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# *In vitro techniques for selection of radiation induced mutations adapted to adverse environmental conditions*

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*IN VITRO* TECHNIQUES FOR SELECTION OF RADIATION INDUCED MUTATIONS ADAPTED TO ADVERSE ENVIRONMENTAL CONDITIONS

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## FOREWORD

The ever increasing human population and dwindling land and water resources worldwide make it essential to produce more food, fibre and fodder from less and less land. During the last century, plant breeding contributed remarkably to increasing food by producing varieties which give higher yield, have improved quality and nutrition, and resist diseases and pests. Nearly 50% of the increase in food production in Asia during the last fifty years can be attributed to the high yielding, short height varieties of rice and wheat, the remaining to the improved agronomic inputs and management. Many crops, such as cassava, potato, pineapple, sweet potato, sugarcane, banana and plantain are vegetatively propagated. Some of these, such as cassava, potato, sweet potato, banana and plantain are major food crops, and others such as sugarcane and pineapple are important to the economies of many developing countries. One of the solutions to have a sustainable and secure food production is to breed varieties which are tolerant of stress conditions during their growth and development. Hence a Co-ordinated Research Project on *In vitro* Techniques for Selection of Radiation Induced Mutations Adapted to Adverse Environmental Conditions was initiated and focused primarily on the improvement of vegetatively propagated plants.

Mutation techniques in combination with tissue culture methods provide a powerful technology to improve clonally propagated plants. The *in vitro* culture of vegetatively propagated crops in combination with radiation induced mutations has proven to be an invaluable method to produce desired variation and to rapidly multiply the selected mutants and parental material in a disease-free condition. It is possible to upgrade well established clones by changing specific traits by inducing mutations. The availability of large populations for mutagenesis is one of the basic pre-requisites to obtain sufficient variation. The *in vitro* techniques provide the mechanism to generate large populations for mutation induction, selection and rapid multiplication of the selected mutants.

Since the inception of this project, several participating scientists established the optimal dose requirement for *in vitro* cultured material. Investigations were carried out on the effect of radiation to alter traits which affect survival under stress conditions and high temperature stress in potato, pineapple, sweet potato and garlic. The possibility to change traits such as tolerance to saline and water logged soils in sugarcane and gene regulation for salinity tolerance were studied. The limited number of available reports suggest that callus cultures are much more sensitive to radiation treatment and require much lower doses (2 to 5 Gy) than stem cuttings or seeds, and that relatively higher doses (15 to 20 Gy) cause necrosis or loss of regenerative capacity.

The success of this CRP can be measured from the development of improved lines and germplasm tolerant to saline and waterlogged soils or high temperature stress in potato, sweet potato and sugarcane. Much of the germplasm is at early stages of breeding, and would require large scale field evaluation before it can be released as an improved variety to the growers.

This publication summarizes the results of the Co-ordinated Research Project, presented at the third and final Research Co-ordination Meeting, which was held at the invitation of the Shanghai Academy of Agricultural Sciences, Shanghai, China. The IAEA officer responsible for this publication was B.S. Ahloowalia of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

## *EDITORIAL NOTE*

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## SUMMARY

## 1. INTRODUCTION

Many crops, such as cassava, potato, sugarcane, sweet potato, garlic, pineapple, banana and plantain are propagated from vegetative parts. These crops are important in many developing countries as a major source of food. Although some of these plants produce seed, the desired recombinants are not easily obtained through sexual crossing. Adverse environmental stress such as high temperature, frost, saline and waterlogged soil, and drought reduces crop yields and impairs quality, leading to food insecurity. Under extreme stress conditions, crops completely fail, which in turn may lead to food shortage, high food prices, migration of populations from villages, and to rural instability. Farmers know only too well the damage to their crops and their suffering when the rainfall is either too much or too little and untimely.

One of the solutions to have a sustainable and secure food production is to breed varieties which are tolerant to stress conditions during their growth and development. Modern day technologies of plant tissue culture allow the production of large populations of plants in a short duration and on a year round basis in the laboratory. Such populations can be irradiated *in vitro* to induce mutations, multiplied and grown in the field for selection of desired genotypes. Using a combination of mutation and *in vitro* techniques, new genotypes can be created in crops, which are propagated from vegetative parts. Because seed progeny of such crops as potato, sweet potato and garlic is highly variable, induction of mutations offers the possibility to produce only a limited number of desired genetic changes in genotypes and varieties, which are adapted to the local eco-climatic conditions.

The *in vitro* culture of vegetatively propagated crops in combination with radiation induced mutations has proven to be a valuable method to produce desired variation, and to rapidly multiply the selected mutants and parental material in disease-free condition. It is possible to upgrade well established clones by changing specific traits by inducing mutations. The availability of large populations for mutagenesis is one of the basic pre-requisites to obtain sufficient variation. Mutation techniques in combination with *in vitro* culture have become an important tool in upgrading locally adapted cultivars. Mutation techniques in combination with tissue culture and molecular methods provide a powerful technology to improve clonally propagated plants such as banana, plantains, apple, pineapple, date palm, potato, sweet potato, cassava, carnation, chrysanthemums, roses and tulips. Nearly all of these plants can be regenerated and multiplied in vitro, allowing the production of large populations in a small space and short time. The irradiation of *in vitro* cultured date palm, apple, potato, sweet potato and pineapple now provides a means to treat large populations, which would not have been possible before. Irradiation of micropropagated plants, axillary and adventitious buds, apical meristems, regenerative callus cultures, anthers and microspores, and somatic embryos provides a miniaturized version of trees and seeds in the Petri dish instead of the field.

Before the development of *in vitro* techniques, many mutants of vegetatively propagated plants were obtained by irradiation of rooted stem cuttings, detached leaves, and dormant plants. Since the effect of mutation in ornamentals was very visible, selection for changed flower colour, shape, and size was easy. Hence, mutation technique became a major tool for breeding ornamental plants. Other recently released mutants include apple with changed skin-colour in Austria, disease resistant mutant of Japanese pear in Japan, seedless mutants with deep red flesh and juice in grapefruit in USA, and 'Novaria', an early ripening mutant with enhanced flavour in

banana in Malaysia. However, the technology has yet to be exploited for the improvement of clonally propagated crops such as potato, sweet potato, yams, plantain, strawberry and date palm.

In vegetatively propagated plants, following mutagenesis, several cycles of propagation are needed to obtain homo-histonts or to "dissolve" chimeras and obtain "solid" mutants. It has been suggested that many of the mutants thus generated are sectorial chimeras. The *in vitro* subculture of irradiated material through $V_2$ to $V_4$ can be achieved rapidly and without loss of any genotype and under disease free conditions. In many plants, such as banana and potato, it can reduce this duration from 5 years of field propagation to less than nine months in the laboratory. In addition, when plants are regenerated from cell suspensions capable of producing somatic embryos, the chances are that many of the regenerants would be solid mutants, since only a few and in many cases single cells may give rise to such embryos. In many mutagenic studies, gamma ray and X ray radiation have been used to induce mutations. The key factor in irradiation of the plant material is the dose, which is the amount of radiation energy absorbed by the material. In case of *in vitro* cultured plant material, since only milligrams of tissues and micrograms of cell suspensions are irradiated, the dose levels are much lower. Hence, a Co-ordinated Research Project was established on "*In vitro* techniques for selection of radiation induced mutations adapted to adverse environmental conditions".

The objective of this CRP was to develop *in vitro* techniques to induce mutations in vegetatively propagated plants, such as cassava, potato, sweet potato, sugarcane, garlic and plantain. This objective was to be realized by developing techniques of plant micropropagation and regeneration, and procedures for *in vitro* radiation and separation of mutated sectors from multi-cellular explants. In addition, the possibility to develop protocols for *in vitro* selection was to be explored based on manipulation of the culture media. It was also important to develop protocols for large scale *in vitro* multiplication for the release of selected mutants to the end users.

## 2. ACHIEVEMENTS

### 2.1. Overall

- During the course of the CRP, the participants developed appropriate *in vitro* culture protocols for potato, sugarcane, sweet potato, pineapple and garlic. The adoption of these techniques has had a spin-off in Pakistan and India, where these protocols have been adopted for the multiplication of disease-free planting material of potato and sugarcane on commercial and semi-commercial scales, and have already reached the farmers.
- All participants carried out radio-sensitivity tests with their respective crops. The optimal gamma ray doses were determined for potato, sugarcane, pineapple, sweet potato and garlic using *in vitro* cultured material. In potato, sugarcane, pineapple and sweet potato in vitro-cultured microplants were irradiated. Three laboratories in India, Pakistan and Egypt reported 20 Gy as the optimal dose for the irradiation of tetraploid potato microcultured plants, and in the Netherlands, 6–8 Gy appeared to be the optimal dose for monoploid material. In Ghana, 45 Gy was reported as the optimal dose for irradiation of pineapple shoot apical meristems, and 20–25 Gy for sweetpotato for plantlet irradiation in China. For sugarcane, Pakistan and Bangladesh reported 20 Gy as the optimal dose for irradiation of the microcultured plants. In some experiments, plant regeneration was attempted from leaf explants and callus cultures.

- The dose required for callus cultures is relatively very low. For example, in garlic, irradiation dose of 8–10 Gy caused callus necrosis, and the optimal dose for sweet potato callus was 5 Gy; higher doses 8–10 Gy were lethal and caused necrosis of callus. The surviving propagules from radio-sensitivity tests were further multiplied through $V_1$ to $V_3$ propagation. In a few cases, the experiments were repeated by using the optimal dose, e.g. in pineapple, potato and sugarcane.

## 2.2. Specific achievements of the CRP

- To select for stress tolerance, in vitro-derived plants were subjected to the appropriate stress conditions. For example, in potato, for selection of tolerance to salinity, *in vitro* material was either cultured on media or grown in soil containing salt. Variants of potato for salt-tolerance were selected in Egypt and Pakistan. However, their stability needs to be investigated. In Egypt, micropropagated potato plantlets, irradiated with 20 Gy, were able to produce microtubers upon subsequent propagation in medium supplemented with 4000 ppm NaCl. One variant line of these microtubers was further propagated as minituber, which gave normal tubers after growth under saline conditions (4000 ppm NaCl).
- In India, selection for resistance to high temperature stress in potato produced promising variants. These were obtained by early planting of tubers under high temperature conditions. Late blight resistant variants were selected by application of fungal spore suspensions and selection by leaf-necrosis and plant death in the field. The obtained variants exhibited segregation of sectors in the successive propagations, and the proportion of resistant variants increased. Further vegetative propagations are required to obtain stable variants. In the Netherlands, the *amf* mutant had been isolated in 1986, and characterized for granule bound starch synthase activity (GBSS). It seems that the mutation is due to one base pair deletion in the GBSS gene, and its inheritance is monogenic recessive. It is now being used for breeding varieties and for selection of new starch mutants.
- In Ghana, the exposure of pineapple plants, obtained after irradiation, to high temperature and drought caused high lethality, and produced reduced fruit size. Severe selection pressure for drought tolerance in the field did not give the desired variants.
- In sweet potato, one promising variant tolerant to heat stress was isolated in China P.R. by subjecting $M_1V_3$ propagules to heat stress in the glasshouse. The sweet potato variant, 91-C3-150, which has improved drought tolerance, was obtained by regeneration of callus, irradiated with 5 Gy. Plants were propagated three times, and had good quality of tubers, with the same yield as the parent variety. *In vitro* protocols were established to induce "globular bodies" for microplant production in garlic, and as a source material for radiation induced mutations. Doses of 5–10 Gy gave bulblets capable of germination.
- Salt-tolerant alfalfa genotypes were obtained in USA, which have remained salt tolerant through successive vegetative propagation for the past nine years since 1989. Selection of mutants at cell level for salt tolerance in alfalfa and rice gave regenerated plants with heritable improvements in salt tolerance. Studies on molecular differences between salt-tolerant and -sensitive alfalfa have led to cloning of several genes that are expressed primarily in roots, and may contribute to the tolerance of the plant to salinity. One of them (*Alfin1*), a transcription factor, when over-expressed in transgenic plants, enhances the expression of an endogenous salt-inducible gene (*MsPRP2*) in callus culture and roots.
- In Bangladesh, by flooding the field of sugarcane, variants with tolerance to waterlogged soil were obtained from the irradiated $M_1V_5$ propagation. The inoculation of $M_1V_3$ to $V_6$ material with red-rot fungus produced two resistant and six moderately resistant variants.

Selection for late flowering in $M_1V_2$ yielded three variants, which flowered 100 days later than the parental variety. These were further selected for cane yield, Brix index and disease response. It is expected that at least one or two mutants might be released as varieties. In addition, three variants were identified, which showed tolerance to waterlogged conditions in $M_1V_6$ generation. Selections were done on the basis of intensity of greening of leaves, growth of the canes, number of nodes bearing adventitious roots, Brix index, cane yield and disease reaction. For selecting delayed flowering/non-flowering mutants, one variant was selected which flowered 3 months after the flowering date of the control. Protocol for induction of callus and regeneration of plants in sugarcane were established. In $R_4$ generation, three hills showed moderate resistance to red-rot disease and three hills were tolerant to waterlogged conditions.

## 3. RECOMMENDATIONS

### 3.1. General recommendations

- There is a need to propagate plant material in large numbers for irradiation. Micropropagation of plant material provides such populations.
- Irradiation of starting material should be carried out with a dose range appropriate for individual crops.
- Callus irradiation for mutagenesis should be carried out only in systems for which good regeneration systems are available.
- Material generated from pilot experiments on radio-sensitivity tests should not be considered as the primary source for selection of mutants.
- Appropriate nomenclature should be used to describe successive propagations. Irradiation of *in vitro* shoots followed by successive propagation is called $M_1V_1$, $M_1V_2$, $M_1V_3$. Irradiation of regenerated plants and subsequent *in vitro* propagation is called $R_1V_1$, $R_1V_2$, $R_1V_3$.
- Induction and establishment of variants should be made under normal growing conditions and selection for mutants afterwards under stress conditions.
- After irradiation, *in vitro* propagation should be carried out to produce large populations of plants (5,000–10,000) to allow separation of chimeras and detection of useful variants. Recommended propagation is to a minimum of $V_4$ before screening procedures are applied. However, those using regeneration and *in vitro* propagation may use the $M_1V_2$ generation.
- It should be recognized that somaclonal variation is likely to contribute additional variation if selection is carried out involving regeneration protocols.
- A complete record of the lineage of each variant should be maintained.
- Statistical analysis on the performance of irradiated materials and subsequent propagations does not make any sense for the selection of the desired trait. Mean performance value of *individual* variants should be used to compare the characteristics with the non-irradiated starting material.
- Selection protocols for stress tolerance need to be optimized for severity of stress to avoid loss of potentially useful variants.
- Final evaluation of variants needs to include appropriate measurements of genotype stability and variant stability should be tested several times, preferably at several locations.
- Evaluation of large plant numbers is essential; testing may be carried out in batches to facilitate screening of large populations.

- The combination of transposon and irradiation induced mutations simultaneously should be investigated as part of future strategy to understand mutations at the molecular level.

### 3.2. Specific recommendations

Potato

Contacts between laboratories in developing and developed countries for the analysis of potato mutants generated should be promoted. More recessive mutations, deficient for enzymes, should be induced and selected for traits, which are not suppressed by antisense expression genes in transgenics. Mutants with altered starch should not only be tested for the industrial use, but also for new food products for direct consumption. An effective and workable *in vitro* system (screen) for selection of salt tolerance is still needed for early selection of variants.

For the induction and selection of starch mutants the protocol consists of irradiation of axillary buds, regeneration of shoots from leaf explants, microtuber formation on individual regenerants, and iodine staining of microtuber starch for selection of altered starch composition.

In Pakistan studies should continue to screen $M_1V_5$ material at multilocation trials on saline soils. The material should be incorporated into Regional and National research programs on utilization of salt affected lands.

In India, it is recommended that stable variants should be evaluated in field trials. In Egypt, further selection should be carried out on the putative tolerant tuber line to assess stability of the genotype. Salt analysis should be done on the variant plants to determine salt accumulation in various tissues. If a stable line is obtained, arrangements should be made with breeders for future release of the line.

Alfalfa

The applicability of *Alfin1* over-expression in regulation of other genes and improved salt tolerance phenotype in alfalfa and other plants should be investigated. Alfalfa homozygous for *Alfin1* transgenes should be produced using molecular markers to determine copy number and in test crosses. *Alfin1* should be re-engineered to study over-expression in a tissue specific manner.

Sweet potato and Garlic

For inducing variation, microplants instead of callus should be irradiated. The selected variant 91C-3-150 should be incorporated into performance trials. In garlic, there is a need to develop $M_1V_5$ bulblets to full size bulbs for large scale testing.

Sugarcane

Advanced generation variants ($M_1V_7$) resistant to red-rot disease and tolerant to water logged soils in Bangladesh have shown promising cane yield, Brix index and other agronomic characters. These variants need further testing before release as varieties. Incase of somaclonal variation and callus irradiation, some plants are now at $R_1V_3$ ($R_3$) and $R_1V_4$ ($R_4$) propagation. Population size should be enlarged in this case. Selection of variants should be done in $R_1V_3$, $R_1V_4$ and $R_1V_5$ propagation. In Pakistan, studies should be continued on propagation and

screening of $M_1V_5$ material at multiple locations on saline soils. The material should be incorporated into Regional and National research programs.

Pineapple

The surviving plants from the drought-stressed populations should be irrigated and further propagated. Suckers should be collected and grown under both the normal and stressed conditions to select the desired variants. If spineless variants are obtained during selection, these should be evaluated and selected as mutants with potential for release as new varieties; their special advantage is that they make fruit harvest easy.

# *In vitro* induction of variability through radiation for late blight resistance and heat tolerance in potato

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**Abstract.** *In vitro* cultured shoots of potato, cvs. 'Kufri Jyoti' and 'Kufri Chandramukhi', were irradiated with 20 and 40 Gy gamma rays. Microtubers, obtained from M1V3 shoots multiplied *in vitro,* were planted in pots. The resulting plants were screened for resistance to late blight, using detached leaf method. In 'Kufri Chandramukhi', 42% plants and in 'Kufri Jyoti' 36% plants, obtained from 40 Gy treatment, showed resistance to late blight. The frequency of resistant plants was lower from 20 Gy treatment. The progenies of putatively resistant plants were grown in field, and inoculated with sporangial inoculum of late blight fungus. The field grown progeny segregated for disease resistance, and approximately 56% plants showed resistance. During the next propagation, the frequency of resistant plants increased to 72%. For developing heat tolerance, microtubers obtained from 20 and 40 Gy treatments and *in vitro* multiplied $M_1V_3$ shoots were cultured at high temperature of 28C. In both varieties, the number of the microtubers per plant was highly reduced and the resulting microtubers had distorted shape but showed better germination (62%), even in early sowing at relatively higher temperature. Of the two radiation doses, the higher dose of 40 Gy gave better results in both the varieties. Heat tolerance was also assessed from chlorophyll persistence. The progenies from putative heat-tolerant plants were tested in field by planting at higher temperature in two subsequent generations. The heat tolerant plants segregated in each generation, but the frequency of heat-tolerant plants increased.

## 1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important food crop in India. From an area of 0.935 million ha under potato, nearly 15.2 million ton tubers are produced annually. The main objectives of potato research in India include breeding for early maturity, high yield, and resistance to late blight and viruses. 'Kufri Jyoti' and 'Kufri Chandramukhi', the two most popular varieties, are susceptible to late blight. Also, the cropping pattern in North India demands an early planting variety in September, so that it matures by November and a following crop of wheat can be grown. However, the temperature during early potato planting period ranges between $20^o$ to $32^o$ C. The present project was initiated to induce variation for resistance to late blight and heat tolerance in the local potato varieties.

## 2. MATERIALS AND METHODS

Cultivar 'Kufri Jyoti' and 'Kufri Chandramukhi' were used as the experimental material. 'Kufri Jyoti' is a medium late maturing (100–110 days) variety and 'Kufri Chandramukhi' is an early maturing (90–100 days) variety. Both varieties are high yielding with slow rate of viral degeneration, wide adaptability and desirable commercial attributes, but are susceptible to late blight and are not suitable for early planting being heat susceptible.

### 2.1. Establishment of *in vitro* cultures and their multiplication

Healthy potato tubers were rinsed in 10% ethanol, and sprouted at 20°C in dark. Sprouted tubers were planted in pots. Nodal sections were excised from stems, and surface sterilized for 5 min. in a solution of 0.1% HgCl2 and 0.1% Sodium lauryl sulphate, and washed three times in sterile water. Nodal sections, 0.5–1.0 cm, were cultured on basal MS medium [1]. The cultures were maintained at 28±2°C under 16/8 light/dark (3000–4000 lux). After 5 to 6 weeks, the shoots were cut into 0.5–1.0 cm long nodal segments, and sub-cultured on fresh medium to produce a second generation of plants. The *in vitro* grown plants with 5 to 6 nodes each were irradiated with 20 and 40 Gy gamma rays. The irradiated plants were cut into nodal sections, and cultured on basal MS medium for 4–5 weeks.

### 2.2. Microtuber formation

*In vitro* cultured, 4 to 5 weeks old plants, were used for obtaining microtubers. The plants were cut into 0.5–1.0 cm long segments, each with one leaf and an axillary bud, and transferred to MS medium with 8% sucrose. The cuttings were placed in 300 ml jam jars with 40 ml medium or 120 ml Watson Module containers with 25 ml medium. The Watson Module system consists of disposable pre-sterilized clear plastic containers with snap on lids. Cultures were kept at 28±2°C (16hr light) and 25±2°C (8 hr dark) for 60 days. They were then transferred to dark at 20±1°C after pouring liquid MS medium supplemented with 10 mg/l BAP and 8 % sucrose. This protocol was tested for 25 cultivars, and was found suitable for most varieties.

### 2.3. Callus induction

Leaf cuttings from *in vitro* plants were cultured on modified LS medium [2] without cytokinins but supplemented with 5 mg/l NAA. Callus initiation and subsequent proliferation was obtained in dark at 28°C. The obtained calli were irradiated with 20 and 40 Gy gamma rays. Regeneration was obtained by transferring calli to modified LS medium containing 50 ml coconut milk, 0.1 mg NAA and 5 mg BAP per litre. Young regenerated sprouts were transferred to B5 medium [3].

### 2.4. Screening for late blight resistance

The microtubers were planted 1.2–2.5 cm deep in a mixture of soil and farm yard manure (1:1) in pots. To screen for late blight resistance, leaves were surface sterilized with 0.1% $HgCl_2$ and 0.1% Sodium lauryl sulphate for 7 min. or obtained from *in vitro* grown plantlets from these plants. The sterile leaves were then placed on the Gamborg medium [3], containing toxin from a single pathotype of *Phytophthora infestans.* The toxin filtrate was prepared from fungal cultures grown on pea extract medium. The cultures were checked microscopically for infection, and cultured in fluid pea extract. After three weeks, the toxin fluid was filtered to remove sporangia.

Tubers from plants classed as resistant or moderately resistant to late blight in 1995 were grown in pots on 15 October 1996, and kept outdoors. Late blight inoculum was prepared by collecting spores from a mixture of pathogen races, and was sprayed on the plants at a concentration of 10–15 spores/drop in distilled water. The plants were covered with polythene bags to provide humidity. The bags were removed after 24 hr, and disease reaction was observed after five days. The plants were graded on a scale of 1 to 6 (Table I). The same method was used for screening for disease resistance in 1997.

**TABLE I. SCALE TO CLASSIFY INFECTION OF FIELD GROWN PLANTS**

| Infection type | Description | Class* |
|---|---|---|
| 0 | Healthy | R |
| 1 | Few specks on the leaves only. | R |
| 2 | One or two lesions on few leaves, plants green. | HR |
| 3 | One or two lesions clearly visible on leaves with specks on stem but plants green. | MR |
| 4 | Lesions uniform on leaves with few leaves drooping, lesions present on stem, plants greenish brown. | MS |
| 5 | Most leaves with brown lesions, up to half of stem showing brown discoloration, plants mostly brownish with few green areas. | S |
| 6 | Plants dead with dead leaves, brown stem and leaves. | S |

*R- resistant; HR- Highly resistant; MR- Moderately resistant; MS- Moderately susceptible; S- susceptible

### 2.4. Selection for tolerance to high temperature

During 1995–96, microtubers were produced at 20°C and 28°C. *In vitro* irradiated plants were cut into nodal sections and cultured on medium for microtuber formation. After proliferation of the shoots, liquid medium was added to culture vessels, which were maintained at 20°C and 28°C. Non-irradiated control plants were propagated in each experiment.

The microtubers obtained from irradiated and control plants at 20°C were planted on 1 and 15 September, and 1 and 15 October for germination under high temperature, and screened for heat tolerance. To study heat tolerance, leaf discs were taken from fully expanded leaves, and placed in Petri dishes lined with wet cotton, sealed with parafilm, and maintained in a growth chamber at 25°C. After four days, leaf discs were scored for damage. A disc was considered damaged when more than 50% area was yellow. The ratio of damaged disc was used as a relative measure of injury.

The nodal sections from plants that showed resistant reaction were used as explant for micropropagation to multiply the mutated sectors. The nodal sections were sterilized with 0.1% $HgCl_2$ and 0.1% SDS, and rinsed three times in sterile distilled water. The explants were then cultured on MS basal medium without hormones. The micropropagated plants were irradiated second time with 20 Gy dose.

## 3. RESULTS AND DISCUSSION

### 3.1. *In vitro* culture and microtuber production

The nodal segments from plants obtained from sprouted tubers planted in soil gave better results than directly from sprouted tubers. A total of 176 explants of ‘Kufri Chandramukhi’ and 162 of ‘Kufri Jyoti’ were cultured. By subculture of 547 *in vitro* grown plantlets, 400 plantlets were obtained in ‘Kufri Chandramukhi’, and from 481 plantlets, 328 plants were obtained in ‘Kufri Jyoti’. Not all the cultured plants survived and thin segments failed to grow into plantlets. The *in vitro* plantlets of both the varieties were irradiated at 20 and 40 Gy. When the plantlets were transferred to medium with 8% sucrose, there was excessive proliferation of shoots, and the leaf-size was greatly reduced.

Microtubers initiated after 10 days of transfer of cultures to dark. Microtubers originated as aerial structures from microstems, although a few microtubers were also formed in the medium. Microtuber size varied from 2 to 15 mm in diameter. The number of microtubers ranged from 1 to 3 per plant. In $M_1V_3$ generation of 'Kufri Chandramukh', 368 microtubers were obtained from 384 plantlets derived from 20 Gy treatment and 278 plants from 40 Gy treatment produced 214 microtubers. In 'Kufri Jyoti', 315 cultured explants from 20 Gy dose gave 298 microtubers and 350 explants from 40 Gy gave 296 microtubers (Table II).

**TABLE II. *IN VITRO* PLANT IRRADIATION AND INDUCTION OF MICROTUBERS AT 20°C**

| Variety | Dose GY | No. of plants irradiated | No. of plants micro-tuberised | No. of microtubers |
|---|---|---|---|---|
| Kufri | 20 | 133 | 384 | 368 |
| Chandramukhi | 40 | 120 | 278 | 214 |
| Kufri Jyoti | 20 | 133 | 315 | 298 |
| | 40 | 115 | 350 | 296 |

### 3.2. Isolation of mutants

Microtubers were sprouted a month before sowing. Sprouting of microtubers improved their emergence. Without pre-sprouting, the microtubers either failed to germinate or germinated very late. Leaves from microtuber derived plants were placed on medium with toxic filtrate. Some of the leaves turned yellow and brownish whereas others remained totally unaffected. When the leaves from the *in vitro* cultured plantlets were placed on the toxic medium, lesions appeared on leaf-surface after 4 days, as expected and reported by others [4]. When toxin filtrate was used at 30 ml/l in the medium, more than 50% of the leaf area was covered with lesions. All the control plants were susceptible. Plants resistant to late blight were observed in both cultivars from the two doses of 20 and 40 Gy.

**TABLE III. REACTION OF *IN VITRO* SCREENED PLANTLETS TO LATE BLIGHT DISEASE**

| Variety | Dose Gy | Disease score* | | | Plants tested No. |
|---|---|---|---|---|---|
| | | R | MR | S | |
| Kufri Chandramukhi | 20 | 36 | 20 | 44 | 39 |
| | 40 | 12 | 8 | 80 | 40 |
| | Control | 0 | 0 | 100 | 6 |
| Kufri Joyti | 20 | 20 | 20 | 60 | 40 |
| | 40 | 9 | 30 | 61 | 47 |
| | Control | 0 | 0 | 100 | 8 |

z* Per cent plants: R- resistant; MR- Moderately resistant; S- susceptible

The progeny of the plants, classed as resistant during 1995, gave resistant reaction. In some cases though, variation was observed, i.e. tubers from the same plant showed varying disease reaction. In case of moderate resistance, progeny included susceptible types. This suggests that chimeras were present and the mutant sectors could be multiplied by rapid *in vitro*

propagation as proposed by Lu et al. [5]. The plants, which gave resistant reaction in 1996, gave resistant reaction in 1997 also. Although, variability was observed in the progeny of the same plant, the frequency of segregation was less as compared with that in the previous year. Whereas in 1996, the segregation was up to nearly 50%, in 1997, the maximum segregation was only 27.7%, thereby confirming that the lines were becoming stable.

### 3.3. Isolation of heat tolerant mutants

The normal temperature for microtuberisation was 20±1°C, hence screening for heat tolerance was done by sub-culturing the irradiated material at 28°C. The plantlets, irradiated with 20 and 40 Gy formed mictubers, but the response was drastically different compared with the control (Table IV). The control plants degenerated and no microtuber were obtained, even after two months of culture. The difference between controls and irradiated material was dramatically less between microtubers produced at 20°C. The effect of high temperature was also reflected in tuber shape.

**TABLE IV. MICROTUBER PRODUCTION FROM IRRADIATED AND CONTROL PLANTS AT 28°C**

| Variety | Dose Gy | No. of Culture Vessels | No. of Microtubers |
|---|---|---|---|
| Kufri Chandramukhi | 20 | 10 | 6 |
| | 40 | 10 | 14 |
| Control | 0 | 5 | 0 |
| Kufri Jyoti | 20 | 10 | 4 |
| | 40 | 10 | 11 |
| Control | 0 | 5 | 0 |

Microtubers, planted on various dates, showed marked difference in their germination (Table V). In early planting, when temperature was high, tuber germination was better in the irradiated ones than the controls (Table VI). Based on chlorophyll persistence [6], plants showing leaf-area damaged less than 40% were categorized as heat tolerant. In 'Kufri Chandramukhi' from 40 Gy treatment, 76 of the 164 plants tested showed less than 50 % damaged leaf-area. From 20 Gy treatment, 70 of 250 plants showed less than 50% leaf injury. During 1997, chlorophyll persistence showed that nearly 50% of the plants in both varieties had less than 20% damaged leaf-area, indicating that these included variants for heat tolerance (Tables VII and VIII). Thus, late blight and heat tolerant variants have been obtained in both the varieties. However, more vegetative generations are required to obtain stable mutants.

**TABLE V. EFFECT OF IRRADIATION AND PLANTING DATE ON MICROTUBER GERMINATION, 1996**

| Date of planting | Variety | Treatment | No. of microtubers | No. of plants | Germination (%) |
|---|---|---|---|---|---|
| 1st Sept. 1996 | Kufri Chandramukhi | 20 Gy | 105 | 58 | 55 |
| | | 40 Gy | 54 | 31 | 57 |
| | | Control | 10 | 3 | 30 |
| | Kufri Jyoti | 20 Gy | 68 | 29 | 43 |
| | | 40 Gy | 68 | 32 | 47 |
| | | Control | 10 | 5 | 50 |
| 15 Sept. 1996 | Kufri Chandramukhi | 20 Gy | 115 | 74 | 64 |
| | | 40 Gy | 64 | 42 | 66 |
| | | Control | 10 | 5 | 50 |
| | Kufri Jyoti | 20 Gy | 72 | 42 | 54 |
| | | 40 Gy | 74 | 43 | 58 |
| | | Control | 10 | 6 | 60 |
| 1 Oct. 1996 | Kufri Chandramukhi | 20 Gy | 69 | 69 | 72 |
| | | 40 Gy | 35 | 35 | 67 |
| | | Control | 8 | 8 | 80 |
| | Kufri Jyoti | 20 Gy | 72 | 56 | 72 |
| | | 40 Gy | 70 | 54 | 77 |
| | | Control | 10 | 8 | 80 |
| 15 Oct. 1996 | Kufri Chandramukhi | 20 Gy | 52 | 49 | 88 |
| | | 40 Gy | 44 | 39 | 89 |
| | | Control | 10 | 10 | 100 |
| | Kufri Jyoti | 20 Gy | 86 | 75 | 87 |
| | | 40 Gy | 84 | 78 | 93 |
| | | Control | 10 | 10 | 100 |

**TABLE VI. GERMINATION OF MICROTUBERS OBTAINED FROM IRRADIATED AND CONTROL PLANTS PLANTED ON DIFFERENT DATES, 1997**

| Planting date | Variety | Dose Gy | No. of microtubers planted | No. of plants obtained | Germination % |
|---|---|---|---|---|---|
| 1 Sept. 1997 | Kufri Jyoti | 40 | 25 | 8 | 32 |
| | | 20 | 20 | 1 | 5 |
| | | Control | 10 | 0 | 0 |
| | Kufri Chandramukhi | 40 | 22 | 4 | 19 |
| | | 20 | 10 | 5 | 50 |
| | | Control | 10 | 0 | 0 |
| 15 Sept. 1997 | Kufri Jyoti | 40 | 42 | 38 | 90 |
| | | 20 | 26 | 16 | 61 |
| | | Control | 10 | 6 | 60 |
| | Kufri Chandramukhi | 40 | 42 | 22 | 52 |
| | | 20 | 36 | 32 | 88 |
| | | Control | 10 | 7 | 70 |

**TABLE VII. CHLOROPHYLL PERSISTENCE IN IRRADIATED PLANTS, 1996**

| Variety | Dose Gy | No. of plants tested | No. of plants with damaged leaves | | | | |
|---|---|---|---|---|---|---|---|
| | | | 20% | 40% | 60% | 80% | 100% |
| Kufri Chandramukhi | 40 | 164 | 28 | 48 | 56 | 20 | 12 |
| | 20 | 250 | 46 | 24 | 66 | 58 | 56 |
| | Control | 26 | - | - | 10 | 8 | 5 |
| Kufri Jyoti | 40 | 207 | 46 | 26 | 54 | 40 | 41 |
| | 20 | 202 | 22 | 40 | 64 | 48 | 28 |
| | Control | 28 | - | - | 12 | 10 | 5 |

**TABLE VIII. CHLOROPHYLL PERSISTENCE IN IRRADIATED PLANTS, 1997**

| Variety | Dose Gy | No. of plants tested | No. of plants with damaged leaves | | | | |
|---|---|---|---|---|---|---|---|
| | | | 20% | 40% | 60% | 80% | 100% |
| Kufri Chandramukhi | 40 | 22 | 10 | 5 | 5 | 2 | - |
| | 20 | 37 | 15 | 10 | 4 | 6 | 2 |
| | Control | 10 | - | - | - | 3 | 7 |
| Kufri Jyoti | 40 | 38 | 16 | 4 | 7 | 5 | 6 |
| | 20 | 16 | 6 | 5 | 1 | 2 | 2 |
| | Control | 10 | - | - | - | - | 10 |

## REFERENCES


[1] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **5** (1962) 473–497.

[2] LINSMAIER, E.M. and SKOOG, F. Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. **18** (1965) 100–127.

[3] GAMBORG, O.L., MILLER, R.A. and OJIMA, K.A. Nutrient requirements of suspension callus resistance of soybean root cells. Exper. Cell Res. **50** (1968) 151–158.

[4] THIND, T.S., MOHAN, C., SOKHI, S.S. and BEDI, J.S. A detached leaf technique for maintenance and multiplication of *Phytophthora infestans* and evaluation of fungicides. Curr. Sci. **58** (1989) 388–389.

[5] LU, S.Y., WU, C.G., LI, W.J. and FENG, Q.H. 1993. "The use of gamma irradiation *in vivo* and combining use of *in vitro* culture to select mutant clones in sweet potato". Proc. Final Res. Coord. Meet. TECDOC-809. IAEA, Vienna 19–35.

[6] MORPURGO, R. Screening of heat tolerance in potato (*Solanum* spp.) an *in vitro* approach. Agricultura Subtropicalee Tropicale **3** (1989) 409–414.

# Induction of recessive mutations in potato using tissue culture techniques

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**Abstract.** In potato, two different *in vitro* approaches were used to generate recessive mutants. In the first method, monoploid plant material was irradiated to isolate and identify amylose-free (*amf*) mutants in potato. For isolating secondary mutants in the *amf* background new monoploids of the *amf* type were developed. A few selected *amf* monoploids showed excellent vigour *in vitro*, large leaves and microtuber formation. A diploid and a monoploid were tested for *in vitro* mutation induction and irradiated with 0 to 16 Gy X rays. The optimal dose for survival and mutation induction was between 4 and 8 Gy and plants were regenrated from irradiated leaf explants. In the second approach, mutants were induced by insertion of transposable elements in the diploids. This method was used to mutate *R* genes for resistance to *Phytophthora infestans*. Diploid heterozygous *Rr* plants with the immobilised *Ds* element, closely linked to one of the *R* genes, were selected. Mobilisation of *Ds* using *Ac* element transposase resulted in the selection of plants with active somatic *Ds* excision frequency of about 10%. *In vitro* protoplast isolation and plant regeneration from such plants enabled the selection of regenerants with new independent *Ds* insertions. Hygromycin selection (*Ds* excision marker on the T-DNA) during protoplast regeneration increased the frequency of *Ds* excision regenerants to 56%. A total of 582 hygromycin resistant plants were regenerated and selected *in vitro.* Preliminary analysis of the regenerants showed re-insertions of *Ds* in the predicted coding sequences of genes.

## 1. INTRODUCTION

Cultivated potato is an autotetraploid, hence, recessive mutations are not easy to isolate. Induced mutations are valuable in plant breeding and for molecular isolation and functional analysis of genes coding important traits. Two different *in vitro* approaches can be used to generate recessive mutants for biochemical pathways, which change biosynthesis of starch. In the first *in vitro* approach, mutants can be induced with X rays. Monoploid plant material is also a prerequisite because of self-incompatibility of potato at the diploid level and high inbreeding depression after selfing of diploids. The *in vitro* selection of an earlier described amylose-free (*amf*) potato mutant, which is mutated in the gene coding for granule bound starch synthase (GBSS), was done by X ray irradiation of leaf explants of a monoploid plant [1]. Amylose alone, loosely branched amylopectin or a mixture of amylose with amylopectin stains blue, but amylose-free starch stains red with iodine. Jacobsen et al. [2] tried to induce revertants of the original *amf* mutant by *in vitro* irradiation and selection for blue staining starch. The blue staining starch is theoretically the result of a reversion of the GBSS-gene, giving rise to normal starch with amylose and amylopectin or of a mutation of the branching

enzyme (BE) causing loosely branched amylopectin or only synthesis of amylose. However, all the selected revertants showed a restored GBSS activity, which was probably caused by the existing chimerism within the originally selected monoploid *amf* mutant. Hence, new stable monoploids of the *amf* type are needed. The required monoploids should also contain excellent *in vitro* vigour, large leaves for regeneration of many shoots after irradiation, and microtuber formation ability. In addition they have to be tested for the optimal irradiation dose in order to have a balance between mutation induction and survival of irradiated cells.

The second approach of mutation induction is the use of homologous or heterologous transposon insertions leading to interrupted genes, which can be cloned. This approach has been successfully used for the isolation of specific genes, for example, those coding for disease resistance [3, 4, 5]. Maize *Ac*/*Ds* transposons, introduced by *Agrobacterium* transformation with *Ac* or *Ds* containing T-DNA, have proven to be functional in crops like potato and tomato [6, 7, 8]. *Ds* is an *Ac* element, which is deleted in the transposase gene, and consequently becomes immobile. It can be mobilised in the presence of the transposase gene of an *Ac* element. In the present studies, the *Ds* element is interrupting the hygromycin resistance gene, which reverts after excision of *Ds*. In previous research, sixty *Ds* containing T-DNA insertions have been introduced in diploid potato and localised by RFLP analysis [9]. Among the population of transformants, linkage was found between some *Ds* containing T-DNA's and *R* genes, coding for resistance to *Phytophthora infestans*. Additional crosses were made to select for recombinants between the *Ds* containing T-DNA and the *R* gene in *cis* position, because the transposed *Ds* elements frequently re-insert nearby on the same chromosome [10, 11]. The *Ac* transposase source was introduced by crossing, and *Ac*/*Ds* plants with relatively high frequencies of independent somatic *Ds* transpositions were selected. *In vitro* protoplast isolation and culture enabled the selection of regenerants with independent *Ds* excision events. A hygromycin assay on regenerated plants resulted in selection of plants with *Ds* excision. Hygromycin selection during protoplast culture resulted in an increased frequency of *Ds* excision regenerants. Selected plants have been used for preliminary molecular experiments to search for gene insertions.

## 2. MATERIALS AND METHODS

### 2.1. Production of monoploids

Monoploid (2n=x=12) *amf* plants ($M_0$) were obtained by prickle pollination of seven diploid (2n=2x=24) *Solanum tuberosum* genotypes (KA91-895, 5002-18, 880004-2, 880004-3, 880004-6, 880004-9 and 880004-11) with *S. phureja* pollinators IVP35, IVP48 and IVP101 [12]. Two of the genotypes, 5002-18 and KA91-895, were homozygous for amylose-free starch (*amf*), and the others were heterozygous *Amf*/*amf*. To obtain monoploids from seed-bulks from crosses between the diploid *S. tuberosum* genotypes and *S. phureja*, seeds without an embryo-spot were selected, and cultured singly *in vitro*. The plantlets with 'nodal bands', controlled by the same genes as embryo-spot, were removed [13]. In non-hybrid seedlings without nodal band, the ploidy level was measured by Flow cytometry [14]. The starch composition was tested by iodine staining of tubers from the monoploids (*amf*/wt). Vegetative propagation was done from cuttings; shoot cultures were cultured *in vitro* on Murashige and Skoog (MS) medium [15], supplemented with 30 g/l sucrose and 8 g/l agar, and maintained at 24 °C, 16 hr light, at intensity of 40 $Tmolm^{-2}s^{-1}$.

## 2.2. *In vitro* culture protocol

Before large-scale experiments were undertaken, a diploid and a monoploid genotype were tested to establish protocol. Adventitious shoots were produced according to the procedure of Hovenkamp-Hermelink et al. [16]. The leaves from sterile shoots were floated on a solution of 147 mg/l $CaCl_2.2H_2O$ and 80 mg/l $NH_4NO_3$, supplemented with 10 mg/l BAP and 10 mg/l NAA. After floating for one night, the leaf explants were cut into 3-4 mm wide segments, and placed on callus induction (CI) MS medium containing 40 g/l mannitol, 10 g/l sucrose, 2.25 mg/l BAP, 0.0175 mg/l IAA and 8 g/l agar. Large-scale irradiation of leaf explants was done one day after culture on CI medium [1]. After 6 days, the segments were transferred to regeneration (RE) MS medium, supplemented with 15 g/l sucrose, 2.25 mg/l BAP, 5 mg/l $GA_3$ and 8 g/l agar. The segments were transferred to fresh RE medium every 3 weeks. Harvested adventitious shoots were cultured on MS medium with 10 g/l sucrose. When a shoot had formed more than five nodes, single defoliated nodes were placed on tuber induction (TI) MS medium, supplemented with 80 mg/l sucrose, 0.5 mg/l ABA and 2.5 mg/l kinetin [16]. The Petri dishes were maintained in dark at 18°C, and the first microtubers were formed after 5 weeks. All Petri dishes and containers were sealed to prevent infection.

## 2.3. Mutation induction with X rays

To find the optimal radiation dose, single nodes were irradiated with X rays at TNO-SCD (Arnhem, Netherlands) from a Röntgen apparatus at 200 kV, 2 mA, without filter from a distance of 80 cm. This resulted in a dose rate of 1.5 Gy/min at the centre of the field (40 cm diameter) and a dose rate of 1.1 Gy/min, measured 20 cm from the center. Seven Petri dishes were placed in a circle at approximately 13 cm from the center of the field. Six doses 2, 4, 6, 8, 10 and 16 Gy were used to determine the optimal dose. For each dose, plant growth and vigour ($M_1$) were scored.

## 2.4. Mutation induction with transposable elements

The genotype Ds (Ds 53-34) is derived from BET92-Ds-A16-416 and contains a *Ds*-transposon-containing T-DNA construct (pHPT:*Ds*-Kan [17]) on chromosome 5 [9]. This *Ds* plant was crossed with TM (TM 17-2), a diploid potato transformed with an *Ac*-transposon-containing T-DNA construct (pMKGBSS*Ac* [8]), and selected for *Ac* excision events during development.

The seeds from the cross DsxTM gave 76 *in vitro* plants on MS medium, supplemented with 30 mg/l sucrose and 8 g/l agar (MS30). After 6 to 8 weeks, one shoot tip from each plant was transferred to fresh MS30 medium, and after 2 weeks transferred to a greenhouse. For testing of resistance to *Phytophthora*, race 0 inoculum was prepared and inoculated according to the procedure of El-Kharbotly et al. [18]. Shoot tips or axillary buds from *in vitro* grown plants were tested for their rooting ability on MS30, supplemented with 100 mg/l kanamycin or 30 mg/l hygromycin. Plant genomic DNA was isolated from greenhouse grown plants according to the method of Dellaporta et al. [19]. Primers designed on the *Ds*-containing T-DNA construct (pHPT::*Ds*-Kan) were used in PCR to detect the presence or absence of the *Ds* in its original T-DNA configuration (Fig. 1).

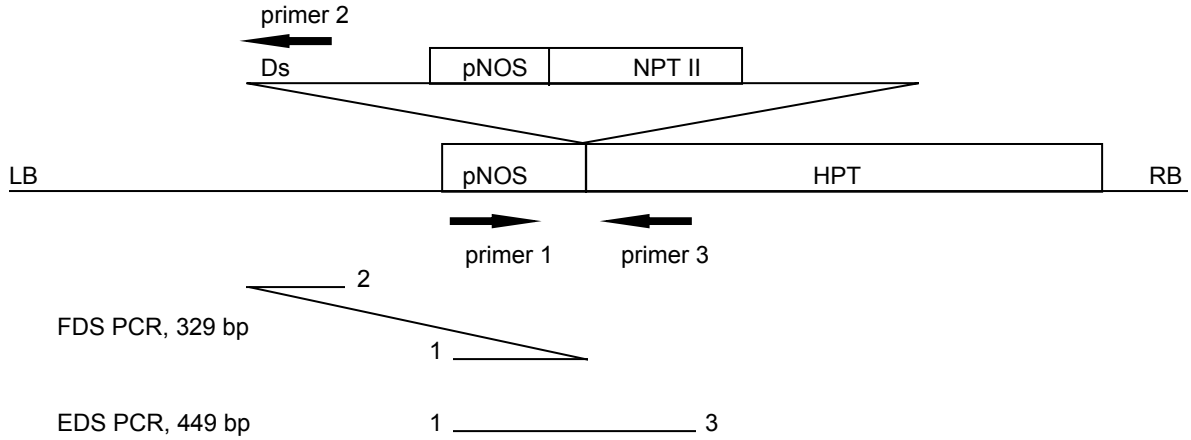


*Fig. 1. Schematic drawing of pHPT::Ds-Kan showing the position of the primers used for detecting Ds element in the T-DNA construct. Primer 1($^{5'}$GCG CGT TCA AAA GTC GCC TA$^{3'}$) and primer 2 ($^{5'}$GTT TCC GTT TCC GTT TAC CGT TTT$^{3'}$) give an amplification product when Ds is in the T-DNA (Full Donor Site = FDS). Primer 1 and primer 3 ($^{5'}$GTC AAG CAC TTC CGG AAT CG$^{3'}$) give amplification product after excision of Ds (Empty donor site=EDS). LB- left border. RB- right border. pNOS- nopaline synthase promoter. NPT II- neomycin phosphotransferase gene. HPT- hygromycin phosphotransferase gene.*

### 2.5. Protoplast isolation and culture

Protoplasts were isolated from four-week old *in vitro* grown shoots according to Uijtewaal et al. [20]. Before use, the plants were placed in dark at 4ºC for 6 hr. After second centrifugation, the protoplast pellet was not purified further but immediately re-suspended in culture medium TM2G [21] to a final concentration of 500.000 protoplasts/ml. After one week, the protoplast cultures were diluted 1:1 with fresh TM2G medium. After 2 weeks the cultures were diluted 1:1 with TMD medium [21]. In the selection experiment, 10 mg/l hygromycin was added. In the control experiments, no hygromycin was added during the whole protocol. After three weeks, the largest micro-calli were transferred to callus growth medium [22], and in the selection experiment, hygromycin concentration was increased to 20 mg/l. This concentration was maintained during regeneration of calli on shoot induction and shoot elongation media [22].

### 2.6. Hygromycin resistance of protoplast regenerants

To compare regeneration with or without hygromycin selection, the regenerated plants were re-tested for their resistance on MS30 medium, supplemented with 40 mg/l hygromycin. The number of roots was scored after 10 days of growth. Regenerants with two or more well growing roots were considered to be hygromycin resistant. Regenerants with no roots or with one small slow-growing root were considered to be sensitive.

## 3. RESULTS

### 3.1. Mutation induction using X ray on monoploids

Prickle pollination of the selected diploid potato genotypes with *S. phureja* produced 480 berries from which 995 spotless seeds (putative monoploids) were selected. On *in vitro* culture, only 604 of the seeds germinated; some seedlings died immediately after germination,

and the others were diploid (Table I). Finally, from 8 crosses, 26 monoploids were obtained. Of the seed parents, 880004-2, 880004-9 and 880004-11 gave more than one monoploid, indicating a clear genotype effect for this trait.

**TABLE I. EFFECT OF SEED PARENT ON MONOPLOID PRODUCTION FROM SPOTLESS SEED**

| Parent | No. of spotless seeds | Seeds germinated No. | Plants without nodal band (No.) | | |
|---|---|---|---|---|---|
| | | | Dead* | Diploids** | Monoploids# |
| 880004-2 | 38 | 24 | 1 | 1 | 3 |
| 880004-3 | 18 | 6 | 0 | 0 | 1 |
| 880004-6 | 21 | 5 | 1 | 0 | 1 |
| 880004-9 | 189 | 120 | 22 | 10 | 14 |
| 880004-11 | 89 | 40 | 2 | 21 | 6 |
| KA91-895 | 19 | 14 | 0 | 1 | 1 |

*Plants died before analysis. **Ploidy level determined by flow cytometry. #Some monoploids could not be maintained *in vitro*.

During *in vitro* propagation, several monoploids were lost due to poor genetic constitution. After one year, 13 monoploids survived and differed markedly in their growth habit. Of these, 10 monoploids were investigated for vigour, leaf size, starch composition and ploidy level from leaf cells by flow-cytometry (Table II). In some plants, it was necessary to determine starch composition of the progeny because the wild type background was heterozygous *Amf/amf*. The vigour and leaf size of the monoploids varied considerably. The amylose-free monoploid genotypes, TH96-1022M-54 and TH96-1022M-118, were vigorous as well as had large leaves under *in vitro* condition. The C-value of the leaf cells varied from 1C to 4C. The frequency of 1C cells was variable and ranged from 34% to 65%. There was no indication for instability in the ploidy level of 10 intensively investigated monoploids.

A selected monoploid, *amf* type 1022M-54, was tested together with a diploid (5002-18) as control. Its regeneration capacity was investigated, and the number of regenerating leaves was counted. In addition, the number of primordia was recorded to determine the potential number of shoots per leaf segment. The monoploid had more regenerating leaf segments (72.2 % versus 65.6 %), but developed far less primordia per leaf segment (13 versus 92) than the diploid (Table III). Tuber induction from the diploid was much more successful than the monoploid. Irradiation experiments with single nodes of both genotypes showed that there was stimulation of growth at low doses but a negative effect on growth at higher doses (Table IV). The monoploid was affected much more by radiation at a lower dose (>4 Gy) than the diploid (>10 Gy). These observations suggested that for mutation induction, the dose between 4 and 8 Gy would be suitable for monoploids.

### 3.2 Mutation induction using transposable elements

#### *3.2.1. Selection for active Ds excision*

Seventy-six vigours plants from the cross *Ds* (*R Ds/r* -) x TM (*r* -/*r*-*Ac*) were tested for *Phytophthora* (race 0), kanamycin (100 mg/l) and hygromycin (30 mg/l) resistance. Kanamycin resistant plants were expected to be the result of the presence of the *Ds* containing and/or the *Ac* containing T-DNA. Hygromycin resistance occurs when the *Ds* element excises

**TABLE II. MONOPLOIDS CULTURED *IN VITRO*, THEIR GROWTH VIGOUR, STARCH COMPOSITION AND PLOIDY LEVEL**

| | | | | C-value (%)[b] | | | |
|---|---|---|---|---|---|---|---|
| Genotypes | Vigour[a] | Leaf size[a] | *amf*/wt[c] | n | 1C | 2C | 4C |
| 5002-18[d] | 4 | 4 | *amf* | 2 | 0 | 86.1 | 13.9 |
| TH98-1017M-15 | 2 | 1 | n.d. | 1 | 34.1 | 49.4 | 16.5 |
| TH96-1022M-47 | 3 | 3 | n.d. | 10 | 51.5 | 40.2 | 8.3 |
| TH96-1022M-54 | 4 | 4 | *amf* | 12 | 34.9 | 44.9 | 20.2 |
| TH96-1022M-66 | 3 | 3 | n.d. | 11 | 45.1 | 45.5 | 9.4 |
| TH96-1022M-76 | 3 | 2 | wt | 9 | 53.0 | 39.2 | 7.8 |
| TH98-1022M-118 | 4 | 4 | *amf* | 13 | 52.6 | 39.6 | 7.8 |
| TH96-1023M-2 | 3 | 2 | n.d. | 8 | 54.4 | 37.9 | 7.7 |
| TH96-1025M-12 | 2 | 2 | n.d. | 2 | 47.3 | 37.9 | 14.8 |
| TH96-1025M-17 | 2 | 1 | wt | 4 | 64.6 | 33.2 | 2.2 |
| TH96-1026M-6 | 2 | 2 | n.d. | 3 | 39.3 | 43.9 | 16.8 |

[a] Scored on a scale of 0 to 4, 0 being poor and 4 being excellent. [b] Ploidy level determined by flow cytometry. [c] n.d. = Not determined. [d] Diploid control

**TABLE III. *IN VITRO* REGENERATION AND TUBER FORMATION IN DIPLOID AND MONOPLOID**

| | 5002-18 (2x) | | TH96-1022M-54 (x) | |
|---|---|---|---|---|
| | | n | | n |
| Regenerating leaf segments[a] (%) | 66 | 50 | 72 | 36 |
| Primordia number (mean) | 5 | 50 | 3 | 36 |
| Tuber formation (%)[b] | 92 | 36 | 13 | 45 |

[a]After 9 weeks of regeneration from non- irradiated leaves. [b]Tuber induction after 9 weeks of transfer on inducing medium from adventitious shoots of non-irradiated leaves

**TABLE IV. INTERNODE NUMBER OF PLANTS GROWN *IN VITRO* FROM SINGLE NODES IRRADIATED AFTER TWO WEEKS OF CULTURE**

| | 5002-18 (2x) | | TH96-1022M-54 (x) | |
|---|---|---|---|---|
| Dose (Gy) | Average No. of internodes* | n | Mean No. of internodes* | n |
| 0 | 4.8 $^{x}$ | 48 | 4.6 $^{w}$ | 47 |
| 2 | 4.9 $^{x}$ | 24 | 4.4 $^{w}$ | 24 |
| 4 | 5.3 $^{w}$ | 24 | 5.0 $^{w}$ | 23 |
| 6 | 4.9 $^{x}$ | 24 | 2.7 $^{x}$ | 24 |
| 8 | 4.6 $^{xy}$ | 24 | 3.0 $^{x}$ | 24 |
| 10 | 4.3 $^{y}$ | 24 | 0.9 $^{y}$ | 24 |
| 16 | 3.2 $^{z}$ | 24 | 0 $^{z}$ | 24 |

*Numbers followed by the same letter do not differ significantly (p= 0.05), based on Duncan's multiple range test

from the *Ds* containing T-DNA. Since, we were interested in the selection of *Phytophthora* resistant plants containing both transposable elements on T-DNA's, the *Phytophthora* and kanamycin resistant plants were tested for hygromycin resistance and analysed by PCR (data not shown). Finally, 20 *Ds* and *Ac* containing plants, resistant (*Rr*) to *Phytophthora infestans*,

were selected by PCR for having ca. 10% cells with somatic excision of *Ds*. These plants were not able to grow on hygromycin (30 mg/l) containing medium.

*3.2.2. Protoplast isolation and regeneration of hygromycin resistant plants*

From the 20 *Ds* and *Ac* containing *Rr* plants, 5 plants (3, 5, 6, 14, and 23) were used for protoplast isolation and plant regeneration to select for hygromycin resistant regenerants after *Ds* excision in individual cells. As a negative control, the plant 21, with only *Ds* T-DNA construct, was used. As a positive control, a kanamycin and hygromycin resistant *Ds* containing segregant was used that resulted from a *Ds* excision event early in embryo or seedling development. This plant was resistant on 40 mg/l hygromycin. Regeneration of at least 25 plants in the control experiments and about 200 plants in the selection experiment was attempted (Table V). As expected, in the negative control no hygromycin resistant plants were obtained whereas this class of plants frequently appeared from protoplasts of the positive control. The five selected plants showed regeneration of hygromycin resistant plants. The use of hygromycin in the culture medium greatly increased the isolation of the resistant plants. Without hygromycin, selection frequency of resistant plants varied between 13 and 40%, and with selection between 43 and 68%. These results clearly indicate the occurrence of *Ds* excision in the tested plants. A total of 582 hygromycin resistant plants are individually tested for instability in resistance to *Phytophthora infestans* as a first indication of insertion in the resistance gene involved. In addition, inverse PCR experiments were started to classify the new independent *Ds* insertion sites. Sequence analysis of the PCR products and database searches showed homology with known coding sequences.

**TABLE V. REGENERATION OF PROTOPLASTS WITH OR WITHOUT SELECTION FOR HYGROMYCIN RESISTANCE FROM DIFFERENT PLANTS AND CONTROLS**

| Plant | Regeneration without selection | | | Regeneration with selection | | |
|---|---|---|---|---|---|---|
| | No. | R* | % | No. | R* | % |
| 3 | 23 | 3 | 13 | 166 | 83 | 50 |
| 5 | 29 | 8 | 28 | 198 | 121 | 61 |
| 6 | 6 | 2 | 33 | 205 | 139 | 68 |
| 14 | 70 | 15 | 21 | 208 | 118 | 57 |
| 23 | 51 | 2 | 40 | 211 | 91 | 43 |
| Total | 179 | 30 | 17 | 988 | 552 | 56 |
| + control | 49 | 22 | 45 | 198 | 98 | 50 |
| - control | 21 | 0 | 0 | 10 | 0 | 0 |

*R- resistant to hygromycin.

## 4. DISCUSSION

The induction and selection of mutants is highly valuable, especially, to obtain biochemical pathway variants. In self-fertilising crops like flax, soybean and rice, the mutational approach has been frequently used. In vegetatively propagated crops like potato, this approach is much more complicated but valuable for generating new material for use in breeding. The existing *amf* mutant is a clear example of this approach. This mutant was isolated in 1986, and it will be 2005 before the first variety is approved based on this mutant. The reason for this long duration of breeding lies in the monoploid nature of the original mutant, and in the need to combine such new recessive trait with many other important traits

at the tetraploid level. In the meantime, new varieties have been developed with amylose-free starch by using antisense gene approach. This approach until now was not successful for the development of plants with amylopectin-free starch. Therefore, the selection of an amylopectin-free mutant is important despite the long duration it takes to produce a new variety.

In the present studies, new *amf* type monoploids were obtained. The frequency of monoploids was influenced by the genotype in specific combinations, as reported elsewhere [13]. With optimised *in vitro* culture, new *amf* monoploid mutants could be selected at tissue culture level. A similar wild type monoploid '79.7322' was described earlier during the induction and selection of the *amf* mutants [16]. Flow cytometry showed that monoploids were ploidy stable during *in vitro* multiplication of these plants, although previous findings had suggested that monoploids can change in ploidy level during multiplication (unpublished). Therefore, testing of new monoploids for the unwanted ploidy change is essential. Moreover, the present investigations that amylose-free monoploids can be obtained, which are not only vigorous with relatively large leaves but also produce microtubers in sufficiently high number. It is important to perform irradiation experiments with optimised material for selecting new mutants either without starch or with amylopectin-free starch. The following procedure is suggested to obtain new starch mutants using *in vitro* techniques in combination with radiation (Fig. 2).

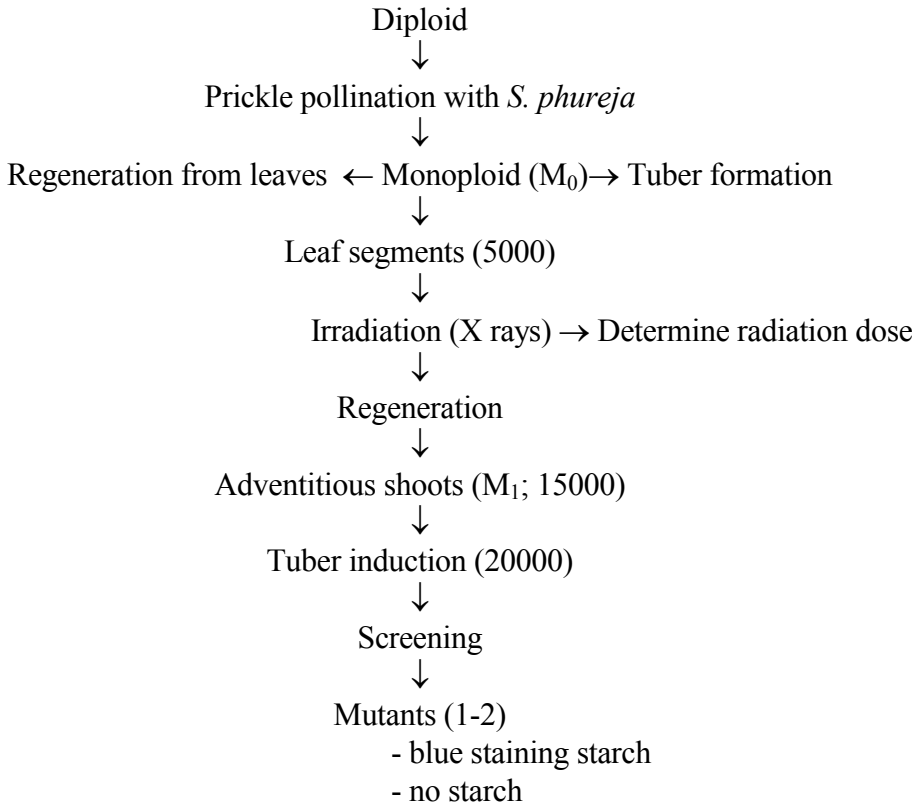


*Fig. 2. A schematic view for obtaining new mutants in potato*

The second method to induce mutations is based on molecular cloning and functional analysis of the desired genes. In this approach, heterozygosity for the particular trait is important at the diploid level. The use of heterologous transposon systems in potato is new, and the isolation of *R* genes, coding for *Phytophthora* resistance, is not yet described. The

material that has been developed during the past eight years is unique, and highly useful for modern molecular biology. *In vitro* induction and selection of independent transposon insertional events is crucial to obtain mutants. The present study showed that a combination of *Ds* and *Ac* elements in one genotype causes mobilisation of the *Ds* element. In the present case, the *Ds* element is closely linked with the *R1* gene and a large number of individual excisions can be isolated in individual plants by regeneration from protoplasts. Presently, more than 1500 hygromycin resistant regenerants have been selected, and are being tested individually for resistance to *Phytophthora infestans*. Preliminary experiments with a number of hygromycin-resistant regenerants showed excision of the *Ds* element and independent re-insertions into coding regions of the potato genome. It is not known if the *Ds* transposition behaviour in the investigated *Ds*/*Ac* plants is restricted to the neighbouring DNA on the same or different chromosome. To isolate *R1* gene, it is crucial that the *Ds* transpositions are located near to one another on the same chromosome. The described material is a good basis for the molecular isolation of the *R1* gene. Both examples discribed in this contribution show the importance of *in vitro* techniques for mutation breeding in vegetatively propagated crops.

## REFERENCES


[1] HOVENKAMP-HERMELINK, J.H.M., JACOBSEN, E., PONSTEIN, A.S., VISSER, R.G.F., VOS-SCHEPERKEUTER, G.H., BIJMOLT, E.W., VRIES, J.N. DE, WITHOLT, B. and FEENSTRA, W.J. Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). Theor. Appl. Genet. **75** (1987) 217–221.

[2] JACOBSEN, E., KRIJGSHELD, H.T., HOVENKAMP-HERMELINK, J.H.M., PONSTEIN, A.S., WITHOLT, B. and FEENSTRA, W.J. Revertants of the amylose-free potato clone 86.040 (2n=1x=12). Plant Sci. **67** (1990) 177–182.

[3] WHITHAM, S., DINESH-KUMAR, S.P., CHOI, D., HEHL, R., CORR, C. and BAKER, B. The product of the tobacco mosaic virus resistance gene *N*: Similarity to Toll and the Interleukin-1 Receptor. Cell **78** (1994) 1101–1115.

[4] JONES, D.A., THOMAS, C.M., HAMMOND-KOSACK, K.E., BALINT-KURTI, P.J. and JONES, J.D.G. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science **266** (1994) 789–793.

[5] ANDERSON, P.A., LAWRENCE, G.J., MORRISH, B.C., AYLIFFE, M.A., FINNEGAN, E.J. and ELLIS, J.G. Inactivation of the flax rust resistance gene *M* associated with loss of a repeated unit within the leucine-rich repeat coding region. The Plant Cell **9** (1997) 641–651.

[6] KNAPP, S., COUPLAND, G., UHRIG, H., STARLINGER, P. and SALAMINI, F. Transposition of the maize transposable element *Ac* in *Solanum tuberosum*. Mol. Gen. Genet. **213** (1988) 285–290.

[7] YODER, J.I., PALYS, J., ALPERT, K. and LASSNER, M. *Ac* transposition in transgenic tomato plants. Mol. Gen. Genet. **213** (1988) 291–296.

[8] PEREIRA, A., AARTS, M., AGTMAAL, S. VAN, STIEKEMA, W.J. and JACOBSEN, E. Waxy variegation in transgenic potato. Maydica **36** (1991) 323–327.

[9] EL KHARBOTLY, A., JACOBS, J.M.E., LINTEL HEKKERT, B. TE, JACOBSEN, E., RAMANNA, M.S., STIEKEMA, W.J. and PEREIRA, A. Localization of *Ds*-transposon containing T-DNA inserts in the diploid transgenic potato: linkage to the *R1* resistance gene against *Phytophthora infestans* (Mont.) de Bary. Genome **39** (1996) 249–257.

[10] JONES, J.D.G., CARLAND, F., LIM, E., RALSTON, E. and DOONER, H.K. preferential transposition of the maize element *activator* in linked chromosomal locations in tobacco. The Plant Cell **2** (1990) 701–707.

[11] OSBORNE, B.I., CORR, C.A., PRINCE, J.P., HEHL, R., TANKSLEY, S.D., MCCORMICK, S. and BAKER, B. *Ac* transposition from a T-DNA can generate linked and unlinked clusters of insertions in the tomato genome. Genetics **129** (1991) 833–844.
[12] HERMSEN, J.G.TH. and VERDENIUS, J. Selection from *Solanum tuberosum* group *phureja* of genotypes combining high-frequency haploid induction with homozygosity for embryo-spot. Euphytica **22** (1973) 244–259.
[13] UIJTEWAAL, B.A., HUIGEN, D.J. , HERMSEN, J.G.TH. Production of potato monohaploids (2n=x=12) through prickle pollination. Theor. Appl. Genet. **73** (1987) 751–758.
[14] JACOBSEN E., TEMPELAAR, M.J. and BIJMOLT, E.W. Ploidy levels in leaf callus and regenerated plants of *Solanum tuberosum* determined by cytophotometric measurements of protoplasts. Theor. Appl. Genet. **65** (1983) 113–118.
[15] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant **15** (1962) 473–497.
[16] HOVENKAMP-HERMELINK, J.H.M., JACOBSEN, E., PIJNACKER, L.P., VRIES J.N. DE, WITHOLT, B. and FEENSTRA, W.J. Cytological studies on adventitious shoots and minitubers of a monoploid potato clone. Euphytica **39** (1988) 213–219.
[17] PEREIRA, A., JACOBS, J.M.E., LINTEL-HEKKERT, W. TE, RUTGERS, E., JACOBSEN ,E. and STIEKEMA, W.J. Towards the isolation of resistance genes by transposon targeting in potato. Neth. J. Plant Path. **98** (1992) 215–221.
[18] EL-KHARBOTLY, A., LEONARDS-SCHIPPERS, C., HUIGEN, D.J., JACOBSEN, E., PEREIRA, A., STIEKEMA, W.J., SALAMINI, F. and GEBHARDT, C. Segregation analysis and RFLP mapping of the *R1* and *R3* alleles conferring race-specific resistance to *Phytophthora infestans* in progeny of dihaploid potato parents. Mol. Gen. Genet. **242** (1994) 749–754.
[19] DELLAPORTA, S.L., WOODS, J. and HICKS, J.B. A plant DNA minipreparation version II. Plant Mol. Biol. Rep. **1** (1983) 19–21.
[20] UIJTEWAAL, B.A., SUURS, L.C.J.M. and JACOBSEN, E. Protoplast fusion of monohaploid (2n = x = 12) potato clones: Identification of somatic hybrids using malate dehydrogenase as a biochemical marker. Plant Sci. **51** (1991) 277–284.
[21] WOLTERS, A.M.A., SCHOENMAKERS, H.C.H., MEULEN-MUISERS, J.J.M. VAN DER, KNAAP, E. VAN DER, DERKS, F.H.M., KOORNNEEF, M. and ZELCER, A. Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum.* Theor. Appl. Genet. **83** (1991) 225–232.
[22] MATTHEIJ, W.M., EIJLANDER, R. KONING, J.R.A. DE and LOUWES, K.M. Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens. Theor. Appl. Genet. **83** (1992) 459–466.

# Improvement of pineapple using *in vitro* and mutation breeding techniques

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**Abstract**. Induction of genetic variation in pineapple (*Ananas comosus* (L.) Merr.), cv. 'Smooth Cayene' and 'Sugar Loaf', was investigated by irradiation of shoot tips with 45 Gy gamma rays. Irradiated shoot tips were sub-cultured *in vitro*, and 2500 plants of each cultivar were generated and acclimatised at 45/40$^{o}$ C day/night temperatures. The plants were transplanted in field, and subjected to prolonged periods of drought and heat. In 'Smooth Cayene', the stress treatments did not produce any variant capable of survival under prolonged drought. In 'Sugar Loaf ', 54% of the plants, derived from irradiated shoot-tips, survived prolonged drought, but produced unmarketable fruits. A method of *in vitro* propagation to generate large numbers of pineapple plants was developed.

## 1. INTRODUCTION

Pineapple (*Ananas comosus* (L.) Merr.) is a tropical fruit, propagated from vegetative parts, such as hapas, bitts, suckers, slips and crowns [1]. It is highly heterozygous, and its genetics is not much studied. It is among the few crops in which all the cultivars have been derived from spontaneous somatic mutations and natural evolution without controlled breeding.

Pineapple is cultivated in regions of high atmospheric humidity near the sea with wide range in rainfall (635 - 2,500 mm per annum). The optimal rainfall for commercial production is 1,000-1,500 mm [2]. It has xerophytic features, and can tolerate drought. However, severe drought causes early withering of the peduncle, resulting in reduced flow of nutrients to the fruit. The fruit pericarp, produced under such adverse conditions, lacks lustre and has corky microfissures. The sugar content is also reduced and thus the taste changes. As a consequence, the market value of the fruit is reduced [3].

In Ghana, pineapple has become the leading non-traditional export crop over the past decade. Average fruit production per year is in excess of 10,000 metric tonnes compared to 6,600 during 1980-1990 [4]. This has resulted from government's policy, aimed at diversification of agricultural crops for export. 'Smooth Cayene' and 'Sugar Loaf' are the main cultivars used for commercial production in Ghana. Cultivation of pineapple is predominantly along the periphery of the forest zone, including regions of the coastal savannah, where temperature ranges from 27 to 35$^{o}$C and annual rainfall is between 1,042 to 1,488 mm [5]. This limits the area of production to a narrow strip in the southern part of the country, which is unsuitable for the cultivation of traditional crops, such as cocoa, coffee and oil palm.

Farmers experience poor harvest when the dry season extends beyond five months in any particular year. The vast arable lands of the northern part of the country are currently unsuitable for pineapple cultivation because of long spells of drought (in excess of five months) experienced annually with accompanying elevated temperatures, sometimes exceeding 40$^{o}$C. Further increases in pineapple production in the country will depend on extending the area of

cultivation to regions currently considered marginal or even unsuitable. This will involve breeding new varieties capable of withstanding the adverse local environmental conditions in these regions. This in turn will lead to increased sustainable use of land, and improve income of many farmers.

In many vegetatively propagated crops, mutation induction in combination with *in vitro* culture is the only effective method for their improvement [6,7,8]. The selection of desired types may be done *in vitro* or conventionally in the field. *In vitro* selection allows screening of large populations in a small space under controlled environmental conditions. Heat and drought are traits amenable to *in vitro* selection. For effective selection, test material could be exposed to temperature ten degrees higher than the maximum temperature for cultivation. The combined use of these techniques could enhance breeding of new varieties of pineapple, suitable for use in the relatively dry and hot parts of Ghana. The objectives of this project were to induce genetic variation for tolerance to prolonged periods of drought and heat, and to modify the existing method of micropropagation of pineapple for generating large numbers of propagules of two cultivars, namely, 'Smooth Cayene' and 'Sugar Loaf'.

## 2. MATERIALS AND METHODS

### 2.1. *In vitro* culture

Pineapple cultivars, Smooth Cayenne and Sugar Loaf were used in the experiments. Young basal suckers, collected from a commercial farm near Kwabenya, were used as explants. They were washed in tap water, followed by cleansing in 7X detergent for about 15 minutes. The explants were then rinsed in distilled water, and surface sterilised for 20 minutes in 20% Clorox containing 2-3 drops of 'Tween 80'surfactant. This was followed by four rinses in sterile distilled water. The shoots were further trimmed down. Shoot tips were cultured on shoot proliferation medium containing MS salts [9], 3.5% sucrose, 3 μM thiamine HCl, 3 μM naphthalene acetic acid (NAA), and varying concentrations (0, 10, 15, 20 and 25 μM) of 6-benzylaminopurine (BAP). Prior to sterilisation of the medium, the pH was adjusted to 5.8. The cultures were placed in a completely randomised design with three replicates for each treatment. Multiple shoot buds were produced in liquid media, agitated on a rotary shaker at 70 rpm, and records taken after 8 weeks of culture. Rooting was induced on solid MS medium, supplemented with 1.5 μM indole-3-butyric acid (IBA), 0.75 μM indole-3-acetic acid (IAA), and 0.8% agar. All cultures were maintained at 27$^{o}$C under 16 hr cool-white fluorescent light at an intensity of 3000 lux.

### 2.2. Radio-sensitivity and mutation induction

Shoot tips generated *in vitro* were irradiated with 0, 15, 25, 45, 60, 80, 100 and 120 Gy gamma rays from a $CO^{60}$ source at rate of 215 Gy/hr. There were 50 explants per treatment, and each treatment was replicated three times. Irradiated shoot tips were transferred to shoot proliferation medium, supplemented with 20 μM BAP (based on results from *in vitro* culture). Radiation response was evaluated in terms of explant survival and shoot proliferation after 8 weeks of culture. Surviving shoot tips were transferred to fresh medium, and sequentially subcultured up to $M_1V_4$. After the radio-sensitivity test, shoots of both cultivars, generated *in vitro*, were irradiated with 45 Gy dose, and subcultured to the $M_1V_4$ via micropropagation method, described above.

### 2.3. Weaning and selection of variants

Plantlets, derived from *in vitro* irradiated material, were transferred to greenhouse for selection of variants tolerant to high temperature. Plants were grown in 30x25 cm black polythene bags, filled with loamy soil. Prior to transfer into soil, plants were gently washed in water to remove all traces of agar on the roots. The potted plants were placed in a heat chamber with day temperature of 45$^{o}$C and night temperature 40$^{o}$C with low relative humidity. The plants were watered with 6.6 mg/ml 'Raizal 400' macro-nutrient solution for two weeks, and subsequently with ordinary water as required. Plants were monitored regularly, and observed for the presence of chlorophyll and spineless variants. The plants were kept in the greenhouse for an extended period in order that field evaluation would coincide with the dry season. After 5 months, the weaned plants were transferred directly to the field for further selection and evaluation.

### 2.4. Field selection for drought tolerance

For each variety, 2500 plants treated with 45 Gy of gamma radiation were planted in the field. There were five plots, each comprising 10 rows of 50 plants at 1.5x1.5 m spacing. Plots were blocked according to the age of the plants (from laboratory to field), and separated by two rows of non-irradiated material (control). Initially, the plants were irrigated once each week to ensure good establishment in the field, and then sparingly to mimic drought conditions. Plots were kept clean by weeding and application of herbicides, as required. NPK fertiliser (15:15:15) was applied three months after planting, at a rate of 15 g per plant. Plants were induced to flower at 18 months. This was achieved by placing 1 g of calcium carbide within the leaf axil of each plant, and adding 200 ml of water to generate ethylene. Data were collected on individual plants for fruit weight, length, and girth, and presence or absence of spines on the leaves.

### 2.5. Data analysis

The data on *in vitro* culture and the radio-sensitivity tests were analysed using the statistical software Statgraphics (STSC Inc., and Statistical Graphic Corporation, USA). The data were subjected to analysis of variance using the general linear model to test the significance of treatments (gamma radiation dose). Least square means were derived, and standard errors were used to test significance. In addition, a Dunnetts' t-test was performed on data to compare the means.

## 3. RESULTS AND DISCUSSION

### 3.1. Shoot proliferation and radio-sensitivity

The experiment established $LD_{50}$ to be 45 Gy. These results are consistent with those reported by other workers on mutation breeding in pineapple [10], and with the previous results on micropropagation and radio-sensitivity [11]. Multiple Range Test ($p=0.05$) showed that concentrations of 10, 15, 20 and 25 μM significantly affected shoot proliferation. More shoot-buds were formed with increasing concentration of BAP in the medium. In both cultivars, the optimal proliferation of shoot buds occurred with 20 μM BAP and rooting was achieved on medium supplemented with 1.5 μM IBA and 0.75 μM IAA. The proliferation of 'Smooth Cayenne' was better than that of 'Sugar Loaf'.

### 3.2. Weaning and selection for heat tolerance

The temperature in the heat chamber was favourable for the acclimatisation of the plantlets derived from all treatments. Plantlets weaned under the higher temperature conditions grew faster than those weaned under conventional conditions (at 22°C humidity chamber for 2-3 months; 23°C cold room for 4-6 weeks; ambient temperature in plant barn for 4-6 weeks). These observations suggest that pineapple plantlets can grow at temperatures up to 45°C and that gamma radiation does not affect their ability to do so.

### 3.3. Field evaluation for drought tolerance

Generally, there was a higher rate of survival in plants from control experiments (i.e. 0 Gy) than in plants from irradiated explants for both varieties. The establishment of irradiated plants under drought conditions in the field was not successful in Smooth Cayenne whereas in Sugar Loaf 54% survival was recorded from material treated with same dose of 45 Gy gamma radiation. Irradiated plants were as vigorous as non-irradiated ones (Table I). However, only 37.5% produced fruits as against 87.5% among non-irradiated plants. Under-sized fruits, weighing 268 to 421 g from irradiated and 288 to 512 g from non-irradiated plants were produced compared with 0.7 to1.5 kg required for export [12].

**TABLE I. PLANT AND FRUIT CHARACTER OF PINEAPPLE CV. 'SUGAR LOAF', DERIVED FROM *IN VITRO* MUTAGENSIS**

| Dose (Gy) | Plant height (cm) mean±SE | Leaf width (cm) mean±SE | Fruit length (cm) mean±SE | Fruit girth (cm) mean±SE | Fruit weight (g) mean±SE |
|---|---|---|---|---|---|
| 0 | 64.4±(4.0) | 3.1±(0.3) | 11.0±(2.3) | 24.0±(3.6) | 350±(162) |
| 45 | 68.3±(4.2) | 4.8±(0.3) | 10.2±(1.1) | 23.0±(1.7) | 344± (76) |

## 4. CONCLUSIONS

An improved system for the rapid multiplication of pineapple was established. In both 'Smooth Cayene' and 'Sugar Loaf', optimal shoot proliferation occurred in liquid medium composed of MS salts, 3.5% sucrose, 3 μM thiamine HCl, 3 μM naphthalene acetic acid, and 20 μM 6-benzylaminopurine. The same medium supplemented with 1.5 μM indole-3-butyric acid and 0.75 μM indole-3-acetic acid, and solidified with 0.8% Bacto-agar induced rooting. The $LD_{50}$ for inducing useful mutations in both cultivars was 45 Gy gamma rays. The *ex-vitro* irradiated plants were successfully weaned, and subsequently acclimatised at elevated temperatures of up to 45°C for five months. However, induced mutations did not reveal any variants capable of surviving prolonged drought conditions in 'Smooth Cayene' or producing marketable fruits in 'Sugar Loaf'.

## REFERENCES


[1] SRIVIVAS, R., DORE, S. and CHACKO, E. K. Differentiation of plantlets in hybrid embryo callus of pineapple. Scientia Horticult. **15** (1981) 235–238.

[2] PURSEGLOVE, J.W. Bromeliaceae. Tropical Crops, Monocotyledons. (1988) 75–91.

[3] PY, C., LACOEUILHE, J.J. and TEISSON, D.C. 1987. The Pineapple: Cultivation and Uses. G.P. Maisonneuve & Larose, Paris, (1987) 127–137.

[4] PPMED, 1997. Agriculture in Ghana: Facts and Figures. Policy Planning, Monitoring and Evaluation Division. Ministry of Food and Agriculture. 30 pp.

[5] GHANA METEOROLOGICAL SERVICES DEPARTMENT, Accra Ghana. 1996. Average annual rainfall in pineapple growing areas, 1961–1995.

[6] NOVAK, F.J. "*In vitro* mutation system for crop improvement". Plant Mutation Breeding for Crop Improvement, Vol. 2, Proc. Symp. IAEA, Vienna, (1991) 327–342.

[7] AHLOOWALIA, B.S. "*In vitro* mutagenesis for the improvement of vegetatively propagated plants." Proc. Joint FAO/IAEA Internatl. Symp. "Induced Mutations and Molecular Techniques for Crop Improvement", IAEA, Vienna (1995) 531–541.

[8] MALUSZYNSKI, M., AHLOOWALIA, B.S. and SIGURBJÖRNSSON, B. Application of *in vivo* and *in vitro* mutation techniques for crop improvement. Euphytica **85** (1995) 303–315.

[9] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiolog. Planta. **15** (1962) 473–497.

[10] LAPADE, A.G., VELUZ, A.M.S. and SANTOS, I.S. "Genetic improvement of the Queen variety of pineapple through induced mutation and *in vitro* culture techniques. Proc. Joint IAEA/FAO Internatl. Symp. "Induced Mutations and Molecular Techniques for Crop Improvement", IAEA, Vienna, (1995) 684–687.

[11] OSEI-KOFI, F., LOKKO, Y. and AMOATEY, H.M. "Improvement of pineapple (*Ananas comosus* (L) Merr.) using biotechnology and mutation breeding techniques", Rep. Second FAO/IAEA Research Co-ordination Meeting on "*In vitro* Techniques for Selection of Radiation Induced Mutants Adapted to Adverse Environmental Conditions", IAEA, Vienna, (1996) 23–28.

[12] ANONYMOUS. Pineapples. Selected European Markets for Tropical and Off-season Fresh Fruits and Vegetables. UNCTAD/GATT, Geneva, (1981) 10–11.

# Development of sugarcane mutants with resistance to red rot, water-logging and delayed or non-flowering through induced mutations

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**Abstract.** Three varieties of sugarcane, 'Isd-2/54', 'Nagarbari' and 'Latarijaba', were irradiated with 20, 30 and 40 Gy gamma rays to induce variation for resistance to red rot disease. The $MV_2$ population was screened for disease resistance by planting infected canes in between the treated material, and selected plants were further propagated. Among the 2,954 $MV_3$ hills, inoculated with red rot spore suspension, 37 resistant and 151 moderately resistant plants were isolated. Selection was carried out in the $MV_4$ to $MV_7$ propagation. Seven $MV_7$ selected variants were tested for yield at two locations. Of these, four promising variants were selected on the basis of cane yield, Brix index and disease resistance. In another experiment, four varieties of sugarcane, 'Isd-2/54', 'Isd-16', 'Nagarbari' and 'Latarijaba' were irradiated with 20, 40 and 60 Gy gamma rays. Approximately, 10,000 $MV_3$ canes were planted in a low-lying field, and subjected to water-logging stress. $MV_5$ and $MV_6$ populations were inoculated with red rot spore-suspension under waterlogged conditions. Five $MV_5$ variants were selected on the basis of greenness of the leaves, growth of the canes, number of nodes bearing adventitious roots, Brix index, cane yield and disease reactions, and grown as $MV_6$ propagation. Three selected variants, SCM-12, SCM-14 and SCM-15, were tolerant to waterlogged conditions in $MV_6$. Two additional varieties, 'I-291/87' and 'I-525/85' were treated with 20, 30 and 40 Gy gamma rays to select for delayed/non-flowering types. Four variants were selected in $MV_3$ for delayed flowering; one mutant, SCM-28 flowered three months later than the parent 'I-291/87'.

## 1. INTRODUCTION

Sugarcane is an important industrial crop in Bangladesh but its yield is much lower than the other sugarcane growing countries of the world. The average yield of sugarcane in Bangladesh is around 40-50 t/ha [1]. The main reason for the poor yield is disease susceptibility of the existing varieties [2]. Red rot, caused by *Colletotrichum falcatum*, is a major problem both in the commercial and seed cane crop [3]. It causes serious yield losses, and damage of the entire crop in a field is not uncommon [4]. Incase of nodal infection, 8-15% loss in germination has been reported [5]. Sugarcane varieties succumb easily to red rot disease under waterlogged condition. During the rainy season in Bangladesh, considerable area under sugarcane is waterlogged, which results in poor soil aeration and reduces oxygen supply to plant roots. The waterlogged condition causes anaerobic break down of sugar that leads to accumulation of ethanol at toxic levels in plants lacking tolerance to flooding [6]. Although, there is no adverse effect of flowering on cane quality up to 60 days, the quality deteriorates after this period [7]. It is not possible to crush all the cane within 60 days. Cane crushing in some sugar mills continues up to 3 to 4 months after the first sixty days. As a result, both the cane yield and sugar recovery deteriorates. All the existing cultivars are susceptible to red rot disease under waterlogged conditions, and deteriorate in yield and quality after 60 days of flowering. Hence, a breeding program was initiated to induce mutants with resistance to red rot disease, tolerance to waterlogged conditions and delayed or non-flowering.

## 2. MATERIALS AND METHODS

### 2.1. Screening for red rot resistance

Three varieties of sugarcane, 'Isd-2/54', 'Nagarbari' and 'Latarijaba' were irradiated with 20, 30 and 40 Gy gamma rays, and $MV_1$ propagation was grown in 1990-91. Two central canes from each $MV_1$ hill were taken, and about one-third portion from the middle of each cane was cut for growing $MV_2$. Red rot infected canes were planted between the treated rows for disease spread. $MV_3$ population of 4,482 hills was grown at BINA, Mymensingh during 1992-93. Two central canes from each of the 2,954 hills were artificially inoculated with red rot spore suspension. The selected 37 resistant and 151 moderately resistant canes were grown at BINA, Mymensingh during 1993-94. Spacing between rows and hills was 100 cm and 30 cm, respectively. Fertilizer was applied at a rate of 280 kg urea, 185 kg TSP, 185 kg MP, and 4 tons cow dung per hectare. The surviving 2,114 canes were again inoculated. During 1994-95, four resistant and 64 moderately resistant canes of $MV_5$ were grown in plant-progeny-rows at two locations, Ishurdi and Magura. In this generation, 2,416 canes were further inoculated. During 1995-96, preliminary yield trial was conducted with 9 selected $MV_6$ mutants (1 resistant and 8 moderately resistant) at two locations, and the population of 1872 two canes was artificially inoculated. The selected variants were renamed as sugarcane mutants (SCM). During 1996-97, an advanced yield trial was conducted with seven $MV_7$ mutants at two locations and 1,586 canes were inoculated with red rot spore suspension. Standard plug method of inoculation was used on 8-9 month-old canes, using spore suspension from 10-15 day-old culture of *Colletotrichum falcatum*, grown on Potato Dextrose Agar (PDA) [8]. The inoculum consisted of about one million spore per ml of water, and 0.2 ml suspension was injected into each cane. The canes were split open after two months, and scored as per disease severity index (0-9) developed by the All India Co-ordinated Research Programme for red rot of sugarcane [9]. Selection was made on the basis of disease severity. Canes showing disease score of 0.0 to 2.0 were selected as resistant (R) and 2.1 to 4.0 as moderately resistant (MR). Data on number of tillers/hill, cane height, cane diameter (base, middle and top), Brix index and cane yield was recorded from the selected lines and the controls in all the experiments.

### 2.2. Screening for tolerance to water-logging

Buds of four sugarcane varieties, 'Isd-2/54', 'Isd-16', 'Nagarbari' and 'Latarijaba' were irradiated with 20, 40 and 60 Gy gamma rays, and $MV_1$ generation was grown at BINA farm, Mymensingh during 1991-92. $MV_2$ generation was planted during the following year. During 1993-94, approximately 10,000 $MV_3$ canes were grown in low-lying plots for selecting resistant/tolerant canes to waterlogged condition. The plot was irrigated from time to time after six months of planting to maintain water level up to 30-50 cm till harvest (November). The canes displaying green leaves were scored as tolerant to water-logging. During 1994-95, 38 $MV_4$ selected lines, tolerant to water-logging along with four controls were grown as plant-progeny-rows at two locations, Ishurdi and Mymensingh. During 1995-96, 9 $MV_5$ mutant lines tolerant to water-logging and four controls were grown again at two locations for screening against red rot under waterlogged conditions. During 1996-97, the selected material of $MV_6$ generation was grown at Ishurdi and five selected mutants were planted in replicated trials. A population of 630 canes was artificially inoculated with red rot spore suspension. Data on the number of tillers/hill, cane height, cane diameter, number of nodes bearing adventitious roots, Brix index, cane yield and disease reactions were recorded.

### 2.3. Selection for delayed or non-flowering

Two varieties of sugarcane, 'I-291/87' and 'I-525/85' were irradiated with 20, 30 and 40 Gy gamma rays to obtain delayed/non-flowering mutants. $MV_1$ generation was grown at Mymensingh during 1994-95, and $MV_2$ was raised in the following year. About 8,500 $MV_3$ canes were grown at BINA farm, Mymensingh during 1996-97. Canes that did not flower after two months of the controls were selected. Normal cultural practices were followed during the entire growing period. Data on the number of tillers/hill, cane height, cane diameter, Brix index and days to flowering were recorded.

## 3. RESULTS AND DISCUSSION

### 3.1. Screening for red rot resistance

In $MV_1$, germination decreased with increase in radiation doses (Fig. 1A), and the results were similar to those reported elsewhere [10]. Seedling height, cane height and diameter of cane (base, middle and top) also decreased with increase in radiation dose except at 20 Gy, where a stimulating effect was obtained in all varieties (Fig. 1 B, C, D, E, F). Similar trend was also observed in $MV_2$ generation for cane height and diameter (Table I). All plants in a hill originating from infected canes showed external symptoms of red rot disease, and died before the harvest. Many of the treated $MV_2$ canes also showed similar symptoms, and died. Sets were cut from surviving healthy canes among the treated materials and any set showing disease inside was discarded. Only those sets with no red rot symptoms were selected for growing $MV_3$.

Of the 2,954 inoculated canes, 37 were resistant and 151 were moderately resistant (Table II). The results on radio-sensitivity and selection in $MV_2$ and $MV_3$ generation have been reported previously [11]. In $MV_4$, of the 2,114 canes only 4 were resistant and 64 were moderately resistant to red rot disease (Table III).

In $MV_5$ generation, only one hill was resistant (R) and 8 were moderately resistant (MR) to red rot disease at Ishurdi (Table IV) and at Magura, two hills were moderately resistant. Seven mutants had higher Brix index than the best control 'Latarijaba' at both locations. The experiment at Magura was not as good as that at Ishurdi due to poor and dry soil conditions.

A total of 936 hills were grown at Ishurdi and Magura to assess their yield potential and screening against red rot disease. At Ishurdi, only one hill was resistant and 6 moderately resistant to red rot disease (Table V). Only one mutant exhibited higher number of tillers/hill than the parental variety 'Latarijaba'. One mutant had taller canes than the parent 'Nagarbari'. There was not much variation among the mutants and the controls in cane diameter. All mutants had higher Brix index than the controls. Only one mutant of Isd-2/54 (from 30 Gy treatment) had higher cane yield than 'Nagarbari'. At Magura, the experiment suffered from poor and dry soil conditions. One mutant clone of 'Isd-2/54' (from 20 Gy treatment) was resistant and five mutants were moderately resistant to red rot. There was no difference between mutants and controls in the tiller number, height, diameter and yield. Four mutants showed higher Brix index than the control 'Isd-2/54'. It is evident from Table VI that the mutant SCM-5 produced the highest cane yield at both locations. At Ishurdi, five mutants (SCM-1, SCM-2, SCM-4, SCM-5 and SCM-6) were found to be resistant. But at Magura, only three mutants (SCM-1, SCM-4 and SCM-5) were resistant against red rot disease. No significant variations were found among the mutants and the controls in number of tillers/hill and Brix index at Ishurdi.

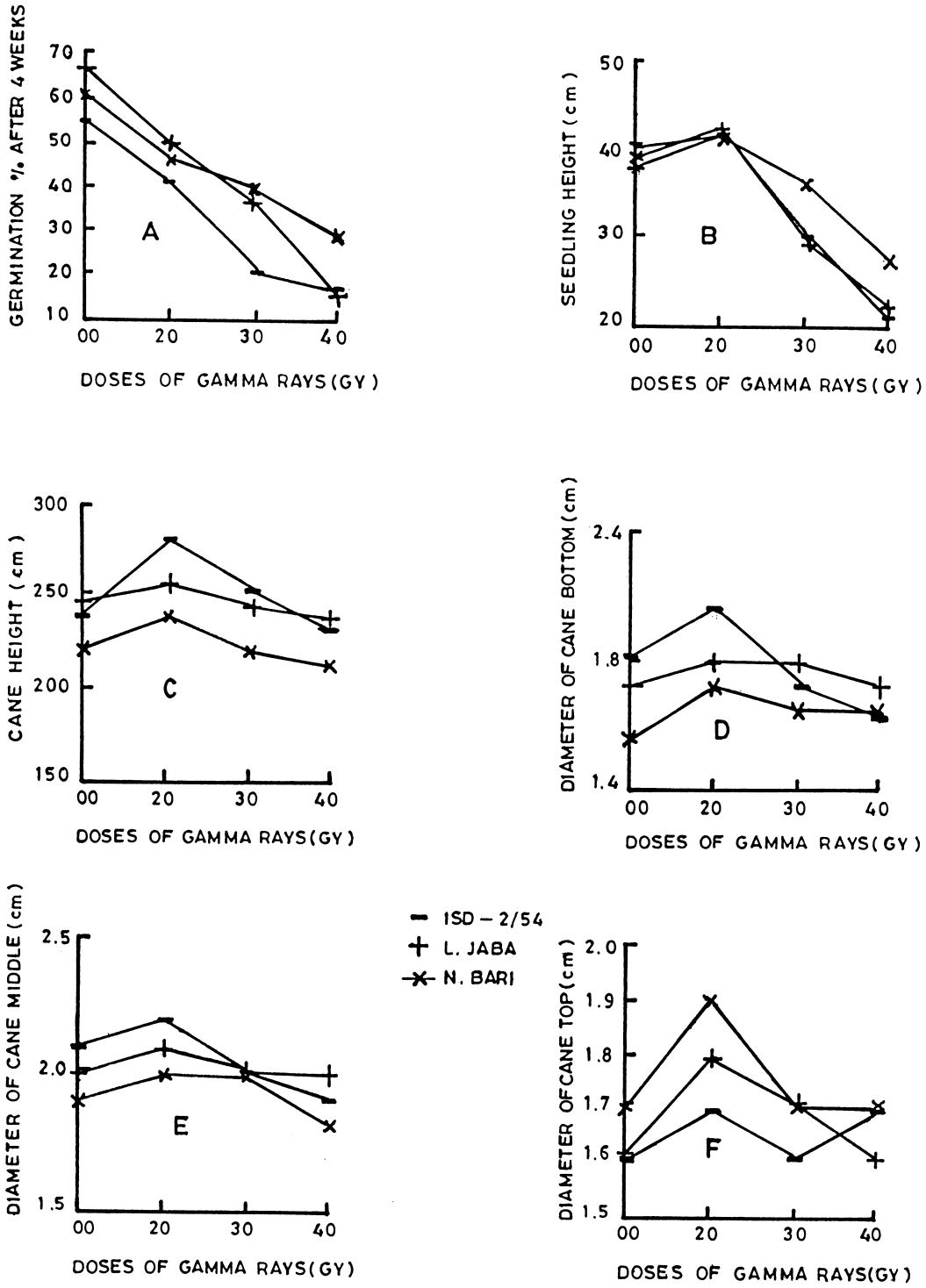


*Fig. 1. Effect of irradiation on six different traits of three varieties of sugarcane in $MV_1$ generation.*

**TABLE I. HEIGHT AND DIAMETER OF $MV_2$ CANES OF THREE VARIETIES AT TWO LOCATIONS (1991–1992)**

| Location variety | Dose (Gy) | Mymensingh | | | | Kaliachapra | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Cane height (cm) | Cane diameter (cm) | | | Cane height (cm) | Cane diameter (cm) | | |
| | | | Base | Middle | Top | | Base | Middle | Top |
| Isd-2/54 | 0 | 195 | 1.6 | 1.8 | 1.7 | 216 | 1.8 | 2.0 | 1.9 |
| | 20 | 218 | 1.7 | 1.9 | 1.8 | 248 | 1.9 | 2.1 | 2.0 |
| | 30 | 207 | 1.6 | 1.8 | 1.7 | 237 | 1.7 | 2.0 | 1.9 |
| | 40 | 187 | 1.5 | 1.8 | 1.6 | 220 | 1.7 | 1.9 | 1.8 |
| Latarijaba | 0 | 200 | 1.6 | 1.8 | 1.7 | 231 | 1.8 | 2.0 | 1.8 |
| | 20 | 239 | 1.7 | 1.8 | 1.7 | 267 | 1.8 | 2.0 | 1.9 |
| | 30 | 221 | 1.7 | 1.8 | 1.6 | 250 | 1.9 | 2.0 | 1.8 |
| | 40 | 185 | 1.7 | 1.8 | 1.7 | 210 | 1.9 | 2.1 | 1.9 |
| Nagarbari | 0 | 204 | 1.5 | 1.7 | 1.6 | 234 | 1.7 | 1.9 | 1.9 |
| | 20 | 224 | 1.8 | 2.0 | 1.9 | 244 | 2.0 | 2.1 | 2.0 |
| | 30 | 217 | 1.7 | 1.8 | 1.8 | 239 | 1.9 | 2.0 | 2.1 |
| | 40 | 190 | 1.6 | 1.8 | 1.7 | 210 | 1.8 | 2.1 | 1.9 |

**TABLE II. FREQUENCY OF RED ROT RESISTANT/MODERATELY RESISTANT CANES IN $MV_3$ SUGARCANE (1992–1993)**

| **Location** | **Variety** | Doses (Gy) | No. of hills | Plants inoculated (No.) | **Moderately resistant** (No.) | Resistant (No.) |
|---|---|---|---|---|---|---|
| Mymensingh | Isd-2/45 | 00 | 153 | 50 | - | - |
| | | 20 | 231 | 155 | 13 | 4 |
| | | 30 | 230 | 175 | 17 | 4 |
| | | 40 | 255 | 175 | 22 | 9 |
| | Latarijaba | 00 | 110 | 50 | - | - |
| | | 20 | 220 | 173 | 11 | 1 |
| | | 30 | 210 | 164 | 11 | 1 |
| | | 40 | 218 | 170 | 11 | 5 |
| | Nagarbari | 00 | 130 | 50 | - | - |
| | | 20 | 230 | 170 | 8 | 1 |
| | | 30 | 235 | 169 | 11 | 4 |
| | | 40 | 240 | 170 | 6 | 0 |
| Kaliachapra | Isd-2/54 | 00 | 130 | 50 | - | - |
| | | 20 | 185 | 127 | 6 | 3 |
| | | 30 | 200 | 127 | 12 | 1 |
| | | 40 | 150 | 125 | 3 | 1 |
| | Latarijaba | 00 | 150 | 50 | - | - |
| | | 20 | 200 | 152 | 4 | 1 |
| | | 30 | 190 | 130 | 1 | 0 |
| | | 40 | 180 | 127 | 6 | 0 |

**TABLE II. (cont.)**

| | | | | | |
|---|---|---|---|---|---|
| Nagarbari | 00 | 170 | 50 | - | - |
| | 20 | 175 | 123 | 2 | 1 |
| | 30 | 150 | 125 | 3 | 1 |
| | 40 | 140 | 122 | 4 | 0 |
| **Total** | | **4482** | **2954** | **151** | **37** |

**TABLE III. FREQUENCY OF RED ROT RESISTANT CANE SELECTED IN $MV_4$**

| Variety | Dose (Gy) | No. of canes inoculated | Moderately resistant | Resistant |
|---|---|---|---|---|
| Isd-2/54 | 00 | 20 | - | - |
| | 20 | 248 | 24 | 1 |
| | 30 | 326 | 14 | 3 |
| | 40 | 414 | 11 | - |
| Latarijaba | 00 | 20 | - | - |
| | 20 | 216 | 1 | - |
| | 30 | 202 | 6 | - |
| | 40 | 196 | 2 | - |
| Nagarbari | 00 | 20 | - | - |
| | 20 | 140 | 2 | - |
| | 30 | 210 | 1 | - |
| | 40 | 102 | 3 | - |
| **Total** | | **2114** | **64** | **4** |

**TABLE IV. PERFORMANCE OF SELECTED RED ROT RESISTANT CANES IN $MV_5$ AT TWO LOCATIONS (1994–1995)**

| Variety | Dose (Gy) | No. of tillers/hill | Cane height (cm) | Cane diam. (cm) | Brix index (%) | Disease score* |
|---|---|---|---|---|---|---|
| Location Ishurdi | | | | | | |
| Isd-2/54 | 00 | 17.6 | 251 | 2.13 | 12.2 | MS |
| | 20 | 18.4 | 266 | 2.11 | 15.5 | R |
| | 20 | 18.3 | 265 | 2.10 | 15.4 | MR |
| | 30 | 16.1 | 288 | 2.20 | 17.0 | MR |
| | 40 | 17.3 | 275 | 2.10 | 17.5 | MR |
| Latarijaba | 00 | 13.0 | 270 | 2.21 | 16.7 | S |
| | 30 | 13.3 | 249 | 2.08 | 17.5 | MR |
| Nagarbari | 00 | 14.6 | 232 | 2.23 | 12.8 | S |
| | 20 | 13.6 | 242 | 2.22 | 18.0 | MR |
| | 30 | 8.0 | 233 | 2.00 | 17.5 | MR |
| | 40 | 16.6 | 238 | 2.22 | 16.5 | **MR** |
| Location Magura | | | | | | |
| Isd-2/54 | 00 | 6.6 | 150 | 1.85 | 16.5 | MS |
| | 30 | 6.2 | 167 | 1.77 | 17.3 | MR |
| | 40 | 8.5 | 154 | 1.78 | 16.8 | MR |

*S (susceptible), MS (moderately susceptible), MR (moderately resistant) and R (resistant)

Most of the mutants had higher Brix index than the controls at both locations. The mutant SCM-5 was taller in height and had thicker diameter, which resulted in higher cane yield. In sugarcane, induced mutations have been successfully used to increase cane yield, higher sugar content and resistance to diseases [12, 13, 14]. It has been reported [15] that in case of vegetatively propagated plants like sugarcane, it is possible to generate desired genotypes through either somaclonal variation or through *in vitro* mutagenesis. On the basis of cane yield, Brix index and disease score, four mutants, SCM-4, SCM-5, SCM-6 and SCM-7, have been selected for zonal yield trials.

**TABLE V. PERFORMANCE OF SELECTED $MV_6$ LINES OF SUGARCANE AT TWO LOCATIONS (1995–1996)**

| Variety/strain | Tillers/hill (No.) | Cane height (cm) | Cane diam (cm) | Brix index (%) | Cane yield (t/ha) | Disease score* |
|---|---|---|---|---|---|---|
| *Location Ishurdi* | | | | | | |
| Isd-2/54 (control) | 7.4 | 310 | 2.1 | 14.8 | 110 | MS |
| Isd-2/54 20 Gy (R) | 7.9 | 312 | 2.1 | 15.8 | 113 | R |
| Isd-2/54 20 Gy (MR) | 6.2 | 303 | 2.0 | 15.9 | 110 | MR |
| Isd-2/54 20 Gy (MR) | 9.2 | 288 | 1.9 | 17.5 | 100 | MR |
| Isd-2/54 30 Gy (MR) | 7.1 | 301 | 2.1 | 15.2 | 124 | MR |
| Nagarbari (control) | 8.4 | 311 | 2.1 | 14.3 | 114 | S |
| Nagarbari 20 Gy(MR) | 6.7 | 295 | 2.1 | 16.7 | 93 | MS |
| Nagarbari 30Gy (MR) | 6.7 | 302 | 2.0 | 17.2 | 90 | MR |
| Nagarbari 40 Gy (MR) | 8.3 | 303 | 2.1 | 15.1 | 101 | MR |
| Latarijaba (control) | 8.6 | 290 | 1.9 | 14.8 | 112 | S |
| Latarijaba 30 Gy (MR) | 8.0 | 275 | 2.1 | 16.5 | 77 | MS |
| Latarijaba 40 Gy (MR) | 7.4 | 286 | 2.0 | 16.5 | 84 | **MR** |
| *Location Magura* | | | | | | |
| Isd-2/54 (control) | 5.2 | 150 | 1.9 | 14.6 | 56 | MS |
| Isd-2/54 20 Gy (R) | 6.6 | 150 | 1.9 | 15.5 | 65 | R |
| Isd-2/54 20 Gy (MR) | 5.3 | 118 | 1.9 | 15.4 | 69 | MR |
| Isd-2/54 20 Gy (MR) | 4.6 | 148 | 1.7 | 16.7 | 58 | **MR** |
| Isd-2/54 30 Gy (MR) | 5.9 | 152 | 1.8 | 16.3 | 63 | **MS** |
| Nagarbari (control) | 5.0 | 128 | 1.8 | 13.2 | 62 | S |
| Nagarbari 20 Gy (MR) | 5.1 | 120 | 1.7 | 12.8 | 61 | MR |
| Nagarbari 30 Gy (MR) | 4.9 | 116 | 1.9 | 14.3 | 59 | MS |
| Nagarbari 40 Gy (MR) | 5.2 | 122 | 1.8 | 12.0 | 55 | MR |
| Latarijaba (control) | 6.3 | 130 | 1.9 | 13.8 | 67 | S |
| Latarijaba 30 Gy (MR) | 6.7 | 122 | 1.8 | 14.0 | 65 | MS |
| Latarijaba 40 Gy (MR) | 4.3 | 145 | 1.8 | 14.5 | 68 | **MR** |

*S (susceptible), MS (moderately susceptible), MR (moderately resistant) and R (resistant).

### 3.2. Screening of mutants for tolerance to water-logging

Of the10,000 $MV_3$ canes, only 38 showed tolerance to waterlogged condition and remained green till harvest. The number of tillers/hill showed the highest coefficient of variation (26.9 %), followed by cane height (16.0%) and cane diameter (Table VII). At Ishurdi, of the 1,575 $MV_4$ hills, only 8 showed tolerance to waterlogged condition. The data on agronomic performance and Brix index of the selected canes and the controls are presented in Table VIII. Five mutants had a higher number of nodes bearing adventitious roots than the controls. Brix index was also higher in some mutants.

**TABLE VI. PERFORMANCE OF SELECTED $MV_7$ MUTANTS OF SUGARCANE AT TWO LOCATIONS (1996–1997)**

| Variety/mutant | No. of tillers/hill | Cane height (cm) | Cane diam. (cm) | Brix index (%) | Cane yield (t/ha) | Disease score* |
|---|---|---|---|---|---|---|
| Location Ishurdi | | | | | | |
| SCM-1 | 7.4a | 248bcd | 2.00a | 17.7a | 81.2d | R |
| **SCM-2** | 7.8a | 240cd | 1.88c | 18.5a | 74.3de | R |
| SCM-3 | 7.0a | 250bc | 1.88c | 16.4a | 65.9e | MR |
| SCM-4 | 6.8a | 256bc | 2.01a | 17.4a | 85.7bcd | R |
| SCM-5 | 7.4a | 259ab | 2.05a | 17.7a | 102.7a | R |
| SCM-6 | 7.6a | 251bc | 1.98ab | 17.8a | 97.8abc | R |
| SCM-7 | 8.0a | 234d | 1.99a | 18.2a | 83.9cd | MR |
| Nagarbari control | 7.4a | 250bc | 2.06a | 16.8a | 89.5a-d | S |
| Latarijaba control | 8.0a | 272a | 1.89bc | 16.9a | 99.8ab | MS |
| Isd-2/54 control | 7.9a | 251bc | 2.00a | 17.3a | 99.2abc | MS |
| Location Magura | | | | | | |
| SCM-1 | 6.1bcd | 199d | 1.91abc | 16.5ab | 79.5c | R |
| **SCM-2** | 6.2cd | 222bcd | 1.64d | 17.6a | 86.6bc | MR |
| SCM-3 | 6.1d | 239bc | 1.79c | 17.5a | 96.2bc | MR |
| SCM-4 | 6.9abcd | 215cd | 1.87bc | 16.6ab | 95.3bc | R |
| SCM-5 | 8.0a | 246b | 2.03a | 16.0abc | 142.3a | R |
| SCM-6 | 7.7ab | 241bc | 1.95ab | 17.6a | 99.8bc | MR |
| SCM-7 | 6.1d | 238bc | 2.05a | 16.5ab | 108.2b | MR |
| Nagarbari control | 7.3abc | 204d | 1.93abc | 14.6cd | 137.8a | S |
| Latarijaba control | 7.9a | 279a | 1.85bc | 14.0d | 130.7a | S |
| Isd-2/54 control | 6.6bcd | 242bc | 2.05a | 15.4bcd | 139.1a | MS |

*Means followed by same letter in a column do not differ significantly at 5% level according to DMRT; NS = Not significant. SCM = Sugarcane mutant.

**TABLE VII. PERFORMANCE OF $MV_3$ CANES SELECTED FOR WATERLOGGING TOLERANCE AT MYMENSINGH (1993–1994)**

| Statistical parameters | No. of tillers/ hill | Cane height (cm) | Cane diameter (cm) | | |
|---|---|---|---|---|---|
| | | | Base | Middle | Top |
| Range | | | | | |
| High | 8.00 | 304 | 2.40 | 2.30 | 2.10 |
| Low | 2.00 | 135 | 1.70 | 1.60 | 1.20 |
| Sd | 1.73 | 35 | 0.22 | 0.18 | 0.20 |
| CV (%) | 26.90 | 16 | 10.90 | 9.50 | 11.70 |
| Isd-16 (control) | 3 | 276 | 1.80 | 2.00 | 1.70 |
| Isd-2/54 (control) | 5 | 211 | 1.90 | 2.00 | 1.90 |
| Latarijaba (control) | 4 | 275 | 1.90 | 2.00 | 1.80 |
| Nagarbari (control) | 4 | 260 | 1.80 | 2.10 | 1.70 |

**TABLE VIII. AGRONOMIC PERFORMANCE AND BRIX INDEX OF SELECTED WATERLOGGING TOLERANT CANES AT ISHURDI (1994–1995)**

| Variety/ mutant | Dose (Gy) | Tillers/ hill No. | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Brix index % | No. of nodes bearing adventitious roots |
|---|---|---|---|---|---|---|---|---|
| Isd-2/54 | 20 | 9 | 173 | 2.11 | 1.98 | 1.75 | 20.0 | 6 |
| | 40 | 10 | 167 | 2.16 | 1.96 | 1.74 | 21.5 | 3 |
| | 60 | 11 | 209 | 2.37 | 2.14 | 1.89 | 20.0 | 5 |
| Isd-16 | 20 | 6 | 197 | 2.33 | 2.10 | 1.91 | 20.5 | 4 |
| | 40 | 7 | 158 | 2.03 | 1.96 | 1.78 | 22.5 | 3 |
| | 60 | 8 | 198 | 2.28 | 2.04 | 1.84 | 23.0 | 4 |
| Nagarbari | 40 | 13 | 168 | 2.00 | 1.86 | 1.69 | 18.5 | 3 |
| Latarijaba | 60 | 11 | 164 | 2.00 | 1.83 | 1.69 | 20.5 | 5 |
| Isd-2/54 | C* | 11 | 159 | 2.13 | 2.11 | 1.81 | 19.5 | 2 |
| Isd-16 | C | 8 | 152 | 2.28 | 2.06 | 1.85 | 20.0 | 3 |
| Nagarbari | C | 10 | 172 | 2.33 | 2.03 | 1.95 | 20.0 | 2 |
| Latarijaba | C | 11 | 174 | 2.23 | 2.02 | 1.78 | 20.5 | 3 |

*Control (C)

**TABLE IX. AGRONOMIC PERFORMACE AND BRIX INDEX OF SELECTED WATERLOGGING TOLERANT CANES AT MYMENSINGH (1994–1995)**

| Variety/ Mutant | Dose (Gy) | No. of tillers/ hill | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Brix index (%) | No. of nodes Bearing adventitious roots |
|---|---|---|---|---|---|---|---|---|
| Isd-2/54 | 20 | 5 | 158 | 1.94 | 1.73 | 1.58 | 17.2 | 4 |
| ,, | 40 | 7 | 147 | 1.95 | 1.85 | 1.62 | 15.0 | 4 |
| ,, | 60 | 6 | 139 | 2.00 | 1.73 | 1.55 | 21.1 | 3 |
| Isd-16 | 20 | 4 | 128 | 2.03 | 2.02 | 1.71 | 18.4 | 5 |
| ,, | 40 | 5 | 133 | 2.13 | 1.86 | 1.76 | 18.6 | 3 |
| Nagarbari | 20 | 6 | 149 | 2.13 | 2.03 | 1.87 | 17.7 | 5 |
| ,, | 40 | 5 | 127 | 2.04 | 1.86 | 1.72 | 20.5 | 7 |
| Isd-2/54 | C* | 5 | 133 | 2.03 | 1.75 | 1.69 | 15.2 | 3 |
| Isd-16 | C | 4 | 138 | 1.90 | 1.74 | 1.64 | 18.8 | 2 |
| Nagarbari | C | 5 | 100 | 2.10 | 1.70 | 1.60 | 17.4 | 3 |
| Latarijaba | C | 6 | 143 | 2.11 | 1.92 | 1.79 | 20.0 | 3 |

*Control (C)

At Mymensingh, of the1380 ($MV_4$) hills only 7 showed tolerance to waterlogging. Five mutants had more nodes bearing adventitious roots than the parent varieties. Three mutants had taller canes than the tallest parent variety 'Latarijaba'. Only two mutants had higher Brix index than the control varieties (Table IX).

In $MV_5$ generation, of the 1,261 hills, six were tolerant to waterlogged conditions. At Ishurdi, five mutants had taller canes than that of the tallest parent 'Isd-16'. Characters such as cane diameter, Brix index and number of nodes bearing adventitious roots did not show much variation, but four mutants had higher cane yield than the higher yielding parent Isd-2/54. Seven mutants were moderately resistant to red rot. At Mymensingh, only one mutant had more nodes bearing adventitious roots than the controls. Three mutants were scored as moderately resistant to red rot. Only one mutant gave higher cane yield than the control varieties (Table X)

In $MV_6$, three mutants, SCM-12, SCM-14 and SCM-15, showed better performance under waterlogged conditions (Table XI). Two mutants, SCM-14 and SCM-15, had taller canes than that of the tallest parent 'Latarijaba'. The mutant SCM-15 differed significantly in cane diameter from the parent 'Nagarbari'. The mutants, SCM-8 and SCM-14, had the highest number of nodes bearing adventitious roots. There was no mutant with higher Brix index than the parent 'Latarijaba'. The mutant SCM-8 gave higher cane yield than the other mutants and controls. All mutants were moderately resistant (MR) to red rot disease.

### 3.2. Selection for delayed or non-flowering

In $MV_2$, three mutants of 'I-291/87' and three mutants of variety I-525/85 fulfilled the criterion of selection for non-flowering (Table XII). The mutant I-525/85 (40 Gy) flowered after three

**TABLE X. AGRONOMIC PERFORMANCE, BRIX INDEX AND DISEASE SCORE OF SELECTED WATERLOGGING TOLERANT CANES AT TWO LOCATIONS (1995–1996)**

| Variety/ mutant | Dose (Gy) | No. of Tillers /hill | Cane height (cm) | Cane diameter (cm) | Brix index (%) | No. of nodes bearing adv. roots | Cane yield (t/ha) | Disease score* |
|---|---|---|---|---|---|---|---|---|
| Ishurdi | | | | | | | | |
| **Latarijaba** | **C*** | 9.1 | 216 | 2.2 | 18.3 | 3 | 46 | MS |
| Latarijaba | 60 | 6.7 | 225 | 1.9 | 16.8 | 2 | 42 | MR |
| Isd-2/54 | C | 9.4 | 191 | 1.9 | 18.8 | 2 | 50 | MS |
| Isd-2/54 | 20 | 8.7 | 253 | 2.3 | 19.0 | 3 | 55 | MR |
| Isd-2/54 | 40 | 5.7 | 248 | 1.9 | 15.7 | 2 | 52 | MR |
| Isd-2/54 | 60 | 8.4 | 199 | 1.9 | 17.0 | 2 | 45 | MR |
| Nagarbari | C | 11.0 | 200 | 2.1 | 17.4 | 3 | 46 | S |
| Nagarbari | 20 | 7.9 | 178 | 2.0 | 16.3 | 3 | 42 | S |
| Nagarbari | 40 | 7.0 | 189 | 2.2 | 16.5 | 3 | 47 | S |
| Isd-16 | C | 5.0 | 223 | 2.2 | 20.5 | 3 | 44 | MS |
| Isd-16 | 20 | 5.1 | 256 | 2.2 | 20.6 | 5 | 69 | MR |
| Isd-16 | 40 | 4.8 | 232 | 2.3 | 19.4 | 3 | 65 | MR |
| Isd-16 | 60 | 4.9 | 103 | 1.8 | 19.5 | 3 | 50 | MR |
| Mymensingh | | | | | | | | |
| **Latarijaba** | **C** | 6.4 | 205 | 1.8 | 16.1 | 2 | 28 | S |
| Latarijaba | 60 | 6.6 | 210 | 1.9 | 16.0 | 2 | 30 | MS |
| Isd-2/54 | C | 9.6 | 230 | 2.1 | 17.3 | 3 | 43 | MS |
| Isd-2/54 | 20 | 8.1 | 240 | 2.0 | 17.8 | 5 | 40 | MR |
| Isd-2/54 | 40 | 8.7 | 246 | 2.1 | 14.0 | 3 | 45 | MR |
| Isd-2/54 | 60 | 7.6 | 151 | 1.9 | 17.0 | 2 | 34 | MS |
| Nagarbari | C | 7.6 | 166 | 2.2 | 18.7 | 4 | 37 | S |
| Nagarbari | 20 | 6.3 | 125 | 1.8 | 18.5 | 2 | 38 | MS |
| Nagarbari | 40 | 5.7 | 133 | 1.7 | 17.1 | 2 | 33 | S |
| Isd-16 | C | 4.0 | 133 | 1.9 | 19.8 | 3 | 39 | MS |
| Isd-16 | 20 | 3.7 | 98 | 1.9 | 15.4 | 2 | 31 | MR |
| Isd-16 | 40 | 7.0 | 153 | 2.2 | 19.9 | 4 | 40 | S |
| Isd-16 | 60 | 6.2 | 140 | 2.1 | 18.0 | 3 | 30 | MS |

*S (susceptible), MS (moderately susceptible), MR (moderately resistant) and R (resistant)

months of the control. The canes of the mutant were taller than that of the other mutants and controls. No mutant was found with non-flowering characteristic in this generation. Of the 8,500 $MV_3$ canes, only three mutants, SCM-27, SCM-28 and SCM-29, of the variety I-291/87 and one mutant, SCM-30, of I-525/85 were selected for delayed flowering character. The mutant SCM-28 flowered after three months of the parent variety, I-291/87. Two mutants had taller canes than that of the tallest parent variety, I-291/87. The mutant SCM-30 showed thicker cane diameter than the other mutants and controls. All mutants had higher Brix index than the parent varieties (Table XIII). The four selected mutants will be further evaluated for their delayed flowering characteristic.

**TABLE XI. AGRONOMIC PERFORMANCE, BRIX INDEX AND DISEASE REACTION OF SELECTED WATERLOGGING TOLERANT MUTANTS AT ISHURDI (1996–1997)**

| Varieties/ Strains | No. of tillers/hill | Cane height (cm) | Cane diameter (cm) | No. of nodes bearing adv. roots | Brix index (%) | Cane yield (t/ha) | Disease score** |
|---|---|---|---|---|---|---|---|
| SCM-8 | 4.8 abc | 101 cd | 1.48 d | 7.1 a | 15.4 bcd | 56 a | MR |
| *SCM-12* | 5.8 a | 95 d | 1.59 bcd | 4.6 bc | 16.9 abc | 52 ab | MR |
| *SCM-13* | 2.7 cd | 88 d | 1.55 bcd | 6.9 a | 15.7 bcd | 45 c | MR |
| SCM-14 | 2.7 cd | 133 a | 1.53 cd | 7.1 a | 16.9 abc | 44 c | MR |
| SCM-15 | 2.8 bcd | 129 a | 1.85 a | 5.8 ab | 18.1 ab | 47 b | MR |
| Nagarbari* | 3.8 abc | 103 bcd | 1.67 b | 3.8 c | 13.9 d | 41 d | S |
| Latarijaba* | 5.0 ab | 123 ab | 1.64 bc | 6.3 a | 19.1 a | 42 d | MS |
| Isd-2/54* | 4.5 abc | 117 abc | 1.64 bc | 5.9 ab | 14.9 cd | 46 d | MS |
| Isd-16* | 1.4 d | 95 d | 1.55 bcd | 6.9 a | 16.6 abcd | 40 d | MS |

*Control, **S (susceptible), MS (moderately susceptible), MR (moderately resistant)

**TABLE XII. AGRONOMIC CHARACTERS OF SELECTED $MV_2$ CANES AT MYMENSINGH (1996–1997)**

| Variety/ mutant | Dose (Gy) | No. of tillers/hill | Cane height (cm) | Cane diameter (cm) | Brix index (%) | Days to flower |
|---|---|---|---|---|---|---|
| I-291/87 | Control | 5.2 | 230 | 1.8 | 14.6 | 346 |
| I-291/87 | 20 | 6.1 | 224 | 1.9 | 14.8 | 406 |
| I-291/87 | 30 | 5.3 | 219 | 1.8 | 15.1 | 436 |
| I-291/87 | 40 | 5.2 | 235 | 1.9 | 15.7 | 421 |
| I-525/85 | Control | 6.3 | 235 | 2.0 | 15.0 | 357 |
| I-525/85 | 20 | 7.1 | 240 | 2.2 | 15.8 | 420 |
| I-525/85 | 30 | 5.8 | 238 | 2.0 | 15.6 | 427 |
| I-525/85 | 40 | 6.8 | 242 | 2.1 | 15.5 | 458 |

**TABLE XIII. AGRONOMIC CHARACTERS OF SELECTED $MV_3$ CANES OF SUGARCANE AT MYMENSINGH (1996–1997)**

| Variety/ Mutant | No. of tillers/hill | Cane height (cm) | Cane diameter (cm) | Brix index (%) | Days to flower |
|---|---|---|---|---|---|
| SCM-27 | 5.1 | 219 | 1.7 | 16.8 | 405 |
| SCM-28 | 4.7 | 217 | 1.6 | 16.1 | 435 |
| SCM-29 | 5.5 | 232 | 1.7 | 16.7 | 418 |
| SCM-30 | 6.2 | 235 | 2.0 | 16.8 | 420 |
| I-291/87 control | 4.2 | 225 | 1.6 | 15.6 | 340 |
| I-525/85 control | 5.3 | 230 | 1.8 | 16.0 | 358 |

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## REFERENCES

[1] IQBAL, M., MALEK, M.A. and RAHMAN, S. Incidence of sugarcane diseases at Sugarcane Research and Training Institute Farm. Bangladesh J. Sugarcane, **8** (1996) 72–74.

[2] AHMED, H.U., KHAN, M.A. and MIAH, I.H. Sugarcane diseases in Bangladesh. SPN. **15/16** (1976) 51.

[3] AGNIHOTRI, V.P. and SINGH, K. Seed piece transmissible diseases of sugarcane and their chemical control. Sugar News **2** (1977) 90–95.

[4] ANONYMOUS. Annual Report for the period 1973–1978 (Special issue). Sugarcane Research Institute, Ishurdi, Pabna, Bangladesh (1979) 95.

[5] AGNIHOTRI, V.P. Diseases of sugarcane. Oxford & IBM Publishing Co. New Delhi (1983) 52.

[6] RAMALINGA SWAMY, K., SWAMY, N.R., JAMUNA, P. and VEERABHADRARAO, K. Screening of some sugarcane cultivars for nutrient (NPK) absorption and yield under waterlogged condition. Bangladesh J. Sugarcane **16** (1994) 171–175.

[7] MIAH, M.A.S. and SARKAR, M.A.A. Effect of flowering on the quality of sugarcane. Bangladesh J. Sugarcane **3** (1982) 25–28.

[8] HUTCHINSON, P.B. A note on disease resistance ratings for sugarcane varieties. Proc. Intnatl. Soc. Sugarcane Tech. **13** (1978) 1087–1089.

[9] ALEXANDER, K.C. Technical Report 1989/90 on plant diseases. All India Co-ordinated Research Project on Sugarcane. Indian Council of Agricultural Research. Indian Institute of Sugarcane Research, Lucknow (UP) (1990) 20–23.

[10] SRIVASTAVA, B.L., BHATT, S.R. and SRIVASTAVA, V.K. Successful mutagenesis in sugarcane. Mutation Breed. Newslett. **37** (1991) 14–15.

[11] SHAIKH, M. A. Q., SHAMSUZZAMAN, K. M., MAJID, M. A. and HOWLIDER, M. A. R. Radiation sensitivity studies in sugarcane and selection of mutants with resistance/tolerance to red rot disease. Bangladesh J. Nuclear Agric. **11** (1995) 37–45.

[12] DARMODOJO, J.R. and SEEDJONO, M. Induction of mosaic disease resistance in sugarcane by gamma ray irradiation. ISSCT Sugarcane Breeders' Newslett. **39** (1977) 4.

[13] JAGATHESAN, D., BALASUNDARAM, N. and ALEXANDER, K.C. Induced mutations for disease resistance in sugarcane. Proc. Res. Coord. Meet. Induced Mutations for Disease Resistance in Crop Plants, IAEA, Vienna (1974) 151.

[14] SINGH, R.K. and SINGH, D.N. Induced mutations for disease resistance in sugarcane. Mutation Breed. Newslett. **42** (1996) 20–21.

[15] AHLOOWALA, B. "*In vitro* mutagenesis for the improvement of vegetatively propagated plants". Proc. Symp. Induced Mutations and Molecular Techniques for Crop Improvement. IAEA, Vienna (1995) 531–541.

# Somaclonal variation and irradiation in sugarcane calli for selection against red rot, water-logged conditions and delayed or non-flowering characters

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**Abstract**. A protocol for callus induction and plant regeneration from primordial leaf culture was established in sugarcane cv. 'Isd-16'. The regenerated ($R_1$) plants were grown in field, and the subsequent propagations ($R_2$-$R_4$) were screened for resistance to red rot disease and waterlogged conditions. Three clones showed moderate resistance (MR) to red rot and 3 clones were tolerant to water-logging in $R_4$. In another experiment, callus cultures were irradiated with 2 to10 Gy gamma rays. The maximum regeneration was obtained from 3 Gy treatment. Of the 768 $R_1$ plants, 50 survived to maturity. $R_2$ and $R_3$ populations were selected for delayed or non-flowering types. Five $R_3$ canes showed delayed flowering.

## 1. INTRODUCTION

Cell and tissue culture techniques offer a great potential for inducing mutants. Somaclonal variants generated through callus culture may provide useful material for breeding programmes. It has been reported that the application of mutagens in tissue culture can play an important role in widening genetic variation. A few reports are available on the response of irradiated plant cells in tissue culture [1, 2, 3]. Regenerants from callus in sugarcane show a wide range in variation [4]. It has been reported that regeneration of desired genotypes is possible through *in vitro* mutagenesis in case of vegetatively propagated plants [5].

During rainy season in Bangladesh, about 50% sugarcane growing areas are submerged under water, which causes decrease of sugar content in canes and increases disease development, specially red rot. The present studies were undertaken to establish a protocol to regenerate plants from callus culture either with or without treatment with gamma rays. The ultimate objective was to produce variants, resistant to red rot disease and tolerance to waterlogged conditions, and with delayed or non-flowering.

## 2. MATERIALS AND METHODS

The explants, taken from the growing point within the leaf-sheath of the variety 'Isd-16', were surface sterilized with 70% ethanol. The explants were cultured for callus induction on modified MS medium [6], supplemented with 3 mg/l 2, 4-D, 10% coconut water, and 20g/l sucrose. Calli, 15 to 20 days old, were subcultured on the same medium for proliferation. After 3–4 weeks of subculturing, the calli were transferred to MS medium supplemented with 5 mg/l IAA and 2 mg/l kinetin for shoot induction. MS medium, supplemented with 5 mg/l NAA and 70 g/l of sucrose, was used for root induction. For shoot and root induction, 12hr photoperiod was maintained with fluorescent tube lighting. In all cases, pH of the medium was adjusted to 5.6.

In the first set, two-month old non-irradiated $R_1$ plants were planted at Mymensingh during 1994–95 for screening against red rot. Further selections were done in $R_2$, $R_3$ and $R_4$

during 1995–96, 1996–97 and 1997–98, respectively. For screening against red rot disease $R_2$, $R_3$ and $R_4$ generations were grown in plant progeny rows at Ishurdi. Row to row and hill to hill spacing was 100 cm and 30 cm, respectively. Artificial inoculation with red rot spore suspension was done on 8–9 month old canes in early generation by using the Plug Method [7]. In the second set, selection was done under waterlogged conditions. To select canes under waterlogged conditions, $R_1$ to $R_4$ generations were grown at BINA farm, Mymensingh during 1994–98. The experimental plots in each generation were kept under approximately 30cm water for 4–5 months. Hills showing green leaves and moderate growth under this condition were selected. Data were recorded in all generations on cane height, number of tillers/hill, cane diameter, number of adventitious roots, Brix index and disease reaction.

In the third set, 3–4 week old calli, obtained from the same variety, were irradiated with doses 2, 3, 4, 5, 6, 7, 8 and 10 Gy gamma rays. Callus survival, number of shoots obtained from callus, and the frequency of regenerated plants were recorded. $R_1$ plants, regenerated from irradiated calli, were grown at BINA farm, Mymensingh during 1995–96 for selecting delayed or non-flowering types. In $R_2$ and $R_3$ generation, the canes, which did not flower after two months of the controls, were selected.

## 3. RESULTS AND DISCUSSION

### 3.1. Selection against red rot disease

Of the 368 $R_1$ inoculated canes, only 3 were moderately resistant, and 2 were moderately susceptible to red rot disease (Table I). Selected canes were further screened in the subsequent propagation. In $R_2$ generation, 5 canes were moderately resistant to red rot disease (Table II). Of these, three were from $R_2P_1$, one was from $R_2P_2$ and one from $R_2P_3$. One cane from $R_2P_2$ was disease susceptible. The co-efficient of variation was the highest for adventitious roots at maturity, and lowest for Brix index.

In $R_3$ generation (Table III) of the 10 hills, only 2 hills were moderately resistant to red rot disease and the rest were susceptible. In $R_4$ generation, only three hills were moderately resistant e and remaining were susceptible (Table IV).

**TABLE I. RED ROT DISEASE REACTION OF $R_1$ PLANTS OF SUGARCANE AT MYMENSINGH, 1994–1995**

| Line No. | No. of hills | No. of plants inoculated | Moderately susceptible | Moderately resistant | Resistant |
|---|---|---|---|---|---|
| 1 | 142 | 70 | 2 | 1 | - |
| 2 | 156 | 75 | - | - | - |
| 3 | 137 | 52 | - | - | - |
| 4 | 165 | 50 | - | - | - |
| 5 | 174 | 73 | - | 2 | - |
| 6 | 162 | 48 | - | - | - |
| **Total** | **936** | **368** | **2** | **3** | **-** |

**TABLE II. AGRONOMIC CHARACTERS AND BRIX INDEX, AND REACTION TO RED ROT OF SELECTED $R_2$ CANES AT ISHURDI, 1995–1996**

| Selected lines | Tiller No. | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Adv. root no. | Brix index (%) | Disease score* |
|---|---|---|---|---|---|---|---|---|
| Isd-16-$R_2P_1H_1$ | 8 | 145 | 2.2 | 2.1 | 1.8 | 3.0 | 20.0 | MR |
| Isd-16-$R_2P_1H_2$ | 6 | 167 | 2.2 | 2.0 | 1.8 | 1.5 | 20.0 | MR |
| Isd-16-$R_2P_1H_3$ | 7 | 208 | 2.2 | 2.2 | 2.0 | 1.5 | 21.1 | MR |
| Isd-16-$R_2P_2H_1$ | 8 | 227 | 2.4 | 2.1 | 1.8 | 1.5 | 19.0 | S |
| Isd-16-$R_2P_2H_2$ | 8 | 200 | 2.4 | 2.2 | 2.2 | 0.5 | 19.2 | MR |
| Isd-16-$R_2$ $P3H_1$ | 10 | 213 | 2.5 | 2.4 | 2.2 | 2.0 | 21.0 | MR |
| Isd-16 control | 5 | 218 | 2.5 | 2.2 | 2.0 | 3.7 | 20.0 | MR |

* susceptible (S); moderately resistant (MR)

**TABLE III. AGRONOMIC CHARACTERS AND BRIX INDEX OF SELECTED $R_3$ CANES SCREENED AGAINST RED ROT DISEASE, ISHURDI, 1996–1997**

| Selected lines | Tiller no. | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Brix index (%) | Disease score* |
|---|---|---|---|---|---|---|---|
| Isd-16 $R_3H_1$ | 9 | 193 | 2.8 | 2.1 | 2.0 | 20.0 | S |
| Isd-16-$R_3H_2$ | 8 | 220 | 2.4 | 2.4 | 2.2 | 19.8 | S |
| Isd-16-$R_3H_3$ | 12 | 140 | 2.1 | 1.9 | 1.1 | 20.0 | S |
| Isd-16-$R_3H_4$ | 9 | 275 | 2.2 | 2.4 | 2.2 | 19.3 | S |
| Isd-16-$R_3H_5$ | 10 | 305 | 2.2 | 2.0 | 1.9 | 19.8 | MR |
| Isd-16-$R_3H_6$ | 7 | 144 | 2.4 | 2.2 | 2.1 | 20.2 | MR |
| Isd-16-$R_3H_7$ | 8 | 290 | 2.2 | 1.6 | 1.8 | 18.0 | S |
| Isd-16-$R_3H_8$ | 9 | 261 | 2.6 | 2.6 | 2.0 | 18.2 | S |
| Isd-16-$R_3H_9$ | 11 | 266 | 2.4 | 2.4 | 2.1 | 19.2 | S |
| Isd-16-$R_3H_{10}$ | 10 | 260 | 2.6 | 2.3 | 2.2 | 20.1 | S |
| Isd-16 control | 9 | 223 | 2.2 | 2.3 | 2.1 | 20.6 | S |

*susceptible (S); moderately resistant (MR)

**TABLE IV. AGRONOMIC CHARACTERS, BRIX INDEX AND DISEASE REACTION TO RED ROT OF SELECTED $R_4$ CANES**

| Selected lines | Tiller no. | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Brix Index (%) | Score |
|---|---|---|---|---|---|---|---|
| Isd-16-$R_4H_1$ | 6 | 140 | 2.0 | 1.8 | 1.6 | 18.5 | S |
| Isd-16-$R_4H_2$ | 5 | 175 | 1.8 | 1.4 | 1.4 | 16.5 | S |
| Isd-16-$R_4H_3$ | 4 | 160 | 1.8 | 1.4 | 1.3 | 19.5 | MR |
| Isd-16-$R_4H_4$ | 5 | 165 | 1.9 | 1.8 | 1.8 | 19.5 | S |
| Isd-16-$R_4H_5$ | 6 | 145 | 2.1 | 2.0 | 1.4 | 19.2 | MR |
| Isd-16-$R_4H_6$ | 4 | 155 | 2.1 | 1.8 | 1.5 | 16.5 | S |
| Isd-16-$R_4H_7$ | 6 | 170 | 1.8 | 2.1 | 1.8 | 17.5 | S |
| Isd-16-$R_4H_8$ | 5 | 163 | 1.7 | 2.1 | 1.4 | 18.5 | S |
| Isd-16-$R_4H_9$ | 6 | 175 | 2.1 | 2.2 | 1.5 | 20.5 | MR |
| Isd-16-$R_4H_{10}$ | 5 | 189 | 1.9 | 1.4 | 1.7 | 14.5 | S |
| Isd-16 control | 4 | 160 | 1.8 | 1.7 | 1.5 | 16.5 | S |

*susceptible (S); moderately resistant (MR).

### 3.2. Selection under waterlogged conditions

From the three canes selected from $R_1$ generation, eight hills survived up to maturity (Table V). Of these, two from $R_2P_5$ and two from $R_2P_6$ were tolerant to waterlogged conditions; the remaining susceptible. The number of adventitious roots showed the highest coefficient of variation (49.1%), followed by cane diameter at the basal portion (18.2%) and top (16.6%).

**TABLE V. AGRONOMIC CHARACTERS AND BRIX INDEX OF SELECTED $R_2$ CLONES UNDER WATERLOGGED CONDITIONS AT MYMENSINGH, 1995–1996**

| Selected lines | Tiller No. | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Adv. roots | Brix index (%) | Score* |
|---|---|---|---|---|---|---|---|---|
| Isd-16-$R_2P_4H_1$ | 5 | 144 | 2.2 | 1.8 | 1.5 | 2.5 | 17.0 | HS |
| Isd-16-$R_2P_4H_2$ | 7 | 152 | 2.4 | 2.0 | 1.8 | 5.0 | 16.0 | HS |
| Isd-16-$R_2P_5H_1$ | 5 | 154 | 2.4 | 2.1 | 1.6 | 7.0 | 18.2 | T |
| Isd-16-$R_2P_5H_2$ | 9 | 141 | 1.9 | 1.8 | 1.6 | 7.0 | 17.2 | T |
| Isd-16-$R_2P_6H_1$ | 8 | 128 | 1.5 | 1.4 | 1.1 | 3.0 | 18.4 | HS |
| Isd-16-$R_2P_6H_2$ | 9 | 105 | 1.8 | 1.4 | 1.2 | 1.5 | 17.2 | HS |
| Isd-16-$R_2P_6H_3$ | 7 | 166 | 2.1 | 1.8 | 1.5 | 6.0 | 19.0 | T |
| Isd-16-$R_2P_6H_4$ | 5 | 173 | 2.7 | 1.8 | 1.5 | 3.0 | 17.3 | T |
| Isd-16 control | 6 | 212 | 2.6 | 2.3 | 2.2 | 2.5 | 19.7 | HS |

*HS- highly susceptible and T- tolerant to water logged conditions.

In $R_3$ generation, of the nine hills only one was tolerant and three were highly susceptible; the remaining were susceptible to waterlogged conditions (Table VI).

**TABLE VI. AGRONOMIC CHARACTERS AND BRIX INDEX OF SELECTED $R_3$ CANES SCREENED AGAINST WATERLOGGED CONDITIONS AT MYMENSINGH, 1996–1997**

| Clone No. | Tiller No. | Cane height (cm) | Cane diameter (cm) Base | Middle | Top | Adv. roots | Brix index (%) | Score |
|---|---|---|---|---|---|---|---|---|
| Isd-16-$R_3H_1$ | 8 | 104 | 1.7 | 1.6 | 1.5 | 1.3 | 20.1 | HS |
| Isd-16-$R_3H_2$ | 10 | 154 | 2.2 | 2.1 | 1.9 | 2.0 | 19.0 | S |
| Isd-16-$R_3H_3$ | 6 | 152 | 2.2 | 2.0 | 1.9 | 1.3 | 17.3 | HS |
| Isd-16-$R_3H_4$ | 7 | 160 | 1.9 | 1.5 | 1.8 | 2.0 | 17.2 | S |
| Isd-16-$R_3H_5$ | 10 | 169 | 2.6 | 2.0 | 1.8 | 3.0 | 13.2 | HS |
| Isd-16-$R_3H_6$ | 8 | 173 | 2.3 | 2.1 | 1.9 | 1.0 | 19.0 | T |
| Isd-16-$R_3H_7$ | 9 | 140 | 2.0 | 1.7 | 1.6 | 1.0 | 19.5 | S |
| Isd-16-$R_3H_8$ | 8 | 170 | 2.3 | 2.1 | 2.1 | 2.3 | 19.1 | S |
| Isd-16-$R_3H_9$ | 7 | 147 | 2.1 | 2.0 | 1.8 | 2.0 | 19.2 | S |
| Isd-16 control | 5 | 153 | 2.1 | 1.9 | 1.9 | 2.0 | 19.0 | HS |

*HS- highly susceptible, T- tolerant and S- susceptible to water logged conditions.

In $R_4$ generation, 10 hills survived up to maturity. Of these, three hills were tolerant, three highly susceptible, and the remaining susceptible under waterlogged conditions (Table VII).

**TABLE VII. AGRONOMIC CHARACTERS AND BRIX INDEX OF SELECTED $R_4$ CANES SCREENED UNDER WATERLOGGED CONDITIONS**

| Clone No. | Tiller No. | Cane height | Green leaves | Cane diameter (cm) | | | Adv. roots | Brix Index (%) | Score* |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Base | Middle | Top | | | |
| Isd-16-$R_4$ $H_1$ | 6 | 173 | 6 | 2.1 | 1.8 | 1.5 | 4 | 20.0 | T |
| Isd-16-$R_4$ $H_2$ | 7 | 205 | 4 | 1.9 | 1.7 | 1.3 | 3 | 19.8 | HS |
| Isd-16-$R_4$ $H_3$ | 8 | 187 | 6 | 1.7 | 1.6 | 1.4 | 5 | 21.3 | T |
| Isd-16-$R_4$ $H_4$ | 6 | 183 | 5 | 2.1 | 1.8 | 1.4 | 4 | 20.8 | S |
| Isd-16-$R_4$ $H_5$ | 7 | 200 | 5 | 2.4 | 1.7 | 1.6 | 2 | 18.5 | S |
| Isd-16-$R_4$ $H_6$ | 5 | 192 | 4 | 2.2 | 1.9 | 1.5 | 4 | 20.5 | S |
| Isd-16-$R_4$ $H_7$ | 7 | 218 | 4 | 2.2 | 1.8 | 1.4 | 3 | 19.2 | HS |
| Isd-16-$R_4$ $H_8$ | 6 | 164 | 5 | 2.1 | 1.9 | 1.5 | 2 | 20.5 | **S** |
| Isd-16-$R_4$ $H_9$ | 7 | 190 | 6 | 2.3 | 1.8 | 1.6 | 3 | 20.9 | T |
| Isd-16-$R_4$ $H_{10}$ | 8 | 195 | 4 | 2.1 | 1.7 | 1.4 | 4 | 20.5 | S |
| Isd-16 control | 6 | 175 | 4 | 1.9 | 1.6 | 1.6 | 2 | 18.0 | HS |

*HS- highly susceptible, T- tolerant and S- susceptible to water logged conditions.

**TABLE VIII. EFFECT OF GAMMA RAYS ON CALLUS REGENERATION IN SUGARCANE**

| Doses (Gy) | Calli irradiated No. | Callus survival after 21 days (%) | Calli regenerating shoots (%) | Plants obtained No. |
|---|---|---|---|---|
| 2 | 90 | 91 | 45 | 202 |
| 3 | 90 | 87 | 50 | 255 |
| 4 | 90 | 78 | 28 | 140 |
| 5 | 90 | 71 | 23 | 92 |
| 6 | 90 | 70 | 11 | 44 |
| 7 | 90 | 66 | 8 | 35 |
| 8 | 90 | 62 | - | - |
| 10 | 90 | 58 | - | - |

## 2.2. Selection for delayed/non-flowering

In $R_1$, derived from irradiated calli, survival decreased with increase in radiation dose (Table VIII). Shoots were regenerated from all callus cultures except those treated with 8 and 10 Gy. The survival ranged from 58 to 91% and regeneration ability varied from 8 to 50%. The maximum regeneration was from 3 Gy dose. Of the 768 $R_1$ plants, only 50 survived up to maturity from calli treated with 2 or 3 Gy dose, and these were propagated further to $R_2$ generation. Of the 50 $R_2$ plants, 9 flowered after 2 months of the control and were propagated further. In $R_3$, only 5 canes flowered after 2 months of the control (Table IX). The mutant Isd-16-$R_3H_4$ flowered after 425 days.

**TABLE IX. AGRONOMIC CHARACTERS OF SELECTED $R_3$ CLONES OF SUGARCANE, MYMENSINGH, 1997–1998**

| Clone No. | Tillers No. | Cane height (cm) | Cane diameter (cm) | Brix index (%) | Days to flower |
|---|---|---|---|---|---|
| Isd-16-$R_3$ $H_1$ | 5 | 212 | 1.8 | 20.0 | 412 |
| Isd-16-$R_3$ $H_2$ | 7 | 217 | 2.1 | 21.5 | 423 |
| Isd-16-$R_3$ $H_3$ | 6 | 215 | 1.7 | 18.5 | 415 |
| Isd-16-$R_3$ $H_4$ | 8 | 210 | 2.0 | 20.5 | 425 |
| Isd-16-$R_3$ $H_5$ | 7 | 220 | 1.9 | 19.5 | 419 |
| Isd-16 control | 6 | 210 | 1.6 | 18.5 | 343 |

## REFERENCES


[1] SRIPICHITT, P., NAWATA, E. and SHIGENAGA, S. Radiation induced mutations by using *in vitro* adventitious bud technique in red pepper (*Capsicum annum* L.) cv. Yaysufusa. Analysis of the variant appeared in $M_1$ generation. Japan. J. Breed. **38** (1988) 141–150.

[2] BALITTO, L.P., HATTORI, K. and FUTSUHARA, Y. Effects of gamma ray irradiation on growth of calli in *Nicotiana* species. Japan J. Breed. **39** (1989) 29–38.

[3] AHMED, G. and ISLAM, M.S. Effect of gamma radiation on callus growth and plant regeneration in *Corchorus capsularis* jute. Plant Tissue Cult. **1** (1991) 67–73.

[4] LIU, M.C. and CHEN W.H. Tissue and cell culture as aids to sugarcane breeding 1. Creation of genetic variation through tissue culture. Euphytica **25** (1976) 393–402.

[5] AHLOOWALIA, B.S. "*In vitro* mutagenesis for the improvement of vegetatively propagated plants." Proc. Joint FAO/IAEA Internatl. Symp. "Induced Mutations and Molecular Techniques for Crop Improvement", IAEA, Vienna (1995) 531–541.

[6] HEINZ, D.J. and MEE, G.W.P. Plant differentiation from callus tissue of *Saccharum* species. Crop. Sci. 9 (1969) 346–348.

# *In vitro* selection of mutants: Inducible gene regulation for salt tolerance

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**Abstract.** Regulation of differentially expressed genes in plants may be involved in inducing tolerance to stress. Isogenic salt-sensitive and salt-tolerant alfalfa lines were investigated for molecular differences in their response to salt. The genes, which are differentially induced by salt in the salt-tolerant alfalfa cells and are also regulated by salt at the whole plant level, were cloned. Both transcriptional and post-transcriptional mechanisms influenced salt-induced product accumulation in the salt-tolerant alfalfa. The salt-tolerant plants doubled proline concentration rapidly in roots, while salt-sensitive plants showed a delayed response. To understand the regulatory system in the salt-tolerant alfalfa, two genes that are expressed in roots were studied. *Alfin1* encodes a zinc-finger type putative DNA transcription factor conserved in alfalfa, rice and *Arabidopsis*, and *MsPRP2* encodes a protein that serves as a cell wall-membrane linker in roots. Recombinant *Alfin1* protein was selected, amplified, cloned and its consensus sequence was identified. The recombinant *Alfin1* also bound specifically to fragments of the *MsPRP2* promoter *in vitro*, containing the *Alfin1* binding consensus sequence. The results show unambiguously binding specificity of *Alfin1* DNA, supporting its role in gene regulation. *Alfin1* function was tested in transformed alfalfa *in vivo* by over-expressing *Alfin1* from 35S CaMV promoter. The transgenic plants appeared normal. However, plants harboring the anti-sense construct did not grow well in soil, indicating that *Alfin1* expression was essential. *Alfin1* over-expression in transgenic alfalfa led to enhanced levels of *MsPRP2* transcript accumulation, demonstrating that *Alfin1* functioned *in vivo* in gene regulation. Since *MsPRP2* gene is also induced by salt, it is likely that *Alfin1* is an important transcription factor for gene regulation in salt-tolerant alfalfa, and an excellent target for manipulation to improve salt tolerance.

## 1. INTRODUCTION

Salt affects approximately a third of the arable land in the world. As more land becomes saline through poor irrigation practices, the impact of salinity on crop production is becoming an increasingly serious problem world wide, and has created a pressing need to improve salt-tolerance in plants. Improvement for salinity and drought tolerance of crop plants by genetic means has been an important but largely unfulfilled aim of modern agriculture. Other approaches have focused on the selection for improved physiological characteristics and metabolic pathways [1, 2].

An alternate genetic strategy has been pursued for improving salinity tolerance by combining selection of salt-tolerant cells in culture, followed by regeneration of salt-tolerant plants, and identification and testing of genes important in conferring salt tolerance. While most of the research work was done on alfalfa, the selection method is also applicable in rice [3, 4]. The regulation of differentially expressed genes is vital during development and differentiation, hence, mutations in these processes have the potential to provide tolerance against plant stress. The hypothesis in selection for salt tolerance is that over-expression of genes for any physiological system that becomes limiting under salt stress is likely to provide increased salt tolerance in cells and the plant. Incremental improvement in salt tolerance could be obtained from enhanced expression of genes in different physiological systems [4, 5]. Therefore, isogenic salt-sensitive and salt-tolerant alfalfa cells and plants were used for molecular studies to

investigate the differences in their responses to salt. The multigenic differences in regulation of endogenous genes [6] are consistent with the concept that salt tolerance is a quantitative trait [7] and the induction of many genes during salt/drought stress of plants [8, 9]. Since the salt tolerance trait in the mutants studied is heritable [10, 11], the multigenic responses indicate that the mutation is likely in the regulatory system. This leads to changes in gene regulation, associated with the ability to survive and grow under otherwise lethal conditions.

Previous studies with rice [11] and alfalfa [12] have demonstrated that cellular salt-tolerance can provide protection at the whole plant level, and can be a useful tool in selection for tolerance. When successful, the cellular selection and regeneration approach relies on identification of mutants optimized for continued survival and productive growth under saline conditions. However, it does not provide ready identification of the genes involved, and requires additional information about altered regulation of the genes [13, 14]. Several genes have been cloned that are differentially induced by salt in cells of the salt-tolerant alfalfa and are also regulated by salt at the whole plant level. In the present study, the root and shoot response to salt stress was investigated for proline accumulation as a measure of osmo-protection in the tolerant phenotype. Both the transcriptional and post-transcriptional mechanisms that contribute to gene product accumulation in the tolerant alfalfa growing in salt were studied. To understand the changed regulatory system in alfalfa, two genes, *Alfin1* and *MsPRP* that are expressed in roots were studied. *Alfin1* encodes a zinc-finger type putative DNA transcription factor, conserved in alfalfa, rice and *Arabidopsis*. *MsPRP2* encodes a protein that might serve as a cell wall-membrane linker in roots. The function of *Alfin1* was established as a transcriptional regulator by *in vitro* binding of the recombinant protein to specific DNA sequences, including fragments of the *MsPRP2* promoter [15]. The transgenic alfalfa over-expressing *Alfin1* from the 35S cauliflower mosaic virus (CaMV) promoter have increased levels of *MsPRP2* transcripts; thus *Alfin1* functions also *in vivo* as a regulator in gene expression [16]. Hence, it was interesting to characterize the role of *Alfin1* in salt tolerance and general gene regulation, since *MsPRP2* is salt-inducible *in vivo.*

## 2. MATERIALS AND METHODS

Alfalfa, *Medicago sativa* L. cv. 'Regen S', salt-sensitive callus cultures, selection of salt-tolerant lines and regenerated salt-tolerant plants have been previously described [10, 17]. Selection of salt-tolerant rice lines from two elite US rice lines (L-202 and M-202) and regeneration and testing of plants from the salt-tolerant lines was done as described previously [11]. Samples for molecular analyses of all tissues were collected at the same time during day to avoid influences of the circadian rhythm. Standard molecular biology protocols were used [18].

Recombinant *Alfin1* was expressed in *Escherichia coli* BL21(DE3), using the PET-29b vector system (Novagen Inc.). The purified S-Tag-Alfin1 fusion protein was used in DNA binding assays. Purified protein was used in the "random DNA binding assay" to identify specific DNA binding sites recognized by the recombinant Alfin1. To select binding sites from a 67 bp DNA, a 25 bp degenerate sequence was flanked by a known sequence of DNA with restriction sites and the template for PCR primers. A single protein bound DNA band was isolated by gel retardation [19] and amplified by PCR. The bound sequence was enriched by sequential selection of *Alfin1* bound PCR amplified DNA. The final round of bound DNA was cloned, and the DNA binding sequence was determined as the consensus sequence represented in the insert of all individual clones that were able to bind recombinant *Alfin1*.

The gene construct for the over-expression and under-expression of *Alfin1* was developed using the *Alfin1* cDNA [12]. The coding sequence was placed downstream of the 35S cauliflower-mosaic virus (CaMV) promoter in the sense and anti-sense orientation. The constructs were transferred into a binary vector that also contained kanamycin-resistance gene, and transformation of alfalfa leaf-discs was accomplished with *Agrobacterium tumefaciens*. Transformed cells were selected on medium containing kanamycin, and transgenic plants were regenerated.

## 3. RESULTS AND DISCUSSION

### 3.1. Cellular selection and regeneration to obtain salt tolerant plants

The cellular selection and regeneration protocol was extended to rice to investigate if the protocol developed for alfalfa [10, 17] was applicable to other species. Cell lines tolerant to 1% (0.171 M) NaCl were obtained from calli initiated from salt-sensitive US elite rice lines L202 and M202, as well as indica rice varieties, 'Pokkali', 'IR28' and 'IR42'. Several plants were regenerated from salt-tolerant lines L-202, M-202 and 'Pokkali'. Seed was collected from four plants regenerated from the salt-tolerant L-202 line and two plants regenerated from the salt-tolerant 'Pokkali'. $R_2$ seedlings were germinated and tested for salt tolerance. Heritable improvement in salt tolerance was obtained in $R_2$ seedlings from one L-202 ($R_4$) plant that had been regenerated after 5 months selection on salt-containing medium. The salt tolerance of these seedlings was comparable to that obtained from 'Pokkali' under conditions where the unselected L-202 seedlings died [11]. The results indicated that improved cellular salt-tolerance could also provide increased salt-tolerance in rice at the whole plant level.

### 3.2. Salt dependent gene regulation in salt-tolerant alfalfa

The characterization of altered gene expression in salt-tolerant alfalfa by salt resulted in identification of a number of genes that are "up-regulated" by salt (Table I). Since, the salt-tolerant phenotype in alfalfa is a heritable semi-dominant trait [10], the altered regulation of many genes must be the result of a mutation in its regulatory pathway. The selected salt tolerant cell lines showed the interesting association of chloroplast activation and increased mRNA accumulation for photosynthesis related genes in the salt-tolerant phenotype. At least some of the mRNAs were translated into functional products. Detailed analysis, comparing steady state mRNA levels for salt induced genes in the tolerant alfalfa with nuclear and chloroplast "run-on" assays, was carried out to measure transcriptional activation. The analysis showed that both transcriptional and post-transcriptional regulation contributed to the increased levels of the gene products when the salt-tolerant cells are grown in salt [13]. It was also demonstrated that regulated tissue-specific expression overrode salt induction of mRNA accumulation for specific genes [20]. Interestingly, several of the genes cloned from callus appeared to function predominantly in the root (Table I).

The correlation of the osmoprotectant proline accumulation with salt-tolerance in the near-isogenic, salt-sensitive and salt-tolerant cell lines and the plants derived from them was investigated. Proline accumulation was studied under salt-stress for productive and non-productive growth in salt-tolerant and salt-sensitive alfalfa (Table II). Although, there was some variability in proline levels between the sensitive and tolerant plants, no consistent correlation could be drawn between high levels of proline in either roots or shoots of the salt-tolerant phenotype. However, both calli and roots [21] from salt-tolerant alfalfa accumulated proline very

rapidly on exposure to salt, while the response was delayed in roots of the salt-sensitive parent plant No. 1 (Table II), and of regenerated control plant, not selected on salt (data not shown). The rapid response in case of salt-tolerant alfalfa may have an early protective function, and apparently form a part of the multigenic response to salt-stress.

**TABLE I. ACCUMULATION OF mRNA IN SALT-TOLERANT ALFALFA**

| Gene | Function[a] | Tissue specificity | | |
|---|---|---|---|---|
| | | Callus | Root | Shoot |
| *Alfin1*[b] | nuclear transcription factor | + | + | - |
| *MsPRP2*[b] | nuclear cell wall protein | + | + | - |
| *pA18*[b] | nuclear unknown | + | - | ± |
| *rbcS*[c] | nuclear Rubisco, small subunit | + | + | nd |
| *rbcL*[c] | chloroplast Rubisco, large subunit | + | + | nd |
| *cab1*[c] | nuclear chlorophyll binding protein | + | nd* | nd |
| *psbA*[c] | chloroplast $Q_B$ binding protein | + | + | nd |
| *H3cI*[d] | nuclear histone H3 (replic. dep.) | + | nd | nd |
| *H3cII*[d] | nuclear histone H3 (replic. ind.) | + | nd | nd |
| *rps11*[c] | chloroplast ribosomal protein 11 | - | nd | nd |
| *Msc27*[d] | nuclear constitutive protein | - | - | - |

*Not determined. [a]Function shown or implied by sequence similarity with proteins of known function.
[b]Expressed in callus and predominantly in roots. [c]Expressed in tolerant callus and predominantly in shoots.
[d]Expressed in callus, roots and shoots.

### 3.3. Two gene products for improved salt tolerance

A major focus of the study was on two novel genes from differential screening of alfalfa cDNA library from mRNA isolated from salt-tolerant cells grown for several months in 171 mM NaCl. One is *Alfin1*, which encodes a 28.8 kDa regulatory factor [12] with the putative zinc finger-binding domain (Fig.1). *Alfin1* transcription and mRNA accumulation is induced by salt in the salt-tolerant cells [13]. In plants, this mRNA is detected primarily in roots.

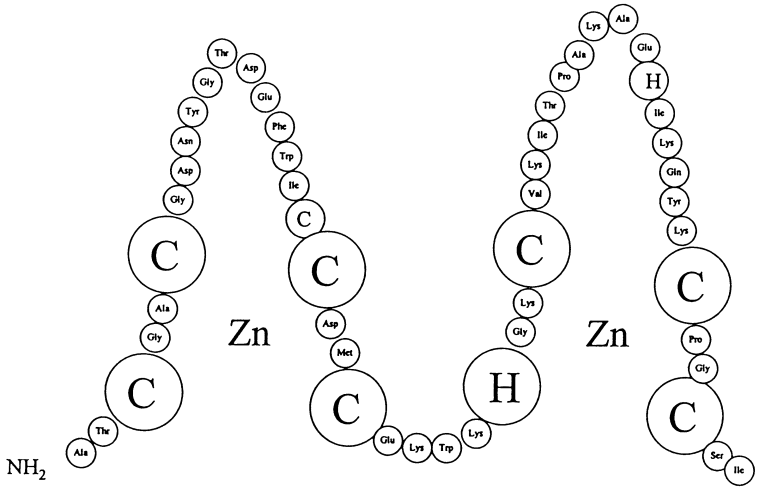
The other gene is *MsPRP2*, which encodes a proline-rich protein with a hydrophobic cysteine-rich carboxy terminus. This gene is also expressed predominantly in roots, and is specifically induced by long-term (i.e., one week) treatment with salt [22, 23]. The proline-rich amino terminal region of this protein has the characteristics of a cell wall component preceded by a cell wall targeting sequence, but the hydrophobic carboxy-terminus contains features characteristic of the nonspecific lipid transfer proteins. The bifunctional domains in *MsPRP2* could play a structural role as a wall-to membrane linker in plants, and thus contribute to structural integrity of the roots when exposed to salt.

The function of *Alfin1* as DNA-binding protein was investigated with the recombinant protein from *E. coli* [15] [Fig. 2] which was affinity purified, and used for selecting specific binding sequences from random DNA as described above.

**TABLE II. PROLINE ACCUMULATION IN ALFALFA EXPOSED TO 171 mM NACL**

| Duration after NaC1 exposure (hrs) | μg Proline/g fresh weight in plants | | | |
|---|---|---|---|---|
| | No. 1 (sensitive) Mean ± S.E. | No. 4T* Mean ± S.E. | No. 5T* Mean ± S.E. | No. 9T* Mean ± S.E. |
| Roots | | | | |
| 0 | 54 ± 44 | 103 ± 89 | 36 ± 15 | 47 ± 21 |
| 24 | 53 ± 29 | 170 ± 13 | 76 ± 13 | 91 ± 24 |
| 28 | 254 ± 168 | 430 ± 150 | 241 ± 173 | 336 ± 156 |
| 72 | 269 ± 27 | 513 ± 153 | 341 ± 181 | 271 ± 169 |
| 192 | 430 ± 118 | 675 ± 46 | 321 ± 86 | 182 ± 66 |
| Shoots | | | | |
| 0 | 126 ± 63 | 102 ± 63 | 97 ± 35 | 119 ± 58 |
| 24 | 206 ± 5 | 353 ± 80 | 530 ± 292 | 472 ± 49 |
| 48 | 407 ± 188 | 706 ± 277 | 1289 ± 188 | 898 ± 82 |
| 72 | 1174 ± 15 | 790 ± 170 | 1381 ± 92 | 921 ± 130 |
| 192 | 1174 ± 80 | 1151 ± 256 | 1163 ± 49 | 1328 ± 311 |

*Tolerant type.




**C**-$X_2$-**C**-$X_{11}$-C-**C**-$X_2$-**C**-$X_4$-**H**-$X_2$-**C**-$X_9$-H-$X_5$-**C**-$X_2$-**C**

*Fig. 1. Putative zinc finger-binding domain in the carboxy-terminus of Alfin1 protein as deduced from cDNA sequence (GenBank accession number L07291).*

Sequence analysis of nine individually isolated clones revealed a consensus binding sequence for *Alfin1* which consisted of two to five triplet repeats of G rich sequences containing at least one GTG element. Both *Alfin1* and *MsPRP2* are expressed primarily in roots. This raised the question about the possible DNA binding sites for the *Alfin1* protein in the *MsPRP2* promoter. Three *TfiI* fragments were isolated from the *MsPRP2* genomic clone containing the promoter (Fig. 3), and reacted *in vitro* with purified recombinant *Alfin1*.

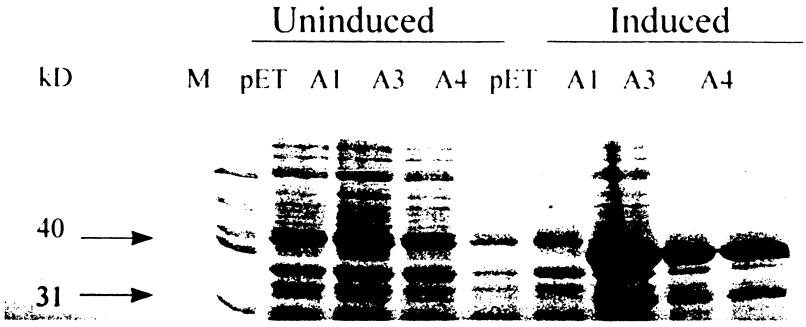



*Fig. 2. Expression of Alfin1 fusion protein in E. coli. SDS-PAGE analysis of proteins expressed in crude extract from clones of E. coli transformed either with pET (vector) or A1, A3 and A4 (pET-Alfin1 construct). Lane 1- Molecular weight markers. Lanes 2,3,4 and 5- Protein from non-induced cells. Lanes 6,7,8 and - Protein from cells induced for 3 hr with IPTG.*

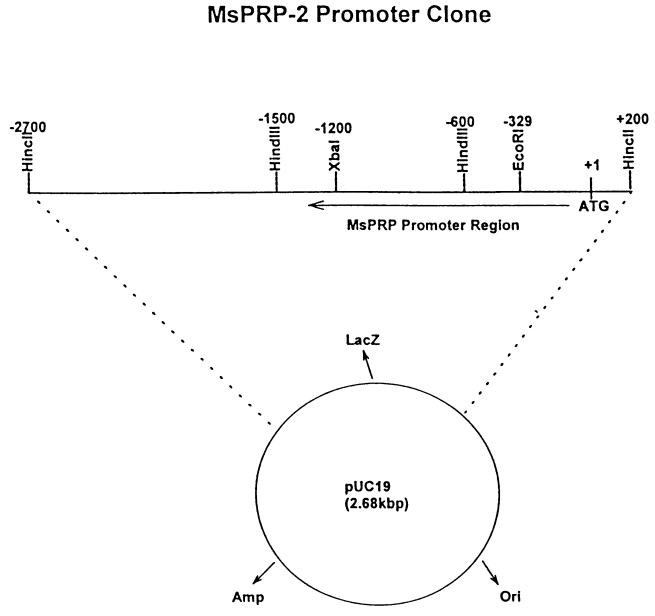



*Fig. 3. Restriction map and organization of MsPRP2 promoter region in alfalfa.*

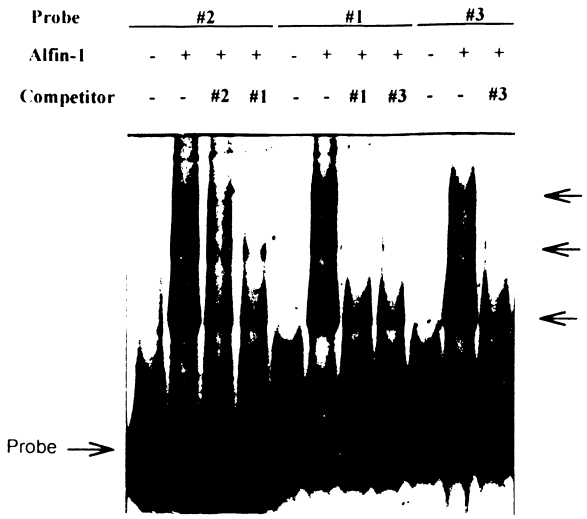



*Fig. 4. DNA binding properties of Alfin1 fusion protein purified from E. coli. Gel retardation assay using 10 ng (20,000 cpm) of each $^{32}P$ labeled TfiI fragment of MsPRP2 promoter with equal amounts of recombinant Alfin1. Competition reactions were performed by pre-incubating with a ten fold excess (100ng) of each competitor fragment and subsequent addition of the $^{32}P$ labeled probe. The three contiguous TfiI fragments start at position -244 and extend to -864 bp as shown in Fig. 3. Probe: No. 1, 211 bp; No. 2, 187 bp; No. 3, 222 bp.2.*

As shown in the gel retardation assay (Fig. 4), each of the *TfiI* fragments was able to bind recombinant *Alfin1* in a specific manner. This was demonstrated by fact that ten fold excess of the same fragment or one of the other fragments were able to compete the binding as shown by the diminished intensity of the retarded band(s). The sequence of each of the *TfiI* DNA fragments from *MsPRP2* was found to contain the *Alfin1* binding consensus sequence. A DNA fragment, which did not contain the *Alfin1* binding sequence, neither bound recombinant *Alfin1* nor was it able to act as a competitor for binding to each of the *MsPRP2* promoter fragments (data not shown). These results showed that *Alfin1* encodes a protein that bound DNA in a sequence specific manner *in vitro* and could act as a transcription factor with the *MsPRP2* gene as a potential endogenous *in vivo* target in alfalfa roots [15].

The *in vivo* function of *Alfin1* is currently being tested in transgenic alfalfa calli and in plants over-expressing *Alfin1* from constructs that transcribe *Alfin1* cDNA from the CaMV 35S promoter. The regenerated sense-plants appear normal despite the ubiquitous expression of *Alfin1* in both roots and shoots. Anti-sense plants develop poorly in soil, indicating that *Alfin1* expression is essential for growth and development. *Alfin1*over-expression in transgenic alfalfa leads to enhanced levels of *MsPRP2* transcript accumulation in callus and roots [16], indicating that *Alfin1* can act as a transcriptional activator for at least one salt inducible gene in alfalfa roots. The results suggest that *Alfin1* may be an important transcription factor involved in gene regulation in the salt-tolerant alfalfa. Future experiments will test the effect of *Alfin1* over-expression on other salt stress regulated genes, and investigate if *Alfin1* over-expression improves salt tolerance in alfalfa.

## REFERENCES


[1] FLOWERS, T.J. and YEO, A.R. Breeding for salinity resistance in crop plants: where next? Australian J. Plant Physiol. **22** (1995) 875–884.

[2] BOHNERT, H.J.and JENSEN, R.G. Metabolic engineering for increased salt tolerance - the next step. Australian J. Plant Physiol. **23** (1996) 661–667.

[3] WINICOV, I. "Enhanced gene expression in salt tolerance of alfalfa cell lines and regenerated plants". Control of Plant Gene Expression (VERMA, D.P.S., Ed.), CRC Press, Boca Raton, (1992) 301–310.

[4] WINICOV, I. New molecular approaches to improving salt tolerance in crop plants. Annals Bot. **81** (in press) (1998)

[5] WINICOV, I. "Gene expression in relation to salt tolerance". Stress Induced Gene Expression in Plants (BASRA, A.S., Ed.), Harwood Acad. Publishers (1994) 61-85.

[6] WINICOV, I., WATERBORG, J.H., HARRINGTON, R.E. and McCOY, T.J. Messenger RNA induction in cellular salt tolerance of alfalfa (*Medicago sativa*). Plant Cell Rep. **8** (1989) 6–11.

[7] FOOLAD, M.R. and JONES, R.A. Mapping salt-tolerance genes in tomato (*Lycopersicon esculentum)* using trait-based marker analysis. Theor. Appl. Genet. **87** (1993) 184–192.

[8] INGRAM, J. and BARTELS, D. The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47** (1996) 377–403.

[9] SHINOZAKI, K. and YAMAGUCHI-SHINOZAKI, K. Gene expression and signal transduction in water-stress response. Plant Physiol. **115** (1997) 327–334.

[10] WINICOV, I. Characterization of salt tolerant alfalfa (*Medicago sativa* L plants regenerated from salt tolerant cell lines. Plant Cell Rep. **10** (1991) 561–564.

[11] WINICOV, I. Characterization of rice (*Oryza sativa* L) plants regenerated from salt-tolerant cell lines. Plant Sci. **113** (1996) 105–111.

[12] WINICOV, I. cDNA encoding putative zinc finger motifs from salt-tolerant alfalfa (*Medicago sativa* L.) cells. Plant Physiol. **102** (1993) 681–682.

[13] WINICOV, I. and KRISHNAN, M. Transcriptional and post-transcriptional activation of genes in salt-tolerant alfalfa cells. Planta **200** (1996) 397–404.

[14] WINICOV, I. and BASTOLA, D.R. Salt tolerance in crop plants: new approaches through tissue culture and gene regulation. Acta Physiol. Plantarum **19** (1997) 435–449.

[15] BASTOLA, D.R., PETHE, V.V. and WINICOV, I. *Alfin1*, a novel zinc-finger protein in alfalfa roots that binds to promoter elements in the salt-inducible *MsPRP2* gene. Plant Mol. Biol. (in press) (1998).

[16] BASTOLA, D.R., PETHE, V.V. and WINICOV, I. *Alfin1*, a transcription factor that regulates the salt inducible *MsPRP2* gene in alfalfa roots. Plant Biology '98 (1998) 798a.

[17] WINICOV, I. Salinity/salt tolerance. Practical Approaches in Plant Cell Culture. (DIXON, R., GONZALES, R., Eds.), IRL Press, Oxford. (1994) 79–86.

[18] SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, (1989).

[19] HUANG, H., MIZUKAMI, Y., HU, Y. and MA, H. Isolation and characterization of the binding sequences for the product of the *Arabidopsis* floral homeotic gene AGAMOUS. Nucleic Acids Res. **21** (1993) 4769–4776.

[20] WINICOV, I. and SHIRZADEGAN, M. Tissue specific modulation of salt inducible gene expression: callus versus whole plant response in salt tolerant alfalfa. Physiol. Plantarum **100** (1997) 314–319.

[21] PETRUSA, L. and WINICOV, I. Proline Status in Salt-tolerant and Salt-sensitive Alfalfa Cell Lines and Plants in Response to NaCl. Plant Physiol. Biochem. **35** (1997) 303–310.

[22] WINICOV, I. and DEUTCH, C.E. Characterization of a cDNA clone from salt-tolerant alfalfa cells that identifies salt inducible root specific transcripts. J. Plant Physiol. **144** (1994) 222–228.
[23] DEUTCH, C.E. and WINICOV, I. Post-transcriptional regulation of a salt-inducible alfalfa gene encoding a putative chimeric proline-rich cell wall protein. Plant Mol. Biol. **27** (1995) 411–418.

# Use of radiation and *in vitro* techniques for development of salt tolerant mutants in sugarcane and potato

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**Abstract.** Sugarcane and potato are propagated vegetatively and are important crops in Pakistan. Protocols were established to initiate callus and regenerate plants in sugarcane and to multiply potato *in-vitro* from nodal segments. Cultures of potato and sugarcane were irradiated with 5, 20, 40, and 60 Gy. Increase in radiation dose above 20 Gy reduced regeneration capacity of sugarcane callus. Doses higher than 20 Gy were lethal to micropropagated plants of potato. Culture of irradiated sugarcane callus on media containing salt was tried, but no regeneration was obtained. Variants for tolerance to salinity were selected, and evaluated under saline field conditions at four locations. The study showed that the selected variants of both sugarcane and potato were sensitive to high levels of salinity. Variants tested within the same salinity treatment did not differ significantly from each other in the traits investigated. Only boron uptake in the variants was much higher on saline soil than on the normal soil. Polymorphism was detected among the variants by DNA fingerprinting using randomly amplified polymorphic DNA (RAPD) markers. RAPD analysis showed that most of the variants reverted back to normal type. It is concluded that a large number of variants need be screened to obtain the desired mutants.

## 1. INTRODUCTION

In Pakistan, of the 21.16 million hectares of cultivable land, 6.68 million ha have been classified as saline or saline sodic [2]. The area of salt affected land is increasing at an alarming rate. Sugarcane and potato are extremely sensitive to salinity that affects almost all phases of their plant growth, and decreases their yield adversely. Many strategies are available to cope with this problem. One durable solution to the problems of salinity and water logging in Pakistan lies in the establishment of an effective and comprehensive drainage system throughout the Indus Basin. These reclamation measures are highly energy and capital intensive. A complimentary strategy would be to modify plants to suit the environment. Germplasm evaluation, breeding for resistance and biotechnological approaches are being used to develop salt tolerant plants and biotic stress [4, 5, 14, 20]. Tissue culture techniques have been used to create genetic variability to improve crop plants [11]. Protocols of various *in-vitro* techniques are available for most crops, although optimization is still needed for some of them. *In-vitro* techniques of protoplast, microspore, anther, ovule and embryo culture have been used to create somaclonal and gametoclonal variation in the existing breeding lines.

Sugarcane and potato are vegetatively propagated, and are important cash crops of Pakistan. Sugarcane plays a significant role in sugar industry, and is grown on 965,000 ha with a production of 42 million tons [2]. Potato is grown over more than 90,000 and has production of

1.30 million tons [2]. *In vitro* culture techniques in association with induced mutations can speed up the creation of variation in vegetatively propagated crops [1, 23]. Keeping in view the importance of these two crops and the climatic conditions of Pakistan, radiation induced variation and *in vitro* techniques were tried to develop salt tolerant genotypes. Use of molecular markers as a tool to identify genetic variability is now a routine [16, 17,19]. The random amplified polymorphic DNA (RAPD) is being widely used because of its simplicity and efficiency [16, 19, 8]. RAPD analysis has been successfully used to detect somaclonal variation [18]. The present studies were undertaken to determine the effect of gamma radiation and saline medium on regeneration of sugarcane callus and on growth of micropropagated potato plants. Attempts were also made to detect genetic variation in the callus and among the regenerated variants of sugarcane.

## 2. MATERIALS AND METHODS

### 2.1. Sugarcane

Field grown plants of cv. 'CP-43/33' (a derivative of Col-54) were obtained from Sugarcane Research Institute, Ayub Agriculture Research Institute (AARI), Faisalabad. Young leaves from the innermost whorls of apical buds were used as explants. Callus cultures were initiated from the explants excised under aseptic conditions. The explants were sliced into 3-4 mm pieces, and transferred to 100 ml Magenta™ jars (Sigma) containing 20ml callus induction medium [15]. Cultures were incubated in dark at $25\pm2^0$C. The calli were maintained and proliferated on the same medium by subculture every 3-4 weeks. Five-week old, yellow to white nodular embryogenic calli were irradiated with five different doses (0, 5, 20, 40 and 60 Gy) of gamma rays from a $^{60}$Co-source at the Nuclear Institute of Agriculture and Biology, NIAB, Faisalabad. Irradiated and non-irradiated calli were transferred to regeneration medium [15], and maintained under 16-hr photoperiod. The regenerated shoots were transferred to rooting medium [15]. After root formation, the plantlets were acclimatized in sand for 3-4 weeks. The regenerated plants were grown in pots under controlled conditions in a greenhouse for 6-8 weeks, and transferred to field in non-saline, fertile soil for large-scale multiplication.

Embryogenic calli of sugarcane were subjected to *in-vitro* salinity stress to study the effect of salt on callus proliferation and regeneration. The calli were subcultured on callus- induction medium [15] supplemented with four concentrations of NaCl. Seven pieces of callus, 128±10 mg each, were cultured in Petri dishes (180x15 mm) on media with 0, 50, 100 and 200 mM NaCl, and subcultured every two weeks by serial transfer on the same media. In each experiment, there were three replicates for each treatment.

DNA was isolated from calli (irradiated, non-irradiated and salt treated) and from the regenerated plants as described earlier [9]. Conditions for PCR amplification were optimized. The purified DNA was treated with RNase and quantified by DyNA Quant$^{TM}$ 200 Fluorometer. DNA was diluted with sterilized distilled water to obtain the final concentration of 12.5ng/l for use in PCR reaction. Random decamer primers (Operon Technologies Inc., Alameda, USA) were diluted in deionized, sterile, double distilled water to give concentration of 15ng/l. The PCR amplifications were performed in a Perkin Elmer Thermal Cycler 480, and 110 primers of Operon kits, OPA(1-20), OPI(1-20), OPJ(1-20), OPM(1-20), OPR(1-20), and OPZ(1-10) were used for amplification.

The regenerated plants derived from irradiated and non-irradiated cultures were grown in field trials at four sites (Table I). $V_0$ plants were transplanted in well-prepared plots with plant to plant space of 61 cm and row to row of 76 cm. The plants were grown under recommended cultural practices. Canal water irrigation was routinely applied, except at Site IV, where ground water containing high level of salts was used. $V_1$ sets, taken from the $M_1V_0$ plants, were replanted in the field for subsequent propagation and multiplication. The sets were planted as single line progenies. The $V_2$ sets, taken from plants at NIBGE campus (Site I), were grown as single line progenies; half the number of sets of each variant were planted in normal soil (Site 1) and the remaining half in saline soil at BSRS, Pacca Anna (Site IV). One field experiment was located on moderately saline soil at the Department of Plant Breeding and Genetics, Gomal University, D. I. Khan, North West Frontier Province (Site III), about 350 km from Faisalabad. This area is becoming increasingly salanized due to canal water seepage. The land in Chak Jhumra (Site II), ca.35 km from Faisalabad, is also moderately saline. Therefore, evaluation of plant material under such condition was highly desirable.

**TABLE I. TIME SCHEDULE OF FIELD EXPERIMENTS OF SUGARCANE**

| Sites | Propagation | | | |
|---|---|---|---|---|
| | $V_0$ | $V_1$ | $V_2$ | $V_3$ |
| I. NIBGE | March, 95 | March, 96 | March, 97 | March, 98 |
| II. Chak Jhumra | - | March, 96 | March, 97 | - |
| III. D. I. Khan | Sept., 95 | Sept., 96 | Sept., 97 | Sept., 98 |
| IV. BSRS | - | - | April, 98 | - |

Chemical analysis, as described by Jones et al. [10], was performed to determine the status of N, Na, K, P, B, Zn and Cu under the normal and saline conditions, planted at four sites (Table I). Third leaf from 4 to 5 month-old plants was taken, and dried for 3-4 days at $60^0$C in an oven. The dried leaves were ground into powder. Digestion was performed by taking the 2 g of the powder which was dry ashed at 450 $^0$C for 5 hrs, and then dissolved in 0.1N HCl and filtered after four hours. Sodium and potassium were determined by using a flame photometer; nitrogen was determined by Kjeldahl's method; zinc and copper were determined by atomic absorption; boron and phosphate were estimated by colorimetric method using agomethine H and Bacton's reagent, respectively, as colour developing reagents.

To determine the effect of salinity level on different growth stages of variants, experiments were also performed in pots (Table II). During March 1997, $V_1$ sets of selected variants were planted in earthen pots (30 cm diam.). Four different levels of salinity (0, 7, 14, 21 $dSm^{-1}$) were applied, and data were collected at three developmental stages (early vegetative, stalk development and maturity stages). Two-factor, completely randomized design (CRD) with three replicates for each parameter was used (Table II).

**TABLE II. DETAILS OF POT EXPERIMENT**

| Parameter | Detail |
|---|---|
| No. of variants (V) | 5 (CP-43/33, 0 Gy- somclonal variant, 5 Gy, 20 Gy and 40 Gy) |
| Level of salinity (S.L.) | 4 (0=2.50 $dSm^{-1}$, 7, 14 and 21 $dSm^{-1}$, mixing of commercial NaCl) |
| Replications (R) | 3 |
| No.of treatments | V x S. L. X R = 5 x 4 x 3 =60 |
| Developmental stages | 3 (early vegetative, stalk development, maturity) |
| Earthen Pot size | 30 cm diam. x 35 cm |
| Soil | 15 kg (60% field soil + 30% sand + 10% FYM) |
| Sets planted | 2 one-eyed pieces of cane ($V_1$) |
| pH | 7.8 |
| EC | 2.5 dSm-1 |
| Irrigation | Canal Water (EC= 0.5 $dSm^{-1}$) |
| Fertilization | 100 ml half-strength Hoagland solution every fortnight |
| Plantation date | March, 1997 |
| Early vegetative | 40 days after planting |
| Stalk development | 140 days after planting |
| Maturity | 240 days after planting |

## 2.2. Potato

True to type, virus-free basic seed tubers of cv. 'Cardinal' were obtained from the Vegetable Section, AARI, Faisalabad. Seed tubers were treated with 50 ppm gibberellic acid (GA3) for 1 hr to break the dormancy, and then surface sterilized with 15% hypochlorite solution for 10 minutes. Single eye-pieces were removed from the tubers with sterilized tools, and planted in plastic cups (4 x 5 in) containing autoclaved mixture of soil, farm yard manure and sand (1:2:1). The eye-pieces were kept in a growth chamber at 25±2 $^0$C at light intensity of 2,500 lux and 16 hr photoperiod. The plants were irrigated with 25% Hoagland solution. The shoot apices were excised after 20 days of germination, and placed at 37 $^0$C in a growth chamber. Axillary buds were removed from the plants after 25 days of heat treatment under aseptic conditions. The buds were disinfected with 10-15% sodium hypochlorite solution for 5-10 minutes, and rinsed 3 times for 10 minutes each with sterile distilled water. Meristem-tips (0.4 mm) were excised from the axillary buds under aseptic conditions, and cultured on MS medium [15]. Micro-shoots were multiplied from meristem tips by nodal fragmentation, as described by Bryan *et al.* [3]. Micropropagated plants were maintained at 25±2$^0$C and 16 hr photoperiod on MS medium [15] in test tubes (150x18 mm). Four to five week-old micro-propagated plants were irradiated with 20 Gy gamma rays from a $^{60}$Co source. Irradiated and non-irradiated micropropagated plants were transferred to wooden trays (30x12x6 in) containing sand, and grown in a green house for hardening. The trays were covered with polythene sheets, which were removed after one week. After 2-3 weeks of hardening, the micropropagated plants were transferred to pots containing 3:1 mixture of peat moss and silt. Apical meristem-tips were removed to break the apical dominance and stimulate growth of the lateral shoots. Stem cuttings, 10-15 cm long, were taken from 5 to 6 week-old mother plants. Stem cuttings were dipped in rooting hormone, and transferred to peat moss for rooting. Well-rooted cuttings were planted

under a plastic tunnel for minitubers production. The minitubers were stored at $4^0$C for braking dormancy, and planted in normal soil at NIBGE campus for multiplication and grown under routine agronomic practices of irrigation, fertilizer application, hoeing, and earthing up.

Stem segments with axillary buds were used for screening variants under *in-vitro* salinity stress. Liquid MS media [15] with 0, 50, 100, 200 mM salt were used for *in-vitro* screening. Ten segments each were transferred to 250 ml conical flasks (8 flasks/treatment) containing 25 ml medium, placed on an orbital shaker at 100 rpm, and kept in a controlled growth room for one month.

Pot experiments were carried out with four levels of salinity (2.5, 3, 6 and 12 $dSm^{-1}$) and three replications in a two-factor, completely randomized design (CRD). Data were collected at two developmental stages- tuber formation and plant maturity. Statistical analysis was performed as described by Gomez and Gomez [7] using MSTAT programme. Randomization, layout and analysis of variance were calculated by using two-factor completely randomized design (CRD) and Duncan's Multiple Range Test (DMRT).

## 3. RESULTS AND DISCUSSION

### 3.1. Sugarcane

Leaf explants initiated white to yellowish white, nodular calli on the cut edges after 2-3 weeks of culture on callus induction medium in dark. Calli were maintained on the same medium in dark by serial subculture. The calli, when transferred to light, turned pink brown. Browning of the calli on transfer from darkness to light might be due to some physical effect of light or some chemical change [12]. Activated charcoal and polyvinylpyrrolidone (PVP) were added to the medium to reduce browning, but no significant effect was observed. Radiation dose affected callus proliferation and regeneration competence. Regeneration frequency drastically decreased with increased radiation dose. At 60 Gy, only 4-5% regeneration was obtained, and the regenerants died during hardening. Likewise, increased radiation dose reduced survival of micropropagated plants of potato. The results from several experiments showed that for both sugarcane and potato, $LD^{50}$ was 20 Gy.

Of the several media tried for regeneration, modified MS medium [15], supplemented with 6% sucrose, 2 mg/L kinetin and 5 mg/L IAA, gave the best results. Modified MS medium [15] also gave excellent *in-vitro* multiplication of potato from stem-nodal cuttings.

The effect of salt stress was studied on sugarcane callus and nodal shoots of potato. Callus weight on induction medium supplemented with different amounts of NaCl was determined after one month. Calli proliferated even at the highest level of salt (200 mM NaCl); however, all calli cultured on salt containing medium, even those on the low level of salt (50 mM NaCl) failed to regenerate when subcultured on regeneration medium without salt. To further investigate the effect of salinity at cell level, irradiated and non-irradiated calli were cultured on regeneration medium supplemented with different amounts of salts (0, 50, 100, 150, 200 and 300 mM NaCl). However, not even a single regenerant was obtained from this experiment, suggesting that sugarcane cells are very sensitive to salinity. Addition of salt to the medium had a drastic effect on shoot development; even a low amount of NaCl (50 mM) resulted in necrosis and decay of shoot tips. High salt concentrations (150 and 200 mM) reduced the number of nodes, branches and roots.

Regenerated plants of sugarcane and micropropagated potato plants required high humidity during acclimatisation. High humidity was maintained by transfer of regenerants in sand, and covering them with a polythene sheet prior to transfer to the soil. Polythene sheet was gradually removed to avoid the drought shock. Sand played a significant role during hardening process as it hardened the delicate roots. The hardening procedure increased the survival of the plants to over 90% as against less than 40%, if high humidity was not properly provided in the initial and crucial stages of transfer to soil. Autoclaved canal water gave better results than Hoagland solution during hardening; the chance of fungal growth increased by using Hoagland solution.

Somaclonal variation occurs among tissue culture regenerated plants. Somaclonal variation may be generated through various types of chromosomal re-arrangement and loss, gene amplification or de-amplification, non-reciprocal mitotic recombination, transposable element activation, point mutations, reactivation of silent genes in multigene families and alterations in maternally inherited characteristics. It is rather difficult to detect genetic variation at the callus stage from morphological features. Therefore, molecular procedures were adopted to detect somaclonal variation. RAPD analysis is widely used to detect variation among somaclones [18]. Irradiated and salt stressed (200 mM NaCl) calli were used for RAPD analysis. Six primers, OPJ-09, OPJ-13, OPJ-15, OPJ-16, OPJ-17 and OPJ-18, were used for PCR amplification. Of these, primers OPJ-13 and OPJ-17 produced polymorphic bands (Fig. 1).

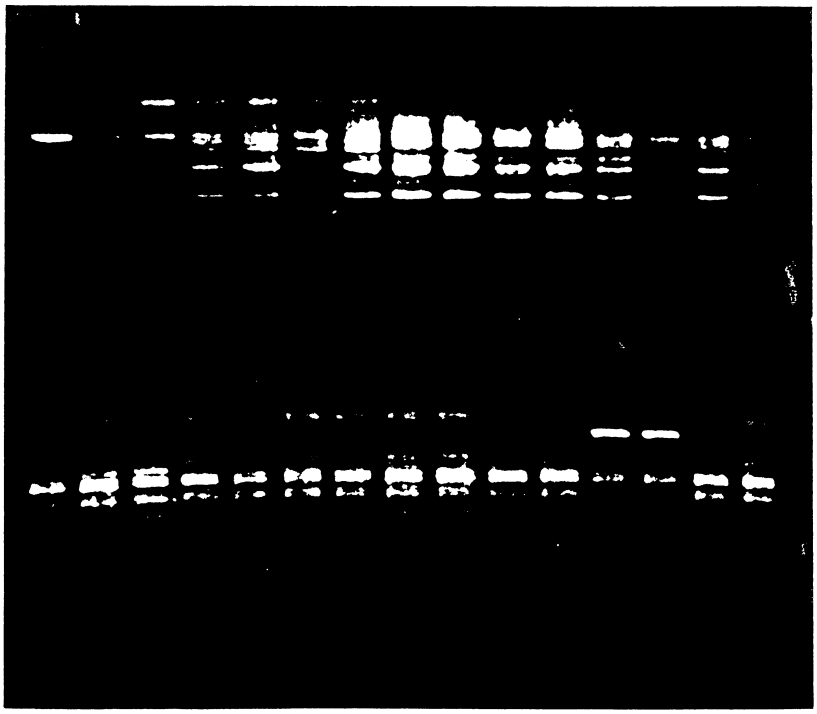

*Fig. 1. RAPD profiles of sugarcane CP-43/33 calli irradiated with gamma rays or subjected to salinity stress. Lanes 1-15, amplification with OPJ-13. 0 Gy: lanes 1, 2, 16 and 17; 20 Gy: lanes 5, 6, 20 and 21; 100 mM salt stressed: lanes 9, 10, 24 and 25; 200 mM salt stressed: lanes 13, 14, 28 and 29. Lanes 16-30: amplification with OPJ-17. 5 Gy: lanes 3, 4, 18 and 19; 40 Gy: lanes 7, 8, 22 and 23; 150 mM salt stressed: lanes 11,12, 26 and 27. Field grown plant: lanes 15 and 30.*

The genomic DNA isolated from irradiated, non-irradiated and field grown control plants of CP-43/33 was amplified and 110 primers were used to detect genetic variation. Of these, 89 primers (80.9%) were successfully amplified the genomic DNA fragments of sugarcane variants with highly reproducible bands. Only 2 of the110 primers (1.8%), OPA-07 and OPA-09, enabled the identification of polymorphism among variants of sugarcane (Figs. 2 and 3).

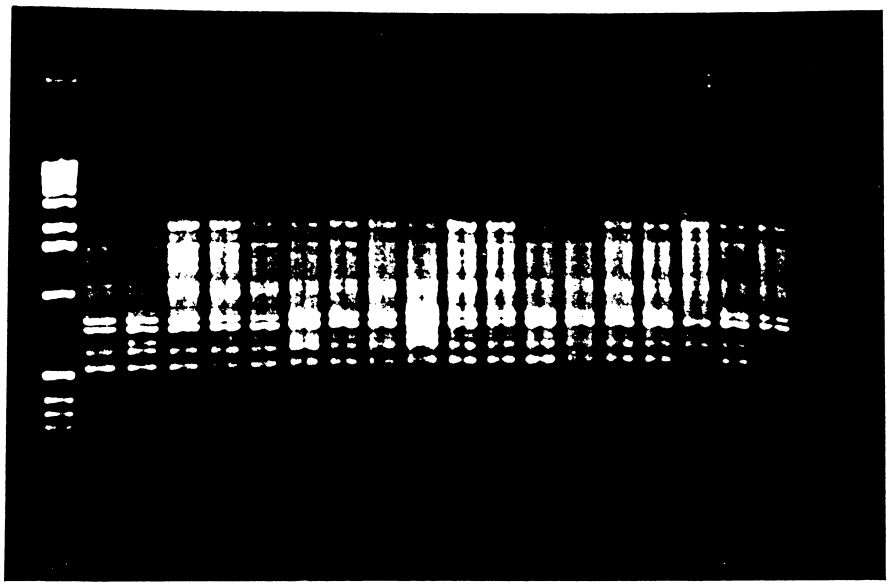

*Fig. 2. RAPD profiles of sugarcane variants with OPA-07. Lanes 2-3: parent CP-43/33; lanes 4-7: 0 Gy; lanes 8-11: 5Gy; lanes 12-15: 20Gy; lanes 16-19: 40Gy; lane 1: marker and lane 20: negative control.*

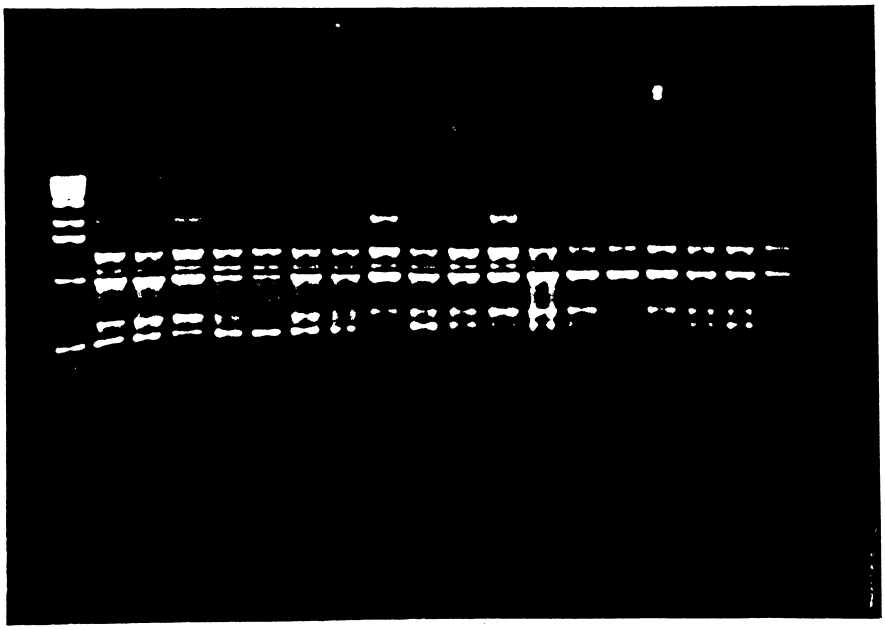

*Fig. 3. RAPD profiles of sugarcane variants with OPA-09. Lanes 2-3: CP-43/33; lanes 4-7: 0Gy; lanes 8-11: 5Gy; lanes 12-15: 20Gy; lanes 16-19: 40 Gy; lane 1 marker and lane 20 negative control.*

OPA-07 showed polymorphism in the variant 40-7 (40 indicates the radiation dose and 7 the regenerant number) in the lane no.17, where a band was missing (Fig. 2). Similarly, a band was missing in the lane no.13 (Fig. 3), where OPA-09 showed polymorphism in the variant no. 20-4. These results indicated that variability was much higher in the callus than among the regenerants. There are two possible explanations: 1. As callus is a mass of many thousand cells, some cells were adversely affected by radiation or *in-vitro* stress, and had a higher mutation frequency than the others. Somehow, only those cells that have low mutation frequency and a high competence were able to regenerate into shoots. This could be the reason that a lower rate of variability was observed among the regenerated variants. 2. It has been reported that photo-inducible DNA repair-mechanism plays a significant role in the reversion of variants to the normal types [6]; hence, there was a low frequency of detectable variation among the regenerants derived from irradiated callus.

In field tests, the $V_1$ plants obtained from irradiated and non-irradiated cultures of sugarcane significantly differed in tiller number and were dwarf as compared with the parent CP-43/33. The variants at $V_2$ stage reverted to the normal types. Some of the physiological changes at $V_1$ stage may have resulted from *in-vitro* stress. Field experiments showed that there was no significance difference among the selected variants of sugarcane with regard to germination, (Table III) and Na and K uptake (Table IV). Germination in normal soil ranged from 72 to 90% in the selected variants; in saline soil germination was drastically reduced and ranged from 7 to 71%. Under saline conditions (Site IV), the germination of selected variants from non-irradiated regenerants was higher than the control and other selected variants. The germination of selected variants from 40 Gy treatment was very low at Site IV (Table III). At other sites, there was no significant difference in germination. The low germination of selected variants of 40 Gy at Site IV might have resulted because a part of field had a high level of salinity. Sodium and nitrogen uptake was also higher in the saline soil (Site IV) than in the normal soil (Site 1) (Table V). There was no significant difference in the uptake of boron, zinc and copper among the variants at all sites (data not shown). However, boron uptake in plants grown on saline soil (Site IV) was ten times higher than those on normal soil (Site I). Commercial cane sugar percentage, total soluble salts and sucrose was lower in the variants than the control (Table V).

Sugarcane variants were also tested at four salinity levels in a pot experiment, but most of the variants died at 21dSm$^{-1}$. Therefore, data were collected for only three salinity levels (2.5, 7 and 14dSm$^{-1}$). The variants did not differ significantly in parameters such as germination, cane yield/plant, number of tillers/plant and plant height at the same salinity level and growth stage (Tables VI to IX). However, there were significant differences within the same mutant at different salinity levels, indicating that increased salinity drastically affected the growth of the plant.

### 3.2. Potato

Irradiated micropropagated plants of potato exhibited remarkable difference in the number of shoots than those from the non-irradiated ones and had less number of shoots. In contrast, plants produced from minitubers obtained from irradiated and non-irradiated micropropagated plants did not show any visual difference. Most of the irradiated and non-irradiated micropropgated plants failed to survive when planted directly in the saline soil. Therefore, minitubers obtained from irradiated and non-irradiated micropropagated plants were grown in the normal soil for large-scale multiplication. Four salinity levels were tested in pot experiment, but most of the minitubers of the control and derived from irradiated material failed to germinate

at 12dSm$^{-1}$. Therefore, data were collected only for three salinity levels i.e. (2.5dSm$^{-1}$, 3 and 6dSm$^{-1}$). Results of the pot experiment showed that the control and those derived from radiation treatments were not significantly different at the same salinity level (Tables X to XII).

**TABLE III. GERMINATION (%) OF SELECTED SUGARCANE VARIANTS (V2) AT FOUR LOCATIONS**

| Variant* | Site I | Site II | Site III | Site IV |
|---|---|---|---|---|
| CP-43/33 | 85 | 87 | 78 | 44 |
| 0-5-1 | 82 | 81 | 82 | 70 |
| 0-6-2 | 88 | 82 | 71 | 57 |
| 0-8-4 | 78 | 86 | 77 | 71 |
| 0-10-3 | 85 | 79 | 82 | 41 |
| 0-11-5 | 87 | 81 | 81 | 26 |
| 5-3-2 | 82 | 80 | 72 | 55 |
| 5-4-1 | 89 | 78 | 77 | 28 |
| 5-5-1 | 79 | 84 | 79 | 34 |
| 5-7-3 | 84 | 89 | 76 | 40 |
| 5-9-2 | 90 | 77 | 77 | 26 |
| 20-1-2 | 74 | 82 | 80 | 50 |
| 20-2-3 | 89 | 83 | 72 | 46 |
| 20-4-5 | 73 | 80 | 71 | 45 |
| 20-5-2 | 86 | 78 | 81 | 40 |
| 20-6-3 | 84 | 88 | 79 | 29 |
| 40-2-1 | 79 | 84 | 76 | 30 |
| 40-3-1 | 92 | 83 | 77 | 12 |
| 40-4-2 | 89 | 81 | 71 | 10 |
| 40-5-6 | 78 | 75 | 72 | 7 |
| 40-7-9 | 72 | 80 | 77 | 22 |

*The first number refers to gamma radiation dose in Gy. Site I = NIBGE Field (Normal). Site II = Chak Jhumra (Moderate). Site III= D. I. Khan (Moderate). Site IV= Pacca Anna (High Saline Sodic).

## 4. CONCLUSIONS

An efficient tissue culture system was developed which allows rapid multiplication of sugarcane and potato. Variation was observed in $M_1V_1$; however, in the subsequent propagations, the observed variation was not of significant magnitude. Polymorphism was detected in the callus by DNA fingerprinting. However, polymorphism was not detected among the regenerants, even by using 110 primers. An effective method is still needed for *in-vitro* selection for salt tolerance. In potato, significant differences were observed between irradiated and non-irradiated micropropagated plants; the irradiated plants had more shoots. In potato, there was no significant difference between plants derived from the irradiated and non-irradiated material grown under normal and saline conditions.

**TABLE IV. SODIUM AND POTASSIUM (%) IN SELECTED SUGARCANE VARIANTS AT FOUR LOCATIONS**

| Variant | Site I | | Site II | | Site III | | Site IV | |
|---|---|---|---|---|---|---|---|---|
| | Na | K | Na | K | Na | K | Na | K |
| CP- 43/33 | 0.04 | 0.9 | 0.03 | 1.0 | 0.05 | 0.9 | 0.5 | 1.1 |
| 0-5-1 | 0.03 | 1.0 | 0.03 | 0.9 | 0.04 | 1.2 | 0.3 | 0.8 |
| 0-6-2 | 0.05 | 0.8 | 0.05 | 0.8 | 0.04 | 0.8 | 0.4 | 1.2 |
| 0-8-4 | 0.03 | 0.8 | 0.04 | 0.9 | 0.04 | 0.9 | 0.3 | 1.1 |
| 0-10-3 | 0.04 | 1.0 | 0.05 | 0.8 | 0.05 | 1.1 | 0.4 | 1.0 |
| 0-11-5 | 0.02 | 0.9 | 0.03 | 0.7 | 0.06 | 1.0 | 0.2 | 0.5 |
| 5-3-2 | 0.04 | 1.1 | 0.02 | 1.0 | 0.04 | 1.2 | 0.4 | 0.8 |
| 5-4-1 | 0.02 | 1.2 | 0.04 | 1.2 | 0.06 | 0.8 | 0.3 | 0.9 |
| 5-5-1 | 0.03 | 1.1 | 0.03 | 0.9 | 0.06 | 1.2 | 0.4 | 1.0 |
| 5-7-3 | 0.02 | 0.8 | 0.02 | 0.8 | 0.08 | 1.1 | 0.4 | 1.1 |
| 5-9-2 | 0.02 | 0.5 | 0.02 | 0.9 | 0.08 | 0.9 | 0.3 | 1.2 |
| 20-1-2 | 0.02 | 0.8 | 0.02 | 0.7 | 0.09 | 0.8 | 0.2 | 1.0 |
| 20-2-3 | 0.02 | 0.6 | 0.02 | 0.8 | 0.04 | 0.9 | 0.1 | 0.8 |
| 20-4-5 | 0.02 | 0.7 | 0.05 | 1.2 | 0.06 | 1.1 | 0.3 | 0.9 |
| 20-5-2 | 0.03 | 1.0 | 0.03 | 1.1 | 0.07 | 1.0 | 0.2 | 0.8 |
| 20-6-3 | 0.03 | 1.2 | 0.03 | 1.0 | 0.08 | 1.2 | 0.1 | 0.9 |
| 40-2-1 | 0.03 | 1.2 | 0.03 | 1.2 | 0.06 | 0.8 | 0.4 | 0.5 |
| 40-3-1 | 0.04 | 0.9 | 0.01 | 0.8 | 0.07 | 0.7 | 0.3 | 0.6 |
| 40-4-2 | 0.02 | 0.8 | 0.02 | 1.1 | 0.07 | 1.0 | 0.5 | 0.9 |
| 40-5-6 | 0.03 | 0.9 | 0.01 | 1.2 | 0.08 | 1.1 | 0.2 | 0.8 |
| 40-7-9 | 0.02 | 0.7 | 0.03 | 1.1 | 0.06 | 1.0 | 0.5 | 0.6 |

*The first number refers to gamma radiation dose in Gy.

**TABLE V. BRIX INDEX, SUCROSE, PURITY AND CCS IN SUGARCANE VARIANTS ($V_2$) GROWN IN FERTILE SOIL, MARCH, 1997**

| Variants | BRIX % | Sucrose % | Purity (%) | CCS % |
|---|---|---|---|---|
| CP-43/33 | 20.2 | 18.7 | 92.7 | 12.7 |
| 0 Gy | 17.2 | 17.5 | 75.2 | 9.1 |
| 5 Gy | 17.2 | 12.8 | 74.3 | 8.6 |
| 20 Gy | 17.2 | 12.8 | 74.3 | 8.6 |
| 40 Gy | 15.1 | 10.7 | 70.7 | 6.8 |

Brix index = total soluble salts; CCS = commercial cane sugar

**TABLE VI. CANE YIELD (G) PER PLANT OF SUGARCANE VARIANTS ($V_1$) AT STALK DEVELOPMENT AND MATURITY UNDER DIFFERENT SALINITY LEVELS**

| Variants | Stalk development | | | Maturity | | |
|---|---|---|---|---|---|---|
| | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ | Control | 7 $dSm^{-1}$ | 14 $dSm^{-1}$ |
| CP-43/33 | 372.3 ab* | 351.0 cd | 263.0 e | 1322.0 a | 1126.0 b | 908.0 c |
| 0 Gy | 375.0 ab | 350.3 cd | 266.3 e | 1359.0 a | 1099.0 b | 881.7 c |
| 5 Gy | 368.7 b | 357.7 c | 258.7 ef | 1324.0 a | 1105.0 b | 912.7 c |
| 20 Gy | 378.7 a | 347.7 d | 246.3 g | 1300.0 a | 1088.0 b | 860.0 c |
| 40 Gy | 367.3 b | 347.7 d | 252.0 fg | 1287.0 a | 1105.0 b | 897.7 c |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT.

**TABLE VII. GERMINATION PERCENTAGE OF SUGARCANE VARIANTS ($V_1$) GROWN IN POTS AT TWO LEVELS OF SALINITY, SEPTEMBER, 1996**

| Variants | Control | 7 $dSm^{-1}$ | 14 $dSm^{-1}$ |
|---|---|---|---|
| CP-43/33 | 86.7ab* | 77.3 de | 41.0 gh |
| 0 Gy | 84.7 b | 76.3 e | 41.7 g |
| 5 Gy | 83.3 bc | 80.3 cd | 42.0 g |
| 20 Gy | 89.3 a | 74.3 ef | 39.7 gh |
| 40 Gy | 81.0 c | 72.0 f | 38.0 h |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT.

**TABLE VIII. PLANT HEIGHT (CM) OF SUGARCANE VARIANTS AT EARLY VEGETATIVE, STALK DEVELOPMENT AND MATURITY STAGES UNDER DIFFERENT SALINITY LEVELS IN POTS**

| Variants | Early vegetative | | | Stalk development | | | Maturity | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ |
| CP-43/33 | 65.7 be | 61.3 dg | 41.0 i | 188.3 bc | 179.0 d | 155.3 e | 188.3 bc | 179.0 d | 155.3 e |
| 0 Gy | 70.7 ab | 64.0 cf | 52.0 h | 203.0 a | 182.7 bcd | 157.3 e | 203.0 a | 182.7 bcd | 157.3 e |
| 5 Gy | 73.3 a | 66.7 bc | 52.3 h | 201.0 a | 180.3 d | 156.0 e | 201.0 a | 180.3 d | 156.0 e |
| 20 Gy | 66.3 bcd | 61.0 efg | 49.3 h | 200.7 a | 180.7 cd | 150.7 e | 200.7 a | 180.7 cd | 150.7 e |
| 40 Gy | 59.0 fg | 57.7 g | 41.3 i | 188.7 b | 178.0 d | 149.3 e | 188.7 b | 178.0 d | 149.3 e |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT.

**TABLE. IX. TILLER NUMBER PER PLANT IN SUGARCANE VARIANTS AT EARLY VEGETATIVE, STALK DEVELOPMENT AND MATURITY STAGES UNDER DIFFERENT SALINITY LEVELS IN POTS**

| Variants | Early vegetative | | | Stalk development | | | Maturity | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ | Control | 7 $dSm^{-1}$ | 14$dSm^{-1}$ |
| CP-43/33 | 3.7 abc* | 3.3 a-d | 2.7 cd | 5.7 ab | 5.3 ab | 5.3 ab | 6.7 ab | 5.7 cde | 5.0 efg |
| 0 Gy | 4.0 ab | 3.7 abc | 2.7 cd | 5.7 ab | 6.0 ab | 4.7 bc | 6.7 ab | 6.3 abc | 5.3 def |
| 5 Gy | 4.0 ab | 3.3 a-d | 2.7 cd | 5.3 ab | 5.7 ab | 5.3 ab | 7.0 a | 6.7 ab | 5.0 efg |
| 20 Gy | 4.3 a | 3.7 abc | 3.0 bcd | 5.3 ab | 6.0 a | 4.7 bc | 6.3 abc | 5.7 cdc | 4.7 fg |
| 40 Gy | 3.33 a-d | 3.0 bcd | 2.3 d | 5.3 ab | 5.3 ab | 3.7 c | 6.0 bcd | 5.3 def | 4.3 g |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT. Planting date = March, 1997; early vegetative = 40 days after planting; stalk development = 140 days after planting; maturity = 240 days after planting.

**TABLE. X. SHOOT NUMBER/PLANT IN POTATO AT TWO STAGES OF GROWTH UNDER DIFFERENT SALINITY LEVELS IN POTS**

| Treatment | Early vegetative stage | | | Mature stage | | |
|---|---|---|---|---|---|---|
| | 2.5 $dSm^{-1}$ | 3 $dSm^{-1}$ | 6 $dSm^{-1}$ | 2.5 $dSm^{-1}$ | 3 $dSm^{-1}$ | 6 $dSm^{-1}$ |
| 0 Gy | 5.3 ab* | 4.7 ab | 2.3 c | 5.7 a | 4.7 ab | 3.0 d |
| 20 Gy | 5.7 a | 4.3 b | 2.7 c | 5.3 ab | 4.3 bc | 3.3 cd |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT.

**TABLE XI. YIELD (TUBER NUMBER/PLANT) OF POTATO AT TWO STAGES OF GROWTH UNDER DIFFERENT SALINITY LEVELS IN POTS**

| Treatment | Early vegetative stage | | | Mature stage | | |
|---|---|---|---|---|---|---|
| | 2.5 $dSm^{-1}$ | 3 $dSm^{-1}$ | 6 $dSm^{-1}$ | 2.5 $dSm^{-1}$ | 3 $dSm^{-1}$ | 6 $dSm^{-1}$ |
| 0 Gy | 11.33 a* | 7.0 b | 4.7 b | 12.0 a | 7.6 b | 4.3 c |
| 20 Gy | 12.3 a | 7.6 b | 4.3 b | 13.3 a | 7.3 b | 5.0 bc |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT. Planting date = September, 1997; early vegetative stage = 40 days after planting; maturity at 80 days after planting.

**TABLE XII. GERMINATION (%) OF POTATO VARIANTS AT DIFFERENT LEVELS OF SALINITY IN POTS**

| Variants | Control | 3 $dSm^{-1}$ | 6 $dSm^{-1}$ |
|---|---|---|---|
| 0 Gy | 93.6 a* | 86.3 b | 72.0 c |
| 20 Gy | 92.6 a | 83.3 b | 70.3 c |

*Means followed by a common letter are not significantly different from each other at 5% level by DMRT.

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## REFERENCES

[1] AHLOOWALIA, B.S. "*In-vitro* radiation induced mutagenesis in potato." The impact of Biotechnology in Agriculture (SANGWAN, R.S. and SANGWAN-NORREEL, B.S., Eds.), Kluwer Acad. Pub. Dordrecht, (1990) 39–46.

[2] ANONYMOUS. Agriculture Statistics of Pakistan 1996-97, Government of Pakistan, Ministry of Food, Agriculture and Livestock, Food and Agriculture Division and Livestock, Economic Advisory Wings, Islamabad, Pakistan.

[3] BRYAN, J.E., JACKSON, M.T., QUEVEDO, M.B. and MELENDEZ, N. Single-node cuttings- a rapid multiplication technique for potatoes. Series I: Rapid Multiplication Techniques for Potatoes. Guide Book I & II, International Potato Centre, Lima, Peru (1981) 1–8.

[4] CHAUDHARY, M.T., *et al*. The regeneration of salt tolerant plants of *Medicago* media from salt selected suspension cultures, Rice Biotech Quat.**17** (1994) 31–34.

[5] DRACUP, M. Increasing salt tolerance of plants through cell culture requires greater understanding of tolerance mechanism. Australian J. Plant Physiol.**18** (1991) 1–15.

[6] FLICK, C.E. Mutagenesis. Handbook of Plant Cell Culture. (Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y., Eds.),Vol.1, Macmillan Publishing Co., New York (1983) 425–426.

[7] GOMEZ, K. A. and GOMEZ, A.A. Statistical Procedures for Agricultural Research. John Wiley and Sons, Inc., Singapore(1984).
[8] IQBAL, M.J., AZIZ, N., SAEED, N.A., ZAFAR, Y. and MALIK, K.A. Genetic diversity evaluation of some elite cotton varieties by RAPD analysis. Theor. Appl. Genet. **94** (1997) 139–144.
[9] IQBAL, M.J., ASAD, S. and ZAFAR, Y. "DNA polymorphisms in banana and sugarcane varieties revealed by RAPD analysis". Proc. Symp "Induced Mutations and Molecular Techniques for Crop Improvement", International Atomic Energy Agency, Vienna, (1995) 309–317.
[10] JONES, J.B. Jr., et al., Plant Analysis Handbook, Micro-Macro Publishing Inc., USA (1991).
[11] LARKIN, P.J. and SCOWCROFT, W.R. Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. **60** (1985) 197–214.
[12] LIU, M., HUANG, Y.J. and SHIH, S.C. The *in-vitro* production of plants from several tissues of S*accharum* species. J. Agri. Assoc. China **77** (1972) 52–58.
[13] MALUSZYNSKI, M., AHLOOWALIA, B.S. and SIGURBJÖRNSSON, B. Application of *in-vivo* and *in-vitro* mutation techniques for crop improvement. Euphytica **85** (1995) 303–315.
[14] MULLET, J. Designing crops for resistance to environmental stress. Ag. Biotech. **2** (1990) 435–436.
[15] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassay with tobacco tissue. Physiolog. Planta. **15** (1962) 473–497.
[16] OROPEZA, M. and DEGRACIA, E. Use of molecular markers for identifications of sugarcane varieties (*Saccharum spp.*). Phyton-Int. J. Expt. Bot. **61** (1997) 81–85.
[17] RITA, H.M., LAWRENCE, J.H. and DUDLEY, J.W. A classification of 148 U.S. maize inbreds. II. Variation of cluster analysis based on RFLPs. Crop Sci. **34** (1994) 852–865.
[18] RIVAL, A., BERTRAND, L., BEULE, T., COMBES, M.C., TROUSLOT, P. and LASHERMES, P. Suitability of RAPD analysis for the detection of somaclonal variants in oil palm (*Elaeis guineensis* Jacq). Plant Breeding **117** (1998) 73–76.
[19] SAGREDO, B., HINRICHSEN, P., LOPEZ, H., CUBILLOS, A. and MUNOZ, C. Genetic variation of sweet potatoes (*Ipomoea batatas* L) cultivated in Chile determined by RAPDs. Euphytica **101** (1998) 193–198.
[20] TOYODA, H., *et al.* Selection of bacterial wilt-resistant tomato through tissue culture. Plant Cell Reports **8** (1989) 317–320.

# *In vitro* technique for selection of radiation induced mutants of garlic

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**Abstract.** *In vitro* culture and radiation techniques were used for obtaining mutants tolerant to high temperature stress in garlic. Callus cultures and globular bodies were initiated from young leaves on MS medium containing 500 mg/l casein hydrolysate, 1000 mg/l yeast extract and 2mg/l 2,4-D. Globular bodies, resembling compact nodular calli, were irradiated with 5 to 10 Gy gamma rays. Plants and roots were obtained from globular bodies cultured on MS medium containing 2mg/l kinetin and 0.5 mg/l IAA. The plants produced bulblets (small bulbs) when cultured on MS medium containing IBA 2 mg/l and maintained at $32^0$C.

## 1. INTRODUCTION

Mutations can be induced through a combination of radiation and *in vitro* culture. Mutants for changed plant type, leaf, flower and fruit have been reported in red pepper (*Capsicum annuum* L.) by irradiation of adventitious buds with gamma rays [1]. A mutant seedling of yellow onion, obtained through tissue culture in combination with irradiation, has been also reported [2]. The purpose of this study was to develop protocols for mutation breeding in garlic through *in vitro* and radiation techniques.

## 2. MATERIALS AND METHODS

A local variety of gralic 'Jiading White' was used in the experiments. Young leaves from germinated bulbs were used as explants. The protective covering and storage leaves were excised from cloves, and surface-sterilized in 70% ethanol for 10 sec. and then in a solution of 0.1% mercuric chloride for 8 min. They were rinsed three times in sterile distilled water. The lower parts of the leaves were cut into 3 mm long pieces, and were cultured on MS medium [3] containing 500 mg/l casein hydrolysate, 1000 mg/l yeast extract and 2mg/l 2,4-D. The cultures were maintained under 16 h photoperiod, 3000 lux intensity at $25^o$C. The cultures were irradiated with gamma rays from $^{60}$Co source with 1, 3, 5, 8 and 10 Gy at a rate of 0.725 Gy/min from a distance of 2.2 m.

## 3. RESULTS AND DISCUSSION

### 3.1. Callus induction and plant formation

Callus was induced from leaf explants cultured on MS medium containing 1mg kinetin (KIN), 1 mg IAA and 2 mg 2,4-D per liter, the frequency of callus induction being 100%. The highest frequency of plant formation occurred from calli cultured on MS medium supplemented with 2 mg BA and 2 mg IAA per liter, the frequency being 77.1% with an average of four plants per callus (Table I).

**TABLE I. EFFECT OF MEDIUM ON PLANT DIFFERENTIATION IN GARLIC LEAF-CALLUS**

| Medium | Growth regulators (mg/l) | | Frequency of shoot formation (%) | Mean No. of shoot per callus |
|---|---|---|---|---|
| 1 | 2 KIN | 2 IAA | 65.7 | 3 |
| 2 | 6 KIN | 2 IAA | 85.7 | 3 |
| 3 | 2 KIN | 6 IAA | 57.1 | 1 |
| 4 | 2 KIN | 2 NAA | 71.4 | 3 |
| 5 | 2 BA | 2 NAA | 71.4 | 3 |
| 6 | 2 BA | 2 IAA | 77.1 | 4 |

### 3.2. Bulblets induction

Small sized bulbs, blublets, were obtained from plants cultured on MS medium containing IBA; their number increased with IBA amount. The number of bulblets induced on the MS medium containing 3mg IBA was four times more than on medium with 1mg/l IBA. However, bulblets induced on the media containing IBA were smaller than those on medium without IBA.

**TABLE II. EFFECT OF IBA ON BULBLET FORMATION**

| IBA (mg/l) | Frequency of bulblet formation (%) | No. bulblets per callus |
|---|---|---|
| 0 | 58 | 2 |
| 1 | 40 | 1 |
| 2 | 78 | 2 |
| 3 | 72 | 2 |

### 3.3. Induction of 'globular body'

The word "globular body" of garlic was proposed by Havranek and Novak [4] for the structural tissue with meristematic capability, but without any primordia for stem or root formation. Zhou et al. [4] suggested that the globular body might contain the primordia for root and stem, and it could differentiate into shoots and roots. In the present study, when leaf explants were cultured on MS medium supplemented with 500 mg/l casein hydzolysate, 1000 mg/l yeast extract, 2mg/l 2,4-D and 5% sugar, detachable globular bodies were produced in the callus. In some cases, ten or more bodies per callus were obtained. Shoots and roots were induced from the globular bodies cultured on MS medium containing kinetin (KIN) and IAA (Table III). Therefore, globular bodies can be used for micropropagation and mutation induction in garlic.

**TABLE III. EFFECT OF MEDIUM ON PLANT REGENERATION FROM GLOBULAR BODY**

| Medium | Growth regulators (mg/1) | | Frequency of plant regeneration (%) |
|---|---|---|---|
| 1 | 2 KIN | 0.5 IAA | 91.7 |
| 2 | 4 KIN | 0.5 IAA | 58.3 |
| 3 | 6 KIN | 0.5 IAA | 62.5 |

### 3.4. Irradiation of *in vitro* cultures

With regard to radiation dose, two experiments were carried out. 1. On response of callus growth to gamma radiation, and 2. On the effect of gamma radiation on the initiation of root and shoot. The results showed that irradiation with 1 and 3 Gy gamma rays stimulated callus growth, and 8 to 10 Gy dose inhibited callus growth remarkably. Especially, the weight of callus irradiated with 10 Gy was 76% less than that of non-irradiated callus (control) (Table IV). Hence, 1, 3 and 5 Gy were used to induce mutants.

**TABLE IV. EFFECT OF RADIATION DOSE ON CALLUS GROWTH**

| Radiation dose (Gy) | Explant No. | Callus weight (mg) | Relative to control (%) |
|---|---|---|---|
| Control | 40 | 0.089 | 100 |
| 1 | 40 | 0.098 | 110 |
| 3 | 40 | 0.096 | 110 |
| 5 | 40 | 0.086 | 97 |
| 8 | 40 | 0.060 | 68 |
| 10 | 40 | 0.021 | 24 |

The results also showed that there was a significant effect of gamma rays on callus differentiation. The shoot differentiation was inhibited by radiation by all doses used. The frequency of shoot induction decreased with an increase in radiation dose. The number of differentiated shoots per callus was affected by radiation dose. In the treatments of 0, 1 and 3 Gy, the mean number of shoots per callus were 10, 5 and 6, respectively, and was much lower at 5, 8 and 10 Gy (Table V).

**TABLE V. EFFECT OF RADIATION ON GARLIC LEAF CALLUS AND REGENERATION**

| Dose (Gy) | No. of explants | Explants forming calli | | No. of plants regenerated | |
|---|---|---|---|---|---|
| | | No. | (%) | Total | Per callus |
| Control | 20 | 20 | 100 | 205 | 10 |
| 1 | 20 | 18 | 90 | 107 | 5 |
| 3 | 20 | 19 | 95 | 123 | 6 |
| 5 | 20 | 11 | 55 | 49 | 2 |
| 8 | 20 | 11 | 55 | 52 | 3 |
| 10 | 20 | 6 | 30 | 25 | 1 |

### 3.5. Selection of mutants for tolerance to high temperature

Garlic leaf explants, cultured on MS medium containing 1mg kinetin and 2mg 2,4-D per liter, were maintained at 25°C for 30 days. The obtained calli were irradiated with 0, 1, 3, 5, 8 and 10 Gy gamma rays, with 40 calli per treatment. After 10 days of irradiation, the calli were subcultured on MS medium containing 6 mg kinetin and 0.5 mg IAA per liter to induce shoots at 25°C. The plants thus obtained were cultured on MS medium containing 2 mg IBA/l to form bulblets at a higher temperature of 32°C. The plants obtained from non-irradiated calli and from

those irradiated with 1 and 3 Gy did not produce bulblets at high temperature. However, the plants from calli irradiated with 5, 8, and 10 Gy, produced bulblets at 32°C, the number of bulblets formed increased with an increase in radiation dose (Table VI).

**TABLE VI. *IN VITRO* BULBLET FORMATION IN GRALIC CULTURES AT HIGH TEMPERATURE**

| Radiation dose (Gy) | Bulblets formed No. | Bulblets germinated | |
|---|---|---|---|
| | | No. | (%) |
| 0 | 0 | - | - |
| 1 | 0 | - | - |
| 3 | 0 | - | - |
| 5 | 14 | 10 | 71 |
| 8 | 25 | 18 | 72 |
| 10 | 53 | 40 | 76 |

It seems that 5 to 10 Gy dose were useful for bulblets production at high temperature and this might allow selection of mutants tolerant to high temperature. However, these results are preliminary, and it would be necessary to repeat the experiments, and test the bulblets by planting under the greenhouse or in the field to observe their tolerance to high temperature.

## 4. CONCLUSIONS

Based on the above results, the optimal system of *in vitro* culture and irradiation, for obtaining mutants was as follow: Callus and 'globular body' was induced from leaf explants on MS medium containing 500 mg casein hydrolysate, 1000 mg yeast extract and 2mg 2,4-D per liter. The globular body was capable of differentiation into shoots and roots and can be used for micropropagation of garlic and mutation breeding. The globular body irradiated with 5 to 8 Gy gamma rays produced plants when cultured on MS medium containing 2 mg kinetin, and 0.5 mg IAA per liter. The plants after subculture on MS medium with 3 mg/l IBA produced bulblets at high temperature, which might include variants for tolerance to high temperature and drought.

## REFERENCES


[1] RIPICHITT, P., NAWATA, E. and SHIGENAGA, S. The effects of exposure dose and dose rate of gamma radiation on *in vitro* shoot-forming capacity of cotyledon explants in red pepper (*Capsicum annuum* L. cv. Yatsufusa). Japan. J. Breed. **38**(1988) 27–34.

[2] ZHEN, H. Yellow onion mutation seedling obtained through tissue culture combined with gamma ray radiation of onion flower head. Acta Agricultural Shanghai **6** (1990) 93–95.

[3] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassay with tobacco tissue. Physiolog. Planta. **15** (1962) 473–497.

[4] HAVRANEK, P. and NOVAK, F. J. The bud formation in the callus cultures of *Allium sativum* L. Z. Pflanzenphysiol. **68** (1973) 308–318.

[5] ZHOU, Y., QIAN, Y. and CAI, Q. Callus induction and plant regeneration from storage leaf of *Allium sativum* L. Acta Botanica Sinica 22 (1980) 402–403.

# *In vitro* technique for selection of radiation induced mutants of sweet potato

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**Abstract.** *In vitro* culture and irradiation techniques were used for obtaining mutants in sweet potato, *Ipomoea batatas*. Callus was initiated from stem explants cultured on MS medium, supplemented with 1 mg NAA, 2 mg IAA, and 0.01 mg BA per litre. Calli were irradiated with 5 Gy gamma rays, and cultured on half-strength MS medium containing 2 mg IAA and 2 mg kinetin per litre. Variants for morphological changes in leaf and tuber skin and flesh color were found among the $M_1V_2$ plants, derived from irradiated calli. A promising clone, 91-C3-15, with improved tuber shape and deep red skin was selected from the 776 $M_1V_2$ population.

## 1. INTRODUCTION

Variation can be induced by irradiation of *in vitro* cultures. If the plants obtained from tissue culture originate from single cells, they would be solid mutants which would avoid chimeras common in mutagen treated material. Irradiation of various tissues produces many mutants, but chimeras seem to occur more frequently with high doses of radiation. Hence, tissue culture combined with low doses of radiation may generate mutants without significant chimeras. Several authors [1,2,3,4] have reviewed the induction of mutations in tissue culture. In this paper, induction of mutations through the combined use of tissue culture and gamma radiation is reported in sweet potato.

## 2. MATERIAL AND METHODS

A local variety of sweet potato, 'Gao-14' was used in the experiments. Explants from stem cuttings were used for tissue culture. The explants were surface-sterilized with 70% ethanol for 10 sec. and then with 3% Sodium hypochlorite for 10 min. and rinsed three times in sterile distilled water. The explants were cut into 3 mm long pieces and cultured on MS medium [5] containing varying amounts of plant growth regulators. The cultures were kept at 25$^{o}$C under photoperiod of 16h light and 8 h dark with light intensity of 3000 lux. The cultures were irradiated with 1, 3, 5, 8, 10, 30, 50, 100, 200 and 250Gy gamma rays from a $^{60}$Co source at a distance of 2.2 m. The dose rate was 0.528 Gy/min.

## 3. RESULTS

### 3.1. Callus and shoots induction

Stem explants, cultured on MS medium containing 0.1 mg BA, 0.1mg NAA, and 0.5 mg IAA per litre, produced callus with roots. The frequency of callus induction was 100%. The calli with roots were cultured on 16 different media to induce shoot formation (Table I).

**TABLE I. SHOOT INDUCTION FROM CALLI ON MEDIA WITH VARYING AMOUNTS OF KINETIN AND IAA**

| Medium | KIN | IAA | Frequency of shoolt initiation (%) | | | |
|---|---|---|---|---|---|---|
| | | | 35 days | 45 days | 55 days | 65 days |
| J1 | 0.1 | 0.1 | 5 | 5 | 10 | 10 |
| J2 | 0.1 | 0.5 | 0 | 0 | 0 | 0 |
| J3 | 0.1 | 1.0 | 0 | 0 | 0 | 0 |
| J4 | 0.1 | 2.0 | 0 | 0 | 0 | 0 |
| J5 | 0.5 | 0.1 | 0 | 0 | 0 | 0 |
| J6 | 0.5 | 0.5 | 0 | 0 | 0 | 0 |
| J7 | 0.5 | 1.0 | 0 | 0 | 0 | 6 |
| J8 | 0.5 | 2.0 | 0 | 0 | 0 | 0 |
| J9 | 1.0 | 0.1 | 0 | 0 | 0 | 0 |
| J10 | 1.0 | 0.5 | 0 | 0 | 0 | 10 |
| J11 | 1.0 | 1.0 | 0 | 0 | 0 | 10 |
| J12 | 1.0 | 2.0 | 0 | 0 | 0 | 6 |
| J13 | 2.0 | 0.1 | 0 | 0 | 0 | 0 |
| J14 | 2.0 | 0.5 | 11 | 17 | 17 | 17 |
| J15 | 2.0 | 1.0 | 10 | 21 | 21 | 21 |
| J16 | 2.0 | 2.0 | 0 | 10 | 21 | 32 |

Only a few shoots were induced on media containing 0.1 mg and 0.5 mg KIN per litre. The frequency of shoot initiation on J1, J10, and J11 media was 10 % and on J7 6%. The frequency of shoot initiation increased with increment of KIN. From these experiments, the best medium for shoot initiation seemed to be the J16 containing 2 mg KIN and 2 mg IAA per litre. The shoot initiation required 30 to 50 days culture, and reached the peak after 55 days of culture.

### 3.2. Irradiation dose

To select optimal dose, two experiments were carried out on the response of callus growth and of root and shoot initiation to gamma radiation. The experiments showed that callus growth was inhibited with 30, 50 and 100 Gy gamma radiation. The growth of callus, irradiated with 100 Gy gamma rays, was 65% less than that of the non-irradiated control (Table II).

**TABLE II. RESPONSE OF LEAF-DERIVED CALLI TO GAMMA RADIATION**

| Gamma ray dose (Gy) | Mean callus weight* (mg) |
|---|---|
| 1 | 2.22 |
| 5 | 2.03 |
| 10 | 2.17 |
| 30 | 1.76 |
| 50 | 1.80 |
| 100 | 1.31 |
| Control | 2.04 |

*After 66 days of culture

The results showed that 5 Gy dose stimulated root development, the mean root weight exceeded that of the non-irradiated control. The root development became less active with the

increasing dose of radiation. The average root weight of 50, 100, 200, and 250 Gy dose were 0.48, 0.15, 0.13, and 0.11 mg, respectively (Table III).

**TABLE III. EFFECT OF GAMMA RADIATION ON ROOT AND SHOOT INITIATION**

| Irradiation dose (Gy) | Root weight (mg) | Frequency of shoot initiation (%) |
|---|---|---|
| 5 | 1.03 | 15 |
| 50 | 0.48 | 0 |
| 100 | 0.15 | 0 |
| 200 | 0.13 | 0 |
| 250 | 0.11 | 0 |
| Control | 0.97 | 25 |

With higher gamma ray dose, the frequency of shoot initiation from stem calli was markedly suppressed. The frequency of shoots in the non-irradiated control and 5 Gy was 25 and 15%, respectively; at higher doses of 50, 100, and 200 Gy, no shoots were initiated.

### 3.3. Variation in $M_1V_2$ population

Between 1992 to 1995, 776 $M_1V_2$ plants were obtained from 1000 calli, derived from stems explants. Various types of morphological variants were observed in $M_1V_2$ population (Table IV) for the ten characters investigated. There were no noticeable mutants for stem length. Among 776 $M_1V_2$ plants, a clone, 91-C3-15 was the most promising, and is being tested at the Chinese Sweet Potato Research Center. The preliminary tests suggest that this clone has improved tuber shape, deep red tuber skin, enhanced flavour, less fibre, and is more attractive than the parent, and would be useful as an edible sweet potato variety.

**TABLE IV. VARIATION IN $M_1V_2$ POPULATION**

| Character | Mutant plants No. | Frequency (%) |
|---|---|---|
| Leaf shape | 6 | 0.8 |
| Pigmented leaf | 3 | 0.4 |
| Pigmented stem | 3 | 0.4 |
| Tuber skin colour | 1 | 0.1 |
| Tuber flesh colour | 1 | 0.1 |
| High tuber number* | 2 | 0.3 |
| Increased tuber weight** | 3 | 0.4 |
| High yield plant*** | 2 | 0.3 |
| Poor growth# | 18 | 2.3 |

*number of tubers was 6 or more per plant. **mean tuber weight exceeded 500 g.
*** tuber yield per plant exceeded 2000g. # tuber yield per plant was under 250 g.

## 4. DISCUSSION

Based on the above results, an optimal protocol for *in vitro* culture and radiation was established for obtaining mutant clones. In this protocol, callus was induced by culture of stem explants on MS medium containing 1 mg NAA, 2 mg IAA and BA 0.01 mg BA per litre. The

optimal gamma ray dose to irradiate callus was 5 Gy. The irradiated calli, when sub-cultured on half-strength MS medium supplemented with 2 mg IAA and 2 mg kinetin per litre, produced plants which included several variants for morphological characters. The preliminary field test suggested that a selected clone, 91-C3-15 is a promising edible sweet potato line, and is being tested for tolerance to drought, resistance to nematode and root rot.

## REFERENCES


[1] BOTTINO, P.J. The potential of genetic manipulation in plant cell culture for plant breeding. Rad. Bot. **15** (1975) 1-16.

[2] DEVREUX, M. "*In vitro* culture and mutation breeding". Induced Mutation in Vegetatively Propagated Plants. Panel Proc. IAEA, Vienna (1972) 41-51.

[3] STREET, H.E. "Plant cell cultures: present and projected application for studies in genetics". Genetic Manipulations with Plant Material (Ledoux, L. Ed.), Plenum Press, New York (1974) 231-244.

[4] ROBERT, M.S. Natural and induced variation in tissue culture. Euphytica **27** (1978) 241-266.

[5] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassay with tobacco tissue. Physiolog. Planta. **15** (1962) 473-497.

# Radiation induced variation in potato for tolerance to salinity using tissue culture technique

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**Abstract.** Meristem-tips of potato *(Solanum tuberosum)* cv. 'Diamant', obtained from tuber sprouts, were cultured on MS medium, and multiplied into plantlets through micropropagation. To induce variation for salt tolerance, the obtained plantlets were irradiated with 0, 20, and 40 Gy gamma rays at 27.7 rad/sec. Irradiated plantlets were cut into single nodes and cultured on MS medium, supplemented with 2000 and 4000 ppm NaCl. Salt tolerant plantlets were transferred for tuberization on MS liquid medium supplemented with the same concentration of NaCl. Micro-tubers, collected after 6 weeks of culture, had fresh weight between 0.03 to 0.3 g. Mini-tubers were obtained by planting micro-tubers in 25 cm pots under insect proof greenhouse. Mini-tuber number per plant ranged from 3 to 6, and the mini-tuber weight ranged from 0.5-3.0 g, depending upon the treatment. Further studies are in progress to produce conventional tubers under salinity stress from the promising variants, specially those tolerant to 4000 ppm, and to assure the stability of the obtained variants.

## 1. INTRODUCTION

Potato is an extremely salt sensitive crop, and soil salinity is a major constraint in the production of this crop in Egypt, particularly in the newly reclaimed desert where plants are irrigated with underground water. This water source is mostly saline and the level of salinity is high; hence, induction of genetic variation for tolerance to salinity is essential for successful crop production. Donini and Micke [1] stated that inducing mutations by gamma rays widens genetic variation, and facilitates selection of desired characters. Also, Chaudhri et al. [2] reported that *in vitro* radiation and other mutagenic agents increased variation and level of stress tolerance among regenerants. Plant cells are totipotent. Tissue culture methods can play a significant role in plant improvement either directly or in association with the traditional methods. Plant tissue culture has a great potential in the improvement of vegetatively propagated species. Potato is one of the crop plants in which *in vitro* culture techniques have been applied with a great success. Cultures can be established from tuber explants, shoot meristems, leaves and stems, which are all capable of plant regeneration.

Micropropagation can be used as an alternative method to conventional propagation of seed potatoes. Pennazio and Redolf [3] reported that16 hr light (1500 lux) and 20-24$^{o}$C was optimal for potato micropropagation. Resende and Paive [4] found that during the stage of culture initiation, MS medium supplemented with 0.01 mg NAA, 0.1 mg GA and 1 mg BA per liter gave better results than MS medium supplemented with 0.4 mg kinetin and 1 mg GA. During shoot growth, the best medium was MS supplemented with 5 mg BA and 0.5 mg GA. During the proliferation and root development stages, MS medium supplemented with 0.01 mg NAA, 0.1 mg GA and 1 mg kinetin per liter gave prolific and healthy shoots. Le [5] mentioned that the best growth capacity after several subcultures was shown by explants taken from the central part of the stem. The explants taken from the lower end of the stem grew better than those from the apex.

At the International Potato Centre (CIP), an *in-vitro* tuber induction method was described by Tovar et al. [6]. This method consists of three steps - initial propagation from single node cuttings, liquid shaker culture and tuber induction. Sladky and Bartosova [7] reported that the induction of axillary micro-tubers on stem segments of four potato cultivars was achieved on medium containing 10 mg BA/l and 8% sucrose. They also stated that the tuber quality was affected by the addition of a mixture of amino acids to the induction medium. Tovar et al. [6] also reported that 8% sucrose was optimal for good tuber size and number. Chandra et al. [8] found that the optimum incubation temperature between 20-25 $^0$C either in dark or at low light intensity (< 500 lux) was the most effective for *in vitro* tuberization.

The present studies were undertaken to obtain new potato genotypes with tolerance to saline water irrigation.

## 2. MATERIALS AND METHODS

### 2.1. Plant material

Tubers of potato (*Solanum tuberosum* L.) cv. 'Diamant' were obtained from the Potato Research Department, Agriculture Research Centre, and were stored in dark at 4$^0$C. The tubers were transferred from the refrigerator and kept at room temperature for sprouting. The sprouts were initiated within two weeks and attained the suitable length (3-5 cm) needed for explant excision.

### 2.2. Explant establishment

The excised sprouts were rinsed in tap water, and cut into single node segments. They were sterilized by dipping in 70% ethanol for 30 sec., followed by immersion in 25% Clorox for 20 min. Thereafter, they were washed thoroughly with sterile double distilled water to remove the excess of Clorox. The surface sterilized sprouts were then aseptically transferred to moistened filter paper in Petri dishes. Five explants each were transferred in glass jars containing 40 ml sterilized solid medium. The pH of the medium was adjusted to 5.7 prior to autoclaving at 115$^0$ C for 20 min. The cultures were maintained at 22+2$^0$ C under 16 hr day-length and 3000 lux intensity, obtained from cool white florescent tubes (40W/32).

### 2.3. Plant multiplication and irradiation

After 6 to 8 weeks, when the plantlets were 8 to 10 cm tall with 6 to 8 nodes, they were cut into single nodes, and the large leaves were removed. Five nodes each per jar were subcultured on the same medium for 2 to 3 weeks till plantlets had 5 to 6 nodes. These plants ($V_1$) were then irradiated with 0, 20 and 40 Gy gamma rays. The $M_1V_1$ plantlets were trimmed by cutting all roots and large leaves, and transferred to liquid MS medium supplemented with 0, 2000 and 4000 ppm NaCl. All cultures were kept for 2 to 3 weeks under conditions as stated above, till plants had 5 to 6 nodes ($M_1V_2$). The surviving plants from the controls and treatments were cut into two pieces, each with 3 to 4 nodes without shoot tip, roots and large leaves. These were transferred to 300 ml containers with 40 ml fresh saline liquid medium, pH 5.7 to obtain $M_1V_3$ plants. These plants were transferred to tuber-induction liquid medium supplemented with the same amount of NaCl. Micro-tubers were collected after 6 to 8 weeks and kept for curing at room temperature, then stored in a refrigerator at 10$^0$C for 3 months to break their dormancy. The weight of micro-tubers ranged from 0.03 to 0.3 g, there being remarkable difference between micro-tubers from different treatments.

## 2.4. Mini-tuber production

The sprouted micro-tubers were cultured in 5 cm pots for 3 weeks in a mixture of peat moss, vermiculite and sand (1:1:1). The obtained seedlings were transferred to an insect proof greenhouse, and grown in fresh soil mixture in 25 cm pots for producing mini-tubers. Hoagland solution was used for irrigation, first at 1/4 dilution, and later gradually increased to full concentration.

## 3. RESULTS AND DISCUSSION

Several morphological abnormalities were observed in the *in vitro* multiplied plants in the successive cycles of *in vitro* culture. These included dwarf plantlets with small leaves, short noded plants with elongated leaves, leaves with pin tips, irradiated plantlets with several branches, malformed leaves, and yellowing and necrosis of leaves. These abnormalities might be partially due to the physiological stress caused by the extended and continuous culture of the plant material under *in vitro* conditions.

Minitubers were harvested after 100 days of planting. The number of mini-tubers ranged from 3 to 6, and the tuber weight varied from 0.5 to 3.0 g depending upon the treatment.

The $M_1V_2$ plantlets grown under salinity stress of 2000 ppm performed better than those grown under non-saline conditions in terms of plant survival (Table I). Plant height was nearly as good as in the controls under both 2000 and 4000 ppm NaCl (Table I). The fresh weight of shoots was lower under salt stress but node number per plant was not affected, and was equal to or better than the controls (Table II). A similar trend was noticed in the production of micro-tubers, the plants derived from 20 Gy dose produced more micro-tubers and had more fresh weight on 2000 and 4000 ppm NaCl than on non-saline medium (Table III). This trend was further observed as shown by the fresh weight of mini-tubers produced on 2000 ppm (Table IV). All these observations suggested that genetic variation for tolerance to salinity had been induced through radiation treatment of *in vitro* cultures.

**TABLE I. EFFECT OF RADIATION AND SALT CONCENTRATION ON NUMBER AND HEIGHT OF PLANTLETS OF POTATO CV. 'DIAMENT'**

| Dose (Gy) | NaC1 concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | No. of plantlets* | | | Height of plantlets (cm)* | | |
| | 0 | 2000 | 4000 | 0 | 2000 | 4000 |
| 0 | 14.7 | 17.5 | 16.2 | 7.0 | 5.3 | 4.1 |
| 20 | 8.7 | 10.2 | 6.3 | 8.5 | 8.2 | 4.2 |
| 40 | 6.8 | 8.2 | 6.8 | 6.7 | 7.0 | 4.3 |

*Each figure is a mean of 100 observations.

**TABLE II. FRESH WEIGHT OF SHOOTS AND NUMBER OF NODES PER PLANT ($M_1V_2$) IN POTATO CULTURED UNDER SALINITY STRESS**

| Dose (Gy) | NaC1 concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | *Shoot wt. (g) | | | *No. of nodes/plant | | |
| | 0 | 2000 | 4000 | 0 | 2000 | 4000 |
| 0 | 0.420 | 0.276 | 0.226 | 6.4 | 4.4 | 4.2 |
| 20 | 0.286 | 0.220 | 0.190 | 6.5 | 6.2 | 4.3 |
| 40 | 0.200 | 0.160 | 0.150 | 6.0 | 5.4 | 4.5 |

*Each figure is a mean of 100 observations.

**TABLE III. NUMBER AND FRESH WEIGHT OF MICRO-TUBERS OF POTATO CV. 'DIAMANT' PRODUCED UNDER SALINITY STRESS**

| Dose (Gy) | NaC1 concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | Micro-tuber number* | | | *Fresh wt. (g) | | |
| | 0 | 2000 | 4000 | 0 | 2000 | 4000 |
| 0 | 7.5 | 9.6 | 6.0 | 1.58 | 1.18 | 2.69 |
| 20 | 4.3 | 5.9 | 5.6 | 1.70 | 4.10 | 2.10 |
| 40 | 5.7 | 4.7 | 4.0 | 2.90 | 2.20 | 1.80 |

*Each figure is a mean of 100 observations.

**TABLE IV. MEAN FRESH WEIGHT OF MICRO- AND MINI-TUBERS OF POTATO CV. 'DIAMANT' PRODUCED UNDER SALINITY STRESS**

| Dose (Gy) | NaC1 concentration (ppm) | | | | | |
|---|---|---|---|---|---|---|
| | *Micro-tuber wt. (g) | | | *Mini-tuber wt. (g) | | |
| | 0 | 2000 | 4000 | 0 | 2000 | 4000 |
| 0 | 0.220 | 0.330 | 0.490 | 6.16 | 8.24 | 11.72 |
| 20 | 0.395 | 0.700 | 0.382 | 10.86 | 17.60 | 8.69 |
| 40 | 0.510 | 0.470 | 0.400 | 10.16 | 11.16 | 9.20 |

*Each figure is a mean of 100 observations.

Further studies are in progress to produce macrotubers (conventional) under insect-proof glasshouse, especially from variants obtained under high salinity (i.e. 4000 ppm). This would also establish the stability of mutants, which will then be multiplied through tissue culture for large-scale testing and release.

## REFERENCES


[1] DONINI, B. and MICKE, A. "Use of induced mutations in the improvement of vegetatively propagated crops". Proc. Regional Seminar on the "Utilization of Induced Mutations for Crop Improvement for Countries in Latin America". TECDOC 305, IAEA, Vienna (1984) 78–98.

[2] CHAUDHRI, M.T. et al. The regeneration of slat tolerant plants of *Medicago media* from salt selected suspension cultures. Rice Biotech Quat. **17** (1994) 31–34.

[3] PENNAZIO, S. and REDOLF, P. Factors affecting the culture *in vitro* of potato meristem tips. Potato Res. **16** (1973) 20–29.

[4] RESENDE, R.O. and PAIVE, P. *In vitro* meristem culture of *Solanum tuberosum*. Ciencia-e-Pratica **10** (l986) 241–251.

[5] LE, C.L. Practical aspects of potato (*Solanum tuberosum*) micropropagation. Rev. Suisse de Agriculture **23** (1991) 357–358.

[6] TOVAR, P., ESTRADA, R., SCHILDE-RENTSCHLER, L. and DODDS, J.H. Induction and use of *in-vitro* potato tubers. CIP Circular, Intern. Potato Centre, **13** (1985) 15.

[7] SLADKY, Z. and BARTOSOVA, ?. *In vitro* induction of axillary potato microtubers and improvement of their quality. Biol. Plantarum **32** (1990) 121–188.

[8] Chandra, R. Dodds, J.H. and Tovar P. *In vitro* tuberization in potato. CIP News Letter **55** (1988) 10–20.

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