml was obtained. The quantity of inoculum was 10 eggs per gram of substrate (initial population, IP).

2.1.6. Nematode evaluation

To identify the nematode resistance of the mutants, the following treatments were established:

(1) Naranjilla plants from irradiated seeds (50 Gy) and inoculated with nematodes;
(2) Control A. 10 plants from non-irradiated seeds without nematode;
(3) Control B. 10 plants from non-irradiated seeds and nematode inoculated.

Increments over one were considered as statistically acceptable.

The plant response to nematodes was determined as in [19] (Table I). In order to determine the efficiency of the host in relation to the nematode reproduction, the index of increment (I=fP/Pi) was considered. The index of nematode increase in population identifies plants

tolerant or resistant to nematodes. Also, the plant height in cm (PH), and the foliage fresh weight in grams (FFW) were scored.

TABLE I. PLANT RESPONSE TO NEMATODE BY [19] WITH MODIFICATION

Reproduction of nematode	Yield compared to control	
	Lower	Equal or higher
Index > 1	Susceptible- No tolerant	Susceptible-Tolerant
Index < 1	Resistant- No tolerant	Resistant-Tolerant

The criteria to evaluate the combination of these variables are as follow:

1. RESISTANT- TOLERANT Plants with low nematode reproduction (fP/Pi < 1) and equal or higher yield than the control.

2. RESISTANT- NON-TOLERANT Plants with low nematode reproduction (fP/Pi < 1) and lower yield than the control.

3. SUSCEPTIBLE- TOLERANT Plants with high nematode reproduction (fP/Pi > 1) and equal or major yield than the control.

4. SUSCEPTIBLE- NON-TOLERANT Plants with high nematode reproduction (fP/Pi > 1) and fewer yields than the control.

After the evaluation, resistant-tolerant naranjilla individual plants were selected, based on increment index between 0.0 to 0.5 and PH and FFW values equal or higher than the control A.

Resistant plants were asexually propagated (preparing an amount of cuttings as large as possible) and labeled properly. The cuttings were treated with rooting hormone, planted in polyethylene bags containing soil and placed in the greenhouse. These plants were used to verify previous results (Fig. 2).

Fig. 2. A. Root-knots from nematode attack in Naranjilla (Solanum quitoense Lam). B. Naranjilla plants growing in greenhouse conditions. C. Naranjilla roots from a resistant plant.

2.1.7. Field experiment

The field experiment was conducted at Palora Experimental Farm (910 masl, 1° 52´S and 78° 3´W).

2.2. Mutation induction of naranjilla by using *in vitro* explants

2.2.1. Radiosensitivity test in vitro

The radiosensitivity test was performed in var. Peluda at the Plant Breeding Unit in Seibersdorf Austria. A preliminary test with doses ranging from 5, 10, 15, 20, 25 Gy to 30 Gy with a dose rate of 28.74 Gy/min was performed. Shoots were placed on filter paper in a petri dish wetted with sterile distilled water and treated with gamma radiation. Immediately after radiation, single node cuttings (devoid of shoot tips) were transferred to fresh culture medium in tubes with two single-node explants per tube and 44 explants per dose, and were cultured in a growth room. The evaluation was performed after 6 weeks.

In vitro cultures of *Solanum quitoense* var. Baeza, which had been irradiated with 5, 6, 7, 8 and 9 Gy were received at *Santa Catalina Experimental Station* from Seibersdorf, Austria. The plant material had been micropropagated after radiation to M_1V_3 generation. Some control plants were also included.

In Ecuador (Ecuadorian Commission of Atomic Energy), a radiosensitivity test (^{60}Co) with doses ranging from 1 Gy to 12 Gy was conducted with shoot tips produced *in vitro*. Monthly evaluations were carried out including variables such as plant height (cm), absence or presence of roots and number of knots per plant.

2.2.2. Greenhouse evaluation

In vitro plants were extracted from the tubes and the agar washed away. Then, after three months of transplanting, naranjilla plants were ready for inoculation and three months later they were ready for nematode evaluation. Resistant plants from the greenhouse evaluation were reintroduced into tissue culture for micropropagation.

In order to facilitate the evaluation of nematode resistance in the greenhouse, we used the classification scale from [19] (Fig 3).

2.2.3. Introduction and micropropagation in vitro

At Santa Catalina Experimental Station in vitro cultures of *S. quitoense* var. Baeza were conducted as follows: For disinfection, naranjilla stem segments were washed with running water, liquid soap and disinfectant (Povidin); Benlate fungicide at 1% (v/v) was also used. Residues from the disinfection process were washed out profusely with distilled water. The Initiation medium consisted of MS formula supplemented with 2 mg/l Calcium panthotenate, 0.25 mg/l GA_3, 10 mg/l Putrescine, 20 g/l Sucrose, 6,5 g/l Agar and 5 g/l Activated Charcoal. The micropropagation medium was as above except that activated charcoal was omitted.

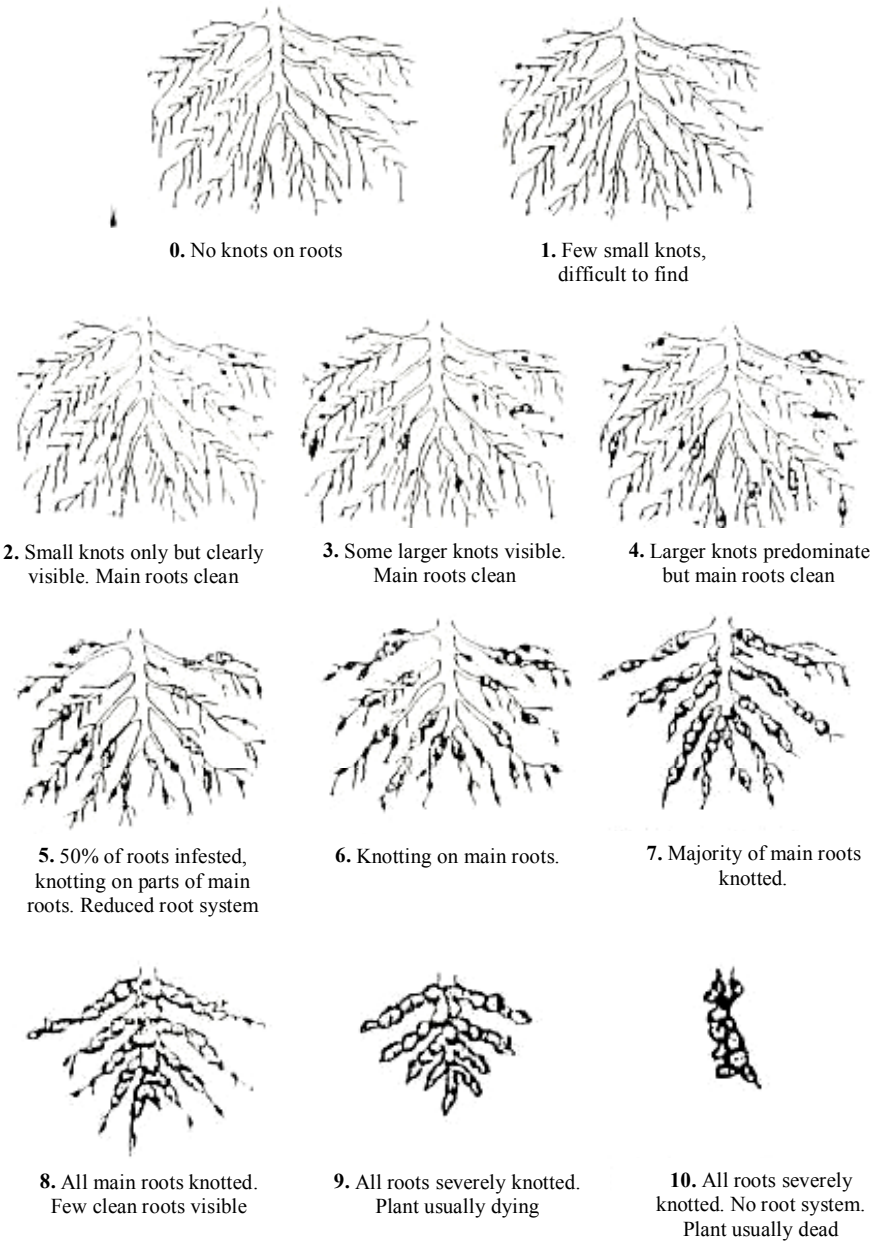

Fig. 3. Root-Knot Nematode Rating Chart taken from [17].

3. RESULTS

2.2.3. Introduction and micropropagation in vitro

At Santa Catalina Experimental Station in vitro cultures of *S. quitoense* var. Baeza were conducted as follows: For disinfection, naranjilla stem segments were washed with running water, liquid soap and disinfectant (Povidin); Benlate fungicide at 1 % (v/v) was also used. Residues from the disinfection process were washed out profusely with distilled water. The Initiation medium consisted of MS formula supplemented with 2 mg/l Calcium panthotenate, 0.25 mg/l GA_3, 10 mg/l Putrescine, 20 g/l Sucrose, 6,5 g/l Agar and 5 g/l Activated Charcoal. The micropropagation medium was as above except that activated charcoal was omitted.

3.1. Mutation induction of naranjilla by using seeds

3.1.1. Radiosensitivity test for seeds

Solanum quitoense varieties showed a similar dose response, and there was no difference in radiation effect on seedling height between doses of 100 and 250 Gy. The germination rates for Peluda were 80, 70 and 60% and for Baeza 60, 70, 80% for control, 100 Gy and 250 Gy, respectively. Germination was delayed by about one week in gamma-treated seeds compared to the control. Doses of 500 Gy and above inhibited germination completely. A more precise radiosensitive test with doses ranging from 50 to 450 Gy and including a higher number of seeds per treatment was recommended.

A mutation experiment conducted in naranjilla at INIAP, including ^{60}Co radiation doses from 50 Gy to 600 Gy, at 50 Gy intervals, showed 50 Gy as the optimum intensity to use. Then, 2,000 seeds of Baeza were irradiated with 50 Gy.

3.1.2. Greenhouse evaluation

By using the conditions described above 1,500 mutated seeds germinated and seedlings were ready for transplanting. These plants and controls were established under greenhouse conditions by using careful aseptic procedures.

Table II shows data from the preliminary experiment conducted with five irradiated seeds and five control plants from the greenhouse previous to the total evaluation. The control plants (non-irradiated) exhibited an increase in the nematode population in a range of 3.6 to 33.6 times, with an average of 10.9. This index of increment is usual under greenhouse conditions and the variability was due to other causes of the trial management. On the other hand, irradiated plants showed different nematode increase index; for example, plant No. 3 presents an (I) value of 0,06 (I=fP/iP=324/5000=0.06), so this plant can be considered resistant (once that the initial nematode population has decreased). Additionally, values of plant height (PH) and foliage fresh weight (FFW) of plant No 3 (38 cm and 104 g) are higher than those of the control (30 cm and 71 g), so that it was also possible to determine that tolerance is present for this plant.

Based on the above results, 1420 out of 1500 plants of naranjilla were also evaluated under the same methodology (data not shown). Then, 162 plants (11.4%) presented resistance-tolerance to nematodes, 566 plants (39.8%) were resistant-non tolerant, 302 plants (21.3%) were susceptible-tolerant, and 390 plants (27.5%) were susceptible-non tolerant (Figure 4).

Of the 162 resistant-tolerant plants, 125 were selected because they presented values of 0.0 to 0.5 (increment index) for nematode reproduction and superior values of PH and of FFW than the control one. Additionally, the irradiated plants presented average plant height of 12 cm, with 4.7 leaves per plant and a leaf size of 12.99 cm long per 11.78cm wide, whereas the control plants were 10.83 cm high, had 4.4 leaves and a leaf size of 11.37 cm long by 10.23 cm wide.

TABLE II. DATA FROM PLANT LENGTH (PL), WEIGH FRESH OF THE FOLIAGE (WFF), INITIAL POPULATION (IP), FINAL POPULATION (FP), POPULATION INCREASE (I) AND RESPONSE OF NEMATODE ATTACK IN IRRADIATED PLANTS AND NON IRRADIATED PLANTS

Treatments	PH (cm)	FFW (g)	Ip (eggs/pot)	FP (eggs/pot)	I	Response
Control No.						
1	36	70	5,000	35,264	7.1	
2	31	83	5,000	168,150	33.6	
3	27	75	5,000	17,840	3.6	
4	26	36	5,000	30,720	6.1	
5	30	90	5,000	41,800	4.2	
Average	**30**	**71**			**10.9**	
Irradiated No.						
1	43	219	5,000	8,308	1.6	S
2	40	109	5,000	48,816	9.7	S
3	38	104	5,000	324	0.06	R
4	35	154	5,000	8,190	1.6	S
5	40	251	5,000	10,560	2.1	S

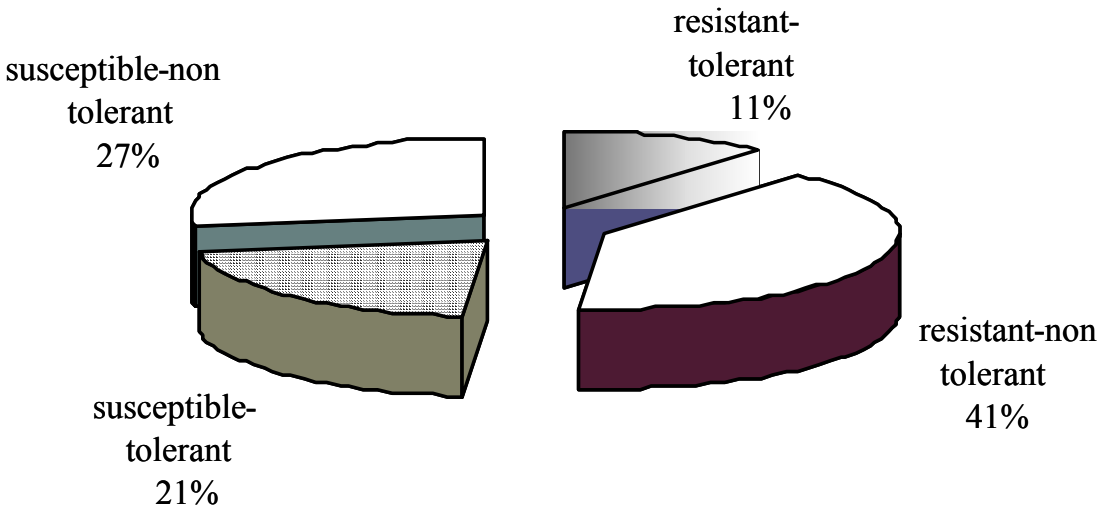

Fig. 4. Results from evaluation of nematode resistance of 1500 plants of Solanum quitoense M1p from irradiated seeds.

The selected plants presented typical phenotypic characteristics to the common Baeza variety (plant height (140 cm in adult plants), anthocyanin pigments in flowers, buds and leaf veins. Spines were absent and hairs present in the leaves, buds, fruits and young stems. However, many plants showed different characteristics from the Baeza type, 8% showed curled leaves, 1% presented atypical spines, 1.4% purple leaves and 0.1% fasciated stems.

3.1.3. Field evaluation

Selected resistant plants from the greenhouse were propagated vegetatively and 576 plants were obtained. The objective of the field plot was to obtain seeds for future characterization and evaluation for nematode resistance. At the beginning, 98% of the plants adapted to the environmental conditions. However, at the end of the experiment only 6% of the plants survived, fruits were collected from 35 resistant plants and seeds were extracted, processed and stored in cold (-15°C).

These materials were lost due to severe attacks of *Fusarium oxysporum* and root wilt (*Pseudomonas solanacearum*). In both cases, the first symptoms appeared after the flowering period, when wilt and chlorosis were observed, followed by flower and fruit drop and, ultimately, the death of the affected plants. In the case of the attack of *Fusarium oxysporum*, the symptoms included dark spots located in the nerves of the plant. Symptoms related to *Pseudomonas solanacearum* were observed as wilt in the root system.

Agronomic characteristics were registered for this material: number of flowers per inflorescence (18), days to flowering (108), days to harvesting (235), weight of fruit (78 g) and green pulp color. The values regarding days to flowering and days to harvesting included the average from plants that produced mature fruits (35 plants) and were morphologically similar to the Baeza variety. These materials have the following codes: 1255, 1201, 1087, 1008, 920, 756, 740, 662, 634, 626, 598, 545, 525, 479, 470, 460, 459, 457, 403, 401, 399, 373, 340, 303, 295, 277, 265, 248, 165 (1), 165 (2), 148, 70 (1), 56, 39 (1), 37 (1).

3.2. Mutation induction of naranjilla by using *in vitro* explants

3.2.1. Greenhouse evaluation

Seventy plants from *in vitro* materials (Vegetative 4) sent from Vienna were evaluated: 33 plants (5 GyV4), 24 plants (7 GyV4), 2 plants (8 GyV4) and 13 plants (9 GyV4). Thirteen plants irradiated at 5 Gy were evaluated as resistant. None of them presented resistance to nematodes (data not included), 94,6% were susceptible non-tolerant and 5,5% susceptible non-tolerant.

From the evaluation of *in vitro* materials with doses ranging from 1 Gy to 12 Gy, we could determine that doses 5 Gy and 7 Gy were optimal.

In vitro multiplication to vegetative 4 (V4) generation was conducted upto 1,000 plants of naranjilla (5 Gy dose). Then, greenhouse evaluation was conducted to these plants obtaining 13 resistant plants (Table III). These plants are maintained in greenhouse conditions and *in vitro* multiplication will be carried out. Table IV, includes data from plants used as controls.

Other 1,000 plants (7 Gy) have been multiplied by using tissue culture and currently are being evaluated in the greenhouse (data forthcoming).

4. CONCLUSIONS

The evaluation of mutated seeds of naranjilla in generation M_1 at Santa Catalina Experimental Stations was a technical error. However, nematode resistance was already observed at this generation. Factors such as the large amount of seeds needed to continue evaluating materials, the large amount of space that this species needs for planting in the field and, also, the life cycle of naranjilla (8 months), made the evaluations difficult. As a consequence, we considered continuing the study with mutated plants by using *in vitro* propagation.

We obtained important resistant material of naranjilla from 5 Gy dose in Vegetative generation 4 (V4), which are being multiplied *in vitro*. We are waiting for the greenhouse evaluation of other 1,000 plants from another one of the optimal doses (7 Gy V4) to continue their evaluation in the field. As nematode resistance must be tested in the field, the last step will be crucial for getting resistant materials to overcome the environmental and technical problems that farmers from the center of production of this species experience in their farms.

TABLE III. NEMATODE RESISTANT PLANTS OF *Solanum quitoense* FROM *IN VITRO (VEGETATIVE 4)* AFTER GREENHOUSE EVALUATION OF 1,000 PLANTS (DOSE 5 Gy)

Code	PH (cm)	FFW (g)	IP (egss/plant)	FP (egss/plant)	I	Reaction
5 GyV4*** Ba 13	31	250	10,000	5,821.2	0.58	RT*
5 GyV4Ba 084	23	50	10,000	9,762.0	0.98	RS**
5 GyV4Ba 126	33	250	10,000	1,826.0	0.18	RT
5 GyV4Ba 163	28	120	10,000	7,404.0	0.74	RS
5 GyV4Ba 165	26	160	10,000	5,860.5	0.59	RS
5 GyV4Ba 206	62	700	10,000	7,855.6	0.79	RT
5 GyV4Ba 248	54	750	10,000	182.8	0.02	RT
5 GyV4Ba 264	41	300	10,000	7,161.0	0.72	RT
5 GyV4Ba 316	68	950	10,000	5,203.2	0.52	RT
5 GyV4Ba 431	40	300	10,000	7,030.8	0.70	RT
5 GyV4Ba 634	73	500	10,000	1,147.7	0.11	RT
5 GyV4Ba 851	33	180	10,000	551.68	0.06	RT
5 GyV4Ba 852	50	500	10,000	395.04	0.04	RT

*RT= Resistant-tolerant; **RS= Resistant-susceptible; ***= Vegetative 4

TABLE IV. DATA FROM NARANJILLA PLANTS USED AS CONTROLS FOR THE NEMATODE RESISTANCE EXPERIMENT

Plants	PH (cm)	FFW (g)	IP (egss/plant)	FP (egss/plant)	I
Control 2	27	250	10,000	16,422	1.64
Control 2	50	150	10,000	24,829	2.48
Control 2	53	146	10,000	58,057	5.81
Control 2	24	91	10,000	216,770	21.6
Control 2	43	169	10,000	207,360	20.74
	39.4	161.2	10,000	104,687.6	10.45

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CHARACTERIZATION OF *Chenopodium* GERMPLASM, SELECTION OF PUTATIVE MUTANTS, AND THEIR CYTOGENETIC STUDY

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Abstract

Forty two lines of *Chenopodium quinoa* Willd., including mutants obtained through radio-induced mutagenesis, and two accessions of the landrace "chía roja" of *Chenopodium berlandieri* ssp *nuttaliae* were characterized in terms of morphology, productivity and saponin content. These studies were aimed at selecting superior genotypes to be offered as alternative crops to peasants inhabiting regions with marginal agricultural conditions in Mexico State. Multivariate analysis allowed distinguishing groups of *Chenopodium* germplasm according to traits related to plant architecture, stem diameter and yield. Three putative mutant lines exhibited high yield and low saponin content in the M_7 generation and the landrace "chía roja" was characterized as a natural low saponin chenopod. Preliminary results on molecular markers (RAPDs) permitted to distinguish low- from high-saponin content germplasm. Cytological studies showed that *C. quinoa* variety Barandales chromosome complement is 2n=4X=36, X=9, with a chromosome length of 1.26–2.05 μm, the total chromatin length being of 57.24 ± 0.21 μm, and with an asymmetry index of TF%=44.86. Flow cytometry techniques determined a 2C DNA content of 2.96 ± 0.01668 pg, and a genome size (1C) of 1413 Mbp.

1. INTRODUCTION

Through history, mankind has met its food needs with only 5,000 plant species, representing 1% of the world flora, and big nuclei of human population depend essentially on three cereals (wheat, rice and corn) and one tuber (potato) for subsistence [1].

As time has elapsed, our dependence on a reduced group of species has increased and the selection and breeding processes have been concentrated on the main crops (wheat, rice, maize, potato, soybean, barley among others) and some crops, such as the pseudo-cereals, have been neglected, notwithstanding that once, in the history of ancient prehispanic cultures, they had a predominant role as staple foods.

Nowadays, when the search for alternative crops with such attributes as high nutritional value, hardiness and low demand of agrochemicals is increasing, these under-utilized crops are being rediscovered as super crops of the future [2].

The term pseudo-cereals is applied to those plants whose grains are like cereals: rich in mealy materials, able to be used in the elaboration of bread, but pertaining to the genera *Amaranthus* or *Chenopodium* [3].

Seeds from species of both genera, which indistinctly received the nahuatl name of huahtli, had a relevant role as food and also in the religious ceremonies of the Aztec civilization, as

documented in the Mendoza Codex, listing the taxes that the 363 villages dominated by the Aztecs had to pay to the Emperor Montezuma. In that list, the *huautli* had an outstanding position as a valuable crop with an annual payment of 7,000 t, quantity excelled only by that of maize, beans and chía (*Salvia hispanica*) [3].

The indigenous landraces huauzontle and chía roja (*Chenopodium berlandieri* ssp. *nuttalliae)* and the amaranth (A*maranthus hypochondriacus* and *A. cruentus*) can be cited among the pseudo-cereals that had their place of origin and dispersion in Mexico [4]. Together with the quinoa (*Chenopodium quinoa* Willd.), these ancient crops are considered nowadays as an alternative with great potential to contribute to solve the malnourishment problems affecting great sectors of population belonging mainly to marginal rural zones [5].

Research on pseudo-cereals, particularly on *Chenopodium quinoa* Willd. as an alternative crop for agricultural marginal conditions in Mexico goes back to the early seventies in the Colegio de Postgraduados at Chapingo Mexico, when the first trials on adaptability of varieties and studies on physiology and genetic variance components were performed [6]. In the late eighties, the Instituto de Investigación y Capacitación Agropecuaria Acuícola y Forestal del Estado de México (ICAMEX) began an evaluation program searching for quinoa varieties able to withstand adverse conditions such as low soil fertility, drought, and frosts, which prevail in many areas of the Mexico State. As result of those evaluations, four varieties emerged as a suitable alternative to be offered to peasants: Isluga, Barandales, Sierra Blanca and Lípez [7]. However, their high saponin content was considered as a negative factor for their diffusion, and a coordinated research program between ICAMEX and the Instituto Nacional de Investigaciones Nucleares (ININ) was therefore launched in 1990, devoted to the application of radio-induced mutagenesis techniques to obtain low-saponin mutants of the variety Barandales.

The approach followed included first a radiosensitivity test concluding that the LD_{50} was 225 Gy [8]. Based upon these results, seeds of Barandales were irradiated at 200 and 250 Gy. Selection of mutants was performed in the second generation (M_2), regarding traits such as earliness, size of panicle, productivity, and saponin content. From the evaluation in M_2, 88 putative mutants were selected and, from M_3 to M_7, a screening process led to the selection of 28 putative mutants [9].

Additionally to the materials generated by mutagenesis, 13 advanced quinoa lines generated by the quinoa breeding program at the Colegio de Postgraduados, were available to our program through a donation made by the National Germplasm Bank from Chapingo [9].

Considering that one of the aims of this research is to generate low-saponin, high- yielding quinoa lines, also activities of exploration, recollection and characterization of native germplasm from *Chenopodium berlandieri* ssp. *nuttaliae* landrace chía roja (the only chenopod whose seeds are consumed in Mexico), were undertaken. This valuable germplasm could thus be incorporated into the breeding program of quinoa, as progenitor in future hybridisations aiming to introduce the low-saponin content trait from chía roja to quinoa.

This paper presents the findings of a research contract partially funded by the International Atomic Energy Agency (10427/RBF) related to the characterization of germplasm of *Chenopodium* and selection of putative mutants, and their cytogenetic study.

2. MORPHOLOGICAL AND YIELD CHARACTERIZATION OF *Chenopodium* GERMPLASM

Aiming to characterize morphologically and regarding to yield 42 lines of *C. quinoa* and two accessions of *C. berlandieri* (Table I), an experiment was carried out during the crop cycles 1999 and 2000, at the Experimental Station of the Facultad de Ciencias Agrícolas from Universidad Autónoma del Estado de México, at Tlachaloya, Toluca, México State. The evaluated germplasm was constituted of 28 lines derived from the Barandales variety and obtained through radio-induced mutagenesis at 200 and 250 Gy, 14 advanced lines and varieties of quinoa donated by the quinoa breeding program at Colegio de Postgraduados, Chapingo, México and two accesions of native germplasm of *C. berlandieri* ssp. *nuttaliae* landrace chía roja, collected from villages near the Patzcuaro lake at Michoacán, where it is grown and consumed without any treatment provided that they have no saponins.

TABLE I. GENOTYPES EVALUATED IN THE EXPERIMENT OF MORPHOLOGICAL CHARACTERIZATION

C. quinoa Barandales Lines from material irradiated at 200 Gy	*C. quinoa* Barandales lines from material irradiated at 250 Gy	Advanced *C. quinoa* lines from the National Germplasm Bank Chapingo	*C. berlandieri* subespecie *nuttaliae* Land race Chía Roja
20 R3–33	25 R1–36	20 Yu–116	Accesion 1
20 R1–27	25 R3–19	44 Tahuaco	Accesion 2
20 R1–9	25 R3–18	15 Sajama	
20 R1–8	25 R2–29	49 Amarilla de Marangani	
20 R3–54	25 R2–12	64–03–04	
20 R3–42	25 R1–7	11–l–240	
20 R1–6	25 R2–38	42 Amarilla de Marangani	
20 R3–7	25 R1–22	17 Yu–42	
20R3–3	25 R3–38	5 Yu –117	
20 R1–41	25 R1–11	4 Yu–115	
20 R1–10	25 R3–17	9 L–111	
20 R3–19	25 R3–11	2 Yu–112	
20 R3–17	25 R2–43	13 Yu 46	
20 R3–47	25 R2–23	10 L–112	
CONTROL	Barandales M5–0		

The experimental design was randomised blocks, with four replications, each plot consisting of three rows three meters long, with a planting distance of 15 cm between plants and 80 cm between rows.

Evaluated variables were plant height (PH), panicle length (PL), panicle diameter (PD), stem diameter (SD), branch number (BN), internode number (IN) and yield (Y). These variables were chosen considering they are good selection indexes [10].

The analysis of variance (ANOVA) was performed and a Tukey test was done. Linear regression was applied in order to determine the relationship among variables. Multivariate analysis of main components was performed to try to explain the existing variability and a cluster analysis, using the mean euclidean distance, was applied to group the genotypes according to similarity. These analyses were performed applying the Statistical Analysis System (SAS) software version 8 [11].

2.1. Morphological characterization

The ANOVA for the seven evaluated variables exhibited statistically significant differences among genotypes for six characters (Table II). Stem diameter had no statistical significance. According to Tukey means test (data not shown), the *C. berlandieri* sbp. *nuttaliae* landrace chía roja, exhibited the tallest height with a mean of 191 cm, and was statistically different from all the genotypes tested in this trial. The minimum height value was for material irradiated at 200 Gy, with a mean height of 80 cm (70 cm for 20 R3–17). It is interesting to note that chía roja is a very tall and vigorous plant whose mean height excels most of quinoa cultivars. Indeed, in an evaluation of 1512 accessions of *C. quinoa,* the maximum height value was 174 cm [12]. Regarding panicle length, both the minimum and maximum values (76 cm and 23.8 cm) corresponded to the irradiated lines 20 R1–6 and 25 R3–17, while for panicle diameter, Sajama exhibited the maximum value with 47 cm and chía roja showed the minimum value with 13.2 cm. Stem diameter had a mean value of 1.1 cm, and there were no significant differences between genotypes.

Regarding the number of branches, the highest value was for line 20 R2–27 the minimum value being for the non branching landrace chía roja which, instead, showed the highest number of internodes (27) contrasting with line 25 R2–43 which had only three internodes on average. The highest yield, estimated on a t/ha basis, was for line 20 R2–27 with 2.016 t/ha, while the lowest (0.193 t/ha) was for line 20 R1–9.

TABLE II. STATISTICAL PARAMETERS FOR THE EVALUATED TRAITS OF *Chenopodium* GERMPLASM

Trait	Range	Mean	SD[1]	CV[2]
Plant height (cm)	69.9–191.0	98.2	14.2	14.2
Panicle length (cm)	23.8–75.9	38.0	4.29	10.6
Panicle diameter (cm)	13.2–47.0	29.3	3.19	10.9
Stem diameter (mm)	0.8–1.6	1.1	0.17	15.8
Number of branches	0–14.5	9.7	2.9	30.3
Internodes number	2.8–27.1	4.9	1.1	23.4
Yield kg/ha	193–2016	708	65.2	9.2

[1]SD= Standard deviation, [2]CV=Coefficient of variation

A correlation analysis (Table III) exhibited a positive association between height and all evaluated traits (panicle length and diameter, internode number, stem diameter and number of branches) except yield, indicating that the evaluated lines have a good architecture and confirming previous results [12]. Panicle length showed a positive association with panicle diameter, stem diameter number of internodes, and number of branches. Similar associations

had been found in a previous study of the pattern of genetic diversity in quinoa [13]. Diameter and length of panicle had a significantly positive relationship as also found in the past in a morphological evaluation of 153 quinoa accessions [14].

TABLE III. CORRELATION MATRIX FOR SEVEN TRAITS IN EVALUATED MATERIAL

	Plant Height	Panicle length	Panicle Diameter	Stem diameter	Branch Number	Internode number
Plant Height	0.4772[a]					
	0.0001[b]					
Panicle	0.36025	0.25989				
length	0.0001	0.0001				
Panicle	0.48176	0.32164	0.47097			
Diameter	0.0001	0.0001	0.0001			
Branch	0.17886	0.16217	0.00749	0.04646		
Number	0.0002	0.0007	0.8760	0.3326		
Internode	0.30152	0.28131	0.01421	0.00774	0.60472	
number	0.0001	0.0001	0.7670	0.8719	0.0001	
Yield	0.07563	0.07669	0.02628	0.04803	0.02076	0.06605
	0.1209	0.1157	0.5904	0.3249	0.6707	0.1756

[a] Correlation coefficient.

[b] Probability ($P<\alpha$)

The first three components account for almost 75% of the total variation (Table IV).

TABLE IV. LATENT ROOTS AND LATENT VECTORS ASSOCIATED WITH THE FIRST THREE PRINCIPAL COMPONENTS

	CP1	CP2	CP3
Latent roots	2.7411	1.3753	1.1135
Percentage of accumulated variance	39.16	58.81	74.72
Latent vectors			
Plant height	0.5083	0.2312	-0.0566
Panicle length	0.4340	-0.0614	0.2670
Panicle diameter	0.3428	0.3419	-0.0241
Stem diameter	0.1949	0.6983	-0.1803
Number of branches	0.4236	-0.4650	0.0357
Internodes number	0.4573	-0.3390	-0.3131
Yield	0.0947	0.0832	0.8904

The first principal component accounted for more than 39% of the total variance. Plant height, internode number, panicle length and number of branches were the variables with the largest positive loading, so this component allows to discriminate for lines with outstanding architecture. The second component accounted for nearly 20% of the total variance. The stem and panicle diameter were the variables with the highest positive loading, so this component allows discriminating for loading resistance. The third component, which accounts for 16% of total variance, was associated with yield, so this component discriminates for traits related to yield.

2.2. Cluster analysis

Figure 1 exhibits the cluster analysis. At a euclidean initial distance of 0.24 and final of 0.81, seven clusters are formed, each containing highly similar materials. Table V presents mean characteristics of each cluster.

TABLE V. COMPARISON PROFILE OF SEVEN GROUPS OF QUINOA LINES (FIGURES ARE MEANS IN EACH CLUSTER)

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Cluster	HP[a] (cm)	PL (cm)	PD (cm)	SD (cm)	NB	NI	Y g/plant
1 (2)[b]	109.90	42.25	34.00	1.29	14.00	5.30	34.46
2 (19)	102.61	42.49	33.58	1.14	12.41	5.19	15.57
3 (5)	107.53	35.42	41.25	1.35	11.35	5.65	8.67
4 (3)	100.78	37.53	36.77	1.48	7.13	3.77	18.97
5 (2)	105.85	40.80	18.95	1.31	7.10	5.50	10.38
6 (13)	85.33	33.73	26.95	1.10	8.62	4.17	12.93
7 (1)	122.50	42.75	24.90	1.12	13.40	8.10	9.00

[a]HP=Plant height , PL= Panicle length, PD= Panicle diameter, SD= Stem diameter, NB= Number of branches, NI Number of internodes, Y= Yield
[b]Number in brackets is the number of lines per cluster

Groups 1 and 2 are formed only with lines obtained by mutagenesis, which indicates the great similarity existing among them. This may be due to their common origin (var. Barandales), and is understandable because radio-induced mutagenesis affects only one ore two characters of a given genotype leaving the rest of the traits almost unchanged. However, it is noteworthy that there is a big difference between the putative mutants belonging to groups one and two and the original variety (M5–0), which is clearly separated from them and belongs to group 6.

This is explained partially by the difference in plant height, because putative mutants from groups one and two are higher than Barandales variety (Table V). Group 3 includes three germplasm lines and two mutant lines, while groups 4 and 5 include only lines and varieties from the germplasm bank, group 6 includes five lines of irradiated material, and the control, six lines from the germplasm bank and one chía roja accession. Finally, group seven is characterised by the remaining accession of chia roja, which, according to the characteristics evaluated, forms a completely distinct group from the quinoa materials (Figure 1).

3. ESTIMATION OF SAPONIN CONTENT

3.1. Afrosimetric estimation of saponins

One of the reasons to incorporate quinoa as an alternative crop for peasants in regions with marginal agricultural and economical conditions is to diminish the degree of malnutrition. Therefore, the evaluation of saponin content is a relevant factor since low-saponin content varieties will have a better acceptance. Thus, the saponin content was estimated in putative mutant lines, following the method established in [15].

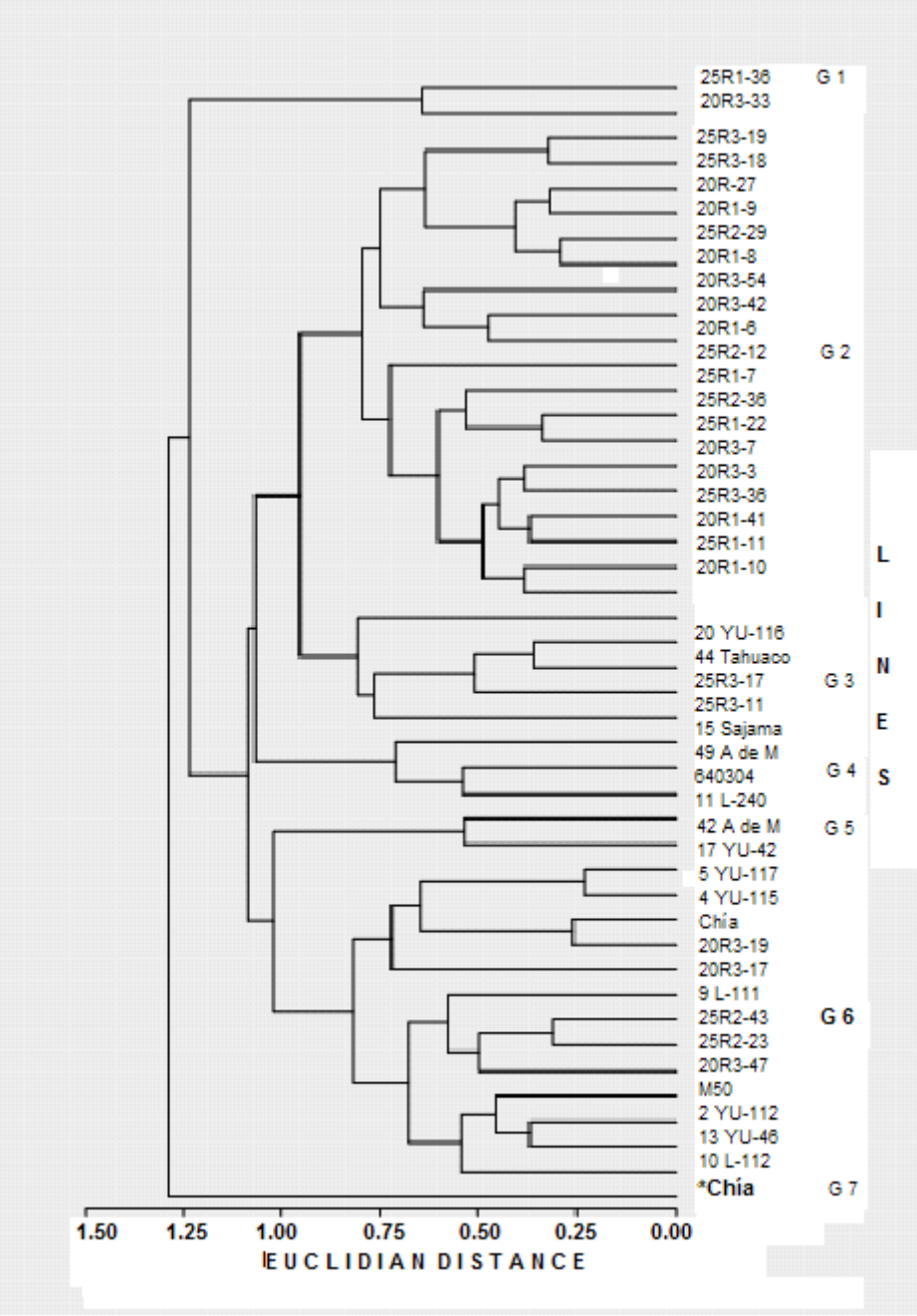


Fig. 1. Dendrogram generated by cluster analysis of seven traits of germplasm studied.

Figure 2 shows the percentage of saponins found in several putative mutants, the landrace chía roja and the varieties Barandales and Sajama. Barandales variety exhibited the highest saponin percentage (2.7%), while Sajama presented a low level of saponins (1%), and putative mutants 25 R3–19, 20 R3–47, 20 R3–54 and chía roja presented the minimum saponin content**.**

3.2. Molecular markers for saponin content

Aiming to identify some molecular markers to differentiate among lines with high and low saponin content RAPD was employed, following methods established in [16]. DNA isolated from quinoa variety Barandales, from putative mutants obtained at 200 and 250 Gy (20 R3–

47, 20 R3–54 and 25 R3–19), and from the Sajama variety, which is considered as a natural low-saponin material, was employed.

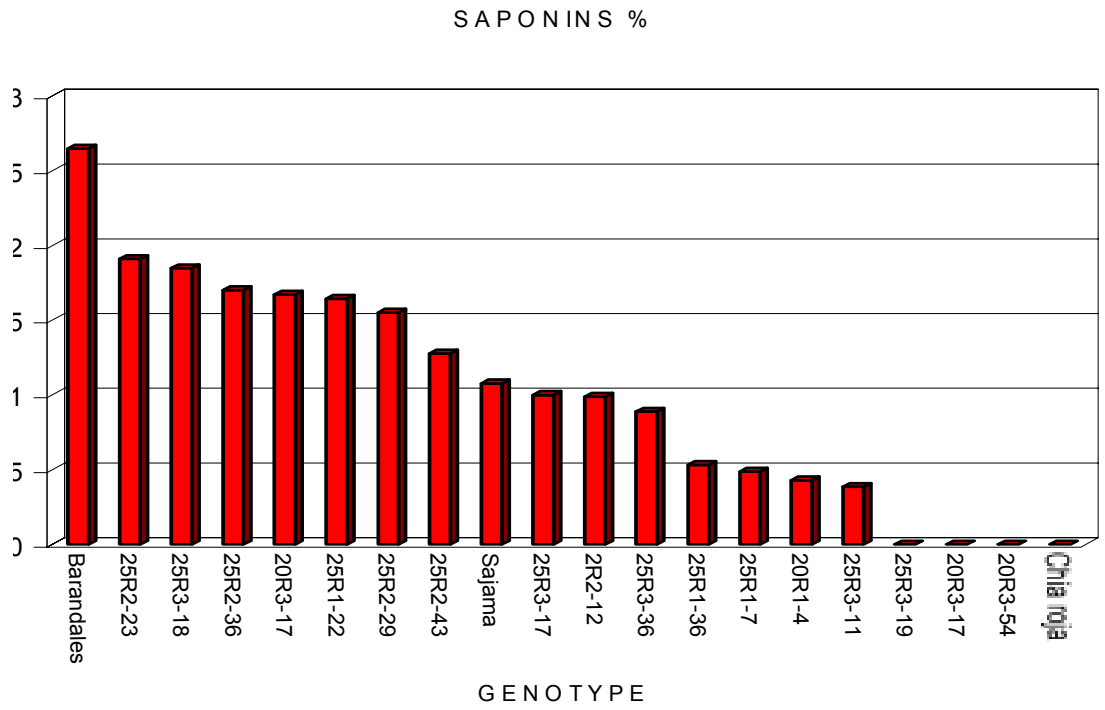


Fig. 2. Estimation of saponin content in putative mutant lines, varieties Barandales and Sajama and the land race chía roja.

Figure 3 shows preliminary results, where two fragments in primer 6 are present in the low-saponin content variety Sajama and also in the low-saponin mutants obtained at 200 and 250 Gy. So, this marker may be useful in order to distinguish among low- and high-saponin genotypes, but further studies must be performed.

4. CYTOLOGICAL STUDIES

4.1. Karyotype

Cytological studies concerning karyotype, genome size and DNA content were performed to characterize the genotypes and to establish affinity among them.

For karyotype determination, 5 plants were studied per taxa, using metaphase cells from root-tips. After the treatment described in [17], cells were observed to photograph the chromosomes for the elaboration of the karyotypes and idiograms.

long arm length, percentage of relative length (L%) were taken as established in [18]. The asymmetry index (TF) was calculated as in [19].

For *C. quinoa* Barandales the chromosome number is 2n=4X=36, X=9 confirming results reported for the specie in Reference [20]. Chromosomes are metacentric and two pairs have satellites (1–2 and 5–6) (Figure 4). The range of chromosome length is 1.26–2.05 µm. Total chromatin length is 57.24±0.21 µm, and the asymmetry index (TF) is 44.86% [21]. Karyotypes from landraces chía roja and huauzontle are in process.

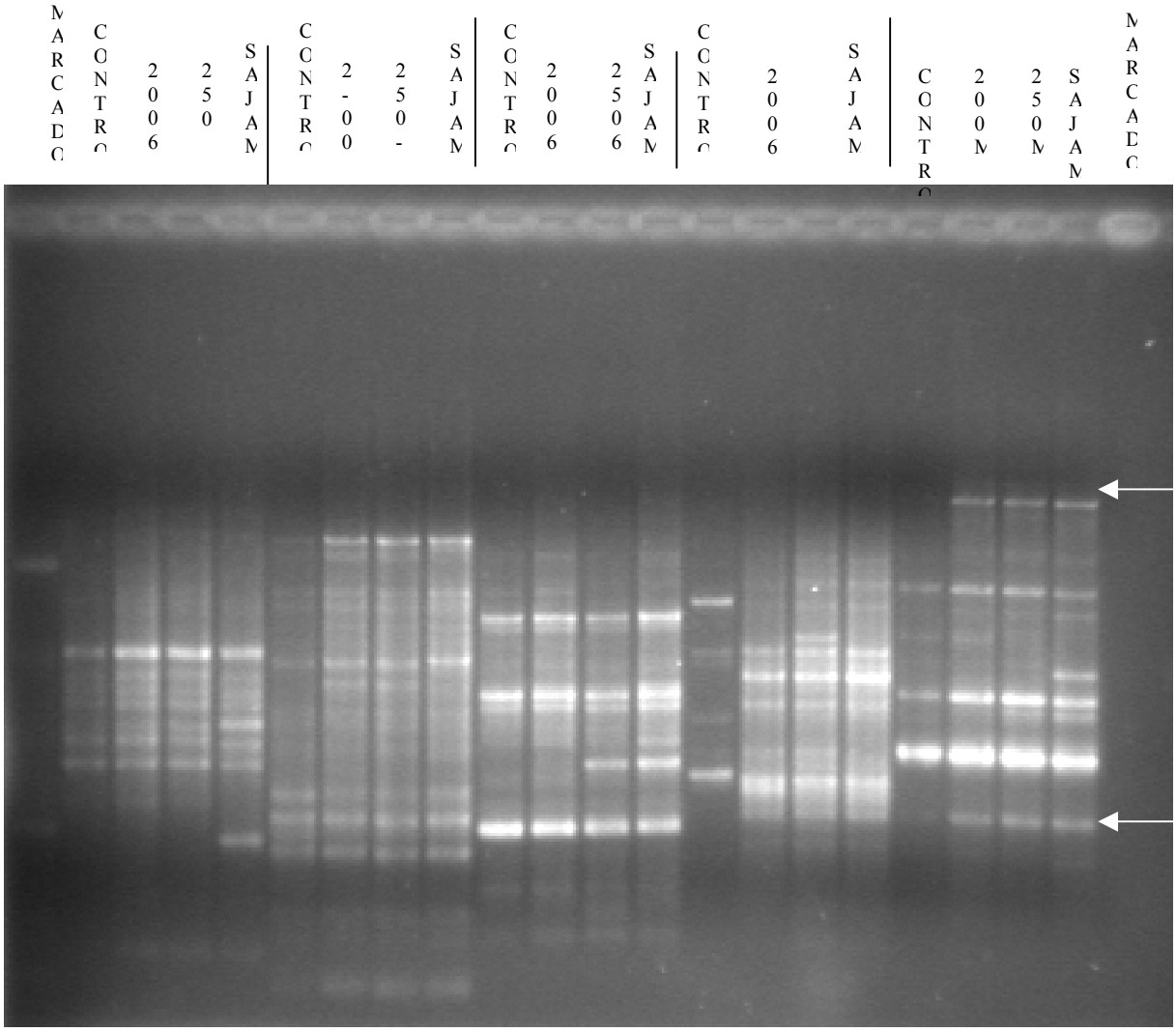


Fig. 3. Electrophoretic pattern of DNA amplified with Gybco primers. Quinoa var. Barandales, control, irradiated to 200 and 250 Gy, and Sajama. The arrows indicate fragments present only in low-saponin genotypes.

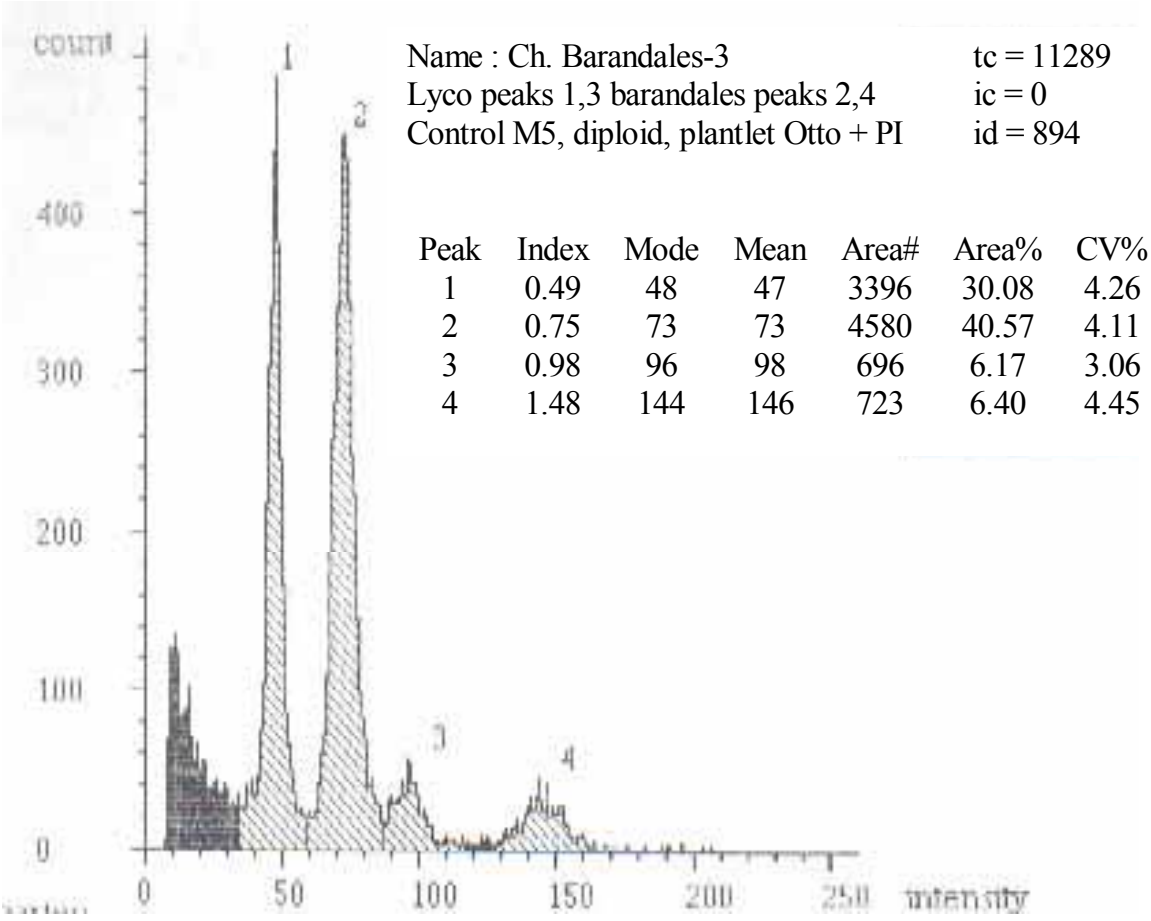
Chromosomes were classed according to centromere position and measurements of short and

Fig. 4. Karyotype of Chenopodium quinoa var. Barandales 2n=36. Escala 10 μm.

4.2. DNA content and genome size

The DNA content (picograms) and genome size in million base-pairs (Mbp) was evaluated applying flow cytometry techniques, after standardization of the process utilizing as control the specie *Lycopersicum esculentum* cv stupicke polni tyckove rane (DNA content 2C=1.96).

The staining material was propidium iodide (PI) and the buffer selected was Otto. Figure 5 shows the DNA histogram of *C. quinoa* var. Barandales. The variation coefficient is less than 5% and the amount of DNA 2C is 2.96±0.01668 pg, and the genome size (1C) is 1413 Mbp [21] Results of studies on putative mutants, varieties and lines of interest of *C. quinoa* and *C. berlandieri* are under analysis.



Peak	Index	Mode	Mean	Area#	Area%	CV%
1	0.49	48	47	3396	30.08	4.26
2	0.75	73	73	4580	40.57	4.11
3	0.98	96	98	696	6.17	3.06
4	1.48	144	146	723	6.40	4.45

Fig. 5. DNA histogram for C. quinoa var. Barandales.

5. CONCLUSIONS

Multivariate analysis allows distinguishing groups of *Chenopodium* germplasm according to traits related to plant architecture, stem (which can be associated with lodging resistance) and yield.

Some mutant lines exhibited high yield and low saponin content in the generation M7 and the land race chía roja is characterized as a natural low saponin *Chenopodium* land race. Preliminary results on molecular markers (RAPD) give the possibility to distinguish among low and high saponin content germplasm.

C. quinoa variety Barandales has a chromosome number 2n=4X=36, X=9, The range of chromosome length is 1.26 – 2.05 μm and total chromatin length is 57.24±0.21 μm, with an asymmetry index TF %= 44.86. Its DNA content 2C is 2.96±0.01668 pg, and the genome size (1C) is 1413 Mbp.

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BREEDING OF BITTER POTATO (*Solanum juzepczukii*) THROUGH MUTATION INDUCTION AND TISSUE CULTURE TECHNIQUES

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Abstract

Potato and other Andean tubers are the most important cultures in Bolivia. Bitter potatoes are cultivated in high areas (4000 m above sea level) and are among the few species that tolerate abiotic factors including freeze. However, the high content of glycoalkaloids is a great inconvenient for their use as food, and using nuclear techniques is an alternative to lower the total glycoalkaloid contents, especially for solanine and solasodine. Thus, tubers of bitter potatoes and cultivated potato were collected in the main centres of genetic diversity in Humanata, Puerto Acosta, Escoma, Ambana, Moco Moco and Italaque. In La Paz, they were submitted to thermotherapy and meristem culture to eradicate viruses eventually present. Thereafter, media were optimised for micropropagation to dispose of enough plantlets to carry out dosimetry studies. Optimum doses were 22 Gy for Bola luk'y and 28 Gy for Luk'y Kheto. Large-scale multiplication was undertaken from M_1V_1 to M_1V_4 in order to eliminate chimeras, and 2000 plantlets were weaned for each variety. Mutant genotypes were identified according to phenotypic changes using a descriptor, and by changes in the level of ploidy. Due to the scarce supply of tubers, they were multiplied in the field. Once this was done, methodologies were developed for the biochemical analyses of the total glycoalkaloid content (TGA). Correlating these variables we identified one Bola luk'y mutant (clone 107) with ploidy changes and 30 mg TGA/100 g fresh tuber, and another one (clone 1564) with less than 50 mg TGA/ 100 g fresh tuber. In addition, for the variety Luk'y Kheto 12 mutant clones were identified. Among them, clones 379 and 399 presented ploidy changes and the most favourable TGA content ranging between 40 and 49 g TGA/100 g fresh tuber, respectively. The other mutant clones, i.e. 568,592, 768, 805, 929, 959, 978, 1005 and 1036 also presented morphological changes but these were coupled with a higher TGA content. This material should now be evaluated under the conditions typical of their natural habitat (high altitude) and to assess their responses when confronted with diseases. Finally, they will have to be characterized molecularly before their release to the market as a new variety.

1. INTRODUCTION

The variability of the phytogenetic resources from the Bolivian Tableland is vast, principally referring to the potato (*Solanum sp.*), oca (*Oxalis Tuberosa)*, papalisa (*Ullucus Tuberosa)* and Isaño (*Tropaelum* tuberosum). Many of these varieties are endangered because of the different kinds of potato varieties marketed in La Paz, Cochabamba and Oruro. The bitter potato has a big importance in the high areas of Bolivia and Peru because of its frost tolerance, but they are not eaten because of their high glycoalkaloid concentration. To eat these potatoes it is necessary to remove the alkaloids from the tubers.

Bitter potato culture in Bolivia is usually found in the departments of La Paz, (Tableland watershed), Oruro (South Tableland), Potosí (North of Potosí) and Cochabamba (Provinces of Bolivar and Tapacari). These four departments account for 72.7% of the cultivated surface and 68.3% of the Total National Production. In La Paz and Oruro cultivation is made at 3820 m over sea level, but above 4000 m over sea level in Potosí and Cochabamba. In these micro-regions the bitter potato diversity is known as Luk'is ch'oquepitus, kaysallas, ajanhuiris and other native aymara names.

About 15% of the total production of potatoes in Bolivia belongs to varieties that are more or less bitter. In the tableland, the culture of bitter potatoes is one of the most important products representing 40% of the Bolivian Central Tableland tubers [1].

The cultivation of bitter potatoes is in a process of deterioration as a consequence of the present day improvements of agriculture**,** that result in the modification of their ecosystem. In this respect, agriculture is now based mainly in the cultivation of forages, so that commodity crops (potato, bean, vegetables, etc) are restricted to less fertile fields. The importance of the cultivation of native potatoes, including bitter potatoes, is decreasing in favour of the sweeter varieties used for the elaboration of chuño and tunta, to the point of their "marginalization". In addition, bitter potatoes are affected by new diseases (*Phytophthora infestans*, *Septoria lycopersici*, etc) and pests (*Premnotrypes* sp and *Globodera* sp) coming from them.

Most of the alkaloids in plants are present as salts of organic acids and, in certain species; there is a special acid associate. Some alkaloids, like those of *Solanum*, are glycosidic forms of rhamnose, galactose and glucose.

The steroid alkaloids present especially in *Solanum* and *Lycopersicum* species are in their majority in glycosidic form. They all possess the skeleton of the colestan with 27 carbons and one nitrogen, with a character of alkamine or alkaloid [2].

The objective of this project is to select mutant genotypes *in vitro* of varieties Bola luk´y and Luk´y kheto (*Solanum juzepczukii*), irradiated for reduced glycoalkaloid content.

The present research work is developed in the plant Biotechnology Laboratory from Centro de Investigaciones Nucleares – Viacha (CIN–IBTEN), located at 34 km from La Paz, between the parallels 16° 39' latitude south and 68° 18' longitude west, at an altitude of 3,850 meters.

2. MATERIALS AND METHODS

2.1. Recollection of genotypes of bitter potato and other varieties

We collected potato genotypes from a part of the Camacho province (north tableland) that was identified as important because of the high potato and tuber diversity. This activity was developed from April to June 1999, and samples were taken from 6 places (Humanata, Puerto Acosta, Escoma, Ambana, Moco Moco and Italaque). The recollection methodology was to profit that the communes of these zones still celebrate country public festivities. The procedure was a direct interview with the farmer [3], and use of the morphological descriptor of CIP [4].

2.2. Characterization of the 32 potato accessions

The follow up and scoring of the vegetative cycle was realized by direct observation and morphological characterization through the predominating morphology as, we could see that there is a great stability, without any changes or segregations that may interfere with the characterization work (Table I).

This material was stored *in vitro* and characterized in pc-GRIN system**.** It would be interesting to know the biochemical and cooking properties of varieties to recommend to country people some varieties for seeding and for selling at higher prices.

TABLE I. NATIVE POTATO ACCESSIONS CHARACTERIZED

No	No Laboratory	NATIVE NAME
1	02	HUAYCHA BLANCA
2	03	HUAYCHA ROJA
3	06	CH'UJU BLANCO
4	07	P''INU
5	16	C'OYU
6	19	WARISAYA
7	22	Unnamed
8	25	CH'OJU NEGRO
9	26	CH'OJU
10	30	PALA
11	32	PALA
12	39	IMILLA ROJA
13	42	CONDOR CAYU
14	43	P'INU
15	44	SURIMANA
16	45	Unnamed
17	46	SURIMANA
18	49	HUARICAYU (1)
19	54	LUK'Y WILA
20	56	PALA BLANCA
21	57	IMILLA BLANCA
22	58	ALL'KA IMILLA
23	63	SACAMPAYA
24	64	PALMA
25	68	Unnamed
26	71	WANA LAK'O
27	72	P'INU
28	73	SAQ'O
29	77	SAQ''O
30	82	AJAWIRI
31	83	IMILLA BLANCA
32	87	PITIQUILLI

2.3. Treatment of bitter potatoes for their introduction *in vitro*

Before the introduction *in vitro* and the characterization in field from the collected material, tubers were sprouted whereby mini-tubers and sprouted shoot apices were introduced *in vitro*.

One of the problems with bitter potatoes is a virus, especially PVX, PVY and PLRV. So, to avoid later problems, meristems of Bola luk'y and Luk'y kheto were introduced into broth to clean material. To achieve this the methodology described by [5] was used.

In vitro introduction. The plant material obtained from the microtubers was meristems (0.4–0.5 mm). The explants were treated with alcohol (70%) for 15 sec and then with sodium hypochlorite (1%) for 3 min, and were rinsed 4 times with sterile water.
The growing conditions were a temperature of 23°C, at 2000 lux, under a photoperiod of 12 h light and 12 h dark. The culture medium was based on MS medium [6], supplemented with 2.5 mg/l of gibberellic acid, 0.65% agar and 3% sucrose.

For virus cleansing, source explants were multiplied and maintained at 20 ºC, under a 16/8 h (light/dark) photoperiod of 3000 lux, with 15 replicates per treatment. The media tested, all based on MS medium with 0.1 mg/l Kinetin added and the pH adjusted to 5.7, were:

MR = MS + NAA 0.08 mg/l + GA_3 0.35 mg/l + CaPant 2.0 mg/l + agar 0.6% + sucrose 3.0%
MV = MS + NAA 0.04 mg/l + GA_3 0.3 mg/l + CaPant 2.0 mg/l + agar 0.6% + sucrose 3.0%
MS = MS + GA_3 0.3 mg/l + CaPant 2.0 mg/l + agar 0.6% + sucrose 3.0%.

Media MV and MS favoured the best plant growth, expressed as height and number of nodes, for both Bola Luk'y and Luk'y Kheto, according to Duncan test with a significance of 5%.

2.4. Radio-sensibility in bitter potato *(Solanum juzepczukii)*

2.4.1. Dosimetry with the Bitter Potato Plantlets

Stem cuttings from the bitter potato varieties were micropropagated in Magenta boxes with medium MV. The radiation doses: were 0 Gy (control 0 min), 15 Gy (9.15 min), 30 Gy (18.32 min) and 45 Gy (27.47 min).

The irradiations were made in the Hospital de Clínicas (Radiotherapy Unit) in La Paz, with an equipment of ^{60}Co pump for superficial therapy, (TERADI made in Argentina, model 8002C, with an activity at irradiation of 4807.25 curies and a potency dose, calculated in air, of 163.79 cGy/min at 73 cm). In Figure 1, the plant response to the different dosimetry treatments above is shown, when the three evaluations were finished at 7,18 and 35 days (one week after the irradiation step). There was a clear dose-response effect of irradiation on plant height, very useful for the determination of the LD 50.

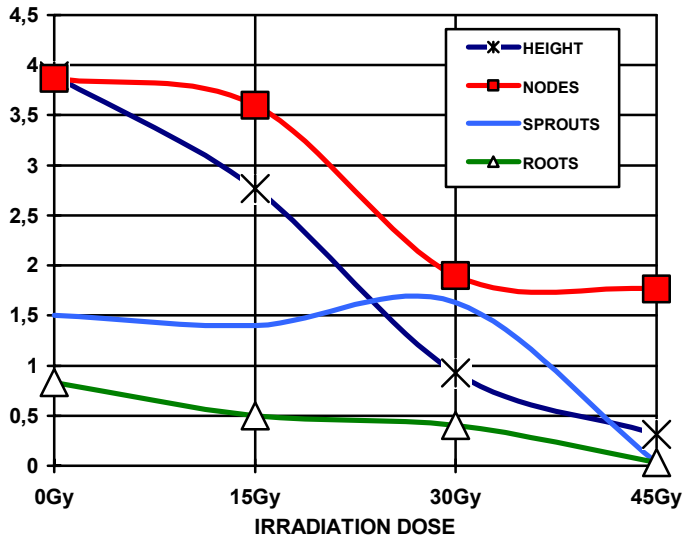

Fig. 1. Radio-sensibility of bitter potato.

2.4.2. Mean Lethal Dose LD50

With the values obtained from the radio-sensibility treatment, the best LD 50 irradiation dose was identified as the one that causes biological damage close to 50% compared to the control. The LD 50 was of 28 Gy for Luk'y kheto and 22 Gy for Bola Luk'y, with a tolerance range of

+/-1 (Fig.1). Sonnino et al [7] determined the best dose for mutation induction in the variety Desiree of *Solanum tuberosum* to be of 30 Gy, and other authors [8] indicated that the best irradiation doses for potato were between 30 Gy and 50 Gy.

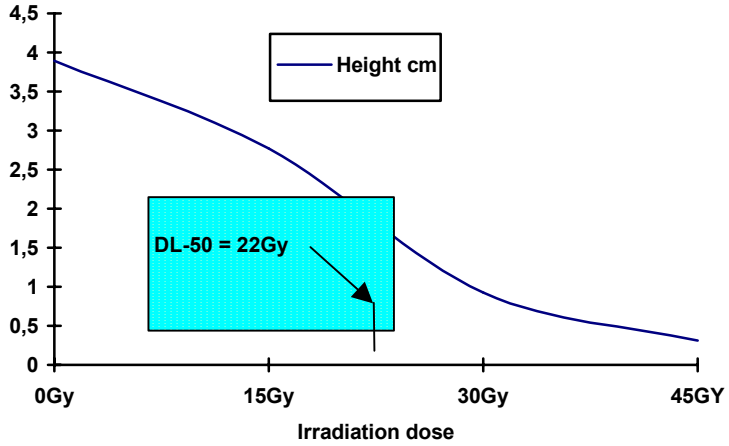

Fig. 2. Optimum irradiation dose for mutation induction for the bitter potato variety Bola luk'y.

2.4.3. Best Irradiation Dose

For this step, the potato plantlets were again multiplied in medium MV to have 1000 plants (40 magenta boxes with 25 plantlets/box) of up to 4 cm. Then, they were irradiated at the best dose (28 Gy and 22 Gy) for both varieties (Fig. 2) about 10.19 minutes, considering that the equipment activity registered 4488.80 curies with a performance of 215.9 (cGY/min) at 62 cm from the samples.

2.5. Multiplication *in vitro* in bitter potato varieties Luk'y kheto and bola Luk'y (*Solanum juzepczukii*) after irradiation

The irradiated samples were micropropagated in fresh MV medium (MS, Kinetin 0.1mg/l, NAA 0.04 mg/l, GA_3 0.3 mg/l, Ca phantotenate 2.0 mg/l, agar 0.60%, sucrose 3.0%, pH = 5.7), excluding the apical part of the plantlet. Responses recorded included: height, number of nodes, number of sprouts and number of roots, as in [9].

For multiplication (M_1V_1 to M_1V_4), stem cuttings of both varieties were cultured in magenta boxes (5x5x10 cm), at 25 plantlets per box, with the idea of eliminating chimeras.

The growth conditions were a mean temperature of 23°C, a light intensity of 3000 lux, and a photoperiod of 16 h light and 8 h dark.

2.6. *In vivo* transfer and acclimatization of *in vitro* plantlets

The micropropagated mutant material of the potato varieties Bola Luk'y and luk'y Kheto (*Solanum juzepczukii)* was acclimated and transplanted to the greenhouse. In this phase a pre-selection was done among the mutant material according to the growth decrease and the morphological characteristics, using potato descriptors with a total of 4000 genotypes from the two varieties (2000 per variety). A large number of micropropagated plants do not survive to the transplant from the *in vitro* conditions to the greenhouse or to the field, and require an acclimatization process to secure survival in soil [10] (Fig. 3).

To avoid tuber mixes during harvest, it was convenient to perform planting in black plastic bags (15x25 cm). Once filled up with the prepared substrate, a vitro plant was placed in each bag and bags were placed in wood beds (300 bags/bed). For transplant, we used the strongest *in vitro* plantlets (6 to 8 cm tall). During this phase we evaluated the percentage of seizure in plantlets of Luk'y kheto and Bola Luk'y varieties (Figure 4), which attained a total of 1572 plants.

Fig. 3. Acclimatization of in vitro plantlets.

Fig. 4. Transplanted plants in the greenhouse.

2.7. Study of the relationship between the number of chloroplasts and ploidy levels

This technique is very useful for a rapid estimate of chromosome number of the material under study, through the determination of the number of chloroplasts in the occlusive cells of the leaf stomata, whose number will give an indication of the ploidy level, according to the scale below (Table II).

TABLE II. NUMBER OF CHLOROPLASTS AND PLOIDY LEVEL

PLOIDY	CHLOROPLAST NUMBER PER GUARD CELL
2X	7 – 8
3X*	9 – 11
4X	12 – 14
5X**	15 – 16

* *S. juzepczukii* (2n = 36)
** *S. curtilobum* (2n = 60)

The ploidy determinations were carried out on the selected genotypes in the Centro de Investigaciones Nucleares de Viacha – IBTEN, following the methodology described by CIP [11].

The study of the chloroplasts in the stomata cells is most adequate, due to the facility of observation and because height is not a problem for the plastid increase. We took special care so that cells were submitted to similar physiological conditions, to avoid wrong readings, as there are cases in which triploid plants have more chloroplasts than tetraploid ones, since their leaves develop slower and they are not comparable. We could identify 8% of mutant genotypes with this trait.

2.8. Morphological characterization of mutant genotypes in the greenhouse

The objective of this work was specifically to characterize and to preliminarily select the mutant material of bitter potato with low glycoalkaloid content through the morphological descriptors (growth decrease, different leaf shapes, different tuber shapes and colours, etc).

The observation and the characterization of the varieties were performed through the morphological descriptor proposed by Huaman [4], compared with the controls. The results have shown variability in some traits such as general shape of tubers, strange tuber shapes, plant height, with a total of 10.8% possible mutants.

2.9. Multiplication of Luk'y kheto and Bola luk'y (*Solanum juzepczukii*) bitter potato plants

In order to produce a large quantity of tubers for the glycoalkaloid analysis, it was best to sow the small tubers in plastic flaks, to secure sprouting and to have 2 to 3 replicates later. Once they attained a size of 15 to 20 cm, they were transplanted to the field, where they finished their vegetative cycle of 180 days.

2.10. Separation of total glycoalkaloids from potato tubers

We adopted the method described by [2] as detailed below

2.10.1. Sample preparation for total glycoalkaloid analysis for maceration and reflux of potato tubers

(1) Take the tubers at random, with a total fresh weight of 50 g
(2) Scrape the tubers (they must not be peeled to avoid variations and biased results)
(3) Macerate tubers for 48 hours in 100 ml of extracting mixture of Methanol:HCl (1N) (9:1, v/v).

2.10.2. Extraction phase

(1) Filter the macerated mixture in one kitasato flask under vacuum with filter paper.
(2) The filtered solution is introduced in a 250 ml balloon where HCl (1 N) is added in a 1:1 v/v ratio, until a volume equal to half of the 250 mL balloon.
(3) This solution is alkalinised to Ph=9–10 adding 20% (v/v) NH_4OH 20% (v/v).
(4) Transfer the alkalinised solution to a decant pear where ethyl acetate is added, shake vigorously and leave to rest until the aqueous phase separates from the ethyl acetate (upper ethyl acetate, down aqueous phase).
(5) Empty the aqueous phase in the test tubes and centrifuge for 15 min at 5000 rpm.
(6) The pellets formed are recovered and dissolved in methanol.
(7) The ethyl acetate supernatant solution is emptied into 100 ml balloon previously weighed and introduced in a rotary evaporator (50°C and 100 mmHg).
(8) The solution is concentrated until it is completely dry.
(9) The balloon is weighed with the extract.
(10) Transfer the dry extract adding Methanol and Ethyl acetate (2:1 v/v) in a previously weighed vial, and the weight of the extract is found and expressed as a % of Glycoalkaloids.

2.10.3. Thin Layer Chromatography

The analysis of the glycoalkaloids of the selected genotypes was carried out in thin layer chromatography, using the thin layer chromatography on silica gel, where the glycoalkaloid assay was obtained in a dichlorometane: methanol, CH_2Cl_2:MeOH, (9:1) system. They were revealed with the Draguendorf's reagent and with sulfuric acid 50% (heating to 70°C for 10 min). The alpha solanine and the solasodine (SIGMA standards), present a pink colour at the beginning, and a few minutes later they turn into dark pink. The same system under UV 366 nm wavelength turns into a bright blue colour.

To obtain the content of Glycoalkaloids in 100 g. of fresh tuber we used the formula

TGA = 100 x PEC/ PTF

Where
TGA = Total Glycoalkaloids (g),
100 = Constant Factor
PEC = Weight of raw extract
PTF = Weight of fresh tuber

2.11. Statistical analysis

In order to evaluate and select mutant genotypes, we used the statistical package SPSS 11.0 regression design and correlation for quantitative and qualitative multi-variables in the morphological traits, ploidy and glykoalcaloids.

3. RESULTS AND DISSCUSION

3.1. Morphological changes in Luk'y kheto

With respect to shape, it was observed that in 16 irradiated plants tubers were round, different to the ovobate shape that is the normal one in this species (Fig 5). Only one plant had fusiform tubers, a change that may have been induced by mutation (Fig. 5).

In terms of leaf shape, only 25 plants presented modifications in the leaves, which changed their rounded form to ovobate (Fig. 5).

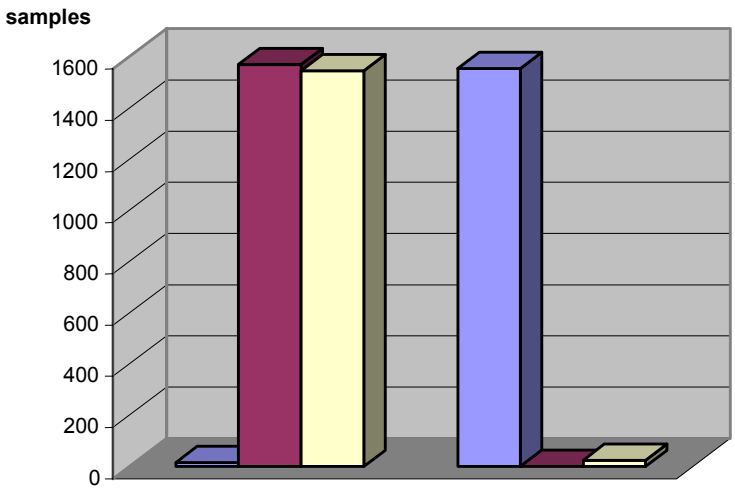

Fig. 5. Normal tubers ■ *, normal tuber shape* □ *and leaf shape* ■

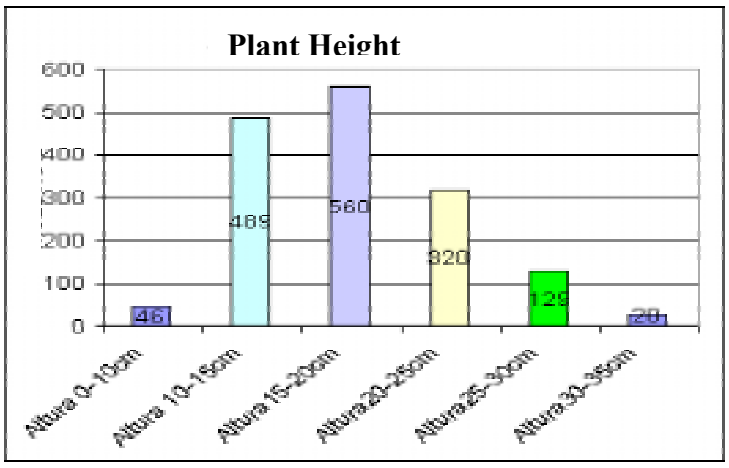

Fig. 6. Plant height.

In terms of plant height, 90% of plants were between 25 and 30 cm tall (Figure 6).

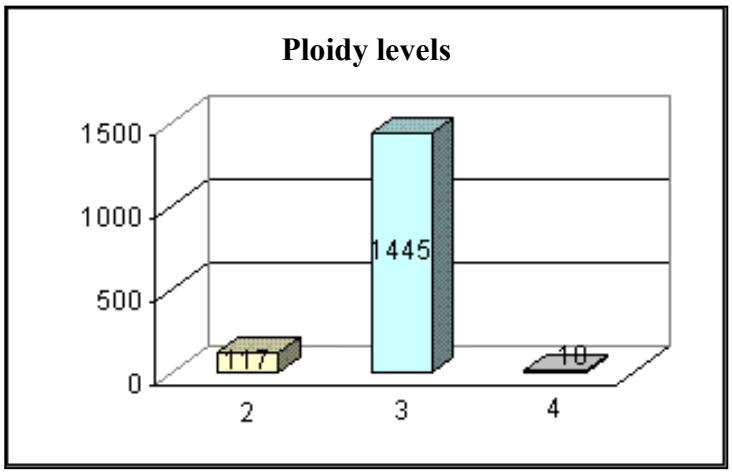

Fig. 7. Changes of ploidy level.

Figure 7 show that 117 plants have changed from triploid (3n) to diploid (2n), and 10 plants to tetraploid (4n). The analysis of this factor would now have to be carried out as well with molecular techniques that allow a better accuracy.

TABLE III. YIELD

Plants	Frequency (%)	Yield (Average g)
Control	98.8	42.2
Mutants	1.2	33.6

We observed that in selected mutants genotypes the tuber yields per plants were smaller than the rest of control plants (Table III).

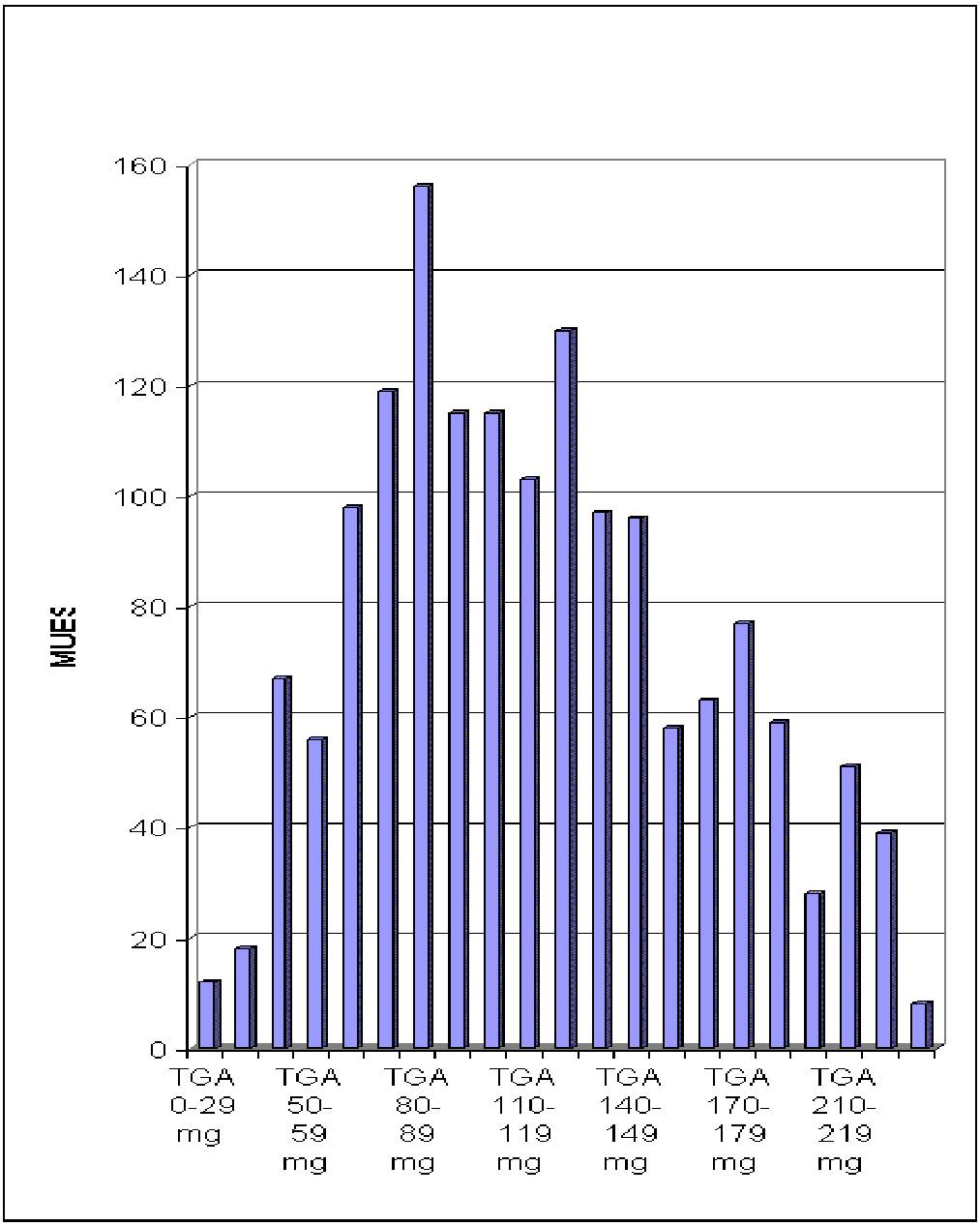

Fig. 8. Total glycoalkaloids (mg TGA/100 GR FT).

The analysis of total glycoalkaloid (TGA) content was performed on fresh tubers (FT) and compared to the control, which possesses 180 mg TGA/100 g FT, and considering that a TGA content beyond that value is considered as toxic. As detailed in Figure 8, 97 of the mutant genotypes obtained showed less than 50 mg TGA/100 g FT.

3.2. Morphological changes in Bola luk'y

Out of 1572 genotypes analysed, we observed 37 plants with abnormal tubers of which 15 were reniform and 22 were fusiform (Fig 9). In addition, 32 of the plants obtained exhibited a leaf shape different from that in the controls (Fig 9).

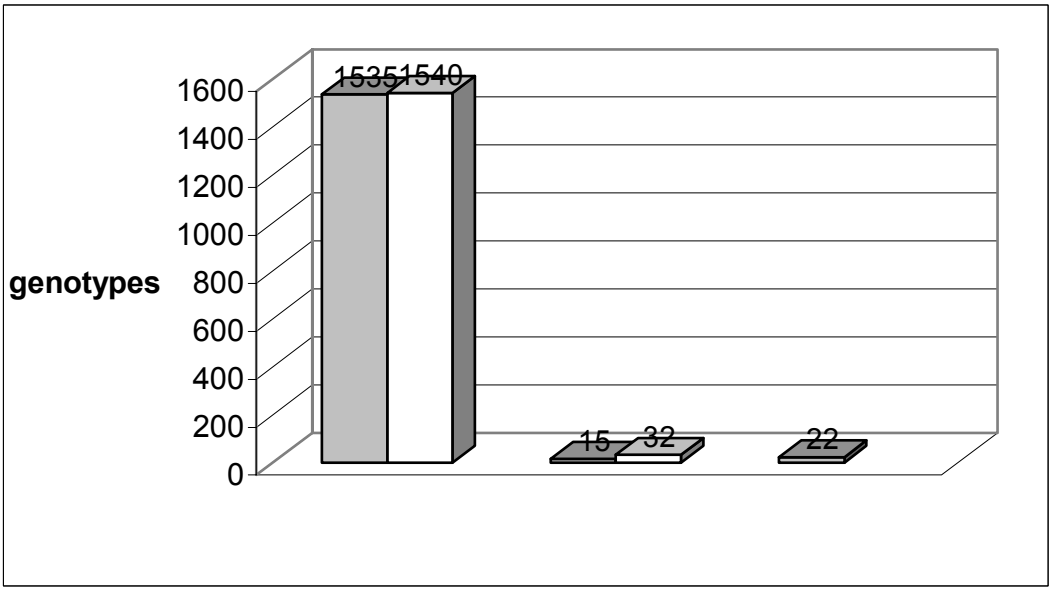

Fig. 9. Tuber and leaf shape.

The plant height showed a large variation compared to the control plants, with 85.6% of smaller plants with a height between 20 and 30 cm and 2.4% of plants that were taller (Figure 10).

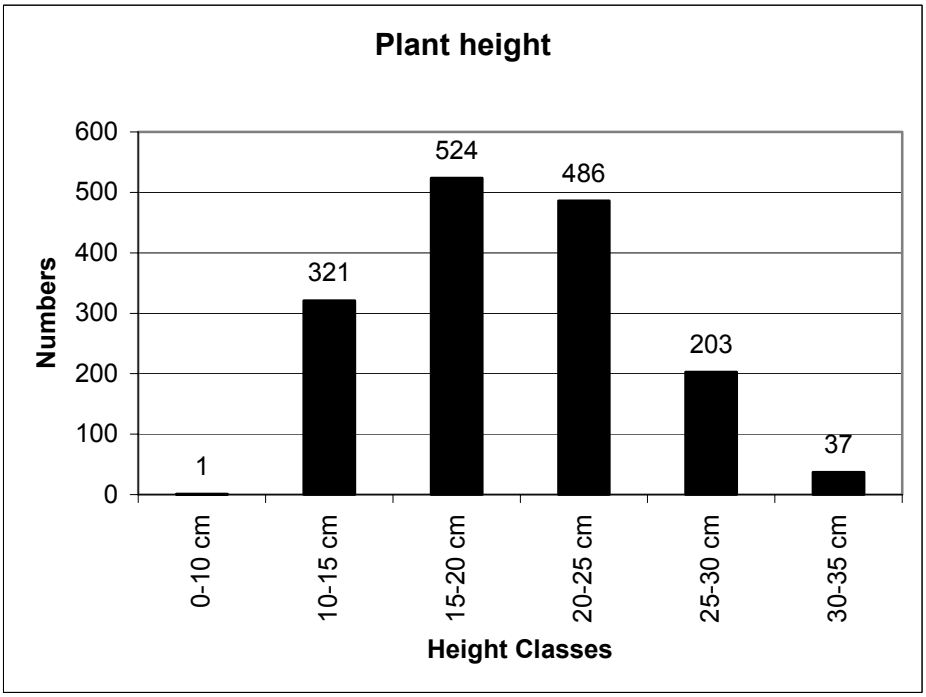

Fig. 10. Height of plants (cm).

Of the 1572 genotypes analysed, 91 irradiated plants exhibited changes in ploidy level from triploid (3n) or tetraploid (4n) (Figure 11).

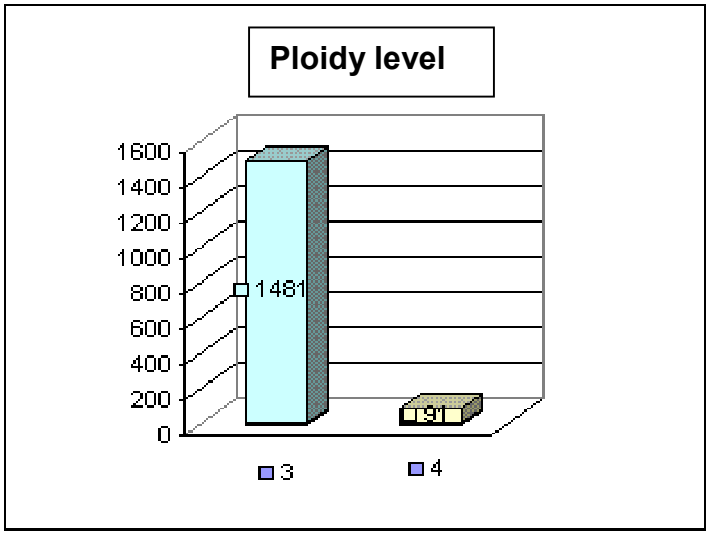

Fig. 11. Changes in the ploidy level.

TABLE IV. YIELD

Plants	Frequency (%)	Yield (Average g/Plant)
Control	99.9	73.6
Mutants	0.1	50.4

The yield of the mutant genotypes was reduced as compared to the control plants (Table IV).

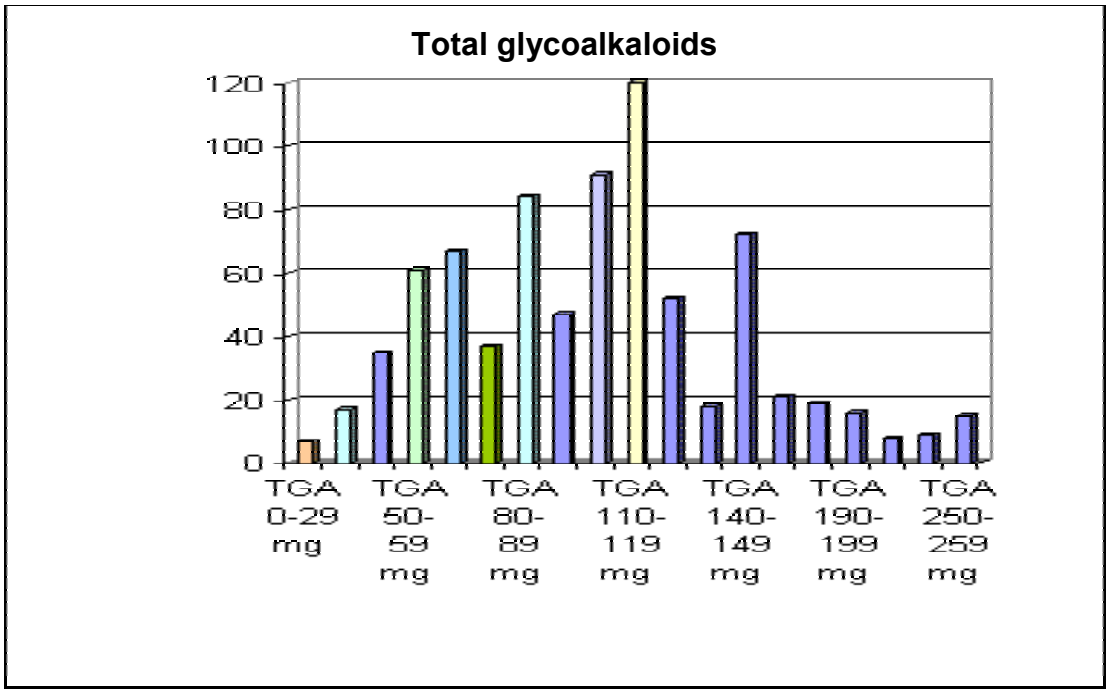

Fig. 12. Total glycoalkaloids (in mg TGA/100 gr FT).

The analysis of total glycoalkaloids showed that 59 plants had a content reduced to 29 to 49 mg/100 g.FT as a result of irradiation, as detailed in Figure 12.

3.3. Mutant genotypes of Bola luk´y

In the variety Bola luk´y we obtained two plants with low glycoalkaloid content, clones 107 and 1564.

In plants of clone 107, we observed a change from triploid (3n) to tetraploid (4n) and a height reduction to 20.2 cm, and this was coupled with the most important reduction of the glycoalkaloid content (to less than 30 mg TGA/100 g of FT).

In plants of clone 1564, a change took place in the leaf shape with a reduction of plant height to 17.9 cm, and coupled with a glycoalkaloid content of less than 50 mg TGA/100 g FT.

The various traits observed for these two clones are summarized in Table V below.

TABLE V. PHENOTYPIC TRAITS OF MUTANT GENOTYPES OF INTEREST IN BOLA LUK'Y

N°	TGA	LS	PH	PLO	AT
107	1	0	20.2	4	0
1564	3	1	17.9	3	0

TGA = Total Glycoalkaloids; LS = Leaf shape; PH = Plant height; PLO = Ploidy level; AT = Abnormal Tuber shape

3.4. Mutants genotypes of Luk´y kheto

In Luk´y kheto, 12 mutant genotypes were obtained, with a low glycoalkaloid content, from 49 to 40 mg TGA/100 g FT, coupled with suitable morphological traits and ploidy levels (Table VI). All plants were smaller than the control plants. Of them, mutants 379, 399 and 559 had a modified leaf shape and changed from triploid (3n) to diploid (2n).

4. CONCLUSIONS

- Before the introduction and characterization of tubers, the pre-treatment of material is important, and sprout culture helped us to preserve most recollected varieties because many of them had various diseases, and this technique helped to clean them from viruses.
- The gathering work and establishment *in vitro* of bitter potato and other varieties was very positive because it allowed us to have a collection of great genetic value, since they are the only potato varieties that can be sown at higher than 4,000 meters above sea level.
- The plant height is one of the most important variables for the selection of mutants when a physical mutagen is applied.
- The yield in selected plants with low glycoalkaloid content decreased approximately by 25% compared to the control plants. Out of 4,000 plants analysed, we have selected fourteen mutant genotypes that showed between 28 and 48 mg TGA/100 g FT with ploidy levels varying between diploid (2n) and tetraploid (4n).
- We should continue with the evaluation of these selected genotypes in order to register them as new bitter potato varieties with low glycoalkaloid content. In order to achieve this, our studies should be completed with a molecular characterization and evaluation of these mutants in their natural high altitude habitat.

TABLE VI. PHENOTYPIC TRAITS OF MUTANT GENOTYPES OF INTEREST IN LUK´Y KHETO

N°	TGA	LS	PH	PLO	AT
399	3	1	23.7	2	0
568	3	0	14.3	2	0
592	3	0	14.3	2	0
768	3	0	16.2	2	0
805	3	0	14.4	2	0
929	3	0	16.1	2	0
959	3	0	21.2	2	0
978	3	0	13.7	2	0
1005	3	0	15.3	2	0
1036	3	0	12.2	2	0
379	3	1	16.7	3	0
559	3	1	24.3	3	0

TGA = Total Glycoalkaloids; LS = Leaf Shape; PH= Plant height; PLO = Ploidy Level; AT = Abnormal Tuber shape

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IMPROVEMENT OF COCOYAM (*Xanthosoma sagittifolium*) USING GAMMA IRRADIATION AND TISSUE CULTURE

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Abstract

Cocoyam (*Xanthosoma sagittifolium*) is an important starchy staple but an under-utilized and neglected crop plagued with the root rot disease, which reduces yields drastically. Resistance to the disease has not been found among cultivated varieties in Ghana. In this study, a radiosensitivity test was first carried out on a promising *Xanthosoma sagittifolium* accession using LD_{30} to select a non-lethal dose, which would generate variability through induced mutations. Shoot tips of *Xanthosoma sagittifolium* accession RAX–93–05 irradiated with the LD_{30} were cultured *in vitro* through four vegetative cycles MV_1–MV_4 and resulting plants were weaned and transplanted to the field. About 2,000 plantlets generated are being multiplied for further work. Root rot causing organisms were isolated from infected *Xanthosoma sagittifolium* plants growing in farmer fields in the Eastern region of Ghana. These were *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., *Penicillium* spp. and *Botrydioplodia* spp. Pure cultures of these organisms have also been established for use in forthcoming inoculations.

1. INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium*) is an economically important starchy staple in tropical and sub-tropical regions particularly West Africa, Asia, Far East, the Pacific and Caribbean regions [1]. The crop originated from Tropical America where it has been cultivated since pre-Columbian times [2]. Cocoyam was introduced into the West African sub-region in 1940 [3]. Following its introduction into Ghana in 1943, the crop has been cultivated throughout the forest belt as a shade crop for young cocoa seedlings. The cormels of *Xanthosoma* are used for human consumption and the corms as propagules. The leaves serve as potherb or spinach and are an important source of proteins and vitamins in the diet of a majority of the people in Ghana [4]. In fact, in some places in Ghana, cocoyam ranks second only to cassava as important source of energy in the daily diet of the population.

In spite of the tremendous value of the crop in Ghana, little research has been done to improve this species. Varieties currently being grown are poor yielding and have poor quality. For many years, production of cocoyams declined significantly in Ghana, due largely to a number of problems including susceptibility to the root rot disease.

The objective of this work was to generate variability through induction of mutations and to identify mutant cocoyam individuals resistant to the root rot disease.

2. MATERIALS AND METHODS

2.1. Plant material

The experimental material, *Xanthosoma sagittifolium* accession RAX–93–005, was selected on the basis of its superior morpho-agronomic characters from 48 accessions of *Xanthosoma sagittifolium* which had been established at the Sinna garden of the University of Ghana in March 1998 as single row plots of ten plants each. These accessions, which were collected from four localities in Ghana (Aburi, Bunso, Legon and Kade) have been characterised in terms of morpho-agronomic traits using IBPGR descriptors [5].

2.2. Radiosensitivity test

A radiosensitivity test was first carried out using LD_{30} to select a non-lethal dose, which would generate variability in cocoyams through induced mutations. Corms of accession RAX–93–005 were cut into minisets (10–12 g), each bearing an adventitious bud. The minisets were treated with a mixture of 5 g/l Kocide + 2 ml/l Actellic + 25 g/l wood ash for 5 min to prevent fungal attack. The treated minisets were pre-sprouted in boxes filled with moist sawdust in a greenhouse. Sprouts excised from the minisets were trimmed and sterilized in 100 ml 15% sodium hypochlorite (bleach) containing a few drops of Tween-20 for 15 min. Sprouts were then trimmed to a uniform weight of 0.3–0.4 g, sterilized again as above and rinsed three times in sterile distilled water.

Sets with ten buds each in a petri dish were exposed to gamma irradiation at 5, 10, 15, 20, 25 and 30 Grays (Gy). A dose rate of 5.6 $Gy.sec^{-1}$ in air at 29±1°C from a ^{60}Cobalt source was used at the Gamma Irradiation Centre of the Ghana Atomic Energy Commission, Kwabenya. In total, about 600 buds were irradiated.

2.3. Culture medium

Irradiated shoot tips were inoculated on artificial basal nutrient medium containing full-strength Murashige and Skoog (MS) formula [6], supplemented with 3 mg/l BA. Subculturing from MV_1 to MV_4 was done at 4–6 week intervals on the same basal medium supplemented with 3 mg/l each of NAA and BA. Cultures were incubated in a growth room at 24±2°C, under a 16 h light photoperiod.

2.4. Weaning

Plantlets were weaned after 16–18 weeks when they had developed enough roots to absorb nutrients from topsoil medium obtained from the farm of the Ghana Atomic Energy Commission. Plants were weaned in Watson containers perforated for a more effective drainage. Plantlets were acclimatized in black polyethylene bags filled with topsoil for two weeks and then transplanted to the field.

2.5. Isolation of organisms causing root rot in cocoyam

A survey was undertaken in the Eastern region of Ghana and samples of diseased plants were collected for laboratory analysis. Samples were cultured on water agar and then subcultured on potato dextrose agar to generate pure cultures.

2.6. Data analysis

For the radiosensitivity test, records were taken on height of shoots and date of first leaf expansion. The total number of leaves produced by each shoot was also recorded. In addition, change in weight of each individual shoot was also taken and changes recorded.

3. RESULTS AND DISCUSSION

3.1. Radiosensitivity test

The performance of irradiated shoot tips six weeks after irradiation was as detailed in Table I.

TABLE I. SOME GROWTH CHARACTERISTICS OF IRRADIATED SHOOT TIPS SIX WEEKS AFTER IRRADIATION

Dose (Gy)	Plant height (m)	Fresh weight (g)	No. of days to first leaf	No. of leaves
0	3.5	0.30	8.0	2.5
5	3.2	0.27	12.5	2.5
10	2.7	0.18	8.0	2.0
15	2.6	0.19	11.0	1.0
20	2.2	0.18	15.0	1.0
25	2.0	0.17	20.0	1.0
30	2.1	0.17	7.3	1.0
LSD	0.9	0.12	4.9	0.5

Shoot tips irradiated with 5 Gy were not significantly different from the control. The only exception was the number of days to first expanded leaf.

The effects of the 10 and 15 Gy treatments, however, were significantly different from the control in the suppression of plant growth rate, height and number of leaves.

Beyond 15 Gy, treatments were not only significantly different from the control, but they also adversely affected the growth and survival of buds and plants. In this respect, most buds treated with dosages between 20 and 30 Gy died after six weeks indicating that the buds tolerated the 10 and 15 Gy doses reasonably well. These doses could, therefore, be used to irradiate large numbers of buds to induce mutations. Selectable characters like size, colour or form could then be readily selected and utilized in a breeding programme.

About 1,950 plants from the irradiation experiments are presently multiplied in the field for prior to their further inoculation with the causal organisms of root rot in the screenhouse.

3.2. Root rot causing organisms

During isolation experiments from the specimens collected, 15 different fungi were identified as provoking root rot disease, as detailed in Table II.

TABLE II. PATHOGENIC FUNGI RESPONSIBLE FOR ROOT ROT DISEASE OF COCOYAM

Organism	No. of samples	Location of sample
Fusarium spp.	4	Begoro, Eastern Region
Penicillium spp.	3	Begoro, Eastern Region
Botryodiplodia spp.	2	Bunso, Eastern Region
Pythium spp.	4	Kade, Eastern Region
Rhizoctonia spp.	2	Kade, Eastern Region

A pathogenicity test is currently being undertaken to establish the severity of infections with each one of these organisms, preliminary to their use in future inoculations.

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GENETIC DIVERSITY IN COCOYAM AS REVEALED BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

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Abstract

Cocoyam (*Xanthosoma sagittifolium*) is an important starchy staple but an under-utilized and research-neglected crop. Farmers have generated a large number of traditional varieties over the years but there is little information on genetic diversity between them. In this study, 70 accessions of *Xanthosoma sagittifolium* from the Eastern and Volta regions in Ghana were analysed using 10 decamer primers to determine genetic diversity in random amplified polymorphic DNA (RAPDs). A total of 122 amplification products were generated with an average of 12.2 per primer and overall polymorphism of 90.5%. Similarity matrices were calculated using Jaccard coefficient and input into cluster and principal coordinate analyses. The similarity indices ranged from 0.15 to 0.84 among the accessions. Cluster analysis identified three main clusters at the 45% level of similarity. Accession BD 96/124 from Awanam Junction in the Eastern region was relatively distant from the other main clusters and separated at the 25% level of similarity. The second major cluster comprised 12 accessions from 11 different localities. All the other accessions including those from unknown localities formed a big cluster with subdivision into three sub-clusters at the 60% level of similarity. The results of the principal coordinate analysis also corroborated this observation but gave a clearer picture of the groupings. The 70 accessions did not cluster according to geographical regions. This indicates active movement of planting material across regions. The wide divergence between some of the accessions indicates genetic differences between them and offers the opportunity to generate variability in offspring of crosses from which selections may be made.

1. INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium*) is an economically important starchy staple in tropical and sub-tropical regions particularly West Africa, Asia, Far East, the Pacific and Caribbean regions [26]. The crop originated from Tropical America where it has been cultivated since pre-Columbian times [6]. Cocoyam was introduced into the West African sub-region in 1940 [22]. Following its introduction into Ghana in 1943, the crop has been cultivated throughout the forest belt as a shade crop for young cocoa seedlings. The cormels of *Xanthosoma* are used for human consumption and the corms as propagules. The leaves of *Xanthosoma* serve as potherb or spinach and are an important source of proteins and vitamins in the diet of a majority of the people in Ghana.

Farmers in Ghana have selected genotypes that best meet their needs and, thus have generated a large number of traditional varieties. An associated problem has been the assignment of different vernacular names to the same varieties depending on ethnic origin. The nomenclature has led to confusion in the exact number of varieties of cocoyam under cultivation in the country, hence the need to characterise the genetic resources of the crop.

Cocoyam characterisation has been undertaken using morphological descriptors [8 and 16] and a combination of morphological characters and isozyme markers and total proteins [1, 13, 21]. These markers, although valuable for cocoyam varietal group identification, reveal limited levels of inter and intra-varietal polymorphism. The similarity of common names and

lack of obvious phenotypic variation among many cocoyam accessions led the authors to suspect a high degree of genetic relatedness.

According to Greene and Pedersen [11] one of the initial steps towards management efficiency of germplasm is the estimation of the genetic diversity within the collection and elimination of duplicate accessions. The Random amplified polymorphic DNA (RAPD) technique has proven to be a useful method for fingerprinting accessions of crop plants. It has been a widely used molecular marker because of advantages in time and cost. RAPD markers, however, have limitations including questionable reproducibility of some bands, a requirement for stringent standardization of reaction conditions, co-migration of different amplification products, and dominance inheritance [3]. Despite these caveats, the RAPD method offers the highest potential for generating large numbers of markers with the greatest ease under limited resource conditions [26]. Tanukari *et al*. [32] demonstrated that 24 out of 28 accessions of cassava could be distinguished using 10 primers. The technique has been used successfully with other vegetatively propagated crops including cocoyam [26], apple [17], grapes [23] annona [25], avocado [9] and yams [2, 20, 24].

Our objective was to determine relatedness among 70 accessions of *Xanthosoma sagittifolium* with the aim of selecting divergent elite lines for a crop improvement programme. Through this study, we hoped to establish the authenticity or otherwise of the different land varieties and to determine any homonyms and synonyms among the accessions. To this end, a protocol for RAPD analysis of cocoyam was adapted and used to screen the accessions. We report here on the results of RAPD marker fingerprinting of the 70 cocoyam accessions.

2. MATERIALS AND METHODS

2.1. Plant material

The 70 accessions of *Xanthosoma sagittifolium* used in this study were received from the Plant Genetic Resources Centre, Bunso, Ghana. These accessions were collected from cocoyam growing regions in the Eastern (ER) and Volta (VR) regions of Ghana and have been maintained routinely but without complete morpho-agronomic data. A list of the accessions indicating their origin, local name and corm characteristics where known is shown in Table I. The accessions were grown and maintained in the Sinna's garden, Department of Crop Science, University of Ghana, Legon.

2.2. DNA extraction

Fresh leaf tissue (100 mg) taken from individual plants was frozen in liquid nitrogen and ground in microcentrifuge tubes. DNA was extracted following the protocol in the DNeasy plant mini kit (Qiagen, Inc.). The concentration of DNA was estimated by comparison with standard markers using the UV transilluminator. The DNA was then diluted to 5 ng/µl for PCR amplification.

TABLE I. ACCESSION NUMBER, CODE NO., LOCAL NAME, LOCALITY/REGION COLLECTED AND SOME TUBER CHARACTERISTICS OF 70 ACCESSIONS OF *Xanthosoma sagittifolium*

Accession	Code no.	Local name	Locality/Region	Remarks
AGA 97/162	1	Mankani fitaa	Oframoase, ER	
AGA 97/161	2	Mankani kokoo	Oframoase, ER	
AGA 97/206	3	Mankani kokoo	Asikan, ER	
AGA 97/029	4	Unknown	Unknown, ER	
RAX 93/008	5	Unknown	Unknown, ER	
RAX 93/006	6	Unknown	Unknown, ER	
RAX 93/005	7	Unknown	Unknown,ER	
RAX 93/004	8	Unknown	Unknown, ER	
RAX 93/002	9	Unknown	Unknown, ER	
RAX 93/001	10	Unknown	Unknown, ER	
TA 97/056	11	Mankani	Avatime, VR	
TA 97/037	12	Mankani dze	Dzolo-Gbogame, VR	
TA 97/018	13	Mankani gye	Matse, VR	
TA 97/017	14	Mankani ye	Matse, VR	
RAX 93/010	15	Unknown	Unknown, ER	
RAX 93/009	16	Unknown	Unknown, ER	
TA 97/140	17	Unknown	Unknown, VR	
TA 97/137	18	Unknown	Unknown, VR	
TA 97/090	19	Mankani	Dodo Pepesu, VR	
TA 97/083	20	Mankani	Kodibonum, VR	
TA 97/074	21	Mankani	Jasikan, VR	
TA 97/068	22	Mankani	Angatse, VR	
SCJ 98/002	23	Mankani	Adjeikrom, VR	Red tuber flesh
TA 97/021	24	Unknown	Unknown, VR	
TA 97/163	25	Mankani	Vakpo, VR	
TA 97/160	26	Unknown	Unknown, VR	
TA 97/54	27	Mankani	Hohoe, VR	
TA 97/146	28	Mankani	Adumadum, VR	
SCJ 98/009	29	Serwaa fitaa mankani	Amoafoasu, ER	

Accession	Code no.	Local name	Locality/Region	Remarks
SCJ 98/007	30	Mankani	Amoafoasu, ER	Red tuber flesh
SCJ 98/006	31	Makani	Akora Darko, ER	Red tuber flesh
SCJ 98/006	32	Makani	Akora Darko, ER	Red tuber flesh
SCJ 98/005	33	Makani fitaa	Akora Darko, ER	White tuber flesh
SCJ 98/003	34	Mankani	Adjeikrom, ER	White tuber flesh
SCJ 98/015	35	Mankani kokoo	Akim- Adukrom, ER	Red tuber flesh
SCJ 98/014	36	Mankani	Akim-Adukrom, ER	White tuber flesh, hard
SCJ 98/013	37	Mankani	Akim-Adukrom, ER	White tuber flesh, hard
SCJ 98/012	38	Mankani	Odumasi, ER	Red tuber flesh
SCJ 98/011	39	Mankani	Feyiase, ER	Red tuber flesh
SCJ 98/010	40	Mankani	Feyiase, ER	Red tuber flesh
SCJ 98/023	41	Mankani	Kwabeng, ER	Red tuber flesh
SCJ 98/022	42	Mankani	Sarbo, ER	Red tuber flesh
SCJ 98/021	43	Mankani	Sarbo, ER	Red tuber flesh
SCJ 98/020	44	Mankani	Sarbo, ER	White tuber flesh
SCJ 98/019	45	Mankani	Sarbo, ER	Red tuber flesh
SCJ 98/106	46	Mankani	Juaso, ER	Red tuber flesh
BD 96/070	47	Mankani	Asiakwa, ER	Red tuber flesh
BD 96/064	48	Mankani	Asiakwa, ER	
BD 96/059	49	Mankani	Bunso, ER	
BD 96/059	50	Mankani	Bunso, ER	
SCJ 98/094	51	Unknown	Unknown, ER	
SCJ 98/024	52	Mankani	Akrofonso, ER	
BD 96/091	53	Mankani kokoo	Amanhyia, ER	
BD 96/085	54	Mankani kokoo	Omenako, ER	
BD 96/079	55	Unknown	Unknown, ER	
BD 96/078	56	Mankani kokoo	Potroase, ER	Red tuber flesh
BD 96/077	57	Mankani kokoo	Potroase, ER	White tuber flesh
BD 96/123	58	Amankani fitaa	Tinkon, ER	
BD 96/118	59	Amankani	Ebedwo, ER	
BD 96/111	60	Unknown	Mamfe, ER	
BD 96/102	61	Mankani kokoo	Awadum, ER	

Accession	Code no.	Local name	Locality/Region	Remarks
BD 96/093	62	Unknown	Amanhyia, ER	
BD 96/092	63	Mankani kokoo	Amanhyia, ER	
BD 96/163	64	Mankani	Asiakwa, ER	
BD 96/158	65	Mankani kokoo	Awenade, ER	
BD 96/145	66	Mankani	Nkawkaw, ER	
BD 96/135	67	Mankani	Enyiresi, ER	
BD 96/126	68	Mankani	Kwasikrom, ER	
BD 96/124	69	Amankani kokoo	Tinkon, ER	
BD 96/183	70	Amankani fitaa	Awanam Junction, ER	

2.3. PCR amplification and DNA analysis

PCR amplifications were carried out using RAPD 10 mer primers (Operon Technologies, Alameda, CA). The 25 μl PCR reaction mixture contained 15 pmol primer, 1.5 μl $MgCl_2$, 19.5 μl autoclaved water and 3 μl template DNA. This mixture was added to PCR beads (Pharmacia) containing 1.5 units of Taq polymerase, 10mM Tris-HCl (pH 9.0), 50mM KCl pH 8.3, 1.5 mM $MgCl_2$ and 200 μl each of dATP, dCTP, dGTP and dTTP. PCR was carried out in a Progene thermo-cycler with heated lid to reduce evaporation. The cycling programme involved an initial denaturing at 94°C for 1 min followed by 45 cycles of 94°C for 1 min, 35°C for 1 min, 72°C for 1 min, and then a final 5 min 72°C extension. The RAPD-PCR products were electrophoresed in 2% agarose (Sigma, St. Loius, MO) gels in TAE (Tris 1.6 M, acetic acid 0.8 M, EDTA 40 mM) at 25 volts for 5 hr. The agarose gel was stained in ethidium bromide, visualised under UV and photographed using a Polaroid MP4 camera. A 100 bp ladder DNA from Giblo BRL (New York, USA) was used as a standard.

2.4. Primer screening

A subset of five accessions, chosen at random, was amplified with 100 RAPD primers. Amplification products were assessed for the number and quantity of polymorphic loci. Ten primers that amplified reproducible polymorphic bands were selected for the analysis. DNA was isolated from each sample in two sets (1 and 2). All of the samples were amplified twice to verify patterns and reproducibility. If ambiguous results were obtained from a given amplification then the amplification was repeated. Only reproducible bands in multiple, independent runs from both extractions (sets 1 and 2) and between replicate amplifications within extractions were scored. A RAPD locus as described here consists of a set of co-migrating RAPD fragments amplified by the same RAPD primer [27]. RAPD loci were scored as either present (1) or absent (0).

2.5. Data analysis

Unambiguous RAPD bands were scored manually as present (1) or absent (0) from the gels. Only polymorphic bands were included in the binary data set and similarities were calculated using Jaccard's coefficient [14]: $S_{ij} = a/(a+b+c)$ where S_{ij} is the similarity between two individuals *i* and *j*, a is the number of bands present in both *i* and *j*, b the number present in *i* but absent in *j* and c the number present in *j* but absent in *i*. Cluster analyses using the UPGMA (unweighed pair-group method with arithmetic averages [29] were carried out on the similarity matrices using the software Genstat 5 statistical package [10] and phenograms (dendrograms) constructed. Similarity matrices from each primer were compared pairwise using a Mantel randomization test (1000 randomisations carried out in each case) based on the product-moment coefficient [18]. This test compares the elements of two matrices and estimates the degree of correlation between the matrices by means of a test criterion, Z and a product-moment correlation, r. In addition, generalized Proscrutes analysis [12] was used to compare the data matrices of the different primers used.

3. RESULTS

A total of 122 amplification products were scored in the 70 accessions with the primers, which exhibited an overall 90.5% polymorphism (Table II). The average number of amplification products observed was 12.2 with a maximum of 17 in OPE–09 and a minimum of 8 in OPAM–05. The size of the amplification products varied in the case of each primer

and the range was 0.3 kb to 3.0 kb. In general, the extent of polymorphism was very high. An example of the level of polymorphism detectable with one of the primers among the accessions is shown in Figure 1. All but two of the primers revealed more than 80% polymorphism. Primers OPB–19, OPD–01, OPE–09 and OPJ–01 were the most polymorphic, showing 100% polymorphism. Primer OPC–17 was the least polymorphic primer showing 69.2% polymorphism.

TABLE II. LIST OF THE OPERON PRIMERS, THEIR SEQUENCES AND SOME CHARACTERISTICS OF AMPLIFICATION PRODUCTS OBTAINED IN 70 ACCESSIONS OF *Xanthosoma sagittifolium* (AP REPRESENTS AMPLIFICATION PRODUCTS AND PP STANDS FOR POLYMORPHIC PRODUCTS)

Primers	Sequence	No. of AP	No. of PP	%Polymorphism
OPAM–03	5'CTTCCCTGTG–3'	9	8	88.9
OPAM–04	5–'GAGGGACCTC–3'	12	11	91.7
OPAM–05	5'GGGCTATGCC–3'	8	7	87.5
OPB–01	5'–GTTTCGCTCC–3'	9	7	77.8
OPB–19	5'ACCCCCGAAG–3'	16	16	100.0
OPC–05	5'–GATGACCGCC–3'	10	9	90.0
OPC–17	5'–TTCCCCCCAG–3'	13	9	69.2
OPD–01	5'–ACCGCGAAGG–3'	16	16	100.0
OPE–09	5–CTTCACCCGA–3'	17	17	100.0
OPJ–01	5'–CCCGGCATAA–3'	12	12	100.0

Cluster analyses were performed with each primer and compared. Mantel tests gave strong evidence that the results from each primer combination are closely associated ($p \leq 0.001$). For each Mantel randomization test performed (1000 randomisations carried out in each case), the value obtained when data matrices from each primer were compared was greater than that obtained from any of the 1000 randomisations. In addition, the 10 individual sets of data for each primer combination were entered into a generalized Procrustes analysis with appropriate scaling applied [4]. No primer appeared to be an extreme outlier. Together with the Mantel tests, these results indicated that the data from the 10 primers could be combined into a single set for analysis.

Jaccard's similarity coefficient values ranged from 0.15 to 0.84. The phenogram produced by UPGMA of the Jaccard similarity matrix from the pooled data of the 10 primers from the 70 samples is shown in Figure 2. Three major clusters were identified from the phenogram. The first major cluster, which was a single accession, BD 96/183 collected from Awaman Junction (ER) was relatively distant from the other accessions. It separated from the two other major clusters at the 25% level of similarity.

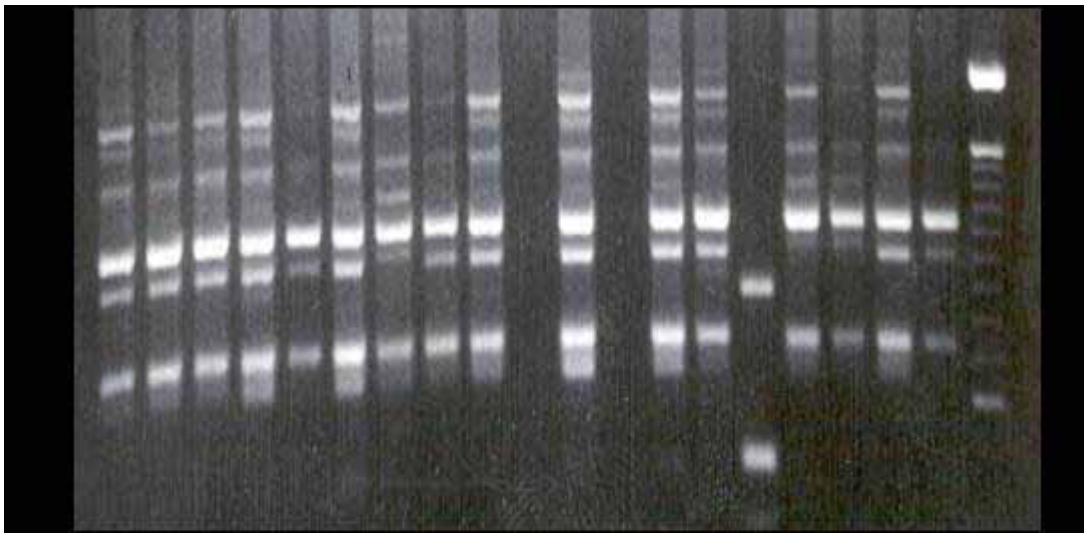

Fig.1. Sample of DNA polymorphism detected in a subset of the 70 accessions of Xanthosoma sagitifolium.

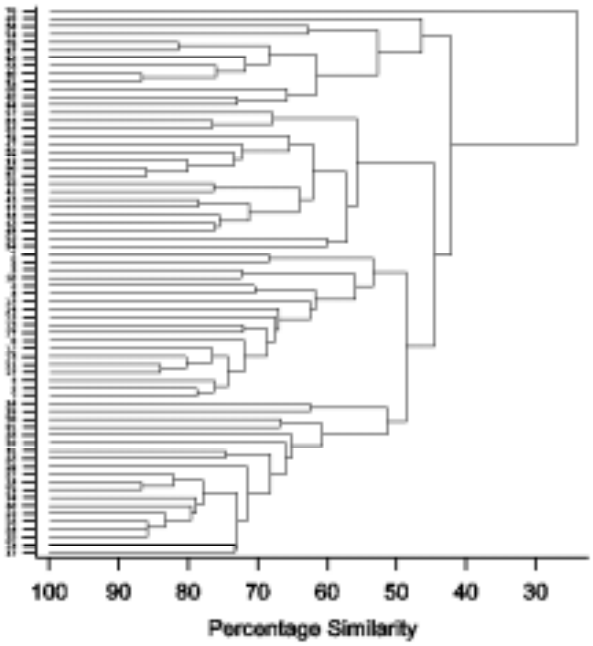

Fig. 2. Phenogram generated using UPGMA analysis showing relationships (based on diversity in RAPDs) among 70 accessions of cocoyams.

The second major cluster was a tight cluster and comprised 12 accessions (BD 96/091, BD 96/123, BD 96/118, BD 96/111, BD 96/102, BD 96/092, BD 96/163, BD 96/158, BD 96/145, BD 96/135, BD 96/126, BD 96/124) collected from 11 different localities (see Table I). The third major cluster was subdivided into three sub-clusters at the 60% level of similarity. Sub-cluster 1 comprised the two accessions (BD 96/078, BD 96/077) from Potroase (ER), the two accessions (BD 96/059, BD 96/059) from Bunso (ER), two accessions from unknown

localities (ER) and three other accessions from different localities (ER). Sub-cluster 2 was made up of 19 accessions, 10 of which were from unknown localities (ER). Of the other nine, two were from Oframoase (ER) and the remaining seven from seven different localities (Table I and Figure 2). The third sub-cluster comprised 20 accessions collected from sixteen different localities (ER) and two unknown places. All of the three accessions from Akora Darko (ER) clustered in this group.

4. DISCUSSION

RAPDs have encountered reproducibility problems [15], however, a number of authors have reported a very high correlation between RFLPs and RAPDs. Thorman *et al*. [33] reported correlations of r = -0.969 between RFLPs and RAPDs for a group of 18 accessions from different *Brassica* species, and Sun et al. (1997) also reported a correlation of r = 0.80 between RAPDs and wheat microsatellite-PCR for 20 accessions of different *Elymus* species. Generally, precision in diversity studies using molecular markers improves as more probes or marker loci are detected in the analysis [19]. Using RAPD markers to analyse annual *Medicago* species, Brummer *et al*. [5] found that at least 10 RAPD primers appear to be satisfactory in order to develop reliable estimates of relatedness among *Medicago* accessions.

To the best of our knowledge, this is the second report describing the use of RAPDs for studying genetic diversity in *Xanthosoma sagittifolium* in Ghana. In a first report [7], we indicated that the *Xanthosoma sagittifolium* collection at our gene bank did not represent the entire genetic resources of the crop and recommended extensive collections to broaden the genetic base. In this study, a larger collection comprising 70 accessions, which had been assembled at the Genetic Resources Centre, was used. Furthermore, we screened several primers and selected 10 very informative ones for the analysis. In our earlier report [7], there was evidence of geographic differentiation among 48 accessions of *Xanthosoma sagittifolium*. Farmers within localities share planting material and over time mutations arising as bud spots could also be selected and given new names and used as new farmers varieties. These may be almost identical genetically to the mother clone. The genetic closeness among accessions from the same locality is, therefore, not unexpected. In the present study, there were a number of cases of accessions from the same locality clustering in the same subgroup with a similarity value of about 80%. A source of confusion is when an accession is moved from one locality to another one and given a different local name. The consequence is the occurrence of several duplicates or genetically closes accessions in totally different and distant localities. In this study, accession BD 96/111 from Mamfe (ER) and BD 96/102 from Awadum (ER) were the closest (90% similarity). Also, accession TA 97/163 from Vakpo (VR) separated from one of the accessions from Akora Darko (ER) at the 85% level of similarity.

Previous studies on cocoyams using morpho-agronomic, isozyme and total protein markers have reported very little variability in the species (see references in the Introduction). Recently, Schnell *et al*. [26] reported very little genetic variation among 18 cultivars of cocoyam from the USDA germplasm collection and recommended the need for introductions to improve the value of the collections as a genetic resource. The clustering pattern did not show any relationship between geographical distribution and genotypic diversity as some genotypes of different geographic origin were grouped in some sub-clusters. This was ascribed to the fact that genetic drift and selection in different environments could cause greater diversity than geographic distances. Sun *et al*. [30] also reported similar findings in *Erymus caninus*. Their conclusion was that geographically distant accessions could be remarkably similar, whereas neighbouring accessions can vary greatly.

DNA based markers have been found to be more useful in detecting genetic diversity in closely related accessions [30]. For any crop species, proper management of the genetic resources depends on the availability of information on relatedness among collections. We have shown that substantial genetic diversity exists in our cocoyam collection. All of the accessions were genetically distinct as no two accessions had the same RAPD profile. The need to exploit this diversity for the improvement of the crop to broaden the genetic base cannot be overemphasized. We are developing root rot resistant cultivars of cocoyam and intend to use RAPD and other DNA markers to determine any associations that there may be between these markers and root rot resistance.

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INDUCTION OF GENETIC VARIATION IN *Xanthosoma* SPP.

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Abstract

Cocoyam (*Xanthosoma sagittifolium*) is an herbaceous perennial plant, which produces edible subterranean cormels, which are consumed in many countries throughout the tropics. In spite of this, it is a neglected crop and its existence is at present hampered by a disease affecting the root system, the root rot disease, whose causal agent in Costa Rica has been identified as *Fusarium* sp., although other reports indicate it is caused by *Pythium myriotylum*. Cultural practices and chemical treatments have not proven effective and plants with a reduced susceptibility have not being obtained by conventional breeding. In the present work, mutation induction was implemented in order to generate genetic variability in cocoyam, specifically resistance to the pathogenic isolate of *Fusarium* sp. attacking cocoyam in Costa Rica. This technique will be combined with micropropagation, which is already developed for this crop. A radiosensitivity assay determined that 4 to 6 Gy is the required dose to induce 30% growth reduction (GR30) in the irradiated *in vitro* grown apices. To determine this dose, several growth variables should be measured for a period of at least 6 weeks. Two hundred apices were irradiated with the selected doses and multiplied *in vitro* for 4 cycles. The resulting 6500 plants were screened in the greenhouse in sterile soil inoculated with the pathogenic strain of *Fusarium* sp. isolated from diseased cocoyam plants. Seventeen of these plants showed reduced severity symptoms of root rot disease. These plants will be further tested in the greenhouse and in the field.

1. INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium*) is a herbaceous perennial plant with a corm or main underground rhizome from which swollen secondary shoots, cormels, and sprout. It belongs to the *Araceae* family and its origin is believed to be the American Tropics [1].

Cormels are an excellent source of carbohydrates and they contain essential amounts of proteins and vitamins [2]. This crop represents a very important staple crop in many countries in Africa, Asia and America. In Costa Rica, cocoyam is cultivated also as a cash crop, exported to Europe and United States.

Cultivation in Costa Rica has increased significantly in the last two decades, from a few hectares to 4.000 ha. during 2002. This increase in cultivated area was also reflected in the value of exports that went from 0.3 million USD in 1983 to 7.9 million USD in 2002.

Associated to this increase in area phytopathological problems have also been observed, namely Dasheen Mosaic Virus (DMV) and root rot disease (RRD). DMV, but not RRD, can be effectively controlled through the use of virus-free plants produced by meristem culture [3,4].

RRD is the main obstacle for worldwide production of cocoyam. In Costa Rica, this disease is responsible for low yields and often, total losses. The characteristic symptoms are the withering and yellowing of the foliage, where only the youngest leaves remain green (Figure

1a); and a partial to total absence of the root system. Infected fields cannot be cultivated again because the pathogen can survive in the soil and disease symptoms rapidly reappear, forcing farmers to move to new areas. Flooding is commonly associated with the appearance of the disease (Figure 1b).

Fig. 1. Infected cocoyam plantation with root rot disease. (A) Typical symptoms of RRD in the front; in the back healthy plants. (B) Infected field showing water accumulation problems, which favor disease appearance and dissemination.

A consensus about the causal organism of this disease does not exist; in reference [5] it is reported that in Cameroon *Pythium myriotylum* was the causal organism, while [6] reported in Costa Rica that *Erwinia* and *Pseudomonas* present in infected cocoyam plants could reproduce the RRD symptoms. Recently, work in our laboratory has found *Fusarium* as the main causing agent. This discrepancy could be explained by the fact that any root disease will cause similar foliage symptoms, and therefore different diseases might have been named RRD. A comparison of root symptoms might help to clarify this matter.

Several control methods have been attempted but none of them have given satisfactory results. Cultural practices such as using drainage canals and planting clean material reduce the risk of infection but when infection appears the disease rapidly disseminates. Farmers because of its high cost do not use chemical control. Conventional breeding has been systematically attempted but no resistant varieties have been obtained [7,8,9], suggesting that this crop probably has a reduced genetic diversity in relation to RRD resistance, probably induced by its almost strict asexual propagation. Its reduced genetic diversity is also evidenced by the fact that most countries cultivate only two varieties, white and red cocoyam. Attempts to cross with wild species, which show resistance, like yellow cocoyam, have failed due to ploidy incompatibility [7].

One approach that has not been attempted in cocoyam to obtain resistance to RRD is mutation induction. This technique allows increasing genetic variation [10], and has successfully been used in wheat, where it has been demonstrated that disease resistance to several diseases such as yellow rust, mildew and brown rust can be obtained by inactivation of genes promoting disease susceptibility [11]. In tomato, resistance to several diseases has been observed in a mutant plant (Sitiens), which shows reduced ABA content [12] caused by a mutation in the ABA biosynthetic pathway.

The aim of the present work is to increase genetic variability in cocoyam (*X. sagittifolium*) through irradiation with gamma rays in order to obtain resistance to RRD caused by *Fusarium* sp. Since micropropagation techniques for this crop are available, both techniques will be combined for the process of irradiation and chimera dissociation [13]. Screenings will be done in the greenhouse with sterile soil inoculated with *Fusarium* sp.

2. MATERIALS AND METHODS

2.1. Selection of material to be irradiated

Individual plants showing high yield (> 1 Kg of cormels), long and straight cormels, without RRD symptoms and without DMV symptoms were selected as the starting material for this work.

2.2. Explant disinfection

Healthy cocoyam cormels from the plants selected in the field were placed in a dark, humid place to induce sprouting, and 3 to 5 cm sprouts were cut off from the cormels. Sprouts were then trimmed to a structure of 1x1 cm in the base and 1 to 2 cm high. These were rinsed in 70° ethanol for 1 min followed by 15 min in a solution of 2% sodium hypochlorite and 1 drop of Tween 20 per 100 ml. Explants were washed three times with sterile water in a laminar flow hood.

2.3. Establishment of *in vitro* cultures and *in vitro* multiplication

With the aid of a dissecting microscope, shoot-tips were trimmed until only the apical dome and one or two leaf primordia remained (~0.5 mm). Each apical meristem was then transferred to 18x150 mm test tubes with 10 ml of establishment medium consisting of Murashige and Skoog (MS) [14]+3% sacarose+25 mg/L indolacetic acid+2 mg/L Kinetin.

Once a plant had regenerated, the apical meristem was removed by cutting off the upper fourth part of the main corm. The remaining corm was then cultivated in multiplication medium (MS+3% sacarose+3 mg/L benzyladenine) to promote axillary bud development. After 4 weeks in multiplication medium the new sprouts were individualized and cultivated in growth medium (MS+3% sacarose) for a period of four weeks [4]. After this step the plants were decapitated again and either placed on multiplication medium or acclimatized in the greenhouse.

Both media were solidified with 0.18% Phytagel®. The pH was adjusted to 5.7 0.1 before autoclaving for 15 min at 121°C. Explants were cultured at 25±2 °C under 12 hr photoperiod. The light source was cool white fluorescent tubes (Phillips 60 W) providing 50 μmol m^{-2} s^{-1}.

2.4. Virus detection

To index the plants for the presence of Dasheen Mosaic Virus (DMV), the enzyme linked immunosorbent assay (ELISA) (double antibody sandwich) technique was used. Antiserum from AGDIA Inc. was used. This detection was done on leaves from regenerated meristems.

2.5. Acclimatization and greenhouse culture

Plantlets were removed from the culture tubes and washed with tap water to remove the gelled medium. Roots were cut off. Plants were placed in 128-compartment styrofoam trays, filled with sterilized substrate composed of soil and coir (coconut mesocarp pith) (2:1). They were maintained under a plastic tunnel (40 cm high) for 2 weeks and then outside the tunnel for another 3 weeks before they were screened. Three days after transplanting, and every week thereafter, the plantlets were fertilized using 20:20:20 (N:P:K) by foliar sprays and micro-minerals as recommended by the suppliers.

2.6. Preparation of explants for irradiation

Virus free plants were dissected removing leaves and leaf primordia until a structure (apex) of 2 mm x 2 mm in the base and 3 mm tall was obtained.

In a first test to determine the irradiation dose, 23 apices were irradiated per dose selected. The selected doses were: 0, 10, 20, 30 and 40 Gy. The power of the irradiation source was at 1.524 Gy/second, therefore the irradiation times were 0, 6.56, 13.12, 19.68 and 26.24 seconds respectively.

After irradiation, the apices were transferred to fresh medium for the development of the apex into a complete plant. After 6 weeks the explants were evaluated for survival, color, height, number of roots and length of the longest root.

A second test compared doses between 0 and 13.3 Gy. The selected doses were: 0, 2.66, 5.33, 7.98, 10.64 and 13.30 Gy. The power of the irradiation source was 1.479 Gy/second, therefore the irradiation times were 0, 1.35, 2.70, 4.05, 5.40 and 6.75 seconds respectively. Thirty apices were irradiated per dose. The variables measured were: height, number of roots, and length of longest root. The irradiation dose selected was the one that induced a 30% growth reduction (GR30) in the plants.

2.7. Screening of plants

Fusarium sp. had been determined to be the main pathogen associated to RRD in several areas in Costa Rica. This pathogen was maintained in PDA medium. For inoculation, the mycelium and conidia from a Petri plate were mixed with water and inoculated to the plants by adding 5 ml of a solution of 100.000 conidia per ml to each plant. Two weeks after inoculation the plants were scored for infection symptoms. Plants that maintained green leaves were selected for further screening.

3. RESULTS AND DISCUSSION

3.1. Determination of irradiation dose

First irradiation. Apices of cocoyam DMV-free *in vitro* plantlets were selected as explants to be irradiated since regeneration is easily obtained from them and, as they are small, a large number of these explants could be irradiated at the same time.

Control (non irradiated apices) developed into complete plants after six weeks of culture. In the irradiated explants survival was not affected by the irradiation doses selected, it was only

reduced to 91% with the highest dose tested (40 Gy). However, the development of the apices into plants was significantly affected as demonstrated by all growth variables: height, number of leaves, number of roots and root length (Table I).

If the survival of explants is taken as the selection criteria for the irradiation dose, higher doses will be required to obtain 50% survival, as recommended for seed-propagated crops. However, it should be considered that even at the lowest dose tested (10 Gy) there is a significant effect in growth. It is possible that doses over 40 Gy will have a larger detrimental effect on growth and it would not be possible to micropropagate the plants.

An alternative solution to select the irradiation dose was to determine it based on explants growth. A 30% growth reduction (GR30) has been recommended for vegetatively propagated crops. It is considered that doses that show further growth reduction have a larger probability of inducing useless mutants. Based on the results in Table I, all growth variables show a reduction in growth larger than 30% even at the lower dose. Only in the case of the discoloration on the explants, at 10 Gy, 30% of the explants were not green; however, this variable is not a direct measure of growth, therefore it was necessary to evaluate a lower range of doses.

TABLE I. EVALUATION OF IRRADIATED APICES AFTER SIX WEEKS FROM IRRADIATION

Dose Gy	% Survival	Plant color		Height Cm	No. Leaves	No. Roots	Root Length cm
		Green	Discolored				
0	100	72	28	1.24	1.56	4.44	2.86
10	91	65	35	0.71	0.22	0.04	0.11
20	100	48	52	0.5	0.09	0.04	0.02
30	96	22	78	0.36	0.13	0.0	0.0
40	91	65	35	0.52	0.04	0.0	0.0
Percentage with respect to non irradiated apices (0 Gy)							
0	100	100	100	100	100	100	100
10	91.0	90.3	125.0	57.3	14.1	0.9	3.8
20	100.0	66.7	185.7	40.3	5.8	0.9	0.7
30	96.0	30.6	278.6	29.0	8.3	0.0	0.0
40	91.0	90.3	125.0	41.9	2.6	0.0	0.0

Second irradiation. A second group of apices were irradiated at a lower range of doses to identify the GR30 dose. After 6 weeks on growth medium, the apices were evaluated for survival, height, and total number of leaf, number of green leaves, total number of roots and length of longest root. Evaluations made before 6 weeks of culture (data not shown) did not show clear differences among treatments.

In figure 2 the effect of the irradiation is visible on the growth of the plants in doses of 7.9 Gy or more (Figure 2b) but that effect is not obvious for lower doses (Figure 2a). However, when growth variables are analysed, it is observed that even the lower doses can have a significant effect on plant growth, for example in plant height (Figure 3a).

Fig. 2. Plants of Xanthosoma sagittifolium irradiated with increasing doses of gamma rays after 6 weeks of growth on MS medium. Doses, from left to right, were: 0, 2.66, 5.33 in Fig. 2a and 7.98, 10.64 and 13.30 Grays in Fig. 2b.

Increasing exposure to gamma irradiation had a negative effect in all variables measured, however not all variables were affected in the same proportion, and this response made the determination of a GR30 more complex. For example, with plant height, a dose between 2.6 and 5.3 Gy should be used to obtain a GR30; for plant survival, the dose should exceed 13.3 Gy (Figure 3b); for the total number of leaves and of green leaves, it should be between 5.3 and 10.6 Gy (Figure 3c and 3d); for the maximal root length, between 2.6 and 5.3 (Figure 3e); and for the total number of roots, between 5.3 and 7.9 (Figure 3f).

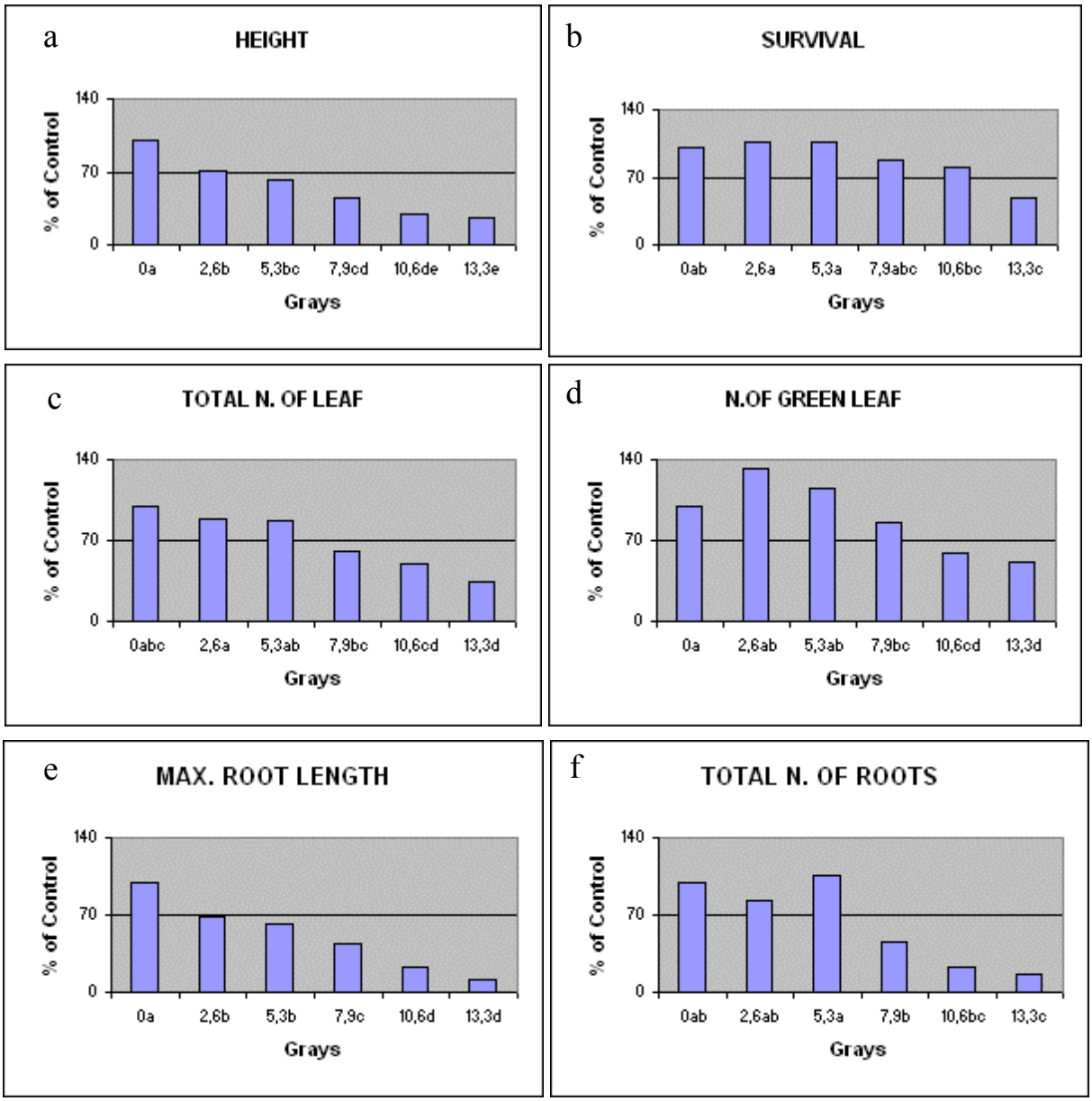

Fig. 3. Effect of increasing exposure to gamma rays on the growth of cocoyam in vitro plants. The values represent the growth of plants compared to the growth in percentage of the non-irradiated explants 6 weeks after irradiation. Treatments with different letters differ significantly according to LSD test at 5%. Calculations were made on the absolute values for each variable.

To help determine the most accurate GR30 a figure including all variables is given in Figure 4. In this figure two variables, survival and the number of green leaves indicate that the GR30 should be over 8 Gy. As deducted from the first irradiation test, survival is not a good indicator for vegetatively propagated crops, so it was not considered.

Plant height and maximal root length show a very similar response indicating that the GR30 should be approximately 3 Gy. The total number of leaves and total number of roots also show a similar response, suggesting that the GR30 should be of approximately 7 Gy. Based on this results and average GR30 should be 5 Gy; however, it was decided that for further irradiations two doses will be selected: 50% of the explants will be irradiated at 4 Gy and 50% at 6 Gy.

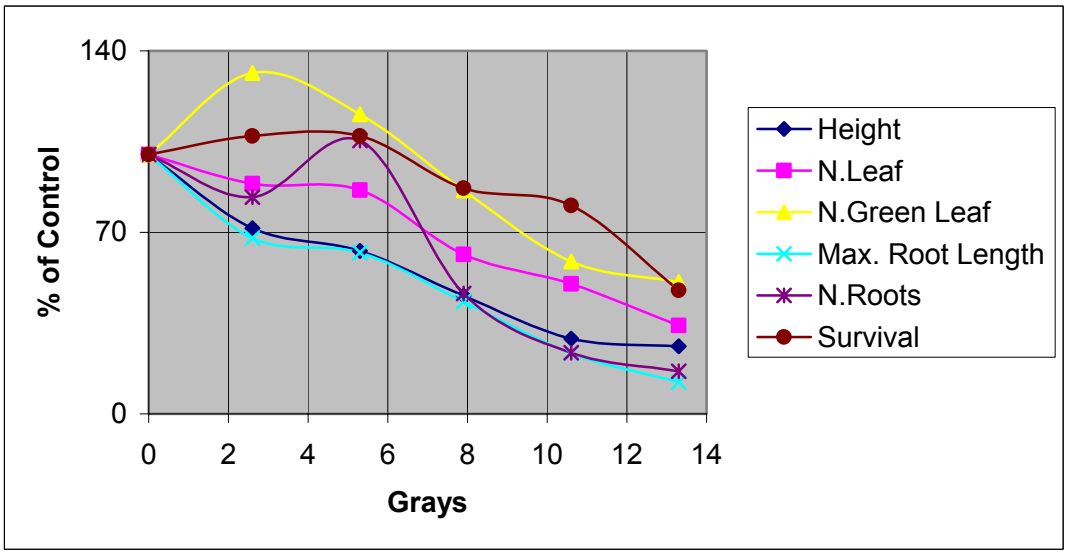

Fig. 4. Effect of gamma irradiation on the growth of cocoyam in vitro plants. The values are given as a percentage referred to the control, non-irradiated, plants.

Two other irradiation tests were made again using doses in the range of 0 to 10 Gy (data not shown). In these tests, the response of apices and plantlets (2 cm) was compared, but no significant differences were found indicating that both type of explants are suitable. Plant height and maximun root length showed similar responses after 6 weeks of culture.

3.2. Multiplication of irradiated apices

The apices or plantlets from the treatments where a 30% growth reduction was obtained (approximately 220 explants) were multiplied *in vitro* for four generations as recommended [13]. *In vitro* multiplication in cocoyam could be obtained through organogenesis [15] or embryogenesis [16]. The organogenic route starts from a 3–5 cm rooted plant from which the meristem is removed by cutting off the apical fourth part of the plant corm. The corm is then subcultured into multiplication medium, normally MS supplemented with benzyladenine, and axillary buds develop after 2–3 weeks. This budded corm could then be either subdivided and maintained in multiplication medium (Large Clump Technique), or the buds could be individualized and allowed to develop into full plants in growth medium before transferring them back into multiplication medium (Small Clump Technique).

The small-clump-technique was used because it allowed a better follow up of the origin of the axillary buds, since in the large-clump-technique it is possible that all the axillary buds develop continuously from the initial irradiated corm and not from successive generations of axillary buds, which favours chimera dissociation and stabilization of mutations [13].

3.3. Acclimatization and screening of plants

After the plants had been multiplied for four cycles they were transferred to the greenhouse for acclimatization. The plants were maintained for two weeks in a high humidity tunnel, to prevent dehydration, and then outside the tunnel for three weeks before screening. Figure 5a shows a tray of acclimatized plants ready to be screened. More than 95% of the plants were successfully acclimatized.

Some mutants were easily detected in the acclimatization trays. Figure 5b shows some of these putative mutants, probably affected in their meristem and developmental program, since they seemed normal during *in vitro* culture and acclimatization, but later they started developing very small leaves.

Screening was done in the greenhouse for three reasons: first, the screening can be done in a shorter period of time, 5–6 weeks after acclimatization; second, the conditions of screening are more homogeneous than in the open field, since all plants will receive the same amount of inoculum; and third, because the pathogen causing root rot disease is not clearly identified, and if there is more than one pathogen involved, it might be possible to miss useful mutants that were resistant to one pathogen but not to others. These mutants would be useful in breeding programs.

The pathogen selected for screening was an isolate of *Fusarium* sp. that had been obtained from diseased plants and had been shown to be pathogenic when re-inoculated in healthy cocoyam plants. It was maintained in PDA medium. Adding 10 ml of distilled water to the plates made conidia suspensions and the suspension was diluted to 100,000 conidia per ml, and 5 ml of this suspension was added to each plant. Approximately 6,500 plants were evaluated.

Development of infection symptoms was affected by the age of the plants in the greenhouse, 6–week–old plants develop root rot symptoms 2 weeks after inoculation, while 12–week–old plants showed symptoms 4 weeks after inoculation, probably because of the larger root system present in older plants (Table II).

Using this strategy, 17 plants showed reduced susceptibility symptoms to the disease (Figure 5e and 5f). These plants maintained the presence of two or more green leaves in inoculated soils, while all other plants lost all green leaves (Figure 5c).

Greenhouse screening presented some difficulties like contamination of sterile substrate. It was common to see that the trays with healthy plants developed disease symptoms before inoculation with the pathogen, although when the soil was analysed *Fusarium* was present. It is possible that the inoculum present in the screening greenhouse was very high and it was not possible to contain it within the infected trays.

TABLE II. INCIDENCE OF *FUSARIUM* SP. ON ROOTS OF IRRADIATED, *IN VITRO* DERIVED COCOYAM PLANTS. FIVE MILLILITER OF INOCULUM WERE ADDED TO EACH PLANT

Plant age	Conidia concentration per ml	% of incidence after inoculation	
		15 days	30 days
15 days	0	0	0
	50,000	0	100
	100,000	40	100
90 days	0	0	0
	50,000	0	0
	100,000	0	100

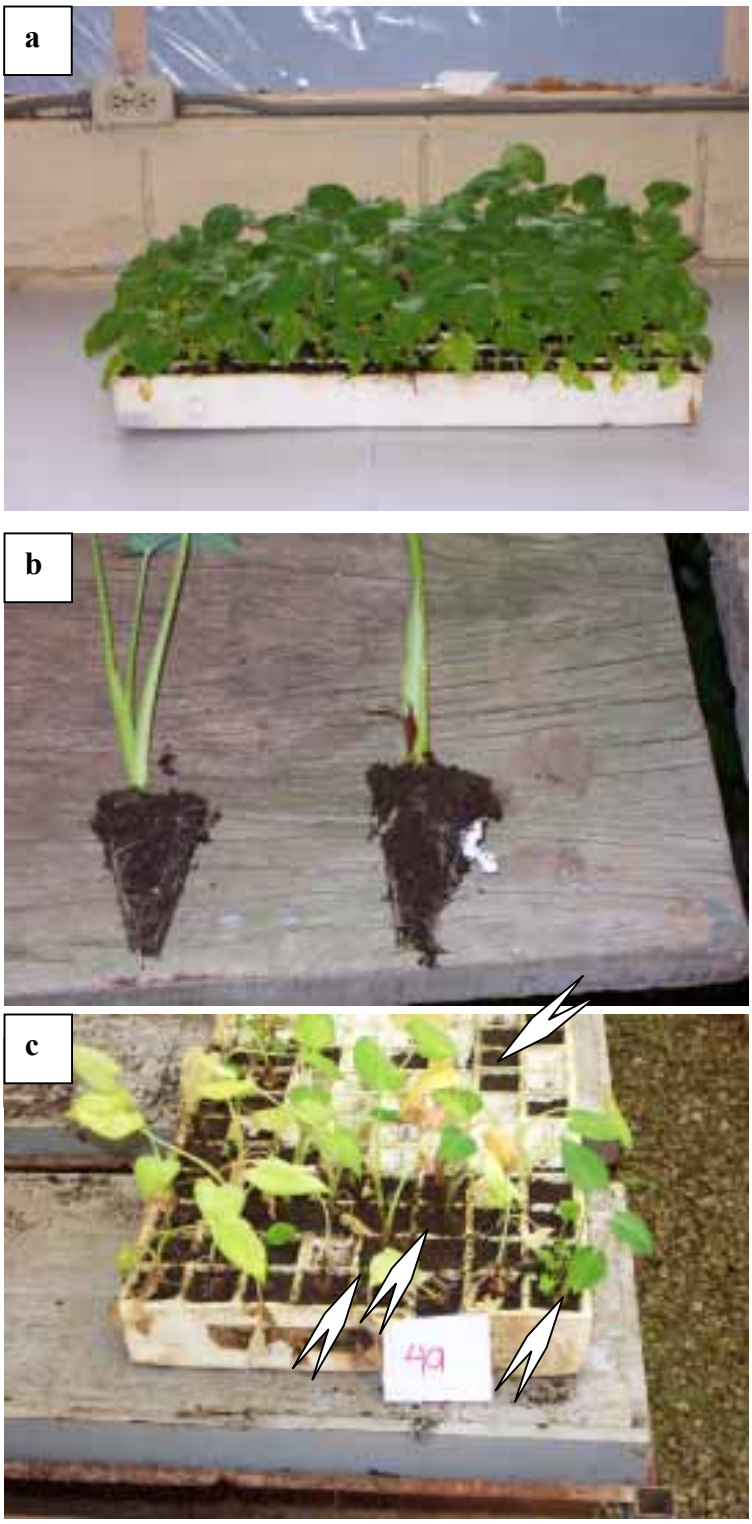

Fig. 5. Acclimatization and screening of cocoyam plants. (A) Tray with acclimatized cocoyam plants ready to be screened. (B) Putative mutants observed among the acclimatized plants. (C) Inoculated tray 30 days after inoculation, possible mutants, with reduced susceptibility to Fusarium sp indicated by arrows.

Another problem that was faced, and that prevented the evaluation of all the 9,000 plants produced, was that the pathogen halted its production of conidia after some time in culture, and therefore it was not possible to quantify the inoculum to screen the remaining plants.

Several growth media such as water-agar, Carnation Leaf-agar and rice, as well as several growth conditions as light/dark, humidity and age were tested to try to induce conidia formation. When new isolates were obtained these soon stopped producing conidia.

At present we are evaluating the inoculation of plants with quantified amounts of mycelium, as it is done with *Pythium myriotylum* by [17], considering that each strand of mycelium can germinate and be infective. Liquid medium for the sporulation of *Fusarium* will also be tested.

Future work will focus on the study of the 17 isolated clones; these will be micropropagated and re-evaluated in the greenhouse and under field conditions. Also new batches of cocoyam apices have been irradiated and are being multiplied and it is expected that more resistant genotypes will be produced. Work will also be undertaken on the optimization of the screening procedure, since it was found to be the most difficult step in this procedure.

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INDUCED MUTATION BREEDING FOR RESISTANCE TO YELLOW VEIN MOSAIC VIRUS IN OKRA

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Abstract

Seeds of Annie and Okura okra varieties were irradiated by gamma rays to induce mutations for resistance to yellow vein mosaic virus disease (YVMD). In experiment I, seeds were irradiated at 400, 600 and 800 Gy and then planted at Huaysai King's Project, Petchaburi Province. Plants with a good plant type and green pods were selected for M_2 generation. M_3 plants were grown at Phichit Horticultural Research Center (PHRC) where YVMD was seriously widespread. 33 plants without disease symptoms were selected for further screening. By using white fly transmission under greenhouse conditions, only four lines showed no disease symptoms. They were transplanted to the field at PHRC. Only Okura irradiated at 400 Gy, designated Rd53–3 showed disease resistance. Subsequent selections were conducted under greenhouse and field conditions up to M_7 generation. Twelve resistant lines showing uniformity of plant type were selected for yield trial observation. All bore reasonable yield but their fruit shape was not suitable for the export market. They will be used as parental lines in further breeding programs. In experiment II, seeds of Annie and Okura were irradiated at doses of 400 and 600 Gy and then planted at Huaysai. M_2 seeds were collected by two methods, the pedigree method by collecting seeds from selected healthy plants, and then planted them at Huaysai. The other method was bulk selection and then seeds were planted at Huaysai and PHRC. The M_3 generation was screened for disease resistance under greenhouse and field conditions. The plants from bulk selection were all infected. By pedigree selection, 34 and 35 lines of Annie and Okura, respectively were obtained. Screening for okra YVMD resistance under greenhouse and field conditions in M_4 generation, it was found that only one plant of Okura irradiated at 400 Gy designated B–21, showed no disease symptoms. Disease resistance screenings under greenhouse and field conditions were performed during M_5 to M_7 generation. Ten lines selected from B–21 showed a good plant type coupled with a desirable fruit shape. They will be used for further yield trials.

1. INTRODUCTION

There are nine species of okra worldwide: *Abelmoschus moschatus, A. manihot, tetraphyllus, A. esculentus, A. tuberculatus, A. ficulneus, A. crinitus, A. angulosus* and *A. caillei* [1]. Okra is grown in India, Sri Lanka, Africa the Caribbean coast and Southeast Asia. In Thailand, *A. esculentus*, commonly known as Krachiab khiew, is cultivated as a home garden crop or on a commercial scale. It is generally cultivated as an annual crop. It flowers within 50 days after

sowing. Petal color is yellow and purple at the base. It has a perfect flower. Young fruits, 6–10 cm. long can be harvested a few days after flowering. The fruit shape is slender, angled to round. Each fruit contains 80 to 200 seeds. Okra fruits provide an important input of vitamins (e.g. A, C) and minerals including calcium [2]. They contain mucilage that gives a slippery texture on cooking. Okra is not only eaten as a vegetable but also used as traditional food for reducing symptoms of peptic ulcers.

The major okra growing area is the central part of Thailand near Bangkok. The total area under okra was increased from 320 ha to 1,600 ha in 1996. The growing area and production was decreased in 1997 by up to 50% due to the disease [3]. Okra plants infected with yellow vein disease display vein yellowing mosaic, yellow leaves and shoots, curling or rolling of shoots and top leaves, and yellow fruits. Severely infected plants are also stunted and they produce fewer fruits. The disease was first observed in 1995/1996 in the growing areas producing for export to Japan. The casual agent of this disease was found to be a geminivirus, which was transmitted by the tobacco white fly (*Bemisia tabaci*) [4]. To solve the problem, a breeding program for okra yellow vein mosaic disease (OYVMD) resistance has been conducted since 1996 by crossing between previously used commercial cultivars and resistant varieties from India. Induced mutation by using gamma radiation is another tool to develop genotypes resistant to OYVM virus as well as high yielding and good quality (shape, color, texture, size) fruits for export.

2. MATERIALS AND METHODS

2.1. Mutation induction by gamma radiation and planting of M_1 generation

Seeds of Annie and Okura okra varieties were irradiated with gamma rays doses of 400, 600 and 800 Gy and planted with non-irradiated ones in November 1997 as experiment I. The other set of okra seeds were irradiated at doses of 400 and 600 Gy and planted with non-irradiated ones in February 1999 as experiment II.

2.2. Selection in M_2 and M_3 generation

M_2 seeds were collected from bagged flowers for selfing. M_2 seeds from plants with good quality phenotype were planted at Huaysai King's Project, Petchaburi Province. Plants showing a healthy performance were selfed and seeds were collected. M_3 seeds were planted at Phichit Horticultural Research Center, Phichit province where OYVMD was seriously widespread.

2.3. Selection in M_4 and M_5 generation

M_3 plants without disease symptoms were selected to collect M_4 seeds (each plant as a line). About 40 seeds of each line were planted and screened for OYVMD resistance by using white fly transmission under greenhouse conditions at Crop Protection Research and Development Office. Plants without disease symptoms were transplanted to the field at PHRC. The remainder seeds of these resistant lines were also planted for field screening at PHRC. The resistant plants were selected to collect M_5 seeds. The same selection techniques in M_4 generation were repeated in M_5 generation.

2.4. Selection in M_6 and M_7 generation

M_6 seeds were collected from M_5 plants showing no disease symptoms in field conditions. Seeds of each line were divided into two groups. One was planted and screened for OYVMD resistance under greenhouse conditions. The other one was planted at PHRC for field screening. The results from both groups were compared. The resistant plants were selected to collect M_7 seeds. Seeds were planted in the field for disease screening. Seeds of selected resistant lines were collected for further yield trials.

2.5. Yield trial of selected resistant lines

The yield trial was conducted at PHRC. Plant height, date of flowering and numbers of plants showing OYVMD symptoms were observed. Fruit quality and yield were also recorded.

3. RESULTS

3.1. In experiment I

3.1.1. Mutation induction by gamma radiation and planting of M_1 generation

Induced mutation in okra was initiated in November 1997. Seeds of Annie and Okura varieties were irradiated by gamma radiation at doses of 400,600 and 800 Gy (200 seeds per dose in Annie; 1,000 seeds per dose in Okura). They were planted with non-irradiated one as control at Huaysai. M_1 plants from irradiated seeds showed mutated characters such as 2–3 shoots per plant, twin fruits and two fruits on an internode. Plant height, number of fruits per plant, fruit shape and color of M_1 plants were recorded. Three fruits from selfed flowers were collected from each M_1 plant. M_2 seeds from plants producing high fruit yields (more than 20 fruits per plant) were selected. 54 plants were selected to collect M_2 seeds (Table I).

TABLE I. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND PLANTS SELECTED IN M_1 GENERATION OF ANNIE AND OKURA GROWN AT HUAYSAI, PLANTING DATE 12–11–97 (EXPERIMENT I)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of plants selected
Annie	0	200	80	4
	400	200	90	2
	600	200	90	5
	800	200	85	2
Total		800	345	13
Okura	0	1,000	689	7
	400	1,000	674	19
	600	1,000	691	10
	800	1,000	658	5
Total		4,000	2,712	41

3.1.2. Selection in M_2 and M_3 generation

In August 1999, 3,280 M_2 seeds were planted at Huaysai. Due to the rainy season, leafhopper and white fly did not infest the plants intensively. The characters of 2–3 shoots per plant and

twin fruits were rarely found in M_2 generation. 63 plants with a good plant type were selected for further screening (Table II).

In April 2000, some M_3 seeds of 63 lines and one resistant variety from Novartis were planted in rows between the susceptible varieties, Phichit 03 at PHRC. Most of M_3 plants were infected with OYVMD and attacked by leafhoppers. Disease rating was performed at 39 days of plant age and the results are shown in Table III. Overall rating was done in July. 33 plants from Okura with good plant type and without disease symptoms were selected to collect M_4 seeds. The number of M_3 plants selected at a dose of 0, 400 and 600 Gy were 17, 8 and 8, respectively.

TABLE II. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND PLANTS SELECTED IN M_2 GENERATION OF ANNIE AND OKURA GROWN AT HUAYSAI, PLANTING DATE 23–08–99 (EXPERIMENT I)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of plants selected
Annie	0	200 (4)	144	0
	400	80 (2)	58	0
	600	200 (5)	113	1
	800	80 (2)	54	0
Total		560 (13)	369	1
Okura	0	280 (7)	193	14
	400	1,490 (19)	756	29
	600	690 (10)	349	17
	800	260 (5)	103	2
Total		2,720 (41)	1,401	62

(..) = No. of selected M_1 plants

TABLE III. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND PLANTS WITHOUT DISEASE SYMPTOMS IN M_3 GENERATION OF ANNIE AND OKURA GROWN AT PHICHIT HRC, PLANTING DATE 11–04–00 (EXPERIMENT I)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of plants without disease symptoms
Annie	600	40 (1)	27	4
Okura	0	560 (14)	381	56
	400	1,160 (29)	797	79
	600	680 (17)	438	62
	800	80 (2)	42	1
Novartis	0	40	26	19
Phichit03	0	2,600	1,660	39

(..) No. of selected M_2 plants

3.1.3. Selection in M_4 and M_5 generation

Seeds of 33 lines in M_4 generation with early five and Phichit 03 as the susceptible varieties were planted and screened under greenhouse conditions in September 2000. Four lines with 21 plants showed no disease symptoms at 37 days of plant age (Table IV). They were transplanted to the field. Only Okura irradiated at 400 Gy, designated Rd 53–3 showed disease resistance.

TABLE IV. OYVMD SCREENING IN GREENHOUSE OF OKRA VAR. OKURA IN M_4 GENERATION (EXPERIMENT I) PLANTING DATE: 28–08–00; INOCULATION DATE: 01–09–00; TRANSPLANTING DATE: 10–10–00

No.	Line	Designation	Dose (Gy)	No. of pods	No. of tested plants	No. of infected plants at 23 days	30 days	37 days	No. of non-infected plants to transplant
1	Rd 4–1	05–9–60–3–13–1	600	4	14	14	14	14	0
2	Rd 7–21	05–10–40–28–6–21	400	2	26	25	25	25	1
3	Rd 8–13	05–12–0–21–4–13	0	2	26	23	24	26	0
4	Rd 9–4	05–12–0–21–5–4	0	3	19	18	18	18	1
5	Rd 10–1	05–12–0–21–6–1	0	2	20	19	19	20	0
6	Rd 10–4	05–12–0–21–6–4	0	2	4	3	4	4	0
7	Rd 10–5	05–12–0–21–6–5	0	3	28	27	27	28	0
8	Rd 10–7	05–12–0–21–6–7	0	3	26	25	26	26	0
9	Rd 11–8	05–12–0–21–8–8	0	3	29	29	29	29	0
10	Rd 12–9	05–12–0–21–10–9	0	4	23	20	23	23	0
11	Rd 13–2	05–12–0–21–14–2	0	2	17	14	16	17	0
12	Rd 20–6	05–12–40–2–31–6	400	1	3	3	3	3	0
13	Rd 24–6	05–19–0–17–3–6	0	7	26	24	26	26	0
14	Rd 25–1	05–19–0–17–6–1	0	4	22	17	20	22	0
15	Rd 25–8	05–19–0–17–6–8	0	3	15	14	14	15	0
16	Rd 26–1	05–19–0–17–8–1	0	1	32	30	32	32	0
17	Rd 26–8	05–19–0–17–8–8	0	3	4	3	4	4	0
18	Rd 27–3	05–19–0–17–10–3	0	5	17	14	16	17	0
19	Rd 29–6	05–19–0–17–22–6	0	3	19	16	18	19	0
20	Rd 30–4	05–19–0–17–2–4	0	3	23	23	23	23	0
21	Rd 32–1	05–19–40–26–16–1	400	5	18	14	18	18	0
22	Rd 34–5	05–19–40–35–3–5	400	4	24	19	20	24	0
23	Rd 34–8	05–19–40–35–3–8	400	4	19	18	19	19	0
24	Rd 37–1	05–19–60–12–6–1	600	1	25	24	25	25	0
25	Rd 38–14	05–19–60–12–9–14	600	3	20	18	18	20	0

No.	Line	Designation	Dose (Gy)	No. of pods	No. of tested plants	No. of infected plants at			No. of non-infected plants to transplant
						23 days	30 days	37 days	
26	Rd 41–3	05–19–60–42–5–3	600	4	16	15	16	16	0
27	Rd 41–8	05–19–60–42–5–8	600	3	26	23	26	26	0
28	Rd 53–1	011–10–40–10–5–1	400	2	8	7	8	8	0
29	Rd 53–3	011–10–40–10–5–3	400	7	27	0	0	9	18
30	Rd 55–12	011–10–40–10–7–12	400	5	22	22	22	22	0
31	Rd 58–10	011–10–60–11–3–10	600	1	16	14	15	16	0
32	Rd 60–1	011–10–60–11–33–1	600	5	21	18	18	20	1
33	Rd 60–4	011–10–60–11–33–4	600	1	2	2	2	2	0
34	Early five		0		18	16	18	18	0
35	Phichit03		0		14	14	14	14	0

The same result was obtained from seed growing of these lines (Table V).

Fruits from each plant were collected separately for further screening. M_5 seeds of 18 plants from transplants and only 13 plants from seed growing were planted and screened for OYVMD resistance in the greenhouse in April 2001. The number of selected plants without disease symptoms from transplants and seed-grown plants were 84 and 57 respectively (Table VI). They were transplanted to the field for OYVMD screening in May 2001.

TABLE V. OYVMD SCREENING OF M_4 SEED PLANTING OF OKURA AT PHRC (EXPERIMENT I) PLANTING 10–10–00

Line	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of infected plants	No. of non-infected plants
Rd 7–21	400	0	0	0	0
Rd 9–4	0	84	48	48	0
Rd 53–3	400	59	14	0	14
Rd 60–1	600	109	59	59	0

3.1.4. Selection in M_6 and M_7 generation

During screening for resistant lines in M_5 generation, from both the seed-grown and transplanting group, 49 lines were obtained. These lines showed uniformity in plant type but fruit shape was too slender. Seeds of each line (plant) were divided into two groups. One was planted and screened for OYVMD resistance under greenhouse conditions with Phichit03 and Starlight as the susceptible varieties. Almost all lines showed disease resistance except Ird 53–3–8–3 and Rd 53–3–10–2. The other group grew in the field. The results of both screenings are shown in Table VII. A total of 33 plants without disease symptoms and with a shorter plant type were selected to collect M_7 seeds. They were planted and screened for OYVMD resistance in field conditions at PHRC as shown in Table VIII. Twelve resistant lines showing uniformity of plant type were selected for further yield trial observation.

TABLE VI. OYVMD SCREENING IN GREENHOUSE AND FIELD OF OKURA IN M_5 GENERATION (EXPERIMENT I)

Planting date 30–03–01 Inoculation date 02,03,10–04–01		Greenhouse evaluation 26–04–01			Transplant date 10–05–01 (PHRC) Field evaluation after 90 days		
No.	Line	Number of plants		% Non-infected plants	Number of transplants		% Non-infected plants
		Infected	Tested		Infected	Tested	
1	Ird 53–3–1	1	25	96.00	1	4	75.00
2	Ird 53–3–2	0	9	100.00	2	3	33.33
3	Ird 53–3–3	1	19	94.74	2	4	50.00
4	Ird 53–3–4	1	21	95.24	2	4	50.00
5	Ird 53–3–5	0	16	100.00	3	6	50.00
6	Ird 53–3–6	0	17	100.00	3	4	25.00
7	Ird 53–3–7	0	15	100.00	4	4	0.00
8	Ird 53–3–8	0	1	100.00	0	0	0.00
9	Ird 53–3–9	0	10	100.00	3	3	0.00
10	Ird 53–3–10	0	25	100.00	2	3	33.33
11	Ird 53–3–11	0	23	100.00	6	6	0.00
12	Ird 53–3–12	1	19	94.74	4	5	20.00
13	Ird 53–3–13	0	27	100.00	4	7	42.86
14	Ird 53–3–14	0	18	100.00	4	6	33.33
15	Ird 53–3–15	0	26	100.00	2	6	66.67
16	Ird 53–3–16	0	23	100.00	1	4	75.00
17	Ird 53–3–17	0	25	100.00	2	7	71.43
18	Ird 53–3–18	0	21	100.00	5	8	37.50
19	Rd 53–3–1	0	22	100.00	4	6	33.33
20	Rd 53–3–2	1	33	96.97	2	5	60.00
21	Rd 53–3–4	0	17	100.00	4	4	0.00
22	Rd 53–3–5	0	22	100.00	1	6	83.33
23	Rd 53–3–6	0	18	100.00	3	4	25.00
24	Rd 53–3–7	0	13	100.00	1	4	75.00
25	Rd 53–3–8	0	23	100.00	3	7	57.14
26	Rd 53–3–9	0	23	100.00	0	6	100.00
27	Rd 53–3–10	0	12	100.00	1	3	66.67
28	Rd 53–3–11	0	1	100.00	0	1	100.00
29	Rd 53–3–12	0	9	100.00	3	3	0.00
30	Rd 53–3–13	0	22	100.00	3	5	40.00
31	Rd 53–3–14	0	16	100.00	3	3	0.00
32	Early five	14	19	26.32	0	0	0.00
33	Phichit03	15	17	11.76	0	0	0.00

Line Ird 53–3–n is collected from M_4 transplanted plant after greenhouse screening. Line Rd 53–3–n is collected from M_4 seed-grown plant

TABLE VII. GREENHOUSE/FIELD SCREENING OF OYVMD IN OKURA M_6 GENERATION (EXPERIMENT I)

	Planting 03–08–01 Inoculation 08–08–01 Greenhouse evaluation 12–09–01			% Non-infected plants	Seed planting 25–07–01(PHRC) Field evaluation after 83 days		% Non-infected plants
No.	Line	Infected plants	Tested plants		Infected plants	Tested plants	
1	IRd 53–3–1–2	0	27	100.00	14	26	46.15
2	IRd 53–3–2–1	0	26	100.00	9	16	43.75
3	IRd 53–3–2–2	0	27	100.00	12	20	40.00
4	IRd 53–3–2–3	0	24	100.00	9	17	47.06
5	IRd 53–3–3–1	0	29	100.00	8	20	60.00
6	IRd 53–3–6–1	0	30	100.00	4	12	66.67
7	IRd 53–3–6–2	0	24	100.00	10	27	62.96
8	IRd 53–3–6–3	0	27	100.00	10	28	64.29
9	IRd 53–3–6–4	0	27	100.00	4	27	85.19
10	IRd 53–3–7–2	0	27	100.00	6	29	79.31
11	IRd 53–3–8–1	0	17	100.00	8	15	46.67
12	IRd 53–3–8–3	1	25	96.00	10	15	33.33
13	IRd 53–3–8–4	0	17	100.00	9	19	52.63
14	IRd 53–3–9–1	0	28	100.00	5	22	77.27
15	IRd 53–3–9–3	0	21	100.00	5	39	87.18
16	IRd 53–3–9–4	0	21	100.00	6	14	57.14
17	IRd 53–3–10–1	0	26	100.00	5	41	87.80
18	IRd 53–3–10–2	0	27	100.00	12	24	50.00
19	IRd 53–3–10–3	0	28	100.00	6	20	70.00
20	IRd 53–3–13–1	0	13	100.00	0	1	100.00
21	IRd 53–3–13–2	0	10	100.00	4	7	42.86
22	IRd 53–3–14–1	0	23	100.00	5	11	54.55
23	IRd 53–3–14–2	0	28	100.00	5	17	70.59
24	IRd 53–3–15–1	0	23	100.00	2	20	90.00
25	IRd 53–3–15–2	0	27	100.00	11	28	60.71
26	IRd 53–3–16–1	0	22	100.00	5	24	79.17
27	IRd 53–3–16–2	0	27	100.00	7	25	72.00

	Planting 03–08–01 Inoculation 08–08–01 Greenhouse evaluation 12–09–01			% Non-infected plants	Seed planting 25–07–01(PHRC) Field evaluation after 83 days		% Non-infected plants
No.	Line	Infected plants	Tested plants		Infected plants	Tested plants	
28	IRd 53–3–16–3	0	19	100.00	1	26	96.15
29	IRd 53–3–17–1	0	26	100.00	8	17	52.94
30	IRd 53–3–17–2	0	27	100.00	9	16	43.75
31	IRd 53–3–18–1	0	30	100.00	2	27	92.59
32	IRd 53–3–18–2	0	13	100.00	4	12	66.67
33	IRd 53–3–18–3	0	23	100.00	3	23	86.96
34	IRd 53–3–18–4	0	27	100.00	1	8	87.50
35	Rd 53–3–2–1	0	25	100.00	7	13	46.15
36	Rd 53–3–2–2	0	26	100.00	4	19	78.95
37	Rd 53–3–2–3	0	23	100.00	3	18	83.33
38	Rd 53–3–4–1	0	25	100.00	6	20	70.00
39	Rd 53–3–8–1	0	24	100.00	6	17	64.71
40	Rd 53–3–8–2	0	21	100.00	2	10	80.00
41	Rd 53–3–8–3	0	17	100.00	6	27	77.78
42	Rd 53–3–10–1	0	24	100.00	9	21	57.14
43	Rd 53–3–10–2	1	28	96.40	11	24	54.17
44	Rd 53–3–10–3	0	25	100.00	4	18	77.78
45	Rd 53–3–11–1	0	19	100.00	8	15	46.67
46	Rd 53–3–11–2	0	30	100.00	4	23	82.61
47	Rd 53–3–11–3	0	26	100.00	2	25	92.00
48	Rd 53–3–13–1	0	27	100.00	10	23	56.52
49	Rd 53–3–13–2	0	25	100.00	9	13	30.77
50	Phichit03	19	20	5.00	840	840	0.00
51	Starlight	27	27	0.00			

TABLE VIII. M_7 SCREENING FOR OYVMD RESISTANCE AT PHICHIT HRC (EXPERIMENT I); PLANTING 06–12–01

No.	Line	Number of infected plants at – days						Number of plants		% Non-infected
		30	44	58	72	86	100	Infected	Tested	
1	Ird 53–3–2–2–2	-	-	-	-	-	-	0	29	100.00
2	Ird 53–3–6–4–1	-	-	-	-	-	-	0	40	100.00
3	Ird 53–3–6–4–2	-	-	-	-	-	-	0	34	100.00
4	Ird 53–3–6–4–3	-	-	-	-	1	-	1	31	96.77
5	Ird 53–3–7–2–1	-	-	-	-	-	-	0	5	100.00
6	Ird 53–3–7–2–2	-	-	-	-	-	-	0	21	100.00
7	Ird 53–3–9–3–1	-	-	-	-	-	-	0	31	100.00
8	Ird 53–3–9–3–2	-	-	-	-	-	-	0	32	100.00
9	Ird 53–3–9–3–3	-	-	-	-	-	-	0	37	100.00
10	Ird 53–3–9–3–4	-	-	-	1	-	-	1	23	95.65
11	Ird 53–3–10–1–1	-	-	-	-	-	-	0	24	100.00
12	Ird 53–3–10–1–2	-	-	-	-	-	-	0	26	100.00
13	Ird 53–3–10–1–3	-	-	-	-	-	-	0	38	100.00
14	Ird 53–3–10–1–4	-	-	-	-	-	-	0	31	100.00
15	Ird 53–3–15–1–1	-	-	-	-	-	-	0	37	100.00
16	Ird 53–3–16–3–1	-	-	-	-	-	-	0	35	100.00
17	Ird 53–3–16–3–2	-	-	-	-	-	-	0	35	100.00
18	Ird 53–3–18–1–1	-	-	-	-	-	-	0	31	100.00
19	Ird 53–318–1–2	-	-	-	-	-	-	0	25	100.00
20	Ird 53–3–18–1–3	-	-	-	-	-	-	0	26	100.00
21	Ird 53–3–18–1–4	-	-	-	-	-	-	0	24	100.00
22	Rd 53–3–2–2–1	-	-	-	-	-	1	1	35	97.14
23	Rd 53–3–2–2–2	-	-	-	-	-	-	0	21	100.00
24	Rd 53–3–2–3–2	-	-	-	-	-	-	0	14	100.00
25	Rd 53–3–8–2–3	-	-	-	-	-	-	0	32	100.00
26	Rd 53–3–8–2–4	-	-	-	-	-	-	0	37	100.00
27	Rd 53–3–10–3–1	-	-	-	-	-	-	0	33	100.00
28	Rd 53–3–10–3–2	-	-	-	-	-	-	0	32	100.00
29	Rd 53–3–10–3–3	-	-	-	-	-	-	0	35	100.00
30	Rd 53–3–11–3–1	-	-	-	-	-	-	0	33	100.00
31	Rd 53–3–11–3–2	-	-	-	-	-	-	0	39	100.00
32	Rd 53–3–11–3–3	-	-	-	-	-	-	0	30	100.00
33	Rd 53–3–11–3–4	-	-	-	-	-	-	0	36	100.00
34	Phichit03	-	4	80	563	381	0	1028	1028	0.00

3.1.5. Yield trial of selected resistant lines

A yield trial was conducted at PHRC in April 2002 (Figure 1). The experimental design was a randomized complete block (RCB) with two blocks and fourteen treatments. The treatments were 12 resistant lines from M_7 generation, Hit 9701 as a resistant check variety and Phichit 03 as a susceptible variety. The results are shown in Table IX. All bore reasonable yield but their fruit shape was not desirable for the export market. They will be used as parent lines in further breeding program.

Fig. 1. M_7 plants of Phichit 03 and mutant line Rd53–3–10–3–3 planted at PHRC.

TABLE IX. PLANT HEIGHT (AT 94 DAYS), DAYS TO FIRST FLOWERING, % NON-INFECTED PLANTS (AT 120 DAYS), FRUIT LENGTH AND WIDTH PER FRUIT, FRUIT WEIGHT AND NUMBER OF FRUITS/RAI (6.25 RAI = 1 HA) OF SELECTED RESISTANT LINES; PLANTING DATE 22–04–02 AT PHICHIT HRC (EXPERIMENT I)

No	Line	Plant height (cm.)	Days to first flowering	% Non-infected plants	Fruit length (cm)	Fruit width (cm)	Fruit weight (Kg/rai)	No. of fruits (x1,000/rai)
1	IRd 53–3–6–4–1	245.9	39.0	88.2	10.1	1.00	4522.8	448.5
2	IRd 53–3–6–4–3	170.9	40.0	94.9	10.5	0.96	5047.3	513.2
3	IRd 53–3–7–2–2	230.4	39.5	89.1	10.0	1.00	4487.3	426.9
4	IRd 53–3–9–3–2	184.2	39.5	93.3	10.1	0.97	5088.9	520.9
5	IRd 53–3–10–1–4	233.4	39.5	88.0	10.2	0.99	4683.9	484.3
6	IRd 53–3–15–1–1	243.4	40.0	78.2	10.0	1.00	4509.9	442.1
7	IRd 53–3–16–3–1	171.7	37.0	97.0	10.3	1.00	4770.8	474.5
8	IRd 53–3–18–1–3	171.3	39.0	96.6	10.2	1.37	4636.1	460.8
9	Rd 53–3–2–2–1	233.5	39.0	89.6	10.0	0.99	4136.9	415.5
10	Rd 53–3–8–2–3	166.1	40.5	92.9	9.8	0.99	4494.4	460.1
11	Rd 53–3–10–3–3	176.6	40.0	89.4	10.3	0.98	4553.3	475.7
12	Rd 53–3–11–3–1	244.7	39.0	93.3	10.2	1.00	4563.2	451.5
13	Hit9701	268.7	39.0	93.0	10.5	0.97	5134.1	529.2
14	Phichit03	131.4	50.0	0.0	9.8	1.00	1827.7	187.2

3.2 In experiment II

3.2.1. Mutation induction by gamma radiation and planting of M_1 generation

In February 1999, seeds of Annie and Okura were irradiated by gamma rays at doses of 400 and 600 Gy (1,000 seeds of Annie and 2,000 seeds of Okura per dose). They were planted at Huaysai with non-irradiated seeds (500 seeds of Annie and 900 seeds of Okura). Mutated characters were also found as in Experiment I. Plants were infested with leafhopper, aphids and white flies. 165 healthy M_1 plants with green fruits were selected to collect M_2 seeds for

pedigree selection (Table X). The rest of M_1 plants were collected as M_2 seeds for bulk selection.

TABLE X. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND PLANTS SELECTED IN M_1 GENERATION OF ANNIE AND OKURA GROWN AT HUAYSAI, PLANTING DATE 16–02–99 (EXPERIMENT II)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of plant selected
Annie	0	500	339	18
	400	1,000	566	45
	600	1,000	574	35
Total		2,500	1,479	98
Okura	0	900	292	12
	400	2,000	683	41
	600	2,000	527	14
Total		4,900	1,502	67

3.2.2. Selection in M_2 and M_3 generation

3.2.2.1. Pedigree selection

In December 1999, 4,628 M_2 seeds of selected plants were grown at Huaysai. Of these, 194 of Annie and 148 of Okura were selected to collect M_3 seeds (Table XI). In August 2000, they were planted for field screening by planting Annie at Huaysai and Okura at PHRC. OYVMD was more seriously widespread at PHRC than Huaysai (Table XII). M_4 seeds were collected from healthy seed setting M_3 plants, i.e. of lines 3, 15 and 16 for Annie and 2, 32 and 1 for Okura, at doses of 0, 400 and 600 Gy, respectively.

TABLE XI. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND PLANTS SELECTED IN M_2 GENERATION OF ANNIE AND OKURA GROWN AT HUAYSAI, PLANTING DATE 13–12–99 (EXPERIMENT II)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of plant selected
Annie	0	442 (18)	288	27
	400	1,115 (45)	727	111
	600	838 (35)	513	56
Total		2,395 (98)	1,528	194
Okura	0	240 (12)	157	13
	400	1,552 (41)	782	109
	600	441 (14)	192	26
Total		2,233 (67)	1,131	148

(..) = No. of selected M_1 plants

TABLE XII. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND NON-INFECTED PLANTS IN M_3 PLANTS SCREENED FOR OYVMD RESISTANCE IN THE FIELD; ANNIE GROWN AT HUAYSAI, PLANTING DATE 01–08–00 AND OKURA GROWN AT PHRC, PLANTING DATE 10–08–00 (EXPERIMENT II)

Variety	Dose (Gy)	No. of seeds planted	No. plants of fertile	No. of non-infected plants	% Non-infected plants	No. of plants selected
Annie	0	810(27)	329	18	5.47	3
	400	3,330(111)	1,177	88	7.48	15
	600	1,680(56)	666	59	8.86	16
Phichit03		1,500	840	95	11.31	0
Okura	0	390(13)	148	2	1.35	2
	400	3,270(109)	1,005	40	3.98	32
	600	780(26)	256	3	1.17	1
Phichit03		2,000	1,091	52	4.77	0

(..) = No. of selected M2 plants

3.2.2.2. Bulk selection

In December 2000, M_2 bulked seeds were planted at Huaysai and PHRC for field screening with the results shown in Tables XIII and XIV, respectively. OYVMD was more seriously widespread at PHRC than Huaysai. So only three M_2 plants of Okura irradiated at 400 Gy planted at PHRC were selected to collect M_3 seeds. They were grown and screened for OYVMD resistance under greenhouse conditions in March 2001. The resistant plants were transplanted to the field at PHRC, where they were susceptible to disease (Table XV). At Huaysai, 77 M_2 plants of Annie and Okura were selected to collect M_3 seeds. They were grown and screened for OYVMD resistance under greenhouse conditions in June 2001. The 89 resistant plants were transplanted to the field at PHRC. They were all susceptible to disease. No resistant plant was obtained from bulk selection.

TABLE XIII. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND NON-INFECTED PLANTS OF ANNIE AND OKURA IN M_2 BULK GROWN AT HUAYSAI, PLANTING DATE 06–12–00 (EXPERIMENT II)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of non-infected plants	% Non-infected plants
Annie	0	200	140	2	1.43
	400	665	337	12	3.56
	600	690	367	39	10.63
Okura	0	200	166	2	1.20
	400	730	451	22	4.88
	600	220	96	0	0.00
Phichit03		500	343	0	0.00

TABLE XIV. NUMBER OF SEEDS PLANTED, FERTILE PLANTS AND NON-INFECTED PLANTS OF ANNIE AND OKURA IN M_2 BULK GROWN AT PHICHIT HRC, PLANTING DATE 20–12–00 (EXPERIMENT II)

Variety	Dose (Gy)	No. of seeds planted	No. of fertile plants	No. of non-infected plants	% Non-infected plants
Annie	0	200	82	0	0.00
	400	665	307	0	0.00
	600	690	312	0	0.00
Okura	0	20	93	0	0.00
	400	730	319	3	0.94
	600	220	76	0	0.00
Phichit03		1,000	804	0	0.00

TABLE XV. OYVMD SCREENING OF M_3 PLANTS OF OKURA FROM M2 BULK UNDER GREENHOUSE CONDITION AND TRANSPLANTING TO THE FIELD AT PHICHITHRC (EXPERIMENT II)

Planting 30–03–01 Inoculation 02,03,10–04–0		Greenhouse evaluation date 26–04–01			Transplant 10–05–01 (PHRC) Field evaluation after 90 days		
No	Line	Number of plants		% Non-infected plants	Number of transplants		% Non-infected plants
		Infected	Tested		Infected	Tested	
1	B–1–0K–40–1	2	18	88.89	0	2	100.00
2	B–2–0K–40–1	10	10	0.00	-	-	-
3	B–3–0K–40–1	11	19	42.11	0	3	100.00
4	B–4–0K–40–1	Non germinated		-	-	-	-
5	B–5–0K–40–2	1	16	93.75	0	1	100.00
6	B–6–0K–40–2	1	3	66.67	0	1	100.00
7	B–7–0K–40–2	Non germinated		-	-	-	-
8	B–8–0K–40–3	1	1	0.00	-	-	-
9	B–9–0K–40–3	3	5	40.00	0	1	100.00
10	B–10–0K–40–3	Non germinated		-	-	-	-
11	B–11–0K–40–3	Non germinated		-	-	-	-
12	B–12–0K–40–3	Non germinated		-	-	-	-
13	B–13–0K–40–3	Non germinated		-	-	-	-
14	Early five	14	19	26.32	-	-	-
15	Phichit03	15	17	11.76	-	-	-

3.2.3. Selection in M_4 and M_5 generation

About 60 M_4 seeds of selected M_3 plants from pedigree selection were grown and screened for OYVMD resistance in the greenhouse in February 2001, while the rest of M_4 seeds were planted directly to the field at PHRC in May 2001. Nine lines with 14 plants of Annie and 24 lines with 59 plants of Okura showed no disease symptoms at 32 days of age (Table XVI). They were transplanted to the field at PHRC. Only one plant from transplanting plants, designed as B–21 (Okura irradiated at 400 Gy or 04–40–34–10–2) showed disease resistance and produced a few seeds. They were planted for field screening at PHRC in July 2001. Only six fertile plants were observed. Five of them showed no disease symptoms and bore desirable fruit shape, but M_6 seeds were obtained from only four such plants (lines).

TABLE XVI. OYVMD SCREENING UNDER GREENHOUSE CONDITION IN M_4 PLANTS OF ANNIE AND OKURA (EXPERIMENT II)

ANNIE planting 12–02–01, inoculation 15–02–01, evaluation 26–03–01					OKURA planting 19–02–01, inoculation 22–02–01, evaluation 26–03–01				
No.	Line	Number of plants		Number of non-infected plants	No.	Line	Number of plants		Number of non-infected plants
		Infected	Tested				Infected	Tested	
1	A1–40–79–4–3	30	30	0	1	O1–40–37–26–8	18	20	2
2	A1–40–109–6–3	30	30	0	2	O1–40–37–26–9	22	23	1
3	A1–40–109–6–7	31	31	0	3	O2–40–3–4–9	27	28	1
4	A1–40–109–15–14	29	29	0	4	O2–40–3–5–7	14	14	0
5	A1–40–109–15–15	29	29	0	5	O2–40–3–40–10	29	30	1
6	A2–0–40–4–20	31	31	0	6	O2–40–3–43–4	31	32	1
7	A2–40–87–4–5	30	30	0	7	O3–40–7–7–14	29	29	0
8	A2–60–46–8–19	28	28	0	8	O3–40–8–2–3	22	22	0
9	A2–60–46–13–2	30	30	0	9	O3–40–11–9–5	27	28	1
10	A2–60–46–13–25	26	29	3	10	O3–40–29–20–1	10	12	2
11	A2–60–57–1–6	35	36	1	11	O3–40–39–7–3	30	31	1
12	A2–60–57–1–8	27	30	3	12	O3–40–39–16–11	27	28	1
13	A2–60–57–1–12	30	30	0	13	O3–40–39–18–9	30	30	0
14	A2–60–57–10–4	30	32	2	14	O3–60–2–1–11	30	32	2
15	A3–0–34–13–3	28	28	0	15	O4–0–51–5–1	25	29	0
16	A3–0–50–3–2	26	27	1	16	O4–0–64–7–6	26	30	4
17	A3–40–51–6–5	30	30	0	17	O4–40–4–15–1	30	33	3
18	A3–40–94–9–3	28	28	0	18	O4–40–4–15–11	31	31	0
19	A3–40–114–11–2	27	28	1	19	O4–40–4–16–2	26	28	2
20	A3–40–114–11–10	28	28	0	20	O4–40–5–4–4	16	16	0
21	A4–40–10–6–8	32	32	0	21	O4–40–34–10–2	25	31	6
22	A4–40–10–6–9	32	32	0	22	O4–40–34–10–6	23	30	7
23	A4–40–10–10–9	25	25	0	23	O4–40–34–15–8	29	30	1

ANNIE planting 12–02–01, inoculation 15–02–01, evaluation 26–03–01					OKURA planting 19–02–01, inoculation 22–02–01, evaluation 26–03–01				
No.	Line	Number of plants		Number of non-infected plants	No	Line	Number of plants		Number of non-infected plants
		Infected	Tested				Infected	Tested	
24	A4–60–56–14–9	30	30	0	24	O4–40–34–15–9	19	21	2
25	A5–40–29–3–4	30	30	0	25	O5–40–18–7–4	28	29	1
26	A5–40–90–6–12	25	26	1	26	O6–40–35–4–7	23	23	0
27	A5–60–16–6–4	34	34	0	27	O6–40–35–8–8	29	29	0
28	A5–60–16–6–12	29	30	1	28	O6–40–35–10–1	46	55	9
29	A5–60–25–3–9	27	28	1	29	O6–40–35–10–2	25	27	2
30	A5–60–25–3–18	31	31	0	30	O6–40–35–10–9	28	30	2
31	A5–60–76–9–7	28	28	0	31	O6–40–35–13–4	28	31	3
32	A5–60–76–9–9	26	26	0	32	O6–40–35–13–5	27	29	2
33	A5–60–76–9–10	28	28	0	33	O6–40–52–2–4	28	30	2
34	A5–60–76–9–14	30	30	0	34	O6–40–76–2–8	26	26	0
35	Early five	28	28	0	35	O6–40–76–5–5	14	14	0
36	Phichit03	30	30	0	36	Early five	27	28	1
					37	Phichit03	28	30	2

3.2.4. Selection in M_6 and M_7 generation

M_6 seeds of four lines of B–21 were planted in the field at PHRC for OYVMD screening in December 2001 (Table XVII). Figure 2 shows such plants.

TABLE XVII. M_6 PLANTS SCREENING FOR OYVMD RESISTANCE IN THE FIELD AT PHICHIT HRC (EXPERIMENT II). SEED PLANTING 06–12–01

No	Line	Code	Number of infected plants – days						Total number of plants		% Non-infected plants
			30	44	58	72	86	100	Infected	Tested	
1	O4–40–34–10–2–6–1	B21–6–1	-	-	-	-	-	-	0	17	100.00
2	O4–40–34–10–2–6–2	B21–6–2	-	-	-	1	8	1	10	31	67.74
3	O4–40–34–10–2–6–3	B21–6–3	-	-	-	-	7	-	7	14	50.00
4	O4–40–34–10–2–6–5	B21–6–5	-	-	-	-	-	-	0	13	100.00
5	Phichit03		-	4	80	563	381	0	1,028	1,028	0.00

The resistant plants showing good plant type and desirable fruit shape were selected to collect M_7 seeds. They were planted in the field at PHRC in July 2002. It rained heavily in September; seeds were collected from some M_7 plants. They were planted in the field at PHRC for OYVMD screening in November 2002. There was no leafhopper and white fly. Plants showed no disease symptoms in all lines.

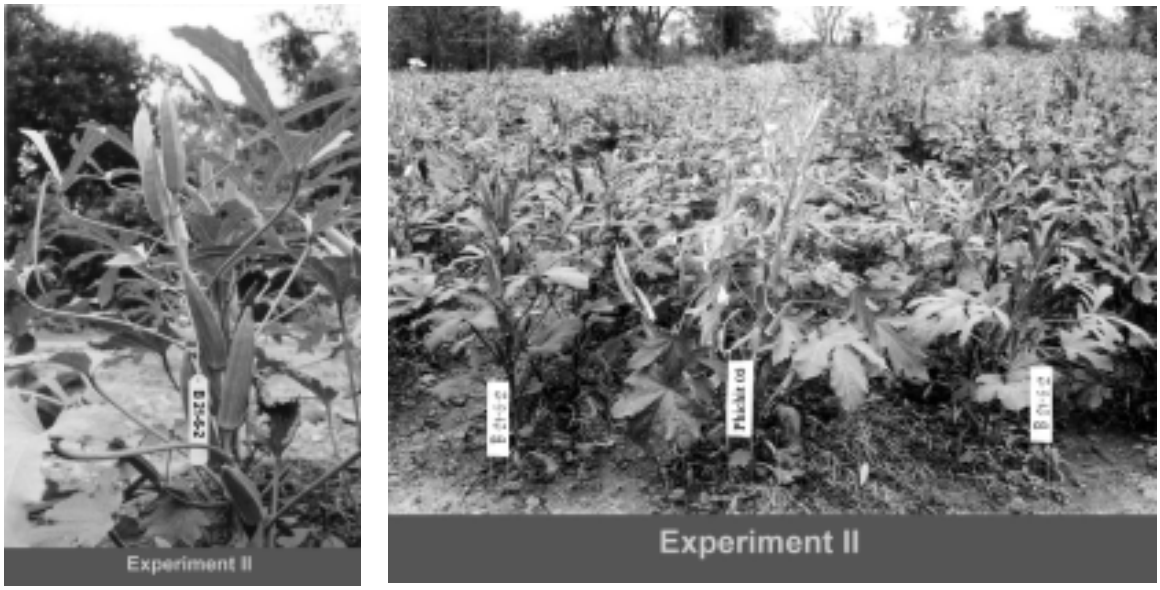

Fig. 2. M_6 plants of Phichit 03 and mutant line B21–6–2 in the field (right) and a close-up of a B21–6–2 plant showing fruits (left).

3.2.5. Yield trial of selected resistant lines

Ten lines of B–21 with good plant type and desirable fruit shape were selected for yield trial observation at PHRC in May 2003.

4. CONCLUSION

The optimal dose of gamma radiation to induce mutation for OYVMD resistance in okra variety Okura was 400 Gy.

- The characters of 2–3 shoots per plant and twin fruits that we found in M_1 generation were not inherited to M_2 generation.

- In Experiment I, twelve resistant lines were obtained from Okura by screening for OYVMD resistance under greenhouse and field conditions up to M_7 generation, and were selected for yield trial observation. They bore reasonable yield but their fruit shape was not desirable enough for the export market. They will be used as parental lines in further breeding programs.

- In Experiment II, ten resistant lines with good plant type and desirable fruit shape obtained from Okura by screening for OYVMD resistance under greenhouse and field conditions up to M_7 generation were selected for yield trial observations.

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DEVELOPMENT OF EARLY MATURING AND LEAF BLIGHT RESISTANT TARO (*Colocasia esculenta* (L.) Schott) WITH IMPROVED TASTE

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Abstract

Shoot tips of taro cv. 'Bentul' plantlets grown in tissue culture and irradiated at 10 Gy produced many variants. The variations included different lamina traits (shape, orientation, undulations, outline of sinus, color), vein junction color, off shoot number, stolon number, plant height, leaf number, petiole color, leaf blight resistance, corm weight, and taste. The predominant lamina characters induced from gamma-irradiated shoot tip of 'Bentul' Taro were a flat shape with dropping edges, a semi-horizontal orientation with entire undulations, a narrow (< 45^{o}) outline of the sinus, green color, whitish green vein junctions, and dark green petioles with purple tips. This was coupled with 1 off shoot and 1 stolon per plant, 31–40 cm tall plants with 4 leaves, a large tolerance to leaf blight disease, 101–150 g corm weights, and an acrid taste. Three variants (B 43, B 63, B 133) with the best characters were selected. They were early maturing (6 months old) with a heavy corm weight (351–400 g), very tolerant to leaf blight disease (little symptom), and had a good taste (sweet and no acrid).

1. INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott) is an important tuber crop grown widely in humid tropics and a source of carbohydrate for many people in Asia, Africa, and the Pacific islands. FAO data recorded that taro area is about 1.4 million ha with a yield of 8.3 million ton per year in the world. In Indonesia, data recorded taro center areas such as West Papua province with 21,952 ha, and two districts of Mentawai islands with 176 ha and with poor yield (2.5–3.4 ton/ha) [1].

This crop has particular potential for marshy, waterlogged, coastal, and salty regions, where it could help overcome food shortage [2]. Taro tuber/corm is a source of carbohydrate used as staple food or snack; taro flour can be produced for soups, biscuits, bread, beverages, and puddings [2]. The leaves and stalks of taro are used as vegetable, as they contain a high protein that is useful for people diet in developing countries. The taro corm contains 63–85% water, 1.4–3.0% protein, 0.2–0.4% fat, 13–29% carbohydrate, 0.6–1.2% fiber, 0.6–1.3% ash, and is a good source of vitamins B and C. The taro leaf contains 87.2% water, 3.0% protein, 0.8% fat, 6.0% carbohydrate, 1.4% fiber, 1.6% ash, and is an excellent source of vitamin C [3].

Indonesia, with about 18,000 islands, is considered to be the centre of origin of taro. There are many wild taros in Indonesia, ordinary palm civets eat their fruits and disperse their seeds [4], having relatively little starch, long stolons, and high acridity [5]. A relationship between cultivars and wild taro was elucidated [6], based on determination of crude protein of tuber and leaves and electrophoresis protein of fresh tuber.

Many developing countries of Asia, Africa, and the Pacific use taro corm as a staple food, including some areas in Indonesia, such as West Papua, Mentawai islands, Maluku, Sangir and Talaud islands [7]. A large number of germplasms (710) have been collected in Cibinong/ Bogor from Java, Bali, Celebes, and Sumatra islands [1]. Taro plants are mostly grown in rice

fields in West Java on low land, even though they can be found on high lands up to an altitude of 1,300 m above the sea level. Prana [8] has collected 335 accessions of taro germplasms from West Java, and identified 62 cultivars. Their isozyme profiles were analysed and 100 zymotypes were found [9]. One of the most popular cultivars is Bentul, which is characterized by dark green petioles with brown streaks, purple petiole tips, few shoots and several stolons, conical corms, 7 months harvesting, and 500–3,000 g corm weights. Bentul is widely grown by farmers in Java because of its early maturing, little care needed and good taste [1].

The expansion of taro farms in the world, including Indonesia is limited because of a leaf blight disease caused by *Phytophthora colocasiae*. An attacked plant shows circular, water soaked, necrotic spots on the leaves, and collapses [10, 11, 12]. Basically, all taro cultivars are not resistant to the disease, although some cultivars appear to be more tolerant than others.

Taro plants rarely produce seed because the flowers are protogynous, i.e. the female flowers mature earlier than the male flowers, also many taro cultivars are triploid (2n = 42) [13], but some cultivars can produce seed naturally in the presence of *Drosophilidae* pollinators [14, 15]. There are many occurrences of wild taro in Indonesia, their fruits are eaten and their seeds are dispersed by ordinary palm civets [4]. Farmers usually propagate taro plants from top shoots, off shoots, stolons, and rarely use seeds because the corms are harvested before flowering. Because of this cultivation practice, genetic diversity is very limited. The quality of taro, especially good taste is very important for the consumers. The most obvious anti-nutritional quality of taro is the acridity, which causes a severe itching, stinging, or burning sensation in the mouth and throat, followed by swelling or a less severe irritation [5].

Taro propagation by traditional means produces limited number of planting materials and almost no genetic variations. Propagation by tissue culture can produce unlimited numbers of planting material, and has been successfully used in taro by several groups [16, 17, 18, 19, 20, 21, 22]. On the other hand, the limited genetic diversity of taro could be widened through irradiation techniques. The aim of this experiment is to obtain early maturing, leaf blight resistant taro 'Bentul' with improved taste through a combination of irradiation and tissue culture techniques and, ultimately, to release these plants to the farmers.

2. MATERIALS AND METHODS

Taro 'Bentul' material was chosen and obtained from markets and fields. Best responding explants were selected among apical bud, axillary bud, stolon, petiole or leaf explants. They were washed with running tap water for about 1 hour. Top shoots were cut out from the corms, the leaves were taken out, buds with their surrounding tissues were cut to about 1 cm^2, offshoots were deprived of their leaves, and buds with surrounding tissues were cut to about 0.5 cm^2. The stolons were cut about to 1 cm length with or without a node. Unfolded leaves were taken; the stalks were cut to about 1 cm long and the leaves to about 1 cm^2. These explants were sterilized by using Clorox or Mercury Chloride and washed with sterilized water.

A preliminary experiment sought to select culture media by using the formulation of Murashige and Skoog (MS) [23] with 1 mg/l 6–Benzylaminopurine (BA) and 0.50 mg/l Indolebutyric acid (IBA), plus agar at 7 g/l or phytagel at 2 g/l as a gelling agent. Shoot tips (3 mm long) from uniform plantlets, were cultured on MS medium with 1 mg/l BA and 0.50 mg/l IBA, and were irradiated at 0, 5, 10, 15, 20, 25, and 30 Gy on an irradiator gamma

cell 220 with Co^{60} at an activity of 156.28 Curie and with a dose rate of 120.86 Gy/hour. LD30 was selected as a non-lethal dose, which would induce mutation of plantlets. The irradiated shoot tips were transferred monthly onto MS media with and without 3 mg/l BA using protocol 2 described in [24]. Once around 2,000 plantlets were obtained (fourth generation), they were taken out to the greenhouse for growth on soil media in plastic bags. The plantlets were acclimated by opening the plastic bags after one week.

Plants were moved to the net house, and planted in the field after 5 months acclimation. A growing area was chosen that was contaminated with leaf blight disease, caused by *Phytophthora colocasiae*, from the previous taro plants. The plants were weeded, recorded plant variations included lamina shape, orientation, undulation, colour, outline of lamina sinus, vein junction colour, petiole colour, off shoot (lateral shoot) number, stolon number, leaf number, plant height, and leaf blight disease symptoms after 2 months in the field. Also, the corm weight and taste after 6 months in the field were recorded, with 20 people to assess the degree of sweetness and acridity of boiled corms of every variant number tasted.

3. RESULTS

Mercury Chloride was more effective than Clorox for sterilization of explants. Apical and axillary buds showed better responses in culture than stolon, petiole, or leaf explants. Phytagel was better than agar as a gelling agent for taro culture.

The radio sensitivity test was based on growth rates, including fresh weight, shoot number, height, and leaf number of the irradiated buds, and showed that LD30 was about 10 Gy. Generally, increasing doses caused decreasing growth rate of taro cultures but there were also variable responses of cultures between and within treatments. There was a negative correlation between shoot number and shoot height. An increasing shoot number caused a decreasing shoot height. This was especially relevant with shoot tip death, which promoted axillary shoot growth. Many cultures were contaminated after several months of incubation.

Taro ‘Bentul’ showed various degrees of resistance to leaf blight disease in the field (Figure 1) where there were inoculant sources in the surrounding soil, from medium tolerant (intermediate symptoms) (Figure 2) up to very tolerant (few symptoms) to the disease. There were leaf colours other than green and yellowish green as in the control, and whitish green, yellowish green, and green vein junctions (Figure 3). The petiole also showed different colours compared with the dark green with brown streaks in the control plants (dark green, light green, brown and red; Figure 4), but the top of petioles was always purple as in the controls.

There were many different leaf characteristics. In terms of shape, they ranged from flat, dropping position of anterior and posterior lobe, cup-shaped, to flat with dropping edges (Figure 5). In terms of orientation, they were semi-vertical with the tip pointing upwards, vertical with the tip pointing downward, semi-vertical with tip pointing downwards, semi-horizontal, and horizontal orientations (Figure 6); they were either entire or presented broad wave undulations (Figure 7); and ranged from a narrow ($< 45^o$) to a wide ($> 45^o$) outline of the lamina sinus (Figure 8).

Fig. 1. Taro 'Bentul' MV4 in the field.

Fig. 2. Intermediate symptoms of leaf blight disease.

Fig. 3. Varied leaf vein colours.

Fig. 4. Varied petiole colours.

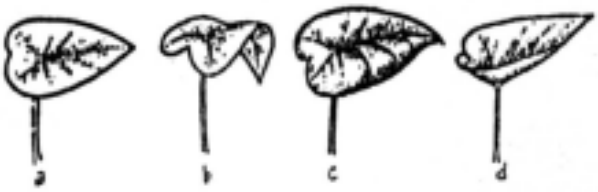

Fig. 5. Leaf shape a. Flat; b. Drooping position anterior and posterior lobes; c. Flat with drooping edge; d. Cup.

Fig. 6. Leaf blade orientation: a. Semi-vertical with tip pointing upwards; b. Vertical with tip pointing downwards; c. Semi-vertical with tip pointing downwards; d. Semi-horizontal; e. Horizontal; f. Horizontal with tip pointing downwards.

Fig. 7. Leaf blade undulation: a. Entire; b. Broad wave; c. Narrow wave.

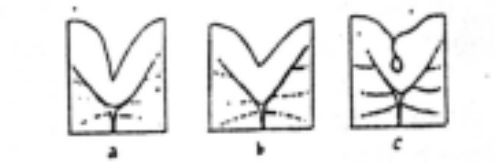

Fig. 8. Outline of leaf blade sinus a. Narrow (< 45^0); b. Wide (> 45^0); c. Overlapping edge

The plants produced 0–5 shoots, 0–5 stolons and 2–6 leaves. The plant heights were 11–51 cm, and several plants produced flowers and chimeras. The corm weights were 7.00–374.40 g. The boiled corm tasted from tasteless to sweet and from acrid to non-acrid.

The dominant variant characters were flat with dropping edge leaf shapes, semi-horizontal and entire leaf blades with narrow (<45^o) outlines of sinus, green with whitish vein junctions, dark green petioles with purple tips, 1 offshoot (Figure 9), 1 stolon (Figure 10), 31–40 cm high (Figure 11), with 4 leaves (Figure 12), very tolerant to leaf blight disease, with 101–150 g corms (Figure 13), and acrid taste.

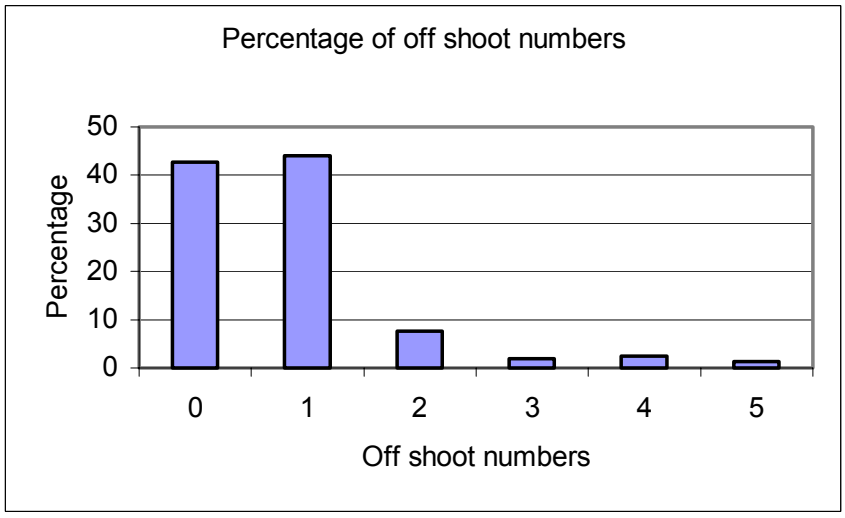

Fig. 9. Percentage of off shoot numbers of irradiated 'Bentul' taro.

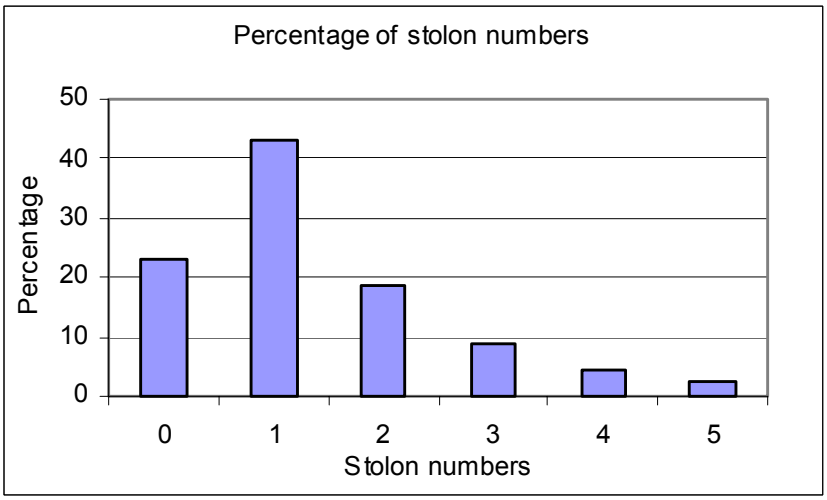

Fig. 10. Percentage of stolon numbers of irradiated 'Bentul' taro.

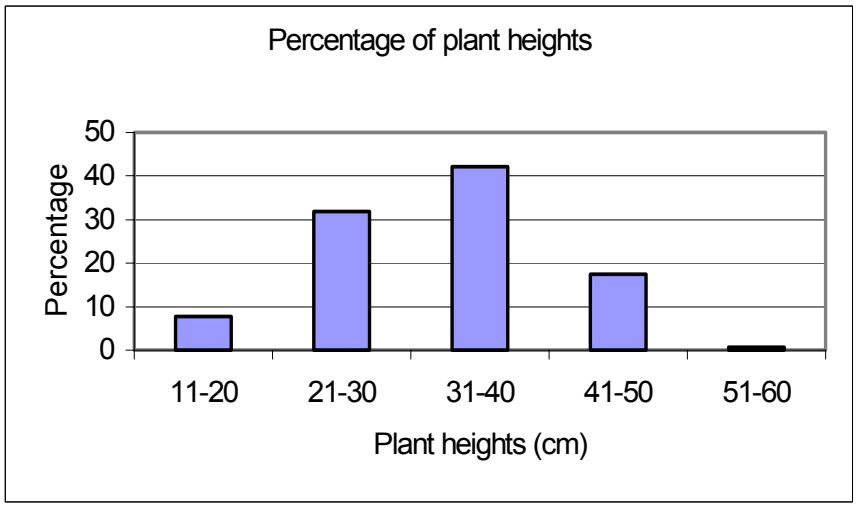

Fig. 11. Percentage of plant heights of irradiated 'Bentul' taro.

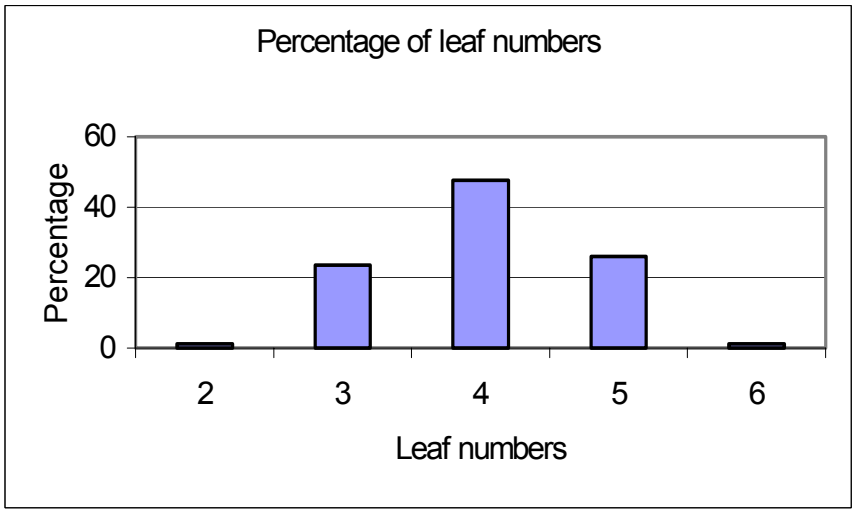

Fig. 12. Percentage of leaf numbers of irradiated 'Bentul' taro.

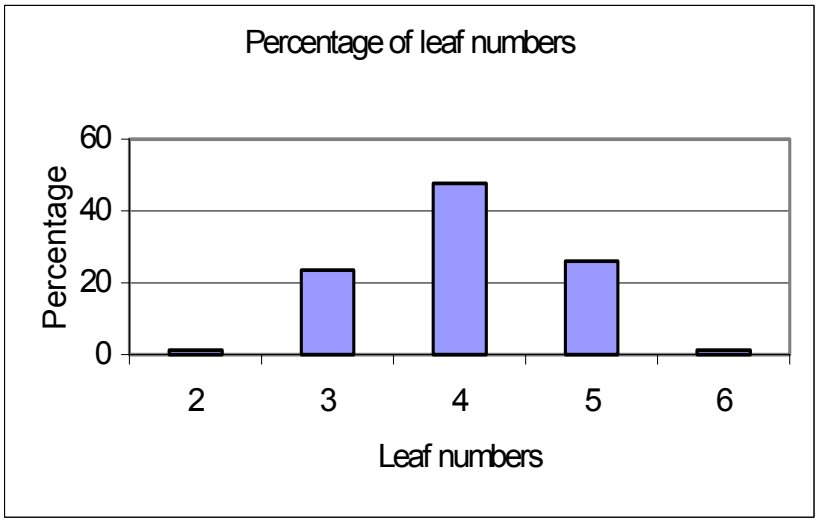

Fig. 13. Percentage of corm weights of irradiated 'Bentul' taro.

Out of all the taro plants derived from gamma irradiation *in vitro*, three variants were selected as having the best characteristics. These were variants number B 43, B 63, and B 133, which were very tolerant to leaf blight disease (few symptoms), and produced heavy corms (353.50–371.00 g) of good taste (sweet and non acrid). Their characteristics are detailed in Table I.

TABLE I. PHENOTYPIC CHARACTERS OF THE VARIANTS SELECTED FROM IRRADIATED 'BENTUL' TARO

Phenotypic trait observed	Variant numbers		
	B 43	B 63	B 133
Lamina shape	flat with drooping edges	flat	cup
Lamina orientation	semi-horizontal	semi-horizontal	semi-horizontal
Lamina undulations	entire	entire	entire
Lamina color	green	green	yellowish green
Outline of sinus	wide (> 45°)	wide (> 45°)	narrow (< 45°)
Vein junctions	green	whitish green	yellowish green
Petioles	light green	dark green	brown
Leaf blight disease	very tolerant	very tolerant	very tolerant
Corm weight (g)	371.00	360.10	353.50
Taste	good	good	good

4. DISCUSSION

A great number of cultures were contaminated with endogenous bacteria and eventually died after several months of incubation. Gunua [25] also detected this problem with taro cultures in Papua New Guinea.

The radio sensitivity test based on growth rate of the irradiated buds showed that LD30 was about 10 Gy. The same radio sensitivity result was obtained on irradiated buds of taro in Ghana [24]. A slightly different result was reported in [26], where it was observed that shoot

numbers still increased on 5 Gy and 10 Gy, and decreased only at 15 Gy and 20 Gy. The use of different cultivars of taro inn each of these studies may explain this discrepancy in results.

The plant heights were 20–51 cm, whereas control taro plants were 125–150 cm tall. However, this difference was caused by a different age, as the plant heights were measured on 2–month–old variants compared to 6–month–old ordinary plants. The measurement of 6–month–old variants could not be done because of a grasshopper attack.

Most of taro plants resulting from gamma irradiation showed very good tolerance to leaf blight disease, to which taro 'Bentul' plants are considered as relatively tolerant only.

The most changing morphological characteristic was dark green, brown, and red petioles, whereas the normal character of taro 'Bentul' is dark green with brown streaks. Likewise, colour variations caused by anthocyanin content in the petioles of irradiated taro 'Egu-imo' have previously been reported in [26].

Another greatly changing characteristic was the corm taste. A great number of gamma-irradiated taro 'Bentul' plants exhibited a relatively good taste (non acrid), compared with the acridity found in corms of ordinary plants. Growing conditions or different maturity at harvest may cause taste changes as reported by [5], gamma irradiation stimulated calcium oxalate content of taro plants provoking an acrid taste.

In addition, several plants produced flowers and chimeras while taro 'Bentul' commonly does not. These plants are not of interest in terms of quality and quantity of corms, but they may be useful for breeding purposes.

The weight of corms ranged between 7.00 and 374.40 g, as compared to 500.00–3,000.00 g normally. The reason for this may be that corms were harvested 1 month earlier than the usual harvest date. Besides, the stolons and offshoots were not cut away, and were attacked by grasshoppers, which further reduced corm weight.

These results showed that a combination of gamma irradiation and tissue culture techniques could produce a broad spectrum of morphological variants of economic and breeding interest in taro.

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APPLICATION OF BIOTECHNOLOGY AND MUTATION TECHNIQUES FOR ANTHRACNOSE RESISTANCE AND COMPACTNESS IN *Dioscorea* sp.

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Abstract

High quality yam corms (*Dioscorea alata*), clone 5969, were collected so they would germinate in a greenhouse to obtain nodal segments to be tested *in vitro*. For regeneration, the medium used Murashige and Skoog, modified with Benzylaminopurine (BAP) and activated charcoal. LD–50 was determined to be between 10 and 20 Gy, and one hundred explants were irradiated with a dose of 15 Gy and were multiplied up to M_1V_6. Then, three different acclimatization tests were carried out. Throughout these tests, attention was focused on survival, number of leaves, and initial and final height. Meanwhile, leaves infected with anthracnose (*Colletotrichum gloesporioides*) were collected. The fungus was isolated, and acervulum and mycelium were produced to obtain conidia. Later, single spore cultures underwent a molecular analysis. The acclimated and irradiated plants were inoculated with 0.5 x 10^6/ml of *Colletotrichum gloesporioides* conidia. These plants were placed within a humid chamber for 48 hours, and survival and the percentage of damaged plants were observed for two months.

1. INTRODUCTION

Yam (*Dioscorea alata*) and yampi (*Dioscorea trifida*) belong to Monocotyledonous class, order *Liliales*, family *Dioscoreaceae*. The genus *Dioscorea* originated from South East Asian jungles, the forest area in Western Africa and the Amazon Basin. Dioscoreas are climbing plants having an underground stem, out of which grow aerial stems, roots and tubers that represent a rich source of carbohydrates [1].

Due to its high quality tuber, *D. alata* is the most important species and the one preferred by farmers from Pococí, Talamanca, and Siquirres, in the Province of Limón (Caribbean Coast of Costa Rica) [2].

Producing yam at a commercial level is of great importance for the economy of developing countries in the tropical areas of Africa, Asia and the Caribbean [3]. Costa Rica reached 14 million kilograms of fresh yam tuber exports, mainly sent to Puerto Rico and The United States. This represented an FOB value of $ 8.890.225,5 (Dirección de Mercadeo, Consejo Nacional de Producción, Costa Rica). Our country has been the mayor yam supplier to the US market [3].

Yam has been mainly used as food for human consumption because it is a good source of carbohydrates and medical compounds of the steroid type [12]. As food, we can find it as puree, or flakes [7]. As a medicine and pharmaceutical product, many wild species contain sapogenins, that have the same chemical structure as corticosteroids (cortisone, sexual hormones, contraceptives) [4].

The most problematic situation in yam culture is its reproduction. Being the fastest and most economical method of reproduction, cloning is the most widespread method. Cloning is carried out by sowing full tubers or crosswise cut tubers that sprout after a period of latency [4–5].

Anthracnose, caused by *Collectrotrichum gloesporiodes*, is the most common disease. It produces black or dark brown spots in a nerve sense on the plant sheath and foliage [6]. However, there are reports of fungi from the genus *Cercospora* and *Gloesporium sp*. that also damage the plant [4–7–8].

The inoculates comes from within the seed. Thus, a healthy seed, chemically treated, as well as resistant varieties must be used in order to get a more effective control [7].

The use of an aerial support represents another obstacle for production since it makes it more expensive. The absence of an aerial support may cause a 30% to 50% decrease in productivity [7–9–10–11].

The use of *in vitro* culture, as a propagation technique has increased in the last years. This has brought forth positive effects to solve the problem of contaminated seed because it permits the reproduction of healthy material, with no pests or diseases [3]. Yam is propagating very fast, mainly because of two tissue culture methods [12]:

- *Meristem tip culture*. In this method, the apical meristem of each tip is cultivated while the foliage sheaths that wrap it are carefully removed with a scalpel. Some authors report that the percentage of plant regeneration with this method is very low [13–14].

- *Nodal segment culture.* This is the most important micropropagation technique for the commercial yam species described in [12]. This technique is used for a fast propagation since it can be done within a 30–day period until the wanted number of plants is obtained.

In order to produce variability, ionic radiation (Co–60) is a practice being developed in some products like banana, plantain and yam, aimed at selecting plants with high quality features.

2. METHODOLOGY

2.1. Collection and propagation of source plants

Dioscorea alata source plants clone 5969, collected in different production areas (Atlantic and Northern zones) were used. The collected corms were disinfected with chlorine at 2% v/v during 12 minutes. Later they were placed in plastic pots containing sow grass, in a greenhouse so that they would germinate.

Once the corms germinated, the nodal segments were used as explants for the material introduction-according Ref [12]. They were disinfected with NaOcl at 4%, diluted at 17% v/v in water for 13 min. Then, they were washed with distilled water and sown in Murashige and Skoog medium (MS) (1962), modified as follows:

- MS medium + 0.5, 1.0, 2.0 mg/L $^{-1}$ of 6–Benzylaminopurine (BAP).
- MS medium + 0 and 1g/L $^{-1}$ of activated charcoal.
- Different combinations between both modifications.

2.2. Mutagenesis treatment and micropropagation

Once the explants were selected and the necessary material was obtained (after four micropropagation cycles), the mutagenesis treatment started.

- A total of 120 explants (10 explants per magenta box in MS medium) were transported to the irradiator at Universidad de Costa Rica School of Biology.
- In order to revalidate the LD–50 established by Victor Madero at the Tissue Culture laboratory from the Universidad Nacional in 1993, treatments of 0, 10, 20, 30, 40, and 50 Gy were carried out with two magenta boxes per treatment.
- In agreement with previous result, 10 to 20 Gy was the best dose.
- 50 shoots were irradiated with a dose of 20 Gy. A very low percentage of regeneration was obtained with this dose.
- The dose was reduced to 15 Gy following OIEA staff advice. A total of one hundred explants were irradiated with this dose.

Once the treatment was over, explants were transferred to MS medium with 1 mg/L BAP and 1 g/L activated charcoal. This medium was used throughout the propagation stage.

A total of six propagation cycles of the material (M_1V_0 to M_1V_6) were carried out and 3,700 plants were obtained. These plants began the process of acclimatization.

2.3. Causal agent collection, purification and molecular characterization

Visits to different production areas, mainly in the Atlantic and Northern zones in Costa Rica, took place in order to collect the causal agent *Collectrotrichum gloesporiodes.*

Once the fungus was isolated, acervuli and micelia were produced to finally obtain conidia. These conidia germinated producing single spore cultures[1].

Molecular characterization followed the protocol below: We took two out of three acervuli from each isolation. Then, we left them in Potato Dextrose and Agar in shaking, for 96 hours. Later, they were centrifuged at 1,200 rpm for 10 min. Finally, we removed the DNA.

A total of 65 single spores were used to extract DNA, following the protocol below:

- Centrifuge and eliminate the supernatant.
- Add to pellet 1 ml TE (Tris–Hcl 10 mM and EDTA 1 mM).
- Centrifuge at 12,000 g for 10 min, mix manually and eliminate the supernatant.
- Add 300 µl of extraction buffer and macerate; them add 300 µl of phenol.
- Mix for immersion and centrifuge at 12,000 g.
- At the watery stage, add 1/3 of sodium acetate 3M pH 5.2 and 2.5 ethanol volume 100% and inoculate at –20ºC for 10 min, then centrifuge at 12,000 g eliminate the ethanol and dry the pellet.
- Resuspend the pellet in 30 µl of TE (Tris–Hcl 10 mM and EDTA 1 mM), add 1 µl of RNAse, at 10 mg/ml, and store at –20ºC.

Then, extractions were submitted to PCR analyses (Perkin Elmer 2400 thermocycler) in presence of many primers. The RAPD technique permitted to preliminary detect polymorphism in some of the evaluated samples, indicating that a range of pathogenic ability could exist among isolates.

2.4. Preliminary test on pathogenic agent acclimatization and inoculation

Three different acclimatization tests were carried out:

[1] Campos Ileana, 2002. Estudio de la distribución espacial de pato tipos de *Collectotrichum gloesporiodes* que atacan el cultivo del ñame (Dioscorea sp) en las principales zonas de producción de Costa Rica. In press.

- Test 1: The first group of 600 plants were acclimated in a 100% sterile soil substrate and in a humid chamber.
- Test 2: The second group of 400 plants were acclimated in a sterile substrate of soil:sand:sow grass (3:2:1), in a humid chamber.
- Test 3: The third group of 120 plants were acclimated by using two types of sterilized substrates and two conditions of humidity. The first substrate was composed of soil and peat moss (1:1). The second substrate was a 1:1:1 soil:peat moss:organic matter mixture. Five days before sowing, both substrates were disinfected with Vitabax (5 g/L) (Active ingredient: Carboxanilide–2,3–dihydro–6–methyl–1,4–oxathiin, EPA Id: 400–115, Uniroyal Chem Co).

Two different conditions of humidity were regulated within the humid chamber by using a covering from a material named pelón (a type of cloth put over plants to maintain the humidity). This material allows water to filtrate and the plants to breath (treatments 1 and 3).

In order to eliminate all agar residues, the roots of *in vitro* plants were carefully cleaned with abundant. Using an automatic timer, programmed to execute two water applications per day, controlled humidity. One application was to be carried out at 6 a.m. for 5 min and the other one at 2 p.m. for 3 min.

For the third test data on survival, number of leaves and initial and final height were taken into account throughout the acclimatization process. This process lasted for about six weeks.

For the preliminary inoculation test, 250 *D. alata* clone 5969 plants were inoculated with 0.5 x 10^6/ml of *Collectrotrichum gloesporioides* conidia, after seven weeks of acclimatization. The inoculation was carried out manually, at 4 p.m. Then, plants were placed in a humid chamber (HR 95%) for 48 hours, and were then placed in the greenhouse where an automatic watering system was used. The plants were observed for two months, data about survival and percentage of plants damaged by the pathogen were taken.

3. RESULTS AND DISCUSIION

3.1. Validation of technology used for micropropagation stage

3.1.1. Mother Plant Collection and Sprouting in Greenhouse

Figures 1 and 2 show corms disinfected and germinated in the greenhouse at Universidad Nacional de Costa Rica.

Fig. 1. (A) Cleaning process of Diocorea alata corms. (B) Disinfection of corms.

Fig. 2. Dioscorea alata corms germinated in the greenhouse.

3.1.2. Introduction of nodal segments in vitro and media test

The disinfection process caused some explants to die (Table I). During the introduction process, some fungi (*Aspergillus sp., Penicilliun sp., Septoria sp.*) and germs were identified.

TABLE I. PERCENTAGE OF SURVIVAL AND CONTAMINATION IN *Dioscorea alata* EXPLANTS INTRODUCED *IN VITRO*

No. of introduction	No. of explants introduced	% of survival	% of cleanness	Explants clean and alive
1	35	85.7	80.0	23
2	40	95.0	90.0	34
3	45	97.8	88.9	39
4	55	94.5	90.9	47
5	65	98.5	90.8	58
6	80	95.0	93.7	71
7	50	88.0	84.0	48
8	45	100.0	97.8	44
9	40	97.5	100	39

Results showed that the protocol described by [13] for yam (*Dioscorea alata*) introduction and micropropagation through nodal segments is 100% effective, with a propagation rate of three new explants per explant per subculture passage.

As results have shown in 9 introductions carried out, 100% of survival was obtained in introduction No. 8. Likewise, 100% of clean leaves were obtained in introduction No. 9. Further, the fungi manifested throughout the different introductions are typical of this propagation.

Table II shows that MS medium with 1 mg/L BAP and 1 g/L activated charcoal or with 2 mg/L BAP and 1 g/L charcoal did not show significant differences with respect to plant regeneration. However, the first medium was chosen because with high BAP concentrations the risk of having somaclonal variations increases.

TABLE II. *Dioscorea alata* EXPLANTS REGENERATING IN DIFFERENT MEDIA

Modified MS		No. of explants to regenerate	No. of explants regenerated at 5 weeks	No. of explants regenerated at 10 weeks	No. of explants regenerated at 15 weeks	No. of explants regenerated at 20 weeks
AC* G/L^{-1}	BAP** mg/L^{-1}					
0.0	0.0	30	0	1	1	2
0.0	0.5	30	0	5	7	10
0.0	1.0	30	15	18	20	20
0.0	2.0	30	17	18	21	21
1.0	0.0	30	0	0	1	1
1.0	0.5	30	10	12	15	17
1.0	1.0	30	25	26	26	27
1.0	2.0	30	26	26	27	29

* Activated charcoal
** 6–Benzylaminopurine

3.2. Variability induction

3.2.1. Variability induction and Micropropagation

Once introduction tests and media tests finished, the micropropagation process started in order to obtain a number of plants adequate to begin with the different mutagenesis treatments. A micropropagation rate of 3:1 every 30 days was recorded.

After exposing the explants to establish LD–50, doses of 10 to 20 Gy were determined to be the best ones. This conclusion agrees with Madero's results in 1993. Figs. 3 and 4 show the LD–50 established for *Dioscorea alata*.

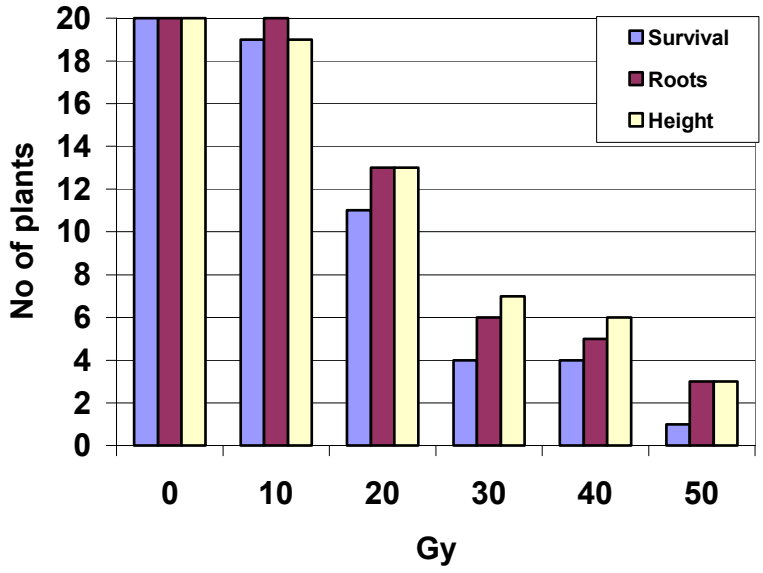

Fig. 3. DL–50 for Dioscorea alata.

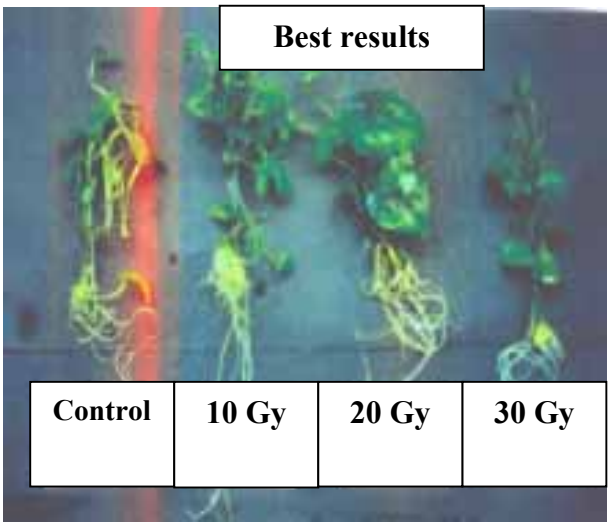

Fig. 4. Best results of LD–50 established for Dioscorea alata.

The first treatment with Gamma Rays was carried out using a dose of 20 Gy; however, the percentage of regeneration was very low. International Atomic Energy staff recommended a dose of 15 Gy. Thus, 100 explants were treated by using 15 Gy, and 100% regeneration was obtained. Figure 5 shows differences in plant regeneration after irradiation at 20 or 15 Gy.

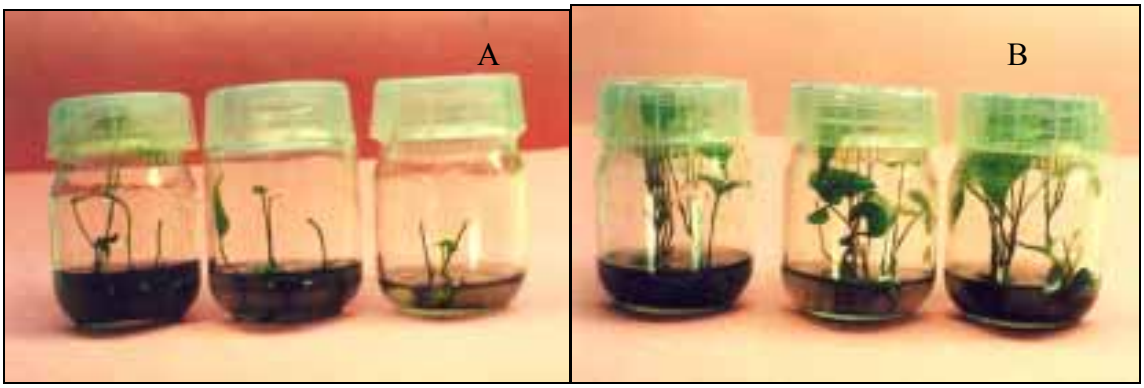

Fig. 5. Treatment effect on Yam explants with 20 Gy (A) or 15 Gy (B).

Plants irradiated with 15 Gy were propagated over six generations (M1 V6). Once this process ended, the plants were rooted and acclimated.

At the plant acclimatization stage, several alterations were observed including variation in the plant shape, changes on leaf pigmentation, shorter internodes and shorter plants (Figure 6). Changes observed on plants that were irradiated, propagated for six generations and afterwards acclimated show the irradiation effects on the phenotype characteristics, i.e. changes in shape and size of the leaves as well as changes in the size of the internodes.

This proves that the irradiation of 15 Gy is effective since the observed changes are not deleterious in the irradiated plants.

Fig. 6. (A) Short plants, (B) and (C) changes in plants shape, (D) shorter distance between internodes.

3.3. Molecular characterization of antrachnosis (*Colletotrichum gloesporioides*)

3.3.1. Collection of plants

Visits to six farms located in the Atlantic and Northern zones took place in order to collect the causal agent. In every plantation, there were plants damaged with *Colletotrichum gloesporioides*. Fifteen samples were collected in each farm.

3.3.2. Isolation and Purification of the Causal Agent

Once the samples were collected, the fungus was isolated and purified in the laboratory. Acervuli and micelia were obtained in order to produce conidia, which, upon germination, gave single spore cultures (Figure 7).

Fig. 7. Colletotrichum gloesporioides single spore culture.

3.3.3. Verification of genetic polymorphism in different fungus isolations

The molecular characterization of the causal agent was carried out by PCR, where the use of RAPDs showed polyphormism in 11 out of the 18 samples tested, proving that there is variation among the isolates of the pathogenic agent.

However, this variability is not very useful during preliminary selection of tolerant plants. It is essential to have a previous selection of a mixture of all possible existing strains of the pathogen in order to be sure that all plants chosen to be placed in the greenhouse are somehow tolerant to the full mixture and not just to a single strain.

Variability observed is very important and we might conclude that this variation depends on an external factor such as an excessive use of agrochemicals. This however, must be confirmed through further research.

3.4. Acclimatization and inoculation of the causal agent

3.4.1. Acclimatization Tests

3.4.1.1. First Test

The first test was carried out with 600 plants. A 100% sterile soil substrate was used, and a mortality rate of 78% was obtained. It was confirmed that the substrate and the percentage of humidity control throughout the process were not the most appropriate. Figure 8 shows the group of plants that survived this process.

Fig. 8. Acclimated Plants on a 100% Soil Substrate

3.4.1.2. Second Test

For this test, 400 plants were used. The substrate was a 3:2:1 mixture of soil, sand and sow grass. A mortality rate of 12% was obtained. For this acclimatization, better quality plants as well as a timer to control humidity were used.

This methodology served to start a program to acclimate plants. The idea was to acclimate 500 plants every month, however, and due to the fact that no more than 1500 plants can be placed in the greenhouse, the program had to be cancelled.

3.4.1.3. Third Test

For this test a total of 120 plants, two substrates (soil:peat moss, 1:1, and soil:peat moss:organic matter, 1:1:1) and two humidity conditions were used.

Figure 9 shows the treatments for the humidity conditions tested.

Fig. 9. Treatment to control humidity: (left) with and (right) without covering

Table III shows a summary of the deviation analysis for the variables: initial and final height, and number of leaves.

TABLE III. DEVIATION ANALYSIS FOR INITIAL AND FINAL HEIGHT, AND NUMBER OF LEAVES

Source of variation	fg	Initial height	Final height	Initial No. of leaves	Final No. of leaves
			Pr > F		
Substratum	1	0.3806	0.2929	0.9287	0.2469
Covering	1	0.0001	0.001	0.0072	0.2765
Substratum + Covering	1	0.9631	0.5825	0.7541	0.1211
C.V (%)	1	27	22	38	51

Results proved that substrate is not a significant factor for survival and growth during *Dioscorea alata* acclimatization stage. However, when different conditions of humidity are analyzed, significant differences arise due to the presence or absence of a cover in the acclimatization bed.

Although, this behaviour was only studied in test 3, test 2 proved that a good control of relative humidity inside the acclimatization bed might strongly diminish the mortality rates.

The age of the plants as well as their possible vitrification are factors not taken into account in any of the acclimatization tests and, even if they do not seem to affect the results, it would be convenient to consider them for future acclimatization tests.

In this test, it was found that the percentage of survival for all treatments was over 40%. Figure 10, shows that T1 (soil: peat moss with no covering) obtained the highest survival rate, 97.6% while T3 and T4 only yielded a survival rate of 40%. These rates were determined at the eighth week of acclimatization.

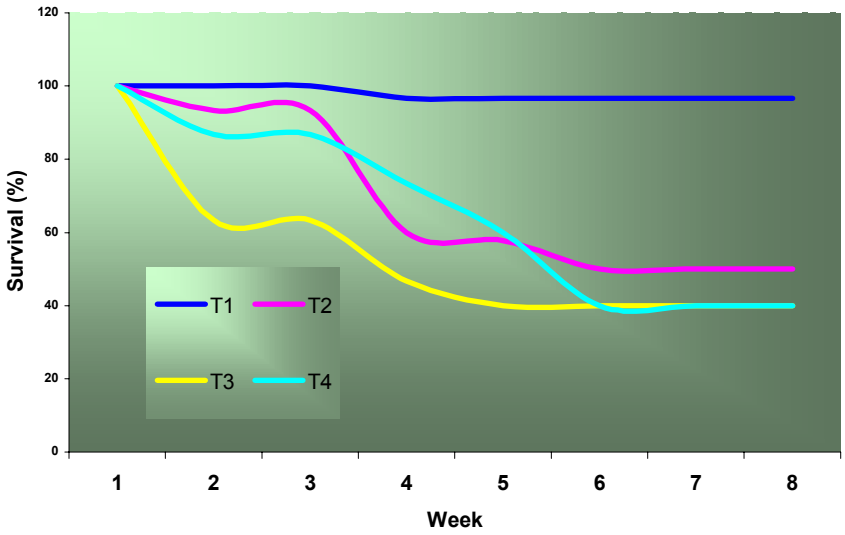

Fig. 10. Percentage of yam survival (Dioscorea sp) per treatment. Experimental farm Santa Lucìa, Heredia. 2003.

3.4.2. Preliminary Inoculation Test

Once this test finished, the pathogenic agent damaged 100% of inoculated plants. It was determined that the age of the plants was not the most convenient for this test. Thus, plants must be six to eight months after acclimatization to be inoculated.

Inoculation with conidia in plants recently acclimated is not the best alternative to have an early selection, as the results showed. Seven–week plants were completely damaged by the fungus. It would be necessary for the plants to have a longer period of time once they have been acclimated so that the inoculation process can start. It would also be useful to execute a series of experiments with different concentrations of conidia in order to establish the optimum dose per ml at the moment of inoculation.

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ENHANCING THE USE OF UNDER-UTILIZED PLANT SPECIES: STRATEGIES, APPROACHES AND EXPERIENCES AT IPGRI

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Abstract

This contribution addresses the work of the International Plant Genetic Resources Institute (IPGRI) in enhancing the use of neglected and under-utilized species within the context of its global mandate to promote better conservation and sustainable use of plant genetic resources. After an introduction on IPGRI's mandate, objectives and strategic choices, the authors present the Institute's strategy on neglected and under-utilized species, considered as powerful allies in strengthening economic development and enhancing nutritional security around the world. An overview of recent contributions promoted by IPGRI in support of neglected and under-utilized species is given along with examples of the role that capacity building and technology transfer have played in the area of germplasm conservation methods, safeguard and promotion of indigenous knowledge, post harvest, processing, add value, marketing and commercialisation of end-products.

1. INTRODUCTION

IPGRI is one of the 16 Centres of the Consultative Groups on International Agricultural Research (CGIAR). IPGRI was established in 1974 with the scope of encouraging, supporting and undertaking activities to improve the management of plant genetic resources worldwide so as to help eradicate poverty, increase food security and protect the environment.

IPGRI's efforts are directed towards the conservation and sustainable use of plant genetic resources (PGR) that are particularly relevant to developing countries [1]. The institute has also special responsibility for bananas and plantains and for supporting the genetic resources work of the CGIAR system. Headquartered in Rome, it has five Regional Offices manned by over 200 staff working from 17 offices around the world, in an effort to reach out its partners and beneficiaries most effectively.

In order to fulfil its global mission IPGRI pursues three main objectives:

(1) Countries can better assess and meet PGR countries needs,
(2) International collaboration in conservation and use of PGR is strengthened, and
(3) Knowledge and technologies relevant to the improved conservation and use of PGR are developed and disseminated.

The Institute is structured around three main components: 1) The PGR Programme, 2) The International Network for Banana and Plantain (INIBAP) and 3) The CGIAR Genetic Resources Support Programme (Policy Advice and System-wide Genetic Resources Program -SGRP).

IPGRI's efforts are grouped under 8 main strategic choices that cover areas where the Institute holds comparative advantage and which are considered to play the most significant role in advancing the status of sustainable conservation and use of PGR. These are as follows:

(1) Strengthening national programmes
(2) Working with networks
(3) Improving conservation strategies and technologies
(4) Increasing the use of plant genetic resources.
(5) Managing and communicating information
(6) Addressing socio-economic and policy issues
(7) Conserving and using specific crops
(8) Conserving and using forestry genetic resources

For more information on IPGRI and its research Agenda, the reader is redirected to the Institute's web page at www.ipgri.org

2. WHAT ARE NEGLECTED AND UNDER-UTILIZED SPECIES?

Neglected crops are those grown primarily in their centre of origin or centres of diversity by traditional farmers, where they are still important for subsistence of local people. Some species may be globally distributed, but tend to occupy special niches in the local ecology and in production and consumption systems. While these crops continue to be maintained by socio-cultural preferences and use practices, they remain inadequately characterized, and neglected by research and conservation. Under-utilized species (NUS) means species whose potential has not been fully realized. Farmers and consumers are using these crops less because in some way they do not compete with other crop species in the same agricultural environment. The eco-geographic decline of these crops may erode the genetic base and prevent the use of distinctive valuable traits in crop adaptation and improvement [2]. Medicinal plants belong to another specific category of NUS, which has been indeed poorly addressed by conservation and use enhancement programs in spite of their recognized use as health remedy by more than 80% of the world population [3].

Though there are various degrees of under-use and neglect that may characterize each of these species, IPGRI uses the term "NUS" when dealing with them, as a way to underpin the intimate link between use and conservation and hence remind constantly that any efforts in support of these species should cover consistently both areas to be sustainable in the long term. For instance, pistachio and oregano are important crops from the use perspective, but they are considered by IPGRI as NUS in view of their poor conservation status, currently largely neglected both *ex situ* and *in situ* [4].

Neglected and under-utilized species are being addressed by IPGRI particularly within its strategic choice no.7. Attention on NUS did not materialize overnight, being in fact the result of a public awareness campaign carried over the last 10 years targeting policy makers and other relevant Stakeholder groups, on the need to broaden R&D efforts so as to include crops left aside by research, technology, marketing systems as well as conservation efforts.

Neglected and under-utilized species are characterized by common features, which can be summarized in the following points:

- Local importance in consumption and production systems
- Highly adapted to agro-ecological niches/marginal areas
- Receive scarce attention by National Policies, R&D
- Represented by ecotypes/landraces
- Cultivated and utilized relying on Indigenous Knowledge

- Scarcely represented in ex situ germplasm collections
- Fragile seed supply systems

In spite of their low profile in most national and international research agendas and even though they do not appear in agricultural statistics at national and international level (e.g. the FAO's Agricultural Year Book), NUS are *de facto* neither neglected nor under-utilized at local level, where communities rely on them for a number of important contributions, including the following:

- Source of diversity rich diet
- Source of essential micro nutrients
- Source of cheap flavouring for food
- Source of income for rural and forest dwellers
- Contribute to maintain cultural identity and traditions
- Source for cheaper and accessible medicines
- Comparative advantage over commodity crops for their adaptation to grow in marginal lands, low input agriculture and in changing environments

At a national level, NUS provide indirect benefits related to their contribution to agricultural diversification, to the broadening of diversity in agro-ecosystems, and to their positive role in reducing imports and enhance self-reliance in agricultural production.

Among the steps that have led to a worldwide recognition of NUS, worthwhile recalling are the 1992 Convention on Biological Diversity (for its recognition of the importance of agro-biodiversity in agricultural development) and the FAO IV International Technical Conference on Plant Genetic Resources for Food and Agriculture held in Germany in 1996 (cf. Activity 12: "Promoting development and commercialisation of under-utilized crops and species") [5, 6]. Instrumental has been also the call made by the Global Forum on Agricultural Research (GFAR) for a coordination mechanism to strengthen research efforts on these species, which lead eventually to the successful launching of a German-supported Facilitation Unit for Under-utilized Species in 2001 [7].

3. IPGRI'S STRATEGY FOR NUS[1]

The ultimate goal of IPGRI's strategy on neglected and under-utilized crops is to strengthen the capacity of stakeholders to maintain and enhance the biological assets of the rural poor by enhancing and deploying a broader range of species adapted to diverse environments and providing new opportunities for better nutrition and income generation. To reach this goal IPGRI has set out three main objectives:

- Develop priority-setting approaches at the local, national and international levels and assist stakeholders to establish priorities for research, development and conservation actions on neglected and under-utilized species that increase their contribution to and impact on sustainable agriculture and livelihoods of the rural poor, and broaden the bases of food security.

[1] This section reports excerpts from IPGRI's strategic action plan for neglected and under-utilized species [8], obtainable free of charge from IPGRI (contact author at <<s.padulosi@cgiar.org>> or downloadable as PDF file from IPGRI's web page <<www.ipgri/cgiar.org/nus>>.

- Enhance the conservation and use of plant genetic resources of neglected and under-utilized species through complementary approaches to genetic resources from production to consumption.
- Strengthen the efforts of other actors working on the documentation, evaluation, and improvement, processing and marketing of neglected or under-utilized species.

IPGRI's efforts will focus on four major areas where neglected and under-utilized species can contribute to sustainable agriculture:

- Food security and better nutrition
  Many neglected and under-utilized species are nutritionally rich and are adapted to low-input agriculture. The use of these species, whether wild, managed, or cultivated, can have immediate consequences on the food security and well being of the poor.
- Increased incomes for the rural poor
  Growing demand from consumers in developed and developing countries for diversity and novelty in foods is creating new market niches for neglected and under-utilized species. These market opportunities can generate additional income.
- Ecosystem stability
  Climate change and the degradation of land and water resources have led to a growing interest in crops and species that are adapted to difficult environments such as poor soil, degraded vegetation, drought, desert margins and so on.
- Cultural diversity
  The use of plants has long been an intimate part of local cultures and traditions. Many neglected and under-utilized species play a role in keeping cultural diversity alive.

IPGRI's efforts on NUS will address eight major areas of work as follows:

3.1. Gathering and sharing information

Information plays a crucial role in enhancing the use of neglected and under-utilized species. Often, little is known about the extent of their cultivation, agronomic requirements, local uses and values, and contribution to local food security and environmental sustainability. Gathering and sharing information among all stakeholders is essential to promote greater use of NUS. IPGRI has already made important contributions to documenting information on NUS and will seek to strengthen its work in this area. IPGRI will provide information on species and also on options, techniques and approaches to support improved conservation and use of NUS. At the national level, ethnobotanic surveys of NUS linked to agricultural and economic development are important first steps. Documenting and disseminating the work of specialists, including those of sister institutes of the CGIAR, will be supported. IPGRI will pay special attention to the maintenance and documentation of local knowledge systems on the uses and management of germplasm of these species. An Internet web site will be maintained to disseminate *inter alia* information on IPGRI's and partners' activities in this area, including publications, databases and any other information related to the uses and conservation of neglected and under-utilized species. The newly established Global Facilitation Unit on Under-utilized crops, supported by the German Government, will advance efforts in this area.

3.2. Priority-setting

The large number of species that need work and the variety of needs and disciplines involved in supporting their conservation and use require the adoption of a structured approach to the

selection of priority species and activities so as to make the best use of limited resources and achieve the greatest development impact. IPGRI will therefore identify and work with key stakeholders to identify priority species and actions. For any given species, there will be different stakeholders at various points in the production, processing, marketing and consumption stages. Working together, using participatory and gender-sensitive approaches, these stakeholders can define priorities and identify all relevant concerns. The activities and species selected will be those that can serve as models for other species of local and regional significance that are important for the livelihood of farmers in similar environments. Priority-setting processes and tools that were developed and tested at IPGRI's Conference on NUS in the Mediterranean region held in Aleppo, Syria in 1998, will be disseminated and further improved to make them more broadly applicable.

Criteria followed to set priorities on NUS and related activities

- Species role in maintaining Agrobiodiversity
- Genetic erosion and conservation needs
- Food Security
- Nutritional value
- Economic security to rural poor

Key approaches in tackling use enhancement of NUS

- Bottom up, participatory methods
- Tools and methods simple, inexpensive and adaptable to different contexts and situations.

3.3. Promoting production and use

Neglected and under-utilized species can be more widely and effectively deployed to address malnutrition, poverty and environmental degradation. They constitute essential biological assets of the rural poor and can contribute to improving the well being of urban populations. Building on its work with communities on diversity management on farms and in home gardens, IPGRI will collaborate with partner institutions on the enhancement and greater use of neglected and under-utilized species. It will support work to assess and realize the nutritional, economic and environmental value of the species. Working with communities, IPGRI will identify strengths and weaknesses within existing production systems. It will work to improve seed supply systems and develop methods to ensure that desired diversity is maintained in production systems. In collaboration with farmers, IPGRI will identify and improve agro-morphological traits needed to enhance the use of NUS in agro-ecosystems and to meet market demands. IPGRI will also support studies of the genetic potential of NUS, crop enhancement work (especially participatory plant breeding) and work on key bottlenecks such as seed production and plant multiplication, including the development of micropropagation techniques. In view of the fact that as crops are improved and become more commercialised loss of genetic diversity may occur, IPGRI will take the lead in assessing both the value and the impact of crop improvement and market promotion on the distribution, use and maintenance of NUS genetic diversity.

3.4. Maintaining diversity

Little is known of the eco-geographic distribution of many neglected and under-utilized species and even less of the extent and distribution of their genetic diversity. Their poor

conservation and high level of genetic erosion call for coordinated efforts to safeguard these resources. Surveys, taxonomic identification and analysis of the extent and distribution of genetic diversity, together with work on local and traditional knowledge, remain priorities for many NUS. Tools to assess genetic erosion will have to be developed and applied, to facilitate these processes. From this information, complementary conservation strategies will need to be developed that give priority to maintenance in production systems (in-situ conservation), with ex-situ conservation providing back-up systems and material for access by other users. Characterization and evaluation can, in many cases, be carried out in the production systems with the communities growing and using NUS. There may also be a need for specific studies on topics such as reproductive biology; *in vitro* conservation and ways of eliminating viruses from vegetatively propagated species.

3.5. Marketing

Strengthened market systems are crucial to the promotion of neglected or under-utilized species. Better commercialisation translates into greater opportunities for income generation by the poor farmers who cultivate these species. The cultural value of NUS is also an important element that can support the markets for these species. IPGRI will seek strategic alliances with agencies or organizations that have experience in marketing, processing and product development of neglected species. Efforts will be directed at (i) identifying opportunities to add value through improved preparation or processing methods and the development of low cost technologies, (ii) marketing activities including user and market niche definition and improving price, distribution and presentation; (iii) creating and identifying opportunities to develop new products and markets, (iv) identifying ways to ensure that the nutritional contributions of selected NUS are recognized and integrated into national nutritional goals, (v) developing public awareness activities for crops and products at local and national levels and integrating such work in development-related activities, for example in-situ and on-farm conservation and home gardens.

3.6. Strengthening partnerships and capacities

Safeguarding the resource base of neglected and under-utilized species requires concerted actions among all stakeholders. Local people and farmers will be the most important actors in reversing the decline in use and arresting the genetic erosion of NUS. However, they will need to be supported by others. Building capacities and partnerships among all the stakeholders at national and regional levels in both the formal PGR institutions and the civil society organizations will be a key element of IPGRI's work. Using participatory approaches, which ensure that the interests of farmers and communities are adequately recognized, IPGRI will promote close collaboration with NARS, NGOs, CSOs, the private sector and international organizations. IPGRI's strategy will be to strengthen existing collaboration and add value to those initiatives that have already delivered relevant outputs in this field. IPGRI's catalytic role will be to focus the scattered efforts and the limited resources that are available to address priority actions to develop and conserve NUS. Strategic partners for IPGRI will be International Centre for Under-utilized Crops (ICUC), FAO and sister Future Harvest centres whose collaboration will be coordinated through SGRP and other system-wide initiatives. IPGRI will work to bring scientists and PGR policy-makers into closer partnerships to realize the full potential of NUS and will devote particular attention to raising the capacities of national programmes to work on these species. IPGRI will include NUS components in its training programmes and in other capacity building activities. Transfer of technology and information from one region to another in the context of building capacities to use and conserve NUS will be pursued whenever possible.

Within this domain of efforts, IPGRI will pursue partnerships through an innovative *"filiere"* approach, meant to bridge traditional PGR actors (gene bank operators, germplasm scientists, breeders, etc.) with production-to-use and policy-involved Stakeholders in order to realize economic development using NUS genetic resources in a concrete, sustainable way (fig. 1).

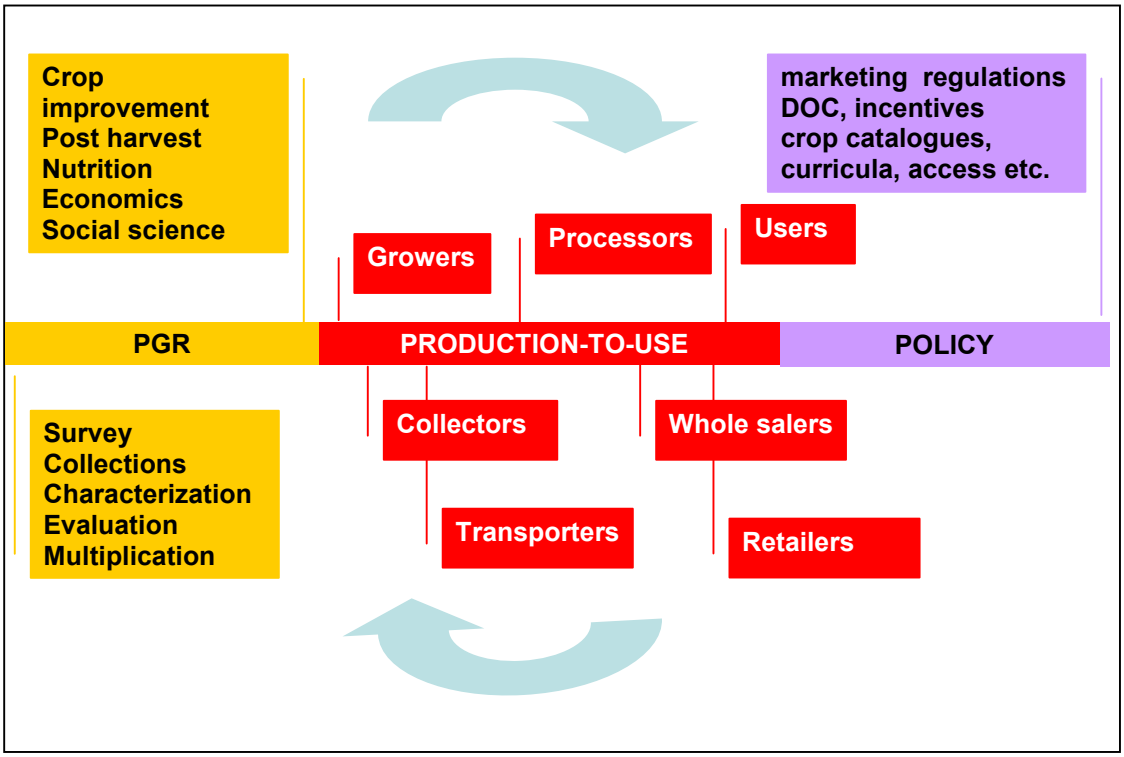

Fig. 1. The "filiere" approach pursued in tackling sustainable conservation and use of NUS (see text for explanation).

3.7.Developing effective policies for NUS

The further deployment, improvement and use of NUS will require taking account of constraints and opportunities that arise from the policy environment in many countries and in the international arena. Better policies and legal frameworks are required to support the research, cultivation and commercialisation of neglected and under-utilized species. As these species are developed and exchanged, IPGRI will work to promote policies, laws and regulations that return benefits from increased use of NUS to the communities that have been custodians of both the genetic resources and the associated knowledge. IPGRI will also work in support of policies that safeguard access to NUS genetic resources by farmers in regions where they are important to food security and incomes. It may well be that policies and procedures developed for major crops or commodities are not appropriate for NUS and IPGRI will use its expertise in policy development to review and address specific aspects that are important to the sustainable use of NUS by farmers and communities.

3.8. Improving public awareness

Raising interest in neglected and under-utilized species is fundamental to creating a more favourable environment for their sustainable production and use. Policy-makers, research institutions, the private sector and users at large should be aware of the concrete benefits that arise from a broader use of these species and should be encouraged to share efforts on

common research goals. IPGRI's role in raising public awareness among partners has contributed to the greater awareness of, and importance given to, research and development of NUS. IPGRI will develop public awareness materials in a variety of media and for diverse audiences in order to build interest and support from public and private sectors. National and international seminars and conferences will be used as opportunities to sensitise stakeholders and the public at large to issues surrounding neglected and under-utilized species. Reversing the neglect and declining use of NUS will also entail work to further the public awareness of the value and utility of these species at all levels, beginning in the local media and cultural institutions where these species are still important. Links to global media and public awareness are also important.

4. EXPERIENCES AT IPGRI

One of the most relevant involvements of IPGRI on NUS is the recently launched IFAD-supported project entitled "Enhancing the Contribution of Neglected and Under-utilized Species to Food Security and to Incomes of the Rural Poor".

The Project's goal is to contribute to raising the incomes and strengthening the food security of small farmers and rural communities around the world through securing and exploiting the full potential of the genetic diversity contained in neglected and under-utilized species. This Project is the result of close cooperation with a diverse range of Stakeholder Groups, including farmers and NGOs from National Programmes of Central & West Asia and North Africa/CWANA (Egypt and Yemen), Asia (India and Nepal) and Latin America (Bolivia, Ecuador and Peru).

The Project aims at the following contributions:

- Greater nutritional security and income generation
- Conservation of genetic diversity
- Enhanced use of local agro biodiversity
- Improved post-harvest, processing and marketing
- Empowerment of local communities and women in managing biodiversity
- Strengthening of production-to-use supply chains

Its expected outputs include the following:

- A secure resource base for the selected crops for use in global development initiatives, including deployment in marginal areas in other regions;
- Knowledge of effective procedures to integrate neglected and under-utilized crops into development actions;
- Publications, including technical guidelines, information on specific crops, their properties and nutritional value;
- Global experience linking plant genetic resources research with local institutions for managing crop resources.

This community-based participatory project focuses on priority species selected by partner countries on the basis of their importance in nutritional security and income generation particularly for the rural poor. These species include medicinal and aromatic plants that are important for the West Asia and North Africa [e.g. argel (*Solenostemma arghel)*, caper

(*Capparis* spp.), oregano (*Origanum syriacum*), mint (*Mentha piperita*), liquorice (*Glycyrrhiza glabra*), aloe (*Aloe* spp.), coriander (*Coriandrum sativum),* cumin (*Cuminum cyminum*) and henna (*Lawsonia inermis*)], Andean grains for Latin America [e.g. quinoa (*Chenopodium quinoa*), canihua (*C. pallidicaule*) and amaranth (*Amaranthus caudatus*)] and nutritious millets for Asia [e.g. finger millet (*Eleusine coracana*), Italian millet (*Setaria italica*) and little millet (*Panicum miliare*)].

The project, in its first year of implementation, is already yielding significant progress, and the following sections reports a summary of relevant outputs so far, along with outcomes from other IPGRI's initiatives undertaken in recent years in this field. These activities are being listed under IPGRI's NUS Strategic Areas, with the intent of giving the reader examples of steps pursued by the Institute in fulfilling its commitment in this important domain.

4.1. Objective: Provide documentation, information and knowledge to users (linked to IPGRI's NUS strategic areas no. 1 and 2)

- **Development of germplasm catalogues:** Activities carried out in China and Bolivia has produced so far germplasm catalogues for a number of species, including safflower [9] and quinoa [10]. The Germplasm Directory for Latin America and the Caribbean is also a useful source of information on NUS holdings in this region [11].

- **Databases:** A germplasm collection database on rocket (*Eruca* and *Diplotaxis* species) was promoted and developed in participation with the Italian Germplasm Institute (National Research Council - CNR) and other partners joining an International Rocket Network coordinated by IPGRI [12]. A database to document the diversity of pomegranate accessions held at the Garygala Research Station, Turkmenistan, was also developed through on-the-job training of local experts. This activity was particularly valuable in consideration of the fact that this field gene bank (established by N.I. Vavilov in the early 1930s and containing more than 1,000 accessions of cultivated and wild *Punica granatum*) had no electronic documentation and precious data related to the collection (passport as well as characterization data) were destined to be lost for ever due to poor documentation.

- **Food recipes:** This is an area gaining increasing importance in recognition of the role that food recipes can play in promoting the use of NUS by appealing to people renewed attention to culture and traditions. IPGRI's partners in Latin America (Bolivia, Ecuador and Peru) have been actively engaging local communities – including women organizations - in the development of booklets in which traditional recipes have been documented and disseminated to users. Andean grains [quinoa (*Chenopodium quinoa*), cañihua *(C. pallidicaule*), amaranth (*Amaranthus caudatus*) and lupin (*Lupinus album*)] have been the focus of these efforts [13].

- **Documenting uses:** Sharing knowledge on uses of NUS is strategic as it relates directly to the maintenance of their genetic resources by the users' communities. Given the increasing erosion and loss of Indigenous Knowledge affecting NUS [2, 14], these activities need to be strengthened decisively. IPGRI's most recent outputs in this domain, include the support for the publication of a book on edible plants of Kenya describing 800 indigenous plant species used for food in one way or another [15] and the ethnobotanic survey on more than 200 indigenous plants in Lebanon used as leafy vegetables, medicinal and aromatic purposes [16,17].

- **Bibliography of NUS:** Access to proper data and experiences is essential to promote uses of NUS and enhancing knowledge and capacities of National Programs around the world. A particularly valuable product in this domain is the bibliography on native fruits of Latin America, produced by IPGRI in 2000 and available on IPGRI's internet web page (<www.ipgri.cgiar.org>). Another useful bibliography to assist researchers in Africa is that on the genetic resources of traditional African vegetables [18]. A specific inventory on under-utilized edible fruits and nuts in their regions of diversity was produced by IPGRI in 1998 [19]. The Institute's partners in Bolivia, Ecuador and Peru are currently compiling all available published literature related to Andean grains and making it available to students and other workers (this valuable resource centre is being created at the PROINPA Foundation library in La Paz, Bolivia). Included in this reference collection are University theses produced by students in Bolivia insofar which have been addressing specifically Andean grains. The compilation and dissemination of research findings on NUS is a very important aspect, requiring greater attention; the so called "grey literature" consisting often of valuable publications poorly known within the scientific community should be indeed better shared among scientists and all other users for enhancing the sustainable use of NUS.

- **Crop monographs:** The provision of comprehensive information, from geographical distribution of genetic diversity and conservation, to breeding, agronomy, post harvest, processing, add value, marketing and uses, is a particularly significant instrument to support National Programs in better realizing the benefits from their biodiversity endowments. Thanks to a German-funded Project, IPGRI has been very active in this domain and 23 publications on priority crops have been produced so far. These monographs can be downloadable for free at IPGRI's internet web page dedicated exclusively to neglected and under-utilized species: <www.ipgri.cgiar.org/nus>.

- **Ecogeographic surveys**: the understanding of the distribution of the genetic diversity of NUS is generally very poor. IPGRI has supported several studies to improve this situation, which resulted into useful scientific publications. Major outputs include the ecogeographic study of the genus *Vicia* [20] and *Corchorus* in Africa [21] and the identification of microcenters of genetic diversity for quinoa, amaranth and lupin in Ecuador [22]. Recent ecogeographic surveys on laurel and pomegranate in Syria were combined with fruit quality analyses of surveyed populations, revealing opportunities for better deployment of existing diversity in income generation [23, 24].

4.2. Objective: Increase availability of germplasm to users (linked to IPGRI's NUS strategic areas no. 3 and 4)

- **Sampling of diversity:** Andean grains have been surveyed and sampled through participatory missions involving experts and community members working closely together. The results of these expeditions carried out in 2002 (50 acc. amaranth, 107 acc. of quinoa and 59 acc. of cañihua sampled in Bolivia, 586 acc. of quinoa in Peru) are used in evaluation and participatory selection activities. Useful wild species from the indigenous Lebanese Flora have been surveyed and sampled in the period 1999–2000 (195 accessions of *Origanum syriacum*, 53 of *Salvia fruticosa*, 102 of *Cichorium intybus* and 82 of *Gundelia tournefortii*) and are now available to farming communities [17]. 37 accessions of pomegranate varieties from Syria were collected in 2001–2002 through an IPGRI-supported MSc Thesis Research effort in collaboration with the University of Aleppo, Syria [23].

- **Rejuvenation/multiplication in gene banks:** During the period 2001–2002, 88 accessions of lupin, 479 acc. of quinoa and 230 of amaranth have been multiplied at the INIAP gene bank in Ecuador whereas 555 acc. of cañihua and 248 of quinoa by the PROINPA's gene bank in Bolivia [25]. Worthwhile to highlight that within the framework of the IFAD-IPGRI Project on NUS, between 50 and 100% of national collections of quinoa, cañahua, amaranth and lupin held in Bolivia, Ecuador and Peru, have been successfully regenerated and multiplied during the period 2002–2003.

- **Provision of NUS material to farmers:** Through the empowerment of local experts (establishment of Local Committees of Agricultural Investigations -CIALs) in Ecuador, the selection of best genotypes and their multiplication and release to farmers, was pursued by IPGRI and its partners in a very effective way. In Lebanon, transfer of *Origanum syriacum* seeds and seedlings to farmers was carried out in cooperation with ICU (an Italian NGO executing development projects in South Lebanon) along with the organization of training courses to capacitate farmers on cultivating techniques.

- **Exchange among farmers:** 490 farmers and 16 private companies attended seed fairs on Andean grains organized by IPGRI's partners in Bolivia, Ecuador and Peru, where champion farmers were also awarded for their contribution in maintaining large crop diversity *on farm* [25, 26].

- **Establishment of field gene banks:** A cherimoya (*Annona cherimola*) field gene bank was established in 1998 at the INIA sub-Station in Ayacucho, Peru, thanks to the support of INIA-Spain, within an IPGRI-led initiative aimed at enhancing the use of this under-utilized fruit tree native to Peru and Ecuador. This collection, made of more than 250 accessions, will represent an invaluable source of material for the Peruvian National Programme engaged in selecting improved material of this crop. A field gene bank of local varieties of Syrian pomegranates was also established in 2002 [23].
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- **Refined technologies:** Improved methods to better maintain and characterize germplasm of NUS were developed in 2002 for the tropical fruits mangosteen, rambutan and jackfruit. This IPGRI's led effort was carried out through the close cooperation of 10 Asian countries participating to an ADB (Asian Development Bank)-funded Project on Tropical Fruits.

- **Enhancing local conservation practices:** The capacitation of communities in conserving their resource base of crop diversity is very strategic, particularly for NUS. Community gene banks have been successfully established in East Asia to safeguard both germplasm and indigenous knowledge of mangosteen, rambutan and jackfruit. Similar efforts in the Andean region, led to the establishment of 6 local gene banks for quinoa and other Andean grains (4 in Peru and 2 in Ecuador). In India, the establishment of local seed banks in close participation with farm communities at Kolli Hills, (Southern India) by M.S. Swaminathan Research Foundation (MSSRF), has helped in promoting *in situ*/on farm conservation of traditional land races of nutritious millets. The communities are encouraged to store the seeds in traditional storage structures called "*dombai*". The seed bank approach has also helped communities in seed sharing to overcome seed shortages, frequently encountered among farmers.

- **Establishment of core collections:** Core collections are useful instruments to promote the use of plant genetic diversity [27]. Core collection of Andean grains has been developed in Ecuador and Bolivia to facilitate the use of *ex situ* germplasm collections of these crops. In

Bolivia, this work was carried out on 2,514 accessions of quinoa using 18 quantitative variables: statistical analyses led to the identification of 6 representative groups of accessions, each group contributing for a specific genetic variation to the core collection made of 267 accessions.

- **Capacitating local producers:** Training courses targeting local producers of Andean grains were carried out in 2002 at the Peruvian gene bank in Puno. Here local growers and processors have learned of the diversity-rich national germplasm collection, and discussed with scientists ways to enhance uses of these crops by making better use of the *ex situ* collection.

- **Enhancing cultivating practices:** In India and Nepal efforts have been spent in 2002 to develop better agronomic operations that would optimize the yield of nutritious millets.

- **Quality standards:** In view of their importance to promote marketing of NUS, quality standards have been addressed by IPGRI's partners consistently both for Andean grains and for nutritious millets. In Bolivia in particular, a *ad hoc* Committee of experts was established in 2002 to elaborate the first quality standard norm for the cultivation and processing of cañihua.

- **Seed storage behaviour:** Often little is known on the seed behaviour of many NUS species conserved in ex situ gene banks. A valuable tool for gene bank managers is the IPGRI's seed storage compendium [28], which complements other gene bank management publications produced also by IBPGR/IPGRI insofar.

4.3. Objective: Enhance cultivation and post-harvest practices for use improvement (linked to IPGRI's NUS strategic area no. 5)

- **Domestication and agronomic requirements:** *Gundelia tournefortii* ("akoub" in Arabic): is used to prepare a famous food delicacy in the Middle East, but unsustainable harvests of young shoots from its wild populations, pose great threats to its own survival [17]. IPGRI has supported a research study in Lebanon to look at its domestication and cultivation practices needed to release the pressure in the wild while creating income opportunities for local communities. Similar genetic erosion threats are being faced by sage (*Salvia fruticosa –a* medicinal/aromatic plant with large economic potentials), oregano (*Origanum syriacum* -an industrial and ornamental species) and chicory (*Cichorium intybus* -a leafy vegetable) all very popular species in Lebanon and in the Middle East. For these species too, domestication efforts and characterization studies have helped considerably the understanding of their cultivation needs: adequate germplasm and cultivation techniques made available to local farmers interested in their better exploitation have contributed to release pressure on natural populations [17, 29].

- **Participatory evaluation:** In 2001 and 2002, IPGRI partners in India carried out the participatory evaluation of 3,486 accessions of finger millet (*Eleusine coracana*), 1,191 of Italian millet (*Setaria italica*), and 452 of little millet (*Panicum miliare*). Demonstration plots for local farmers were also established during these activities. Screening for mildew-resistant, early maturing and high yielding quinoa was also carried out in Ecuador, Bolivia and Peru in 2002 and 2003. In Bolivia, 4 best quinoa genotypes were eventually selected and are currently being multiplied for their release to farmers. Participatory selection of best genotypes has been carried out also for a number of tropical fruit species i.e.

mangosteen, rambutan and jackfruit within the framework of an IPGRI-led International effort supported by the Asian Development Bank.

- **Enhancement of cultivating/add-value techniques:** Improvement of cultivating practices for yield optimisation in nutritious millets has been achieved in India and Nepal by a team of agricultural experts working closely with farmers. Organic agriculture investigations focusing on Andean grains and medicinal and aromatic plants in Ecuador and Egypt, respectively, have been also carried out in early 2003 to investigate on add value techniques to enhance the competitiveness and income generation of these species.

- **Post-harvest technologies:** The IPGRI-IFAD Project on NUS has deployed special efforts to make processing of these crops by local communities more effective. Its Andean component has so far developed a prototype of a simple manual threshing machine wit of crops and hence in promoting their sustainable use. They are even more strategic for NUS, in view of the fact that these species lack most of those instruments necessary to guide characterization and evaluation activities easily available for commodity crops. Descriptors lists produced so far by IPGRI and its partners amount to more than 80, those that specifically address NUS include: bambara groundnut, buckwheat, jackfruit, kodo millet, lupin, quinoa, safflower, winged bean and rocket. IPGRI's partners in Latin America recently developed the first descriptor list for cañihua. The development of descriptor lists in close partnership with National Programs has benefited researchers around the world in a number of ways: significant is the transfer of skills necessary in their production and the strengthening of cooperation and networking among countries gained by all participants in the development processes [31].

- **Development of better agronomic operations** from nutritious millets carried out in India and Nepal;

4.4. Objective: Create capacities on use enhancement (linked to IPGRI's NUS strategic area no. 6)

- **Capacitating courses**: Training in commercialisation and marketing were organized for local entrepreneurs in Peru in 2002. 479 local producers attended these courses, focusing on cañihua, from 7 local communities. Skills developed by the producers during these courses will strengthen their capacities in marketing Andean grains at local and regional level.

- **Adding values:** IPGRI partners in India, Nepal and Latin America have organized several workshops and training courses for both professionals and local community members to investigate and transfer capacities aimed at adding values to Andean grains and nutritious millets and make them more competitive on the market. Strategic add value approaches have included the development of food recipes, which blended traditions with innovative processing technologies.

- **Low cost processing technology:** Capability-building for local community, particularly women in using grain processing mills to do away with the drudgery associated with the traditional method of milling has been carried out in India in 2002. Participants have been trained by food processing experts from University experts on grain malting, milling, flour processing and blending for preparation of various food items to promote consumption of millets as home food, school meals and healthy foods.

- **Analyses of production-to-use chains:** These were carried out along with cost-benefit studies on commercialisation and marketing of Andean grains in Ecuador and Peru.

- **Strengthening agricultural assistance programs:** Courses on more efficient agronomic operations have been carried out in India, Nepal and Latin America.

- **Strengthen operational links in "*filieres*":** Local and regional fairs for Andean grains to strengthen networking at local level were organized in Bolivia Ecuador and Peru in 2002. These events proved to be very useful occasions to consolidate networking among farmers and representatives of the private sector. Meetings with private enterprises to discuss value-added strategies to improve local markets of Andean grains took place in Bolivia in 2002.

- **Mobilization of local authorities:** Local workshops with representatives of Municipalities to discuss on strategies for Andean grain products promotion were organized in Bolivia in 2002. These meetings contributed to also raise awareness on the nutritional values of Andean grains and canvass additional support of local authorities for these crops.

- **Establishment of "Self Help Groups" (SHGs):** Village communities in India have been organized as SHGs to promote nutritious millets cultivation, processing, value addition and marketing. SHGs will be playing a critical role in capacity building interventions and capital supply through micro-credit and other income generation initiatives in support of community members.

- **Empowerment of local processors:** IPGRI's partners in Bolivia pursued the establishment of community-based enterprises to process effectively Andean grains in 2002.

- **Woman workshops:** Women play a fundamental role in processing NUS [8, 32] and their capacitation in the area of food preparation is hence most strategic to enhance the use of these species. IPGRI has been supporting several courses targeting specifically women in Latin America (focusing on Andean grains), India and Nepal (focusing on nutritious millets), where participants improved their own skills in product transformation, value-adding preparations, and nutrition enhancement.

- **Capacitating agricultural students and technicians:** Development of training material to capacitate students in cultivation and post harvest practices took place in Bolivia in 2002 and 2003: 139 students have been trained in the area of germplasm collection of Andean grains.

- **Strengthening capacities at university level:** On-the-job training of graduate and postgraduate students is a priority in IPGRI's Agenda. Recipients of IPGRI's recent support include students of Ecuador and Bolivia working on Andean grains and students from Syria and Turkmenistan working on pomegranate genetic resources.

4.5. Objective: Raise public awareness and improve policies (linked to IPGRI's NUS strategic areas no. 7 and 8)

- **Video** by Austrian Channel One (ORF) on NUS produced and broadcasted in 2002; video on values of Andean crops produced in Bolivia, several newspaper articles published in

India on role nutritious millets, TV program broadcasted in India; 6 one-day farmer workshops held in Ecuador along with TV interviews; radio interviews in many countries across regions, posters and fact sheets produced to raise awareness over importance of NUS.

- **Festival on Andean grains** organized in Bolivia and Peru;
- **Exhibition** of typical food products held in Peru and attended by 48 organizations;
- **Competition for best food** preparations using cañihua (involving 60 participants) organized in Peru.

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ANNEX 1

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