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Induced mutations in connection with biotechnology for crop improvement in Latin America

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FOREWORD

This publication results from the second Co-ordinated Research Project (CRP) on Plant Breeding and Genetics organized on a regional basis in Latin America. The present CRP and the previous one were initiated and implemented in response to the pressing need to enhance the productivity of economic plants, viz. food crops, fruits and ornamentals. Improvement of crop production has become the highest priority in most countries of Latin America, as in other regions. Breeding superior varieties is often the only feasible solution where inputs are limited; well adapted varieties are required to meet specific agro-environmental conditions. Such varieties provide yield stability on an economically required level. The most important and common factors limiting crop production are abiotic, e.g. cold, salinity, soil aluminium toxicity and drought; as well as biotic, e.g. diseases and pests. Modern biotechnology and induced mutations offer new means and significant potential to breed desired varieties in a relatively short time. Additionally, both approaches facilitate the breeding of some vegetatively propagated crops which until now were improved mainly through selection of rare spontaneous mutants in natural or cultivated populations.

Using some of these techniques it recently became possible to produce, in some crops, true-to-type mutated lines or clones within a few months. Biotechnology can also facilitate selection, description and molecular characterization of promising mutants. Currently used DNA markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) as well as other polymerase chain reaction (PCR)-based techniques, were included in this CRP to benefit the important crops of this region.

Also included in this CRP were doubled haploids (DH), which are obtained from anther or microspore cultures and are very suitable biotechnology methods. In connection with radiation-induced mutations, they can speed up conventional breeding programmes of seed propagated crops. Induced mutations, and to some extent also somaclonal variation, are practically the only new sources of variation available to breeders of vegetatively propagated crops because of the lack of genetic segregation. Micropropagation, somatic embryogenesis, protoplast fusion and protoplast regeneration, all techniques in which induced mutations were already successfully used to generate desired genetic variation, were employed in this CRP.

The objectives of the CRP were mainly the following:

— To enhance regional co-operation in the field of radiation-induced mutations and related biotechnology and to stimulate induced mutation activities leading to the improvement of the productivity, yields and reliability of local cultivars by increasing their adaptability and tolerance to biotic and abiotic stresses.
— To investigate suitability of various local cultivars and methods for doubled haploid production (genotypic response), develop homozygous (DH) mutant lines with desired characters and initiate genetic and molecular analysis of promising mutants.
— To implement and develop protocols for *in vitro* induction of mutations in local cultivars of vegetatively propagated crops, evaluate stability and effectiveness of various *in vitro* culture methods for mutation induction and plant regeneration, evaluate frequency of selected mutants, develop true-to-type mutated clones and to initiate genetic and molecular analysis of promising mutants.

This CRP made significant contributions in terms of improved, officially released, varieties and in producing promising mutant lines and clones which are currently under evaluation before release. Notable achievements were made in developing and adapting

techniques for mutation induction using radiations and chemicals, *in vitro* cell and microspore cultures, and micropropagation. The significant achievements of this CRP cover a range of plants and issues dealing with major food crops (rice, wheat and barley), with the neglected cereals of South America (quinoa and kiwicha), and vegetatively propagated fruits (citrus, banana and avocado) and ornamentals. Important additional achievements were the formation of a network of co-operating scientists and the training of researchers. Finally, the know-how gained in this CRP is relevant to the same crops, and to other plants elsewhere. Thus, it will also benefit additional parts of the world.

This TECDOC was jointly prepared by A. Ashri, Israel, and the Scientific Secretary of the final Research Co-ordination Meeting, M. Maluszynski, who is the IAEA officer responsible for the publication.

EDITORIAL NOTE

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SUMMARY

1. INTRODUCTION

Improvement of crop productivity is becoming ever more important in view of population growth on one hand, and the lack of new arable land to bring into cultivation and water for irrigation on the other hand. It is imperative to increase the yields per unit area in order to produce sufficient food, fiber, edible oil and so many other plant products. It is also necessary to grow crops in marginal agro-climatic areas and/or on poor soils, e.g. infertile, shallow, saline or aluminum-toxic. Plant breeding, which in essence is the science and art of developing superior genotypes, has produced in recent decades better varieties in many crops, fruits and ornamentals.

More recently the classical "tools" of plant genetics and breeding have been reinforced by the wider adoption of radiation-induced and chemical mutagen-induced mutation techniques and by the array of related, innovative biotechnological approaches. While the induction of desirable mutants, their selection, evaluation and dissemination to the farmers is very important in seed crops, it is perhaps even more important in vegetatively-propagated plants. In the latter, hybridization cannot be used to generate new genetic variation, the "raw material" for selection and development of improved varieties. Only chance spontaneous mutants ("sports") have been the source of new types of fruits, flowers etc., in these plants. Therefore, the use of mutation induction in breeding vegetatively propagated crops is very critical.

This CRP covered different crops, fruits and ornamentals, including some neglected ones. Its participants employed different breeding methods, radiations and chemical mutagens, and mobilized innovative DNA markers research, doubled haploids techniques and various *in vitro* culture protocols. The efforts of the participating scientists yielded significant achievements, which are described below. They also arrived at some specific and some general conclusions and recommendations, which are detailed below. The findings of this CRP confirm and expand the role of induced mutations in plant breeding.

2. ACHIEVEMENTS

This CRP covered a wide range of issues and characters in a very diverse range of plant species: some annual and some woody perennials, some sexually reproduced and others vegetatively propagated, some major crops and some neglected, some food plants and some ornamental. This CRP shows both general achievements, with wider implications, and specific ones dealing with a crop or a procedure or a trait. The major findings and achievements are described below.

2.1. General achievements

2.1.1. Molecular markers

Some molecular techniques were applied through the CRP to study induced mutants, somatic hybrids for root stocks, segregating populations and pathogens. Some approaches were refined and some procedures were improved.

Characterization of rice mutants through isozyme diversity was conducted in Cuba and in Brazil. Characterization of somatic hybrids from protoplasts fusion using “Cleopatra”

mandarin (*Citrus limonia*) and "Rangpur" lime (*Citrus reshni*), was performed using RAPD molecular markers and isozyme marker analysis. Genetic diversity of the fungus *Pyricularia grisea* was studied in Cuba in cooperation with CIAT through molecular markers, and 4 lineages of the pathogen were identified.

2.1.2. Doubled haploids

The regular production of doubled haploid (DH) plants through anther culture was implemented in rice, wheat and barley and has became part of the national breeding programmes in Chile, Colombia, Cuba, Guatemala, Peru and Uruguay. The doubled haploid plant production approach is currently used by these programmes to reduce the time required by both conventional and induced mutation methods to develop new varieties and for improving different agronomic traits.

Some of the DH lines proved very promising and were included in advanced yield trials. Yields exceeding the parent cultivars were obtained in some of these lines.

Low culturability of anthers was shown to be the biggest limitation of this approach. Genotypes with high anther culture response were identified as well as low responsive materials, whose anther culturability improved when stressed by chemical or physical mutagenic treatments.

2.2. Specific achievements

2.2.1. Cereals

Deus, Cuba (7762/RB) — rice

- Induced mutations generated new semi-dwarf germplasm with useful agronomic traits.
- In observational yield trials, the yields of 22 advanced mutant lines from Basmati 370 and 12 from Gloria variety exceeded the original cultivars (2.2–3.5 t/ha more).
- Ten mutant lines were selected for further evaluation in replicated yield trials.
- The mutant line B 10-3-1-1, from Basmati 370, gave the highest yield and had good resistance to *Pyricularia grisea* and lodging.
- The high frequency of semi-dwarf mutants in this experiment demonstrated that induced mutations could be successfully used for improving the plant type in rice.

Blanco, Uruguay (7620/RB) — rice

- Promising semi-dwarf mutants with desirable grain shape were developed from the traditional variety EEA-404. Methodology for DH production through anther culture was adjusted and is being applied in selected crosses. Promising DH lines were advanced to National Uniform Trials.
- DNA pooling strategies for detection of polymorphism in a population segregating for disease resistance to the aggregated sheath disease (*Rhizoctonia oryzae*) and Oryzica Llanos 5 (partially compatible with lineage SRL-4) were tested; some indications of bulk-specific patterns of DNA amplification using random primers were obtained.

Correa-Victoria, Colombia (8132/RB) — rice

- The genetic structure of blast pathogen populations was characterized in six genetic families.
- The virulence spectrum for each genetic family of pathogen was determined.
- The relationship between genetic structure and virulence was determined.
- Specific interactions between genetic lineage/virulence/resistance were identified.
- Sources of resistance to different genetic lineages in commercial rice cultivars were characterized and identified.
- Mutations were induced in seven commercial rice cultivars of Colombia by gamma rays (cultivars with resistance/susceptibility to different genetic lineages of the pathogen).
- Mutants with complementary resistance to the different genetic lineages of the pathogen were identified in controlled greenhouse inoculations.

Bastos, Brazil (7611/RB) — rice

- Gamma irradiation of rice seeds increased the genetic variability for tolerance to blast.
- Mutants selected under field conditions maintained the basic long grain characteristics of the original rice cultivar and had higher yield potentials and tolerance to blast.

Molina, Guatemala (7618/RB) — rice and wheat

- The regular production of rice and wheat DH plants as a component in the varietal improvement programmes of these crops was achieved. Highly responsive anther culture genotypes were identified and are being used for increasing the efficiency of DH plant production; enhancement through gamma irradiation of the anther cultures was demonstrated.

Romero Loli, Peru (PER/5/024) – barley, quinoa and kiwicha

Barley
- An induced mutant variety, UNA La Molina 95, was released for the Peruvian highlands, following treatment with 300 Gy, with good yield, earliness and naked grain.
- Doubled haploid mutant lines, superior to the parent material in grain yield were developed, using treatments with N-methyl-N-nitrosourea (MNH) and sodium azide (NaN_3).
- New mutated populations of UNA 80 and Yanamuclo varieties (M_1 and M_2) were generated.

Quinoa (*Chenopodium quinoa*)
- Suitable doses of gamma rays and sodium azide (NaN_3) for seed treatment were established. The recommended doses for gamma rays are: 150 to 250 Gy, and for sodium azide 1.0 to 2.0 mM for 30 minutes at room temperature.
- Earlier and more vigorous plants were selected.

Kiwicha (*Amaranthus condatus*)
- The optimal dose for seed treatment with gamma rays proved to be 400–600 Gy.
- There was no response to sodium azide.
- A head color mutant was selected.

Zapata, Bolivia (7610/RB) – barley and quinoa (*Chenopodium quinoa*)

Barley
- Suitable doses for X ray treatment of seeds of local varieties were established.
- Advanced mutant lines from cooperating scientists were tested, a promising Argentine mutant line proved too late.

Quinoa
- Suitable doses for quinoa seed treatments using X rays and sodium azide (NaN_3) were explored.
- A protocol for internode and bud explants which gives good micropropagation was developed.
- A basal culture media yielding good sprout proliferation was verified.

2.2.2. Vegetatively propagated plants

Tulmann Neto, Brazil (7612/RB) – banana, citrus, *Chrysanthemum*, *Stromantha* and *Calathea*

Banana
- In the M_1V_4 generation derived from banana shoot tips irradiated with gamma rays, there was an increase in genetic variability for leaf morphology and bud color. Selection for tolerance to Fusarium wilt was successful.
- A somaclone resistant to yellow sigatoka disease (*Mycosphaerella musicola*), selected in Venezuela, was tested in Brazil and it proved resistant.
- A banana somaclonal variant with reduced plant height was selected. The first observation yield trial showed that the mutant maintained the reduced plant height.

Citrus
- Following bud gamma irradiation, putative citrus mutants were selected for characteristics such as plant height, yield and reduced number of seeds.
- The preliminary results of the yield trial in the field showed that the citrus mutant with a lower number of seeds retained the basic agronomic characteristics of the original cultivar.
- The protocols for protoplast isolation, plant regeneration and protoplast fusion of two rootstocks were established. A somatic hybrid was obtained, as proved using molecular markers.

Chrysanthemum
- Gamma irradiation of young plants of a variety with light pink flowers, followed by the cutting back procedure, led to the identification of two flower color mutants, which have good agronomic characters. These two mutants were released to commercial production as: Christiane — white flowered mutant and Ingrid — dark pink mutant.
- Following gamma irradiation of young plants of the mutant variety Ingrid (see above) 4 new flower color mutants were selected: burgundy, tea rose, bronze and variegated. These mutants have a potential to be released as new cultivars in Brazil.

Stromantha
- Following gamma irradiation of rhizomes a gray rachis mutant was found in the ornamental *S. sanguinea*. It may be released as a new cultivar suitable for export.

Calathea

- Gamma irradiation induced in the ornamental *C. louisae* a variegated yellow leaf mutant. It may be released as a new cultivar for export.

Barrera-Guerra, Mexico (7763/RB) — avocado

- Promising results with *in vitro* propagation studies indicate that protocols for large-scale multiplication facilitating mutagenic approaches may be developed in the near future.

3. RECOMMENDATIONS

Several general and specific recommendations were developed in view of the experience gained in this CRP through the individual research projects and in the interchanges of the participating investigators during RCM's, and in view of the innovations in plant science in recent years.

3.1. General recommendations

- The participants in this CRP should be encouraged to maintain their research contacts in order to enhance their national programmes and bring to fruition their breeding efforts.
- Collaborative groups should be initiated along crops and along methodological approaches.
- Biotechnology is a powerful tool that can be used in several ways in association with mutation induction such as to advance *in vitro* the materials after mutagenic treatments and to obtain non-chimeric mutants through *in vitro* adventitious buds.
- It is recommended to use or develop methods that can reduce the costs when tissue culture techniques are used in combination with induced mutations.
- The judicious use of induced mutations, which have proved beneficial in many of the breeding projects in this CRP, should be adopted more widely.
- Doubled haploids offer additional breeding options. Therefore, the wider implementation of this approach should be encouraged. These options include:
  - To obtain homozygous offspring from F_1 plants and from M_1 plants and thus accelerate breeding efforts.
  - To obtain in homozygotes fixation of F_1-like heterotic performance.
  - Use of combined DH and mutation techniques in order to strengthen breeding programmes.
  - Use of anther culture in interspecific crosses of cultivated rice and barley with wild relatives to overcome sterility problems.
  - Use of anther culture method for the identification of molecular markers associated with resistance to different pathogens or other traits of interest.
- Genotypes which are highly responsive to anther culture should be identified and exchanged to increase anther culturability rate. Other factors related to this trait such as mutagenic stress, culture medium, physiological conditions of donor plants and sowing time should also be considered.

3.2. Specific recommendations

- Selected blast resistance mutants in rice should be crossed to obtain segregants with better and more comprehensive resistance.
- Selected rice mutants exhibiting complementary resistance to different genetic lineages of the blast pathogen should be utilized in further studies of the disease.

- Research on neglected, native crops, such as quinoa (*Chenopodium quinoa*) and kiwicha (*Amaranthus caudatus*), which are important in the highlands of Peru and Bolivia, should be intensified as should the use of other innovative approaches to improve these crops and make them more competitive. The application of mutagenic agents to induce mutations to improve such native crops is recommended.
- In chrysanthemum, a high percentage of solid flower color mutants can be obtained through the use of mutagenic treatments (gamma rays, EMS) of pedicels *in vitro*. Genotypes that can regenerate plants from cultured pedicels should be identified. For chemical mutagen treatments, the maximum period they can be submerged and still regenerate should be established. Research in Brazil showed that for EMS a concentration of 0.75 M for 105 minutes and post washing in water for 15 minutes can be used.
- In chrysanthemum, the roots must be protected in order to avoid radiation damage during gamma ray treatments of young rooted plants (6 axillary buds). Furthermore, the cutting back method should be continued for several vegetative generations (M_1V_1 to M_1V_6) to increase the frequency of periclinal flower color mutations .
- In chrysanthemum as in other plants, the materials to be treated should be chosen carefully since the frequency of mutants may be affected.
- Re-irradiation of chrysanthemum mutants proved successful in yielding more mutants and is recommended.
- In *Calathea* and *Stromantha*, rhizomes can be used for gamma ray treatment, applying doses giving 40–50% M_1V_1 survival. The M_1V_3 new tillers should be used for the screening of mutants.
- In banana, to avoid a high frequency of somaclonal variants, the maximum number of *in vitro* multiplications is about 6. For the screening of mutants, an advance to M_1V_4 generation is recommended; to select for desirable somaclonal variants and for induced mutants, the M_1V_6 generation is useful.
- In citrus, when induction of mutations using gamma rays is adopted, it is recommended to ascertain that the materials are free of diseases, especially virus. It is recommended to use a gamma ray dose (40 Gy) reducing survival by 10% and plant growth by 25% in M_1V_1 branches originating from irradiated buds grafted on rootstocks. In spite of the application of the cutting back method it is recommended that for fruit characteristics, samples from 4 different parts of the plants are evaluated. Five to seven buds from the selected putative mutant plants should be taken for grafting and to begin the field evaluations, which should last for at least 4 years, and use several replications.
- In avocado, in order to employ the induced mutations approach much research should first be devoted to the development of methods for reliable *in vitro* mass propagation. These research efforts should encompass choice of plant genotypes, media, growth regulators, sources of explants, sterilization of tissues, rooting promoting substances and culture conditions.

BIOTECHNOLOGY-ASSISTED BREEDING TECHNIQUES IMPLEMENTATION FOR RICE IMPROVEMENT IN URUGUAY

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Abstract

Mutagenic treatment was highly effective in reducing plant height and growth duration in the parent variety EEA-404. Important variation was also observed in grain and leaf pubescence and in grain shape. Several M_4 lines with desirable plant type and grain shape were selected for yield testing. Aggregated sheath spot (ASS) — *Rhizoctonia oryzae sativae* — disease pressure on M_3 and M_4 lines from INIA Tacuarí was not sufficient to allow selection for this character. However, important variability in resistance to the physiological disorder Straighthead was observed among the lines. The regeneration percentage obtained with the methodology of anther culture facilitated application of this technique in a larger number of crosses. Some of the doubled haploid (DH) lines from resistant/susceptible crosses showed good yield potential and milling quality, and low incidence of stem diseases. Sequential Bulked Typing may provide an alternative to individual typing for a large number of markers, improving DNA pooling techniques based on phenotypic evaluations of quantitative traits.

1. INTRODUCTION

Rice is a major crop in Uruguay, where 190000 ha of irrigated long grain rice are grown and 90% of the production is exported. In the crop seasons 1995/96 and 1996/97, average yield was 6.4 t/ha. And the main varieties were El Paso 144 and INIA Tacuarí, of Indica and Japonica background, respectively. Grain quality is one of the basic characters for the local breeding program, as well as cold tolerance and short growing duration. The increasing incidence of some fungal stem diseases, as ASS, caused by *R. oryzae sativae*, and stem rot (SR), caused by *Sclerotium oryzae*, is limiting rice frequency in the rotations.

The variety INIA Tacuarí, that combines high yield potential, grain quality and cold tolerance, reached 38% of the acreage in 1997/98, but it is highly susceptible to ASS. At the same time, interest in short-grain varieties, to be exported to special markets, increased in recent years. However, a traditional medium-grain variety, EEA-404, as well as introduced short-grain varieties, are susceptible to lodging and have limited yield potential.

The purpose of the project was to combine mutation techniques and other biotechnologies, as anther culture and molecular markers, to speed up the development of high yielding cultivars with resistance to ASS and SR, and adapted short- and medium-grain cultivars.

The objectives of the research were: a) To induce mutations in susceptible high-yielding cultivars and in lodging susceptible medium-grain varieties; b) Adjust procedures for DH production through anther culture in selected populations; c) To evaluate molecular techniques to test genetic diversity in the resulting populations.

2. MUTATION TECHNIQUES

Mutagenic treatment of INIA Tacuarí was done at the beginning of this project in order to develop mutant lines with resistance to ASS and to SR. In the case of the traditional variety EEA-404, the objective of mutagenic treatment was to reduce plant height and growth duration.

2.1. Methodology and description of research carried out

2.1.1. Mutation techniques in EEA-404

Seeds were irradiated at Centro de Investigación Nuclear (CIN), Montevideo, with 250 and 350 Gy. The populations were managed in the following way:

- M_1: 1000 plants were grown in 1994/95 and high sterility was observed.

- M_2: 1000 headrows were grown in 1995/96. In general, the population showed lodging, and 360 panicles from 66 rows were selected, based on agronomic traits.

- M_3: 360 headrows were grown in 1996/97. Heading date and plant height were recorded. At harvest, the selected lines were not bulked, because some of them were still showing some degree of variability. A total of 337 panicles were selected from 115 M_3 lines.

- M_4: 337 headrows were grown in 1997/98. Plant height, heading date, grain shape, lodging, leaf angle and pubescence were recorded, and 118 M_4 lines were selected for yield and grain quality testing in the following season.

2.1.2. Mutation techniques in INIA Tacuarí

Seeds were irradiated at CIN with 250 and 350 Gy. Population management was as follows:

- M_1: 1000 plants were grown in 1994/95 and moderate sterility was observed.

- M_2: 1000 headrows were grown in 1995/96 and a random sample of 1000 panicles was taken.

- M_3: Culture of 845 lines in 2 m headrows under artificial inoculation with the ASS fungus, in 1996/97, to enhance disease incidence. The pathogen was grown on a rough rice grain : rice hulls mixture (2:1). Inoculation was done at internode elongation, after water dilution, at a rate of 50 ml/m^2. However, because of soil and climatic conditions, inoculation was not very effective and a high incidence of SR and of the physiological disease Straighthead was observed. One panicle was collected from each of the selected 499 M_3 lines.

- M_4: In 1997/98, 499 headrows were grown under artificial inoculation with the ASS pathogen. However, SR was again the prevalent disease. A total of 86 M_4 lines were selected for yield and disease resistance testing in the following season.

2.2. Results obtained

2.2.1. EEA-404

M_3 lines from EEA-404 showed high variability in growth duration, plant height, pubescence and grain shape. The parent variety required 100 days to heading and the M_3 lines showed a range of 80 to 110 days. About 35% of the M_3 lines required less than 95 days to heading, and 44% required from 96 to 100 days (Figure 1).

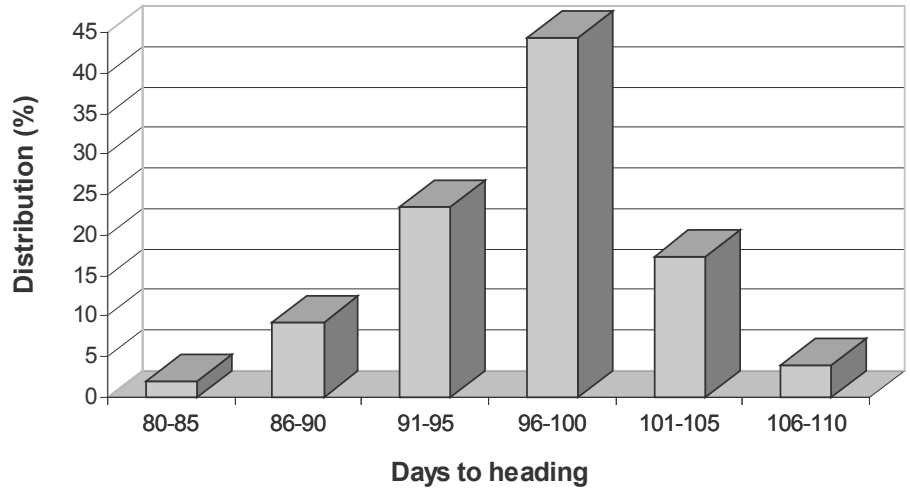

FIG. 1. Days to heading (from planting) — distribution of M_3 lines from the variety EEA-404 (parent variety required 100 days to heading).

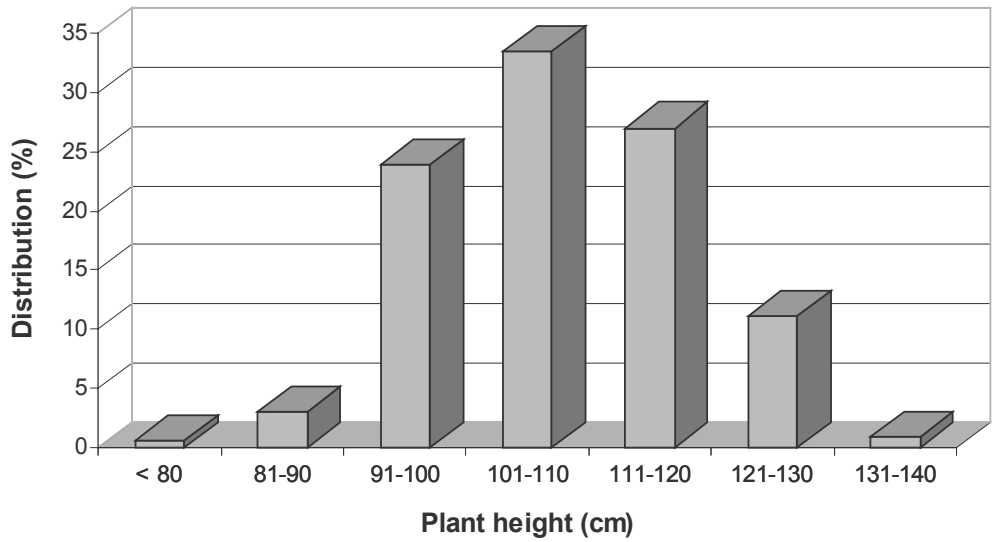

FIG. 2. Plant height — distribution of M_3 lines from the variety EEA-404 (mean height of parent variety was 1.22 m).

Plant height of EEA-404 was 1.22 m and M_3 lines ranged from 0.64 to 1.36 m, with 88% of them shorter than 1.2 m and 28% shorter than 1 m (Figure 2). Thirty semi-dwarf M_4 lines were backcrossed to the parent variety to recover the parent grain type and the populations may be used for genetic studies of dwarf genes involved.

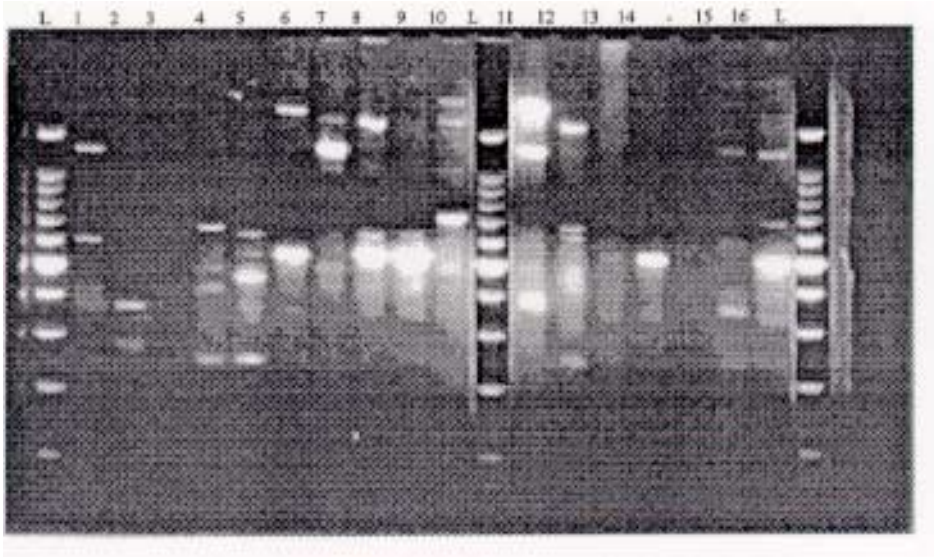

FIG. 3. DNA pooling strategies for detection of polymorphism differences between resistant and susceptible bulks. Lanes: 1–5 bulks of 2 susceptible individuals, –-10 bulks of 2 resistant individuals, 11–12 bulks of 5 susceptible individuals, 13–14 bulks of 5 resistant individuals, 15 bulk of 10 susceptible individuals, 16 bulk of 10 resistant individuals (arrow indicates 600 bp polymorphic RAPD band).

2.2.2. INIA Tacuarí

The ASS incidence was not sufficient to have a reliable characterization of M_3 and M_4 lines. Environmental conditions during 1996/97 and 1997/98 crop seasons favored the development of SR and inoculation with the ASS pathogen was not completely successful, because of competition between both pathogens. However, the material showed wide differences in Straighthead tolerance and some variability in SR and lodging susceptibility. The selected 86 M_4 lines will be tested in 1998/99 on plot basis under inoculation with the ASS fungus in a field with a history of low SR incidence.

3. ANTHER CULTURE AND DH PRODUCTION

3.1. Methodology and description of research carried out

After receiving training at Centro Internacional de Agricultura Tropical (CIAT), Colombia, the Biotechnology Unit at INIA Las Brujas adjusted anther culture procedure on five populations from crosses between resistant/susceptible to ASS-SR parents:

Sel 1855 (Nrx L79/V2-84//Lmnt///EP94)/L933
L933/L578
El Paso 144/L1034
INIA Tacuarí/Katy
INIA Tacuarí/L1034

About 2600 anthers were cultured per treatment, following the adjusted CIAT procedure, detailed in first Progress Report. Efficiency was high, with 2.3-4.5% calli and 1.3–2.4% green plants per anther. A total of 99 DH lines were obtained from those populations. In the 1995/96 crop season, seeds from regenerated plants were planted in the greenhouse, and later transplanted to the field, in INIA Treinta y Tres, for seed increase.

In 1997, anther culture was applied in populations from new crosses involving parents with resistance to ASS, as well as from crosses among introduced short-grain cultivars. Anther culture procedure was completed, obtaining seeds from regenerated individual plants of 16 populations:

Fuzi 102/L1919
L1919/L1762
Koshihikari/INIA Tacuarí
Sasanishiki/INIA Tacuarí
L1919/INIA Caraguatá
INIA Cuaró/L1919
L1167/INIA Tacuarí
L1919/L1172
INIA Cuaró/Chui
L1919/L1415
Koshihikari/Pecos
L1919/Chui
Koshihikari/S-201
Sasanishiki/RU8801121-229
INIA Cuaró/L1762
Sasanishiki/Pecos

However, seeds from the regenerated plants were not obtained in time to be grown in the field in 1997/98. To avoid damage by low temperatures, seed increase was deferred to the following season.

Anther culture procedures were conducted also in 1998 in several populations derived from crosses involving blast resistant Indica parents with local susceptible Indica varieties, as well as in some heterotic crosses among long-grain Japonica cultivars.

Field evaluation of the first group of DH lines from anther culture was carried out in replicated trials in 1996/97 and 1997/98. A total of 99 DH lines from the 5 crosses involving resistant/susceptible to ASS-SR parents, were tested in preliminary trials with 2 and 3 replications in 1996/97 and 1997/98, respectively. The plots had 6 rows, 3.5 m long. Determinations included yield, agronomic traits and milling quality.

3.2. Results obtained

Environmental conditions reduced the yields in 1996/97 and 1997/98. In the first case, heavy rains after planting caused poor stands. In the second case, reduced sunshine during the season and a severe storm before harvest affected grain yield. However, some of the 99 DH lines from the first group showed good yield potential and grain quality (Table 1).

Grain yield of the best DH lines was 10 to 14% higher than that of INIA Tacuarí, while milling quality and growth duration were similar to those of the check. The DH lines also showed better scores for grain length and stem diseases than INIA Tacuarí. Based on this information, 3 DH lines were to be included in the National Uniform Tests for the 1998/99 crop season.

TABLE I. MEAN PERFORMANCE OF THE 5 AND 10 BEST YIELDING DH LINES AND CHECK VARIETIES IN FIELD TESTS IN 1996/97 AND 1997/98

Cultivar	Grain yield		Days to heading	Whole grain %	White belly %	Grain length[a]	SR[b]	ASS[b]
	Kg/ha	% Tacuari						
5 DH > % yield	6322	114	103	61.2	3.6	1.4	4.8	3.0
10 DH > % yield	6049	110	102	61.1	4.2	1.7	5.0	4.0
INIA Tacuari	5249	100	102	61.3	5.1	3.0	6.9	5.3
El Paso 144	5202	99	112	62.3	4.6	3.0	6.0	3.3
Bluebelle	4129	79	107	56.1	5.8	3.0	6.0	3.0

[a]Standard Evaluation System: 1 Extra long, 3 Long.
[b]Standard Evaluation System: 1 Resistant, 7 Susceptible.

Some of the DH lines were not uniform and were discarded. The most promising DH lines were from the population L933/L578, showing good yield potential and milling quality, and low incidence of stem diseases. The parents used in this cross have contrasting reactions to ASS disease, L933 being moderately resistant and L578 (sister line of INIA Tacuarí) highly susceptible.

4. MOLECULAR MARKERS

4.1. Methodology and description of research carried out

Different RAPD molecular markers for detection of genetic polymorphisms among rice cultivars and breeding lines were evaluated, with the objective of applying this technique in analyzing contrasting DNA bulks from populations segregating for ASS and SR resistance. From preliminary results obtained on amplification of bulked DNA samples and using different software, simulations were developed on sample size and probability of detection of RAPD alleles that co-segregate with a phenotypic character ("bulk segregant analysis").

A search for rice genome regions associated with disease resistance was done on the data bases Rice Genes and Rice DB in order to develop a characterization of contrasting lines through PCR amplification of these sequences.

An important group of F_7 breeding lines from one population showed important variability in SR incidence in preliminary trials conducted in 1997/98. ASS was also present in this group, but incidence was much lower than SR. The population also showed good plant type and high yield potential and was derived from a cross between F_5 and F_6 breeding lines: C266 F5/C219 F6 (C266: Nwbt/Nrx L79//L91, C219: Leah//L58/El Paso 144).

To evaluate DNA pooling strategies for identification of genomic regions associated with tolerance to SR, two groups of rice breeding lines were selected representing the phenotypic extremes of resistance/susceptibility in the above mentioned cross.

Two main strategies have been envisaged to select chromosomal regions that contain genes affecting quantitative traits: a) selective genotyping, and b) pooling DNA from several individuals. DNA bulks can provide specific PCR products when pooling the individuals with

the highest and lowest scores of a defined character, when looking for a trait showing continuous variation.

Based on computer simulations, the phenotypic difference between alternative genotypes has to be at least more than two standard deviations, otherwise the probability of finding both genotypes is very high across all of the phenotypic range. If actual frequencies of all the marker alleles can be inferred in the amplified pool, bulked analysis can be directly applied to quantitative traits, either using the quantitative densitometry of allelic bands, or using a procedure called "sequential bulked typing" (SBT).

The experimental design included 3 types of DNA pooling from each group of resistant and susceptible F_7 lines: a) 5 bulks of 2 individuals, b) 2 bulks of 5 individuals, and c) a single bulk 10 individuals. Two 10-mer primers were used to evaluate polymorphisms among the tolerant/susceptible bulks. Amplifications were performed under standard RAPD protocols and resolved in 2% agarose gels.

4.2. Results obtained

Bulked sample analysis (BSA) profiles representative of the above mentioned approach are shown in Figure 3.

The experiments reported here provide some evidences of bulk-specific patterns of DNA amplification using random primers, allowing us to continue searching for additional candidate marker bands. These procedures are being used primarily as an initial screen to detect large effect QTL's associated with resistance/susceptibility in this breeding population. Besides this particular application, SBT would allow to target chromosomal regions containing candidate genes for resistance to SR, providing an alternative to individual typing for a large number of markers and improving DNA pooling techniques based on phenotypic evaluations.

INDUCED MUTATIONS TO DEVELOP SOURCES OF RESISTANCE TO RICE BLAST, *Pyricularia Grisea* Sacc.

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Abstract

Rice blast caused by *Pyricularia grisea* is the most important disease limiting yields worldwide. The pathogen has many virulent forms or pathotypes, hence durable blast resistance is lacking. Studies on strategy to develop durable blast resistance based on defining the genetic structure of the population, using DNA-fingerprinting, and virulence diversity are described. This strategy is leading to the identification of resistance genes/sources against all isolates within a genetic family of the pathogen. Combinations of genes showing complementary resistance to different genetic families of the fungus exclude any compatible interaction with a blast isolate. Identification of complementary resistance genes is based on detecting those virulence factors whose combinations in individual isolates within the pathogen population have a frequency near zero. Identifying and combining resistance genes to which combinations of corresponding virulence genes are absent in the pathogen population should confer more durable resistance than that previously obtained. The use of induced mutations in the development of resistance was limited, since in most cases single gene changes were responsible for the induced resistance against all the pathogen population. The main objective here is to develop many mutants, each with a gene resistant to just one or a few families of the blast pathogen; and crossing them can accumulate the different resistance genes. A total of 201 Latin American commercial cultivars, including Cuban, Brazilian and Venezuelan were analyzed with different genetic families of the blast pathogen to identify potential sources of resistance to blast and identify complementary resistance sources. Characterization of the resistance of 37 mutants of the Colombian rice cultivar Oryzica 1 was conducted in collaboration with the INEA in Colombia. Results suggested that mutations for resistance to genetic families to which Oryzica 1 is susceptible were induced, although one isolate in the most common genetic family (SRL-6) of the pathogen was compatible with all mutants. These results suggest that a larger population of mutants is needed to increase the probability of identifying a gene resistant to all members of this family; and that mutations in a variety, initially resistant to this genetic family (SRL-6) but susceptible to a different family, could have better probability of success. Seven additional commercial rice cultivars from Colombia were irradiated and selection of mutants, differing in their resistance to the different genetic families of the pathogen has been conducted during 1997 and 1998. Several different resistant mutants have been selected and will be used in crosses to combine the various resistance genes.

1. INTRODUCTION

Rice blast (*P. grisea* Sacc., the anamorph of *Maganaporthe grisea* (Hebert) Barr), is a major factor limiting yields of rice (*Oryza sativa* L.) worldwide, particularly under rained and upland conditions prevalent in Latin America. The pathogen produces necrotic lesions on the seedlings' leaves and on the leaves, nodes, necks and panicles of mature plants, causing severe yield losses. Development of resistant cultivars is the preferred means of controlling the disease, as fungicides are expensive and not environmentally friendly. However, resistance is overcome by the pathogen shortly after the resistant cultivars' release. Developing durably resistant cultivars is then a high priority at CIAT and where blast occurs.

The rice blast pathogen reproduces asexually and is noted for the large number of pathogenic races, this variability being cited as the main cause of the breakdowns in resistance. Nevertheless, it has been proposed that continuous challenge to resistant rice

breeding lines by a diverse pathogen population and detailed genetic information on the population structure to understand the virulence dynamics of the blast pathogen would reduce the risk of resistance breakdown and allow identification of more stable resistance.

In order to develop more sustainable production systems in agriculture through the development of more durable resistance to diseases, CIAT with funding from the Rockefeller Foundation, the Colombian Government, and in close collaboration with the IAEA, has been developing strategies directed to the protection of the rice crop. This is being achieved by understanding of the genetic structure of blast populations in Colombia, and thus developing a model that can be applied in all Latin American countries. Such studies are allowing understanding the pathogen changes and mechanisms leading to the breakdown of resistance. Finally, the resistance genes selected for developing a more durable blast resistance will depend on the genetic diversity and virulence composition of a pathogen population. This paper introduces the potential application of induced mutations in developing sources of resistance leading to a more durable blast resistance.

2. GENETIC AND VIRULENCE STRUCTURE OF THE RICE BLAST PATHOGEN IN COLOMBIA

The sustainability of agricultural production systems constantly threatened by the development of epidemics caused by different plant pathogens, can be maintained by developing breeding strategies that facilitate more durable and stable forms of resistance to these pathogens. Accomplishment of this objective is possible by understanding the genetic and virulence diversity of the pathogen. Controlled inoculations of the blast pathogen collected from different sources in Colombia have yielded more than 50 international races. Virulence factors compatible with at least 13 known resistance genes were detected in this population, with no cultivar susceptible to all isolates. Accumulation of a large number of virulence factors to most resistance genes in an individual isolate is common, making it more difficult to identify potential sources of resistance to the fungus. Virulence diversity studies suggest that new resistance genes, or combinations of resistance genes, that can be overcome only by part of the pathogen population, are needed to control rice blast. Combinations of resistance genes could be generated from crosses between complementary groups for which the pathogen does not accumulate the corresponding virulence genes. Long term observation of virulence/avirulence frequencies and accumulation of virulence factors would be needed to monitor this strategy. Absence of certain virulence combinations could be due to deleterious effect of such combinations on the pathogen. In other words, as virulence is expressed after the loss of an avirulence gene, there are apparently certain avirulence gene combinations that if lost are detrimental to the fungus. One of these effects could be a reduced fitness in the population.

A repeated DNA probe (MGR 586) obtained from a rice blast fungus genome was found to be useful for grouping blast populations in genetic lineages, each with a particular spectrum of virulence characteristics. The complex virulence structure in the blast fungus pathotypes found in Colombia was classified into six distinct genetic lineages (SRL1 to SRL-6) using the DNA-MGR 586 probe. The average similarity among all the lineages was 49%, while isolates within each lineage expressed very similar fingerprints, with similarities ranging from 92 to 98%. In general each genetic lineage was composed of several pathogenic races; one race could be present in different genetic lineages, and one lineage could be recovered from different cultivars. However, if *P. grisea* populations are generally composed of a limited number of lineages and if there is a close relationship between the lineage and virulence characteristics of the constituent individuals, population analysis at the lineage level

could aid in directing resistance breeding projects that target the pathogen population in question.

Characterization of the genetic lineage structure, together with the virulence spectrum and the virulence frequencies within the whole blast pathogen population, should provide a more reliable estimate of the durability of cultivar resistance than only consideration of virulence or lineage alone. Results indicate that a given resistance gene may be overcome by isolates from different genetic lineages. On the other hand, several virulence factors are shared among isolates within the same genetic lineage and may be accumulated in a single blast isolate, or are present in individual pathotypes that are a subset of virulence, corresponding to the full virulence spectrum of that lineage. Certain specific virulence-lineage-resistance gene interactions are observed where a resistance gene seems to be effective against all the individuals of entire lineages yet is susceptible to most individuals of other lineages. Although genetic lineage SRL-6 in Colombia exhibits a wide spectrum and accumulation of virulence genes to most commercial cultivars released in the past, a high specific interaction between certain rice cultivars and genetic lineages has been observed. Some commercial cultivars are resistant to all the isolates tested from some lineages, yet, they exhibit a susceptible reaction in the field. Greenhouse studies have demonstrated that in some cases such susceptibility is only to isolates from one genetic lineage, and therefore the cultivar carries resistance genes to other families of the pathogen. Durable blast resistance such as the one exhibited by the commercial cultivar Oryzica Llanos 5 in Colombia is apparently controlled by the combination of complementary resistance genes derived from parents which are susceptible to different genetic lineages of the blast pathogen. Combinations of genes showing complementary resistance to different genetic families of the fungus should exclude any compatible interaction with any blast isolate in any lineage.

The resistance in Oryzica Llanos 5 did not emanate from just one of its ancestors, since all were susceptible in the field and to at least some of the isolates to which this cultivar is resistant. None of the genetic lineages of the blast pathogen in Colombia exhibit compatibility with all the parental lines. Instead, complementary resistance to all the lineages is observed among the different parents. Thus, it seems that development of stable resistance is possible by combining different sources that exhibit susceptibility to some segment of the blast population. Useful resistance genes would be those for which combinations of the matching virulence factors are absent in the pathogen population. Therefore, identification of these potential gene combinations requires continuous characterization of the genetic structure and monitoring of the virulence frequencies of the blast pathogen as well as germplasm screening for the identification of complementary resistance genes.

3. POTENTIAL APPLICATION OF INDUCED MUTATIONS IN DEVELOPING DURABLE BLAST RESISTANCE

Although this technique has been successfully implemented in many cases in the identification of resistance to diseases, rapid breakdown of mutation-induced resistance has also been reported very often. One of the reasons for the failure of resistance obtained through mutation is that in most cases, a single gene change (monogenic resistance) is responsible for the induced resistance against all the pathogen population. In this research agreement we have considered the alternative of developing mutants which are resistant only to a few genetic lineages of the pathogen. The idea is to identify as many mutants as are needed so that targeted crosses between these mutants will facilitate combining different resistance genes to exclude all the genetic lineages of the blast pathogen. We believe that the possibility offered pathogen is a worthy alternative for developing a more stable resistance in rice.

We evaluated at CIAT the reaction to rice blast of 37 mutants of the commercial cultivar Oryzica 1 obtained by gamma ray irradiation of the seeds at the INEA (Instituto de Ciencias Nucleares y Energias Alternativas) in Colombia. The results demonstrated that mutants resistant to the isolates to which the non-irradiated cultivar was susceptible could be obtained. However, we detected in the most predominant genetic family of the pathogen (SRL-6) one isolate compatible with all the mutants. The results suggested that evaluating a larger population of mutants was needed to increase the probabilities of identifying a resistance gene to all members of this family. Results also suggested that mutations in a variety initially resistant to this genetic family (SRL-6) but susceptible to a different family could have better probabilities of success. Resistant mutants identified are being evaluated under field conditions for other traits and used in crosses with other rice cultivars.

A total of 201 commercial rice cultivars from Latin America were analyzed for their reaction to different genetic families of the blast pathogen to identify potential sources of resistance to blast and identify complementary resistance sources. Dr. Adolfo Alvarez irradiated seven of these rice commercial cultivars obtained from Colombia at the INEA institute with gamma rays (300 Gy). Selection of mutants differing in their resistance to different genetic lineages of the pathogen has been conducted during 1997 and 1998. Three of the cultivars, Oryzica Caribe 8 (susceptible only to lineage SRL-4), Cica 8 (susceptible only to lineage SRL-5), and Oryzica Llanos 5 (partially compatible with lineage SRL-4), exhibit resistance to genetic lineage SRL-6. Several mutants exhibiting resistance to every one of those lineages have been selected and will be used in crosses to generate segregating populations for selecting lines combining the corresponding complementary resistance genes. Selection within a larger sample of mutants in the cultivar Oryzica 1 yielded several mutants resistant to lineage SRL-6. The total number of resistant mutants selected were: 26 of Oryzica 1 with resistance to lineage SRL-6; 9 mutants from cultivar Oryzica Caribe 8 with resistance to lineage SRL-4; and 2 mutants from cultivar Cica 8 with resistance to lineage SRL-5.

The future objectives of this project are: 1) Corroborate resistance to blast in M_4, M_5, and M_6 lines of each commercial cultivar (Cica 8, Oryzica Caribe 8, Oryzica 1, and Oryzica Llanos 5). 2) Design crosses (simple, double, triple) among selected resistant mutants to combine complementary resistance genes excluding all pathogen lineage/virulence. 3) Plant segregating lines under field conditions for evaluation and selection of blast resistant lines. 4) Conduct genetic studies of resistance in selected mutants exhibiting blast resistance.

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ISOZYMES VARIABILITY IN RICE MUTANTS INDUCED BY FAST NEUTRONS AND GAMMA RAYS

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Abstract

The isozyme variability of a group of rice mutants induced through gamma and fast neutron (14 MeV) irradiation was studied. Polymorphisms were detected using esterase, peroxidase, polyphenol oxidase and alcohol dehydrogenase systems. The mean value of genetic similarity among the different cultivars, which arose from isozymes, was 0.75. The dendrogram was constructed based on genetic similarity matrices, designed with isozyme data using the unweighed pair group method arithmetic average (UPGMA) method. The efficiency of the UPGMA model for the estimation of genetic relationship among cultivars was supported by cophenetic correlation coefficients. Such values indicate that the distortion degree for the estimated similarities was minimal. It was found that both gamma rays and fast neutrons generated a wide range of variability which can be detected by means of isozyme patterns, even in closely related cultivars.

1. INTRODUCTION

Practical application of plant mutation breeding has made good progress. This breeding technique has yielded genetic variants in many plant species and a certain proportion was found to be useful [1]. Among them, rice has received special attention: already by 1991, 251 new varieties had been introduced into the production [2].

In Cuba, a rice breeding program using fast neutrons and gamma radiation was initiated in 1988. In this program nearly thirty new mutants have been obtained [3].

However, there is very little information about the genetic variability generated by mutagens on these materials, which is crucial for the effective use of genetic diversity resources in breeding programs.

Biochemical methods of investigation, especially isozyme studies, have provided valuable tools for rice geneticists. Electrophoretically identifiable isozymes have often been utilized for the classification of varieties within *O. sativa* [4] and they have proved useful in diversity surveys of rice induced mutants [5]. In the present work the enzymatic polymorphisms in a group of rice mutants was studied.

2. MATERIALS AND METHODS

2.1. Plant material

Three varieties and 8 mutants, 2 to 4 from each variety, were studied (Table I); 4 mutants were induced by fast neutrons and 4 by gamma rays.

TABLE I. GENOTYPES USED IN THE SURVEY AND THEIR ORIGIN.

Variety/line	Origin	Radiation used[a]
Jucarito-104	IR-480-5-9-2/IR-930-16-1	--------
3762	Mutant of Jucarito-104 (20 Gy)	FN
3763	Mutant of Jucarito-104 (20 Gy)	FN
3764	Mutant of Jucarito-104 (20 Gy)	FN
3263	Mutant of Jucarito-104 (20 Gy)	FN
Gloria	--------	--------
G-C_{10}-2-1-7	Mutant of Gloria (300 Gy)	GR
G-C_{10}-2-1-8	Mutant of Gloria (300 Gy)	GR
Basmati	--------	--------
B-14-2-2	Mutant of Basmati (200 Gy)	GR
B-14-2-3	Mutant of Basmati (200 Gy)	GR

[a] FN = Fast neutrons, 14 meV; GR = ^{60}Co, gamma rays.

2.2. Isozyme assays

Electrophoretic assays were performed in vertical polyacrylamide gels with discontinuous buffer system basically as described earlier [6]. The staining techniques used for the esterase and peroxidase systems were reported by Iglesias and Gonzalez [7].

2.3. Data analysis

For bands' scoring, polyacrylamide gels were replicated at least three times. Only consistent and reproducible bands were considered.

Polymorphisms were scored for the presence or absence of bands and the data was analyzed using the NTSYS-PC version 1.8 [8]. The average proportion of alleles that are shared between any two of the accessions screened, was used as the measure of similarity. For inbreeding species, such as the present Cuban rice varieties, this corresponds to using the Nei and Li coefficient [9] or their algebraic equivalent, Dice's coefficient [10] for co-dominant marker data (isozymes). Cluster analyses were based on similarity or distance matrices using the UPGMA and relationships between accessions were visualized as dendrograms. The Mantel matrix correspondence test [11] was used to compare cophenetic and similarity matrices in order to define the congruence degree in the estimation of genetic relationships.

3. RESULTS AND DISCUSSION

The esterases and peroxidases isozyme systems were chosen because they revealed a good level of polymorphism in a previous study on fast neutron and γ-induced mutants [5]. In the present survey higher levels of polymorphism were detected with peroxidases rather than with esterases. Different authors [12, 13] have also reported this isozyme system as the most polymorphic one in plants.

In the peroxidase system, there were eleven polymorphic bands out of twelve, whereas esterase showed six polymorphic bands out of eight. Likewise, whereas the peroxidase system showed eight isozyme patterns among the analyzed genotypes, in the esterase system only six different patterns appeared (data not shown). In this survey, both disappearance and appearance of new bands were observed.

The number of isozyme patterns found in the peroxidase system, related to the Jucarito-104 genotypes, was higher than that obtained in previous studies [5], in which eleven cultivars were used. However, in the esterase system relatively less polymorphism was detected.

The wide distribution of genotypic variants across the different isozyme patterns is good evidence that a high degree of genetic variability was generated (Table II).

TABLE II. FREQUENCY (%) OF THE ISOZYME PATTERN VARIANTS OBTAINED.

Variant[a] No.	Isozyme system	
	Peroxidase	Esterase
1[b]	18.2	18.2
2	9.1	27.3
3	18.2	9.1
4	9.1	18.2
5	9.1	9.1
6	9.1	18.2
7	9.1	-
8	18.2	-

[a]Data not shown
[b]Variant in which the parental profiles are included.

Table III shows the similarity matrix calculated using the Dice index [10]. The genetic similarity mean value from isozymes data was S = 0.75. In general, the isozyme systems used could detect the existing variability, differences between the surveyed lines. These results agree with those obtained in other studies, where the genetic core of the Cuban rice germplasm bank was analyzed [14].

TABLE III. SIMILARITY MATRIX CALCULATED WITH DICE INDEX, USING BINARY DATA OBTAINED FROM PAGE ISOZYMES PROFILES.

	1	2	3	4	5	6	7	8	9	10	11
1	1.00										
2	0.81	1.00									
3	0.88	0.94	1.00								
4	0.81	1.00	0.94	1.00							
5	0.88	0.81	0.88	0.81	1.00						
6	0.79	0.64	0.73	0.64	0.73	1.00					
7	0.86	0.73	0.80	0.73	0.80	0.94	1.00				
8	0.85	0.71	0.73	0.71	0.73	0.81	0.88	1.00			
9	0.66	0.57	0.60	0.57	0.73	0.69	0.77	0.69	1.00		
10	0.66	0.71	0.66	0.71	0.80	0.62	0.71	0.69	0.84	1.00	
11	0.66	0.71	0.66	0.71	0.80	0.62	0.71	0.69	0.85	1.00	1.00

The dendrogram that was constructed based on genetic similarity matrix using isozymes data and the NTSYS-PC computer pack [8] is shown in Fig. 1. All the studied varieties conformed to three well defined clusters. The first one included the J-104 cultivar and mutants, the second included Gloria and related mutant lines. Both mutant lines can be considered as Group I following Glaszmann's classification [4], which includes Indica rice type. The third one, however, is the more distant cluster (68% of genetic similarity with the rest of analyzed lines). The Basmati varieties are included in Group V of Glaszmann's classification [4]. This group holds intermediate type varieties, between the Indica and

Japonica morphological types, which are clearly differentiated from Group I [4]. This explains the large differences found in our survey between Basmati type varieties and the other cultivars.

Likewise, the efficiency of the UPGMA in the estimation of genetic relationships between varieties was corroborated by means of the cophenetic correlation coefficient (0.86, $P < 0.01$) calculated using Mantel test [11]. Such a value indicates that the distortion degree in the estimated similarity relationship was minimal. Furthermore, the results support the conclusion that the dendrogram in Fig. 1 shows a proper representation of the associated similarity matrix.

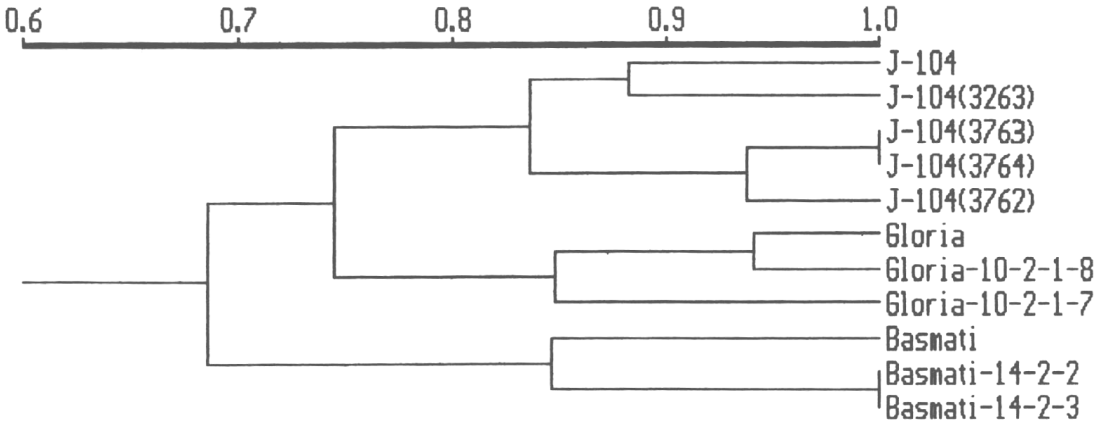

FIG. 1. Dendrogram corresponding to the similarity matrix (Table III) constructed using UPGMA analysis.

The dendrogram generated by the isozyme data agreed well with the genealogy of the rice materials studied (Table 1), showing the usefulness of isozyme markers in diversity surveys of induced mutants in rice.

The results in the present paper suggest that isozyme polymorphisms should be used in varietal certification. However, based on our experience with induced mutants in rice, good polymorphisms are not always obtained. The differential response of plant genotypes to ionizing radiations and the low loci number that can be surveyed by isozymes, should be the determining factors in the degree of the obtained polymorphism (data not published).

Analyses of data bases obtained from different markers types such as AFLP, RAPD and isozymes, offer great potentials in varietal validation, particularly in fast neutron - and gamma-induced mutants.

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IMPROVEMENT OF RICE THROUGH SOMACULTURE AND INDUCED MUTATIONS IN SÃO PAULO STATE

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Abstract

Rice (*Oryza sativa L.*) is one of the most important crops in São Paulo State, Brazil, from both the production and mainly the consumption standpoint. It is cultivated predominantly under upland conditions in areas that have high temperature and humidity, excellent environment for rapid spreading of the blast disease (*Pyricularia grisea*). The objective of this research was to induce mutations in connection with somaculture, for resistance to blast and a high yield potential, retaining the other qualities of IAC 201, a commercial upland variety. Seeds were irradiated with ^{60}Co gamma rays at 300, 350 and 400 Gy, and boots and seeds from M_1 plants in the 300 Gy treatments were harvested for mutation *in vitro* and *in vivo* techniques. Selection was not done in M_2 generation; panicles were harvested and the offsprings were evaluated in the M_3 generation for blast reaction. From 10340 panicles screened, 267 lines were selected with a possible blast tolerance. In the M_4 generation these populations were again evaluated for blast reaction and for agronomic traits. Fifty-three possible mutant lines with tolerance to blast were selected, with more than 15% increments in yield potential. From these lines it is expected that at least one will be released in the near future as a new, resistant upland rice cultivar for São Paulo State, Brazil.

1. INTRODUCTION

Rice (*O. sativa* L.) is grown on every continent and is an important food staple, providing food for almost two thirds of the world's population. Also for Brazil, in São Paulo State, rice is a very important crop from both the production and mainly the consumption standpoints. Most of the rice in São Paulo is cultivated as an upland crop and depends on rainfall for moisture, but there is also some irrigated acreage. The rice growing areas of São Paulo State are characterized by high temperature and humidity, excellent environment for the rapid spread of the blast disease pathogen (*Pyricularia grisea*) [1]. The rice blast, is considered the most important disease in São Paulo State causing severe losses in yield and rice quality. Unfortunately, blast resistance in rice varieties can be lost quickly after large scale cultivation, due to the development of new races.

The main objectives of our rice breeding program, beside yield, are grain quality and blast resistance. We released an upland variety with excellent grain quality and agronomic traits, but this variety is susceptible to blast. The objective of this project was to induce genetic variability in rice cultivar IAC 201 by irradiating seeds with ^{60}Co gamma rays and by using biotechnology approaches, as reported elsewhere [2, 3, 4, 5, 6, 7]; thus obtain and select plants tolerant or resistant to the blast disease having good agronomic characteristics and grain quality, as in the IAC 201.

2. MATERIALS AND METHODS

2.1. First phase

Seeds of IAC 201, an upland rice variety developed by the Instituto Agronômico de Campinas (IAC), were irradiated with 300, 350 and 400 Gy, using a ^{60}Co source of gamma rays. About 4500 seeds per dose were planted in field plots to obtain the M_1 generation, for a mutation programs *in vivo* and *in vitro*. According to the results shown in Table I, only plants from the 300 Gy treatments were used, because the other doses were considered too high. At the boot stage the surviving plants from 300 Gy and control IAC 201 plants were harvested for somaculture. For callus induction 450 tillers from 300 Gy and 300 from non-irradiated plants were harvested, and small panicles (immature) were used with about 1.5 cm long or less as explant material. All the regenerated plants were grown in the greenhouse until maturity and harvested individually. Seeds from plants in the field plot with 300 Gy treatment were also harvested as the M_2 generation. We also harvested the non-irradiated IAC 201 plants from the control plots to be used as checks in the next step. The harvested plants were threshed by hand and the seeds were dried to 13% moisture. There were five seed populations according to the sterility rate (Table II).

TABLE I. SURVIVAL OF PLANTS AFTER SEED IRRADIATION AT THREE ^{60}CO GAMMA RAY DOSES

Material	Doses			
	300 Gy	350 Gy	400 Gy	Unirradiated
Seeds planted, No.	4500	4500	4500	4500
Surviving plants, No.	1576	756	125	4155
Surviving plants, %	35.8	16.8	2.7	92.3

TABLE II. M_2 POPULATIONS ACCORDING TO THE STERILITY RATE IN M_1 GENERATION

Population	Sterility rate
M_2 -1 (A)	Plants with 50 - 80 seeds (mutant *in vivo*)
M_2 -2 (B)	Plants with more than 100 seeds (mutant *in vivo*)
M_2 -3 (C)	Plants with less than 50 seeds (mutant *in vivo*)
SM_2 (D)	Somaclones from 300 Gy (mutant *in vitro*)
R_2 (E)	Somaclones from control IAC 201

2.2. Second phase

The M_2 populations A, B, C, D and the E population (Table II) were sown in a pathogen-free field in a single row per plant, a total of 431 rows, with a total 30617 plants surviving. A check row with the original variety was planted every 10 mutant rows, a total of 3089 plants in 34 rows. No selection was made in M_2 generation, only some albinism was observed and recorded. At maturity one panicle was harvested from each plant in each row, which constituted the M_3 generation seeds, a total of 25000 panicles. All these panicles were threshed by hand and dried to 13% moisture. The seeds of the best panicles were placed in small paper bags and recorded. In the M_3 generation there were 10340 such panicles.

2.3. Third phase

At this phase all the 10 340 panicles in M_3 generation was evaluated for blast tolerance, using The Standard Nurseries for Homogeneous Reaction to Leaf Blast.

In November 1995 two continuous rows were planted, using a susceptible rice variety (IAC 165) all around the area planned for the trial, to ascertain presence of enough inoculum at the evaluation time. In January 1996 the seedbeds were prepared for tests in the M_3 generation. Seeds from the 10340 panicles were sown in short 20 cm long rows and 10 cm apart. Every 10^{th} row was a check with seeds of the original variety (IAC 201). The first and the last five rows were planted with the mixture of susceptible varieties to promote the disease spread, as were the 2 border rows. The total M_3 generation consisted of 52 plots with 200 rows each. All plots were fertilized with 240 kg N/ha applied 1/3 during the planting, 1/3 fifteen days after germination and the other 1/3 twenty five days later. The evaluation was done forty days after germination according to the visual symptoms and rated from one to nine as was adopted at the Symposium on The Rice Blast Disease in 1963, IRRI. The original parent material (IAC 201) was rated 6 (considered susceptible), therefore all rows that rated 3 or less were selected; a total of 267 disease free rows at the leaf stage. All of them were transplanted to another field plot to be evaluated for neck blast. All lines were harvested and naturally dried to 13% moisture. The panicles of each row (line) were threshed separately by hand at maturity, kept in paper bags and stored as the M_4 generation.

2.4. Fourth phase

This phase covered two seasons (1996/97 and 1997/98). In both seasons there were a yield trial and a blast test experiment in the field.

2.4.1. Field experiment

All the 267 lines selected for blast tolerance screening were planted under upland conditions. The experiment was laid out in seven blocks with 40 plots in each and one block with 16 plots, a total of 295 plots. Each plot contained 3 rows, 1.5 m long and 40 cm apart. After every 10 plots with mutant lines one plot with IAC 201 was planted, as a control plot. These experiments were grown as normal for upland rice, harvested by hand at maturity and the data recorded.

2.4.2. Blast test

Seeds from one panicle from all 267 lines in the M_4 generation, were sown as was described for the third phase, replicated in two locations, using the Standard Nurseries for Homogeneous Reaction to Leaf Blast. The two sites covered almost 95% of all the blast races occurring in São Paulo State. The complete experiment contained 3 plots with 200 rows in each and replicated for two seasons.

In this fourth phase data were recorded on blast tolerance reaction and on field traits viz.: panicle length, number of filled grains per panicle, number of empty spikelets per panicle, 1000-grain weight at 13% moisture, estimated yield potential, number of days to flowering, grain shape. At natural infection the field plots experiment did not show panicle blast reaction. The field plots were harvested by hand and panicles were dried to 13% moisture.

3. RESULTS AND DISCUSSION

3.1. First phase

In all irradiated M_1 populations the germination rate was lower than 50% (Table I). These data suggest that a low dose, e.g. 200 Gy would be better. Also, establishing the radiosensitivity for each rice cultivar, before implementation of mutation induction in a breeding program is desired.

Although 300 Gy was considered a little bit high, the population from this treatment was used to continue this study. During the somaclonal work no difference was found between boots that came from 300 Gy population or from the control population and callus was induced very well in both. However, regeneration rate was very poor: there were 33 plants from the control population and 91 plants from the 300 Gy population; this may indicate that irradiation increased the regeneration rate. At maturity a high level of sterility was observed in all somaclones, so we decided to harvest the plants according to the sterility rate and separate them into five M_2 populations (Table II).

3.2. Second phase

Table III presents the data for all five M_2 populations that were planted in the upland field. There were no differences in the survival rate among the population nor in albinism rate (Table III). Only in the somaclonal population no albino plants were found. From the 30 617 plants, 25 000 panicles were harvested and the best 10 340 were chosen visually to constitute the M_3 generation.

TABLE III. M_2 SURVIVAL AND ALBINO FREQUENCY IN FIVE POPULATIONS PLANTED IN UPLAND CONDITIONS

Population	Rows planted No.	Seeds sown No.	Surviving plants, No.	Albinos No.	Albinos %
A	62	3100	2577	61	2.31
B	113	11300	8868	207	2.28
C	65	6500	5754	101	1.73
D	31	3100	2352	60	2.49
E	126	12600	11066	---	---
IAC 201(control)	34	3400	3089	---	---
Total	**431**	**36600**	**30617**	**429**	**---**

3.3. Third phase

The evaluation and selection for blast tolerance were done forty days after germination (Fig. 1A). The leaf blast infection was severe - the sensitive materials were completely burned by blast (Fig. 1B) - and disease-free rows were identified.

Table IV presents the M_3 selection in each population. The population M_3-A gave a high number of selected mutants for blast tolerance and M_3-E was the worst one. Comparing all the populations, 210 mutants were selected from mutations *in vivo*, 41 from mutations *in vitro* and only 6 from somaclonal variation.

FIG. 1. Rice blast evaluation in the M_3 generation using the Standard Nurseries for Homogeneous Reaction to Leaf Blast. A - General view of the plots forty days after germination; B.- Border lines completely burned by blast and selected disease free rows.

TABLE IV. NUMBER OF PANICLES HARVESTED FROM SURVIVING M_2 PLANTS AND NUMBER OF MUTANTS SELECTED FOR BLAST TOLERANCE IN M_3 GENERATION

Population	M_2 plants surviving, No.	M_3 panicles harvested, No.	M_3 mutants *in vivo*, No.	Selected *in vitro*, No.	Selection pressure, %
A	2577	1013	120	--	0.12
B	8868	3820	75	--	0.19
C	5754	1682	25	--	0.02
D	2352	611	--	41	0.07
E	11066	3182	--	6	0.002

This study suggests that the induced mutations in connection with somaculture increased the selection rate for blast tolerance. Data also showed that for mutation *in vivo* compared with mutation *in vitro* the ratio was 5:1. All 267 mutants selected for improved tolerance to leaf blast also show potential tolerance to panicle blast.

3.4. Fourth phase

Fig. 2 presents the frequency distribution for blast reaction for all mutant lines in the M_4 generation. The mean grade in 28 readings for IAC 201, the susceptible cultivar used as a control, was 6.3. 194 lines, 65.8% of the population, had grade 4.0 considered as partial resistance and 60 lines, 20% of the population, were graded 5.0, i.e. moderately susceptible, 35 lines, 11.9% were like the original variety and only 2% were considered highly susceptible. In the M_3 generation blast was the only character selected, hence this M_4 generation distribution. It is also an indication of the efficiency of the methodology used. These results suggest that further selection in further generations should be practiced to ascertain improvement of blast tolerance and identify mutants that could be released as new upland rice cultivars for São Paulo State.

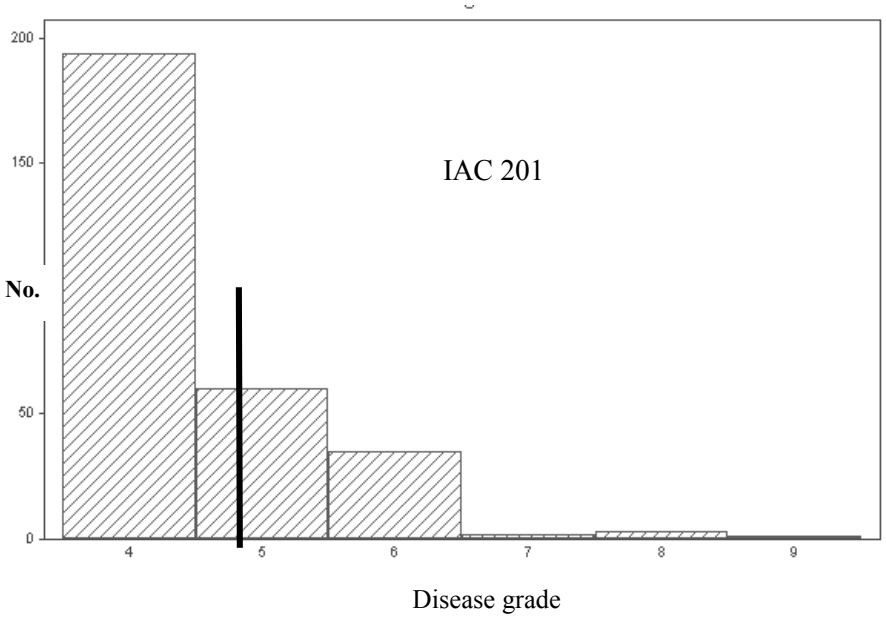

FIG. 2. Frequency distribution of mutants for tolerance to leaf blast (grade 1-9).

Figs. 3, 4, 5, 6 present the frequency distributions for panicle length, 1000-grain weight, % sterility and days to flowering. They show that all these characters studied in the 267 mutants in M_4 generation had a normal distribution. In all characters measured the original cultivar fit exactly around the mean.

Table V presents performance data for all traits evaluated. It is evident that the estimated yield potential was normally distributed and the mutant populations means were close to those of the original cultivar.

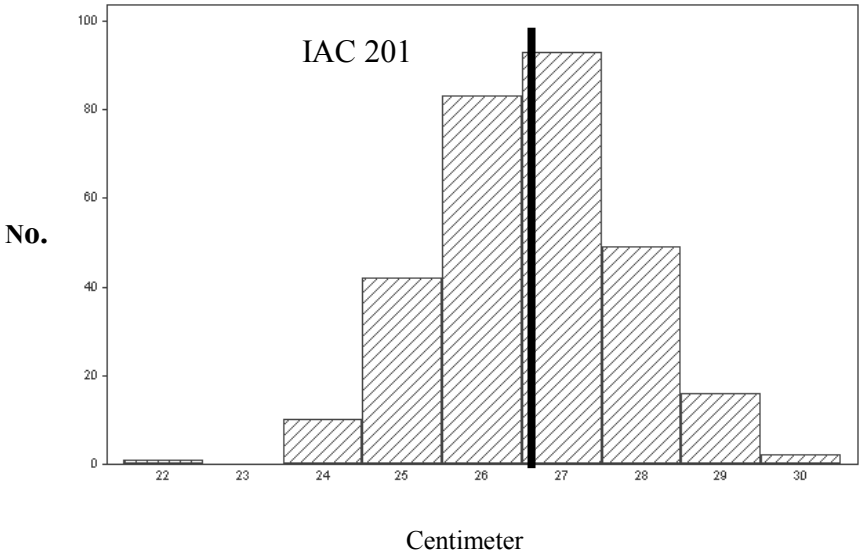

FIG. 3. Frequency distribution of the M_4 mutants for panicle length.

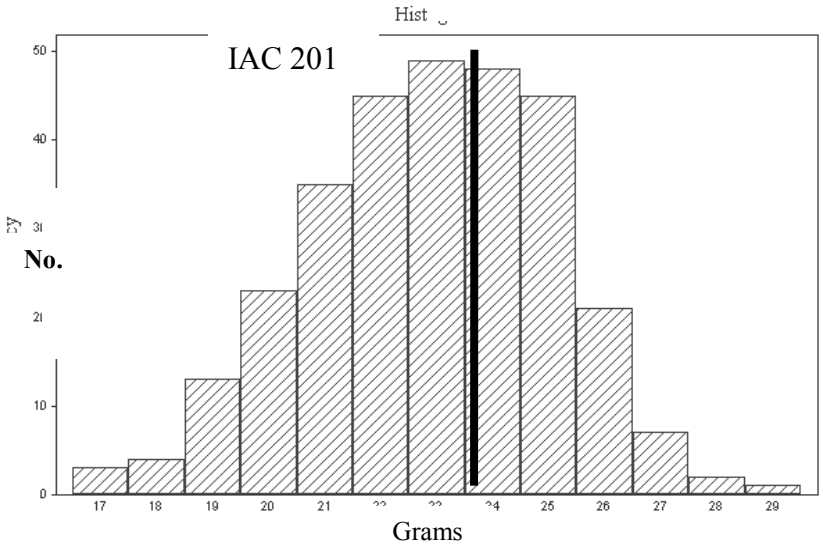

FIG. 4. Frequency distribution of the M_4 mutants for 1000 grain weight.

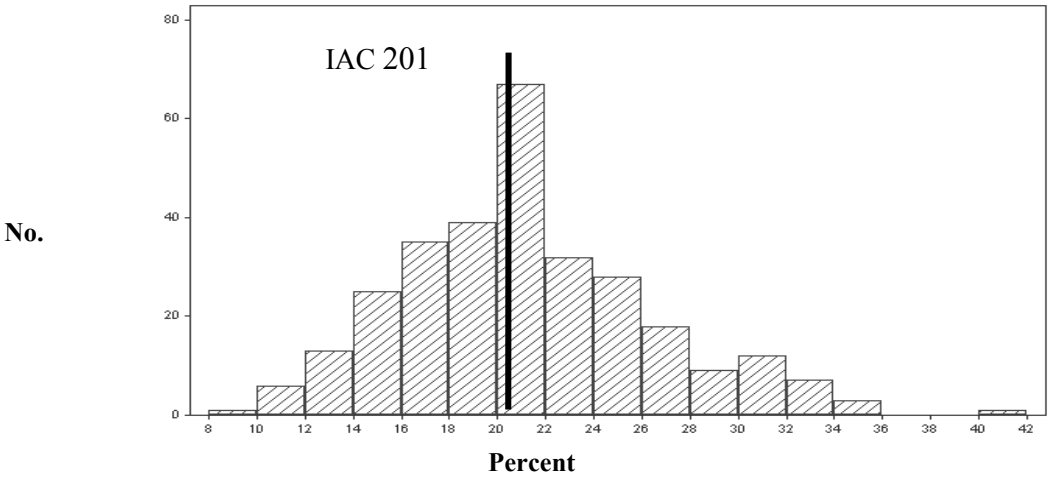

FIG. 5. Frequency distribution of the M_4 mutants for sterility.

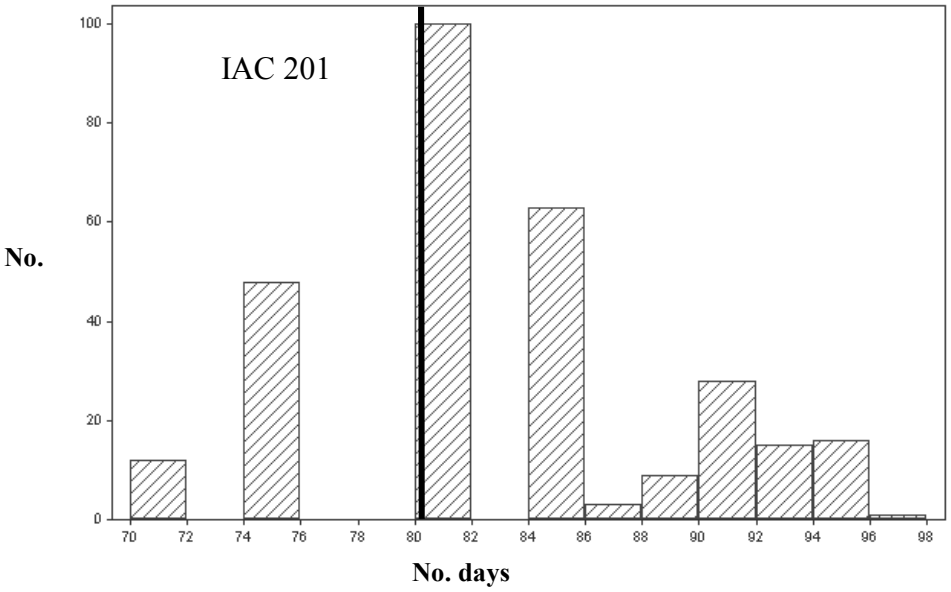

FIG. 6. Frequency distribution of the M_4 mutants for number of days to flowering.

TABLE V. MEAN AGRONOMIC PERFORMANCE OF THE M_4 MUTANTS COMPARED WITH IAC 201 CONTROL

Trait	IAC 201[a]	Mutants				
		N	Mean	SD	Minimum	Maximum
Panicle length (PL)	26.7	296	26.6	1.23	22.0	30.0
1000-Grain weight (1000 GR W.)	22.8	296	22.9	2.19	17.0	29.0
Sterility (STER)	20.3	296	20.8	5.21	8.0	40.0
Number of filled grain, 4 panicles (NFG)	813.0	296	723.0	--	471.0	1298.0
Days to flowering (DF)	80.0	295	82.6	6.32	70.0	96.0
Leaf blast (LB)	6.3	295	4.5	0.85	4.0	9.0
Estimated yield potential (EYP)	2960.0	295	2722.0	837.29	920.0	5280.0

[a]Means of 28 entries.

TABLE VI. MUTANTS SELECTED IN M_5 GENERATION AND THEIR BLAST TOLERANCE, AGRONOMIC PERFORMANCE AND GRAIN SHAPE

Mutant No.	Population	Pedigree		Leaf blast (1 - 9)	Yield potential kg/ha	Days to flowering No.	1000 grain weight (g)	Panicle length (mm)	Sterility %	Grain shape			
										Length (mm)	Width (mm)	Thickness (mm)	Ratio L/W
M96020	POP - A	L 9 -	P13	4.0	3080	75	25.7	28.0	18.6	6.72	2.07	1.73	3.25
M96022	"	L 10 -	P 1	5.0	4320	85	25.4	25.2	29.2	6.77	1.99	1.70	3.40
M96023	"	"	P 3	4.0	4080	75	24.2	26.6	20.2	6.52	1.95	1.66	3.34
M96024	"	"	P 4	5.0	3220	75	24.8	24.9	23.2	6.69	2.06	1.74	3.25
M96036	"	L 24 -	P 2	4.0	3440	85	25.9	24.4	16.1	6.77	1.99	1.70	3.40
M96037	"	"	P 3	4.0	3360	85	23.7	26.4	15.9	6.61	2.02	1.72	3.27
M96043	"	L 34 -	P16	4.0	3960	75	28.9	20.6	24.4	2.49	1.97	1.67	3.29
M96076	"	L 56 -	P 5	4.0	2720	80	25.8	25.5	15.9	6.51	1.99	1.69	3.27
M96077	"	"	P 6	4.0	3140	75	26.1	25.3	18.6	6.62	2.01	1.70	3.29
M96078	"	"	P 7	4.0	3920	80	24.2	28.5	21.0	6.69	2.01	1.71	3.33
M96079	"	"	P 8	4.0	3300	80	26.3	27.9	21.8	6.90	2.00	1.70	3.45
M96080	"	"	P 9	4.0	4140	75	26.5	28.4	33.9	6.41	1.93	1.67	3.32
M96081	"	"	P10	4.0	2940	93	24.7	28.0	20.8	6.47	1.97	1.66	3.28
M96083	"	"	P14	4.0	4160	88	24.3	28.2	30.3	6.38	1.98	1.68	3.22
M96084	"	"	P15	4.0	3640	80	21.5	25.9	23.2	6.63	1.95	1.67	3.40
M96085	"	"	P16	4.0	4280	80	23.2	27.4	23.3	6.80	1.95	1.65	3.49
M96086	"	L 57 -	P 1	4.0	4480	75	23.2	27.0	21.4	6.77	1.99	1.69	3.40
M96093	"	"	P 8	4.0	2940	80	24.0	25.4	12.4	6.58	2.08	1.58	3.16
M96094	"	"	P 9	4.0	3760	75	28.6	25.5	12.1	6.68	1.99	1.69	3.36
M96096	"	"	P11	4.0	3340	75	27.1	25.3	11.1	6.90	2.03	1.73	3.40
M96097	"	"	P13	4.0	4680	75	26.3	25.6	13.1	6.31	2.01	1.70	3.14
M96098	"	"	P14	4.0	3540	80	24.5	25.7	20.8	6.49	1.95	1.68	3.33
M96099	"	"	P15	4.0	3440	80	25.4	24.3	15.0	6.60	1.97	1.69	3.35
M96100	"	"	P16	4.0	2980	80	21.8	27.2	15.8	6.66	2.04	1.72	3.26
M96101	"	"	P17	4.0	3220	85	24.4	25.9	23.7	6.63	1.96	1.64	3.38
M96102	POP - A	"	P18	4.0	3260	75	25.1	25.8	19.5	6.55	1.97	1.69	3.32

TABLE VI. (CONT.)

Mutant No.	Population	Pedigree		Leaf blast (1 - 9)	Yield potential kg/ha	Days to flowering No.	1000 grain weight (g)	Panicle length (mm)	Sterility %	Grain shape			
										Length (mm)	Width (mm)	Thickness (mm)	Ratio L/W
M96139	POP - B	L117 -	P27	4.0	3760	80	23.0	25.3	26.1	6.43	1.93	1.64	3.33
M96154	"	L159 -	P16	4.0	3900	80	19.3	27.3	15.4	6.08	2.00	1.70	3.04
M96160	"	L162 -	P 2	4.0	4420	80	21.8	26.7	17.5	6.25	1.93	1.66	3.24
M96161	"	"	P 3	4.0	4620	70	19.0	26.3	19.4	6.19	1.90	1.66	3.26
M96165	"	L162 -	P 7	8.0	3640	80	21.6	26.6	12.3	6.21	1.93	1.66	3.21
M96167	"	"	P 9	5.0	3900	80	22.2	26.2	18.2	6.49	1.99	1.68	3.26
M96178	"	L184 -	P 8	4.0	3840	75	20.7	27.1	26.4	6.19	1.97	1.67	3.14
M96184	"	"	P14	4.0	3880	80	21.7	26.1	18.8	6.32	2.00	1.71	3.16
M96185	"	"	P15	5.0	4200	85	21.2	25.8	18.5	6.56	1.99	1.69	3.30
M96186	"	"	P16	4.0	3400	90	20.5	27.1	18.7	6.47	1.99	1.69	3.25
M96189	"	L188 -	P 6	5.0	3380	85	23.3	29.0	13.9	6.47	1.98	1.68	3.26
M96190	"	"	P19	5.0	3500	80	20.7	25.0	29.2	6.00	1.94	1.64	3.09
M96191	"	"	P20	4.0	3300	95	21.7	27.5	21.4	6.32	1.97	1.68	3.21
M96192	"	L190 -	P 1	4.0	3320	80	23.3	26.1	7.8	6.28	1.99	1.68	3.16
M96193	"	"	P 2	4.0	3580	85	22.6	28.1	20.1	6.61	1.97	1.69	3.35
M96198	POP - C	L196 -	P14	5.0	2960	80	24.6	28.0	14.9	6.13	1.92	1.60	3.19
M96202	"	L217 -	P 4	4.0	3880	92	19.7	27.0	31.5	--	--	--	--
M96211	"	L248 -	P25	6.0	3400	93	22.6	27.0	14.6	6.52	1.95	1.70	3.34
M96219	POP - D	L274 -	P19	4.0	3820	75	21.1	27.9	23.8	6.02	1.92	1.63	3.13
M96220	"	"	P22	5.0	4700	70	23.3	26.0	19.9	3.64	2.01	1.69	3.30
M96231	"	L276 -	P10	5.0	4000	80	22.0	25.6	13.4	6.16	1.98	1.67	3.11
M96233	"	"	P12	4.0	5280	80	23.9	27.0	14.1	6.31	2.01	1.70	3.13
M96234	"	"	P13	5.0	4220	80	18.9	27.4	29.7	6.19	1.97	1.68	3.14
M96244	"	"	P27	4.0	3900	95	21.3	24.5	19.9	6.72	2.00	1.70	3.36
M96245	"	L277 -	P 1	4.0	4200	70	19.6	25.3	30.7	6.37	2.06	1.78	3.09
M96257	POP - E	L213 -	P 2	5.0	3440	80	22.3	27.5	26.1	6.51	2.00	1.71	3.26
M96258	"	"	P 3	4.0	4180	75	21.2	26.6	23.2	6.13	1.95	1.70	3.14
IAC 201	IAC 165 // LABEL			6.3	2722	80	22.8	26.7	20.3	6.59	1.99	1.71	3.31

The 53 promising mutants selected for improved tolerance to blast and good agronomic performance in M_5 are shown in Table VI. The original cultivar IAC 201 has a lower yield potential, so we selected mutants exceeding by 15% the mean of the original cultivar. Early and late plants were selected but in further generations selection will be for the early ones, that will be better suited for upland conditions. In grain shape the mutant lines selected were not different from the original cultivar (Table VI) which was one of the objectives in this project. Finally, looking at the Table VI it can be seen that 26 selected mutants came from population A in the M_2 generation (Table II), 15 from population B, 3 from population C, 7 from population D and 2 from population E. Thus, 44 selected mutants were induced *in vivo* and only 9 were generated from mutation *in vitro*. These results suggest that, in a rice breeding program, it is not necessary to use somaculture in connection with mutation technology, when the objective is to select for improved blast tolerance and agronomic traits.

4. CONCLUDING REMARKS

The germination rate for all irradiated treatments was below 50%, but the 300 Gy dose was acceptable and our results confirm previous findings in rice [4, 8]. However, the results point to the need to establish the radiosensitivity and the optimal dose for each rice cultivar. The data presented indicated that the application of ^{60}Co gamma ray irradiation with 300 Gy had a stimulating effect on the rate of regeneration. This result was compatible with findings in barley [9].

In the M_3 generation, it was found that selection for blast resistance mutations *in vivo* was better than *in vitro*. From 52 rice mutants *in vivo,* 37 lines were moderately resistant to blast, as reported earlier [8].

The results described for the fourth phase, showed that the population selected in the M_3 generation was normally distributed for all other characters and that as indicated earlier [10] there was genetic variability in both the positive and negative directions. Thus, selection for the traits studied can improve the probability of finding mutants, which will make it feasible to achieve the main objective of the project.

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DEVELOPMENT OF NEW SEMIDWARF SOURCES FOR RICE WITH DIFFERENT CYTOPLASMS (CV BASMATI 370 AND GLORIA)

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Abstract

In Cuba semidwarf rice varieties grow on 98% of the area. Virtually all carry the same Dee-geo-won-gen dwarfing gene. Also, most if not all the cultivars have the same cytoplasm. The induced mutations approach was undertaken in order to generate alternative genetic sources of dwarfing with different cytoplasms and to improve the grain quality of Cuban rice. The seeds of two varieties, Basmati 370 and Gloria, were irradiated with 200 and 300 Gy of ^{60}Co gamma rays. In several generations of selection, progeny testing and preliminary yield tests 10 mutants (6 from Basmati 370 and 4 from Gloria), whose yields exceeded the source cultivars, have been advanced to replicated yield trials. Some of the mutant lines are also resistant to lodging and to blast (*Pyricularis grisea*). It is concluded the induced mutations can be used successfully to improve plant type and other agronomic traits in rice. The induced mutants will be used also in hybridization programs.

1. INTRODUCTION

In Cuba, 98% of rice area is cultivated with semidwarf cultivars. Virtually all carry the same semidwarfing gene of IRRI varieties that comes from dwarf Chinese variety Dee-geo-won-gen (DGWG) [1, 2]. Cuban rice breeders adopted the semidwarf plant type concept, as a breeding objective in the early 70's, and the use of semidwarfs as parents intensified year by year, increasingly crossing semidwarf parents with other semidwarf.

Most of these parental varieties employed in the hybridization programs originated from the same maternal parent, implying that the components of their cytoplasm are similar [3]. This situation highlights the need for alternative sources of dwarfing and for broadening the maternal genetic base in the new cultivars.

During the last 20 years the selection pressure for cooking quality was not so strong. Today the rice cultivars present a good plant type, with a high yield potential, good resistance to the main pests and diseases and they also possesses good milling quality. The cooking quality and the different cytoplasm bases used in the hybridization programs, are not satisfactory. In addition, the gene for semidwarfness is transferred with other genes which reduce the grain quality.

Mutation breeding is an important tool to be used for such programs. This research has the following objectives: (a) To identify alternative genetic sources of dwarfing with different cytoplasmic bases by using mutation techniques. (b) To improve the grain quality of the Cuban rice varieties using the high grain quality Basmati 370 from India and Gloria from Cuba.

2. MATERIALS AND METHODS

The experiment was initiated in 1993 with the varieties of Basmati 370 and Gloria (Table I).

TABLE I. PRINCIPAL AGRONOMICAL TRAITS OF THE VARIETIES USED

Variety	Basmati 370	Gloria
Type	Tall indica	Tall indica
Yield potential	Low	Low
Maturity	Medium	Medium
Lodging	Highly susceptible	Susceptible
Aroma	Aromatic	Standard
Character to improve	Semidwarf, resistant to lodging	Semidwarf, resistant to lodging

The breeding scheme is shown in Fig. 1. The seed were exposed to 200 and 300 Gy of ^{60}Co gamma rays. The irradiated seeds (3600 seeds per treatment) were sown in a nursery and transplanted to the field, one plant per hill, spaced 15 × 15 cm apart. The first 2 panicles were harvested from each plant. The progenies from each M_1 were raised as the M_2 line, each M_2 population was transplanted and grown in a pedigree row (5 m long), one plant per hill, spaced 25 × 30 cm apart.

A total of 52600 M_2 plants per treatment in Basmati 370 variety and 43200 plants per treatment in Gloria variety were sown. At maturity the selection was made for following:

- Semidwarf and intermediate plant type.
- Earliness.
- Healthy plants.

The M_3 and M_4 generations were sown by direct seeding in 5 m rows. One control row of each of the respective source variety was grown every twenty rows of the mutant lines.

The observational yield trials were concluded in the M_5 and M_6 generations. Small plots with 8 rows 5 m long were utilized. Every ten plots three control varieties were sown, the source varieties Basmati and Gloria and the commercial cultivar J-104. They were planted in three locations to evaluate their field performances in different ecological zones.

3. RESULTS AND DISCUSSION

In the M_2 generation much variability was found, mainly in plant height. A total of 153 plants, 91 semidwarf and 62 intermediate plant type, were selected.

A higher degree of variability was found in the Basmati variety with a radiation doses of 200 Gy (Table II). Different types of mutation were identified such as types of grain (bold and medium long grain) and late maturity mutants.

On the basis of the data and visual comparison, 71 true breeding lines (58 from Basmati and 13 from Gloria) were selected for further screening and evaluation (Table III). Many of the M_2 selections segregated and did not breed true in the M_3.

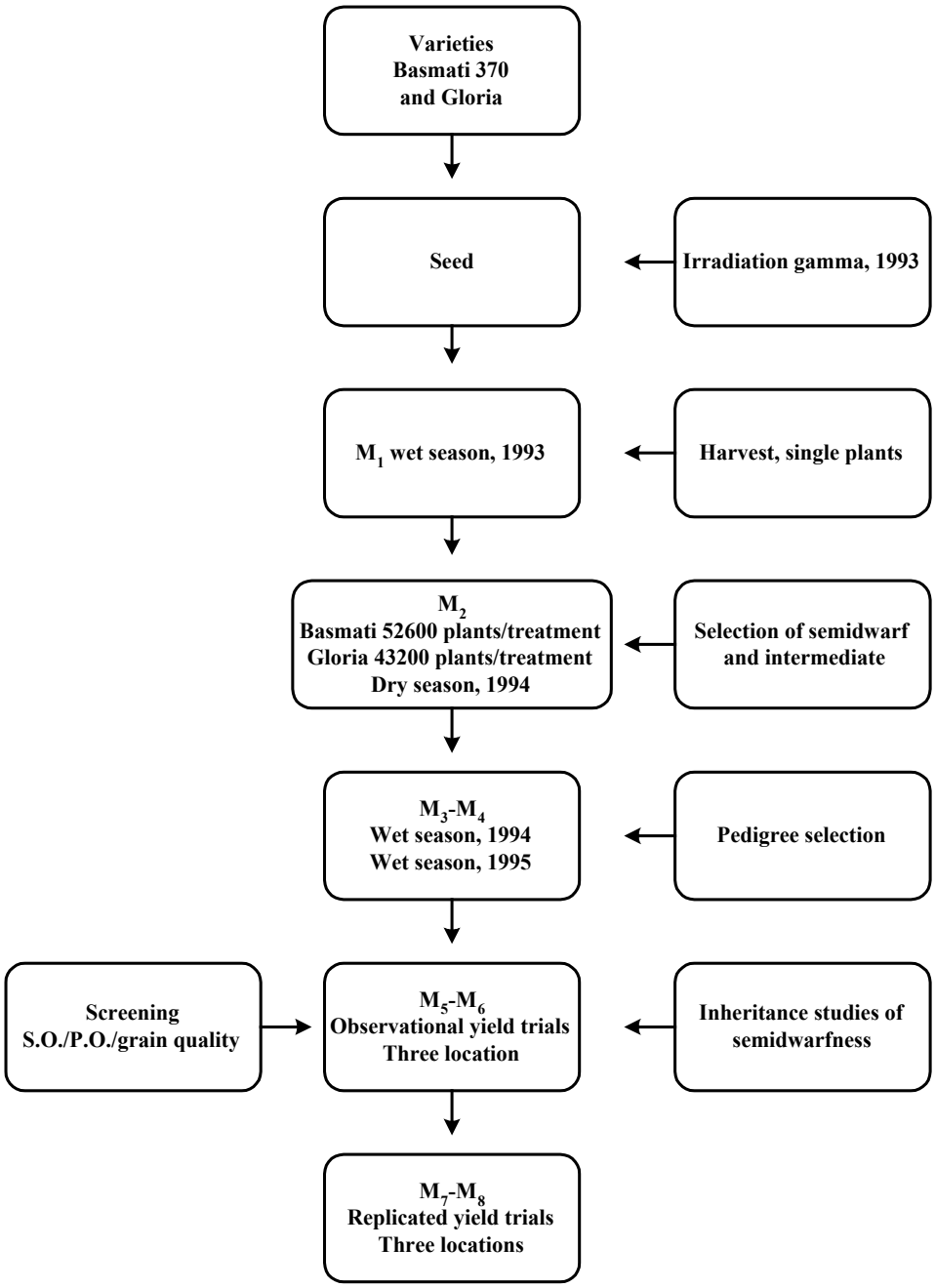

FIG. 1. Breeding scheme.

TABLE II. NUMBER OF SELECTED M_2 PLANTS AND THEIR PROPERTIES, DRY SEASON

Variety	Gamma dose (Gy)	Plants selected, number			
		Semidwarf	Intermediate	Early	Total
Basmati 370	200	38	35	6	79
Basmati 370	300	23	19	8	50
Gloria	200	19	3	2	24
Gloria	300	11	5	5	21
Total	---	91	62	21	174

TABLE III. NUMBER OF TRUE BREEDING M_3 MUTANT LINES SELECTED FOR REDUCED HEIGHT AND EARLINESS

Variety	Gamma dose (Gy)	Semidwarf	Intermediate height	Early	Total
Basmati 370	200	36	3	4	43
Basmati 370	300	17	2	4	23
Gloria	200	7	1	4	12
Gloria	300	5	-	3	8
Total	---	65	6	15	86

TABLE IV. YIELDS OF SEMIDWARF MUTANT LINES SELECTED FROM OBSERVATIONAL YIELD TRIALS IN THREE LOCATIONS (D = DRY SEASON; W = WET SEASON)

Mutant lines	Source	Yield t/ha							
		I.I.A.		*S. Spirits*		Jucarito		Mean	
		D	W	D	W	D	W	D	W
B_{300}-10-1-3-1	Basmati γ300Gy	7.9	5.6	6.9	4.9	6.7	5.0	7.2	5.2
B_{300}-4-2-1	Basmati-γ300Gy	6.6	5.4	4.2	4.0	7.2	5.1	6.0	4.8
B_{200}-15-3-1	Basmati-γ200Gy	5.5	5.0	6.0	4.7	5.2	4.7	5.6	4.8
B_{300}-10-2-2	Basmati-γ300Gy	4.9	4.6	7.0	4.4	4.1	4.0	5.3	4.3
B_{300}-10-1-4	Basmati-γ300Gy	5.4	4.9	6.0	4.3	6.1	4.3	5.8	4.3
B_{200}-14-2-1	Basmati-γ200Gy	5.0	4.7	5.3	5.1	5.5	4.4	5.3	4.7
G_{300}-10-2-1-7	Gloria-γ300Gy	5.2	4.5	7.9	5.0	4.0	4.1	5.7	4.5
G_{300}-10-2-7-3	Gloria-γ300Gy	4.4	4.1	6.3	4.8	4.5	3.9	5.1	4.3
G_{300}-10-2-2-3	Gloria-γ300Gy	4.6	4.2	5.2	4.7	5.1	4.1	5.0	4.3
G_{200}-10-2-1-6	Gloria-γ200Gy	5.5	4.4	4.4	4.6	5.0	4.0	5.0	4.5
Control varieties									
Basmati 370	Unknown	1.9	1.3	1.5	1.2	1.7	1.0	1.7	1.2
Gloria	Unknown	3.1	2.2	3.0	2.1	2.7	1.9	2.9	2.5
J-104	IR480-5-9-3/ IR930-10-1	8.4	6.1	8.9	6.3	8.5	6.2	8.6	6.2

In the observational yield trials, performed in three different locations, 22 lines from Basmati-370 and 12 from Gloria, showed higher yields than the source cultivars (between 2.2 and more than 3.5 t/ha).

On the basis of their field performance and yield data, ten mutants were selected for further evaluation in replicated yield trials (Table IV). The mutant line B10-1-3-1 showed the highest yield with good resistance to *Pyricularia grisea* and lodging.

The high frequency of semidwarf mutants in this experiment demonstrated that induced mutations can be successfully used to improve plant type in rice as has been reported previously [4, 5, 6, 7].

Induced mutations generated new semidwarf germplasm with useful agronomic traits. These new lines will be used for hybridization breeding as semidwarf parents with different cytoplasms and genetic background.

TABLE V. AGRONOMIC DATA FOR THE TEN SELECTED SEMIDWARF MUTANT LINES, MEAN VALUES OF THREE LOCATIONS (D = DRY SEASON; W = WET SEASON)

Material	Source	Vigor[a]	Lodging[b]	Tago-sodes[c] LE	Maturity (days)		1000 grain wt. (g)	Plant height (cm)	Panicle length (cm)
					D	**W**			
B_{300}-10-1-3-1	Basmati γ300Gy	4	R	MR	132	112	23.9	84	26.2
B_{300}-9-2-1	Basmati-γ300Gy	4	R	MR	136	112	23.8	91	25.2
B_{200}-15-3-1	Basmati-γ200Gy	4	MR	MR	135	111	25.7	93	26.4
B_{300}-10-2-2	Basmati-γ300Gy	4	MR	MR	134	113	23.1	91	24.3
B_{300}-10-1-4	Basmati-γ300Gy	4	MR	MR	138	113	24.1	90	25.0
B_{200}-14-2-1	Basmati-γ200Gy	4	MR	MR	133	112	23.7	92	26.5
G_{300}-10-2-1-7	Gloria-γ300Gy	2-3	R	MR	130	110	26.4	100.2	25.8
G_{300}-10-2-7-3	Gloria-γ300Gy	2-3	R	MR	128	110	27.2	100.5	27.4
G_{300}-10-2-2-3	Gloria-γ300Gy	2-3	R	MR	136	113	26.8	101.2	27.2
G_{200}-10-2-1-6	Gloria-γ200Gy	2-3	R	MR	131	111	26.9	97.3	24.2
Control varieties									
Basmati 370	Unknown	3-4	HS	MR	136	112	23.4	147	27.2
Gloria	Unknown	2-3	S	MR	132	111	27.2	154.0	26.7
J-104	IR480-5-9-3/ IR930-10-1	3	HR	MR	145	118	31.2	95.5	25.1

[a]a higher score denotes higher vigor.
[b]R = resistant, MR = moderately resistant, HR = highly resistant, S = susceptible, HS = highly susceptible.
[c]Reaction to *Tagosodes onizycoles*.

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EFFECT OF THE GENOTYPE AND GAMMA IRRADIATION ON THE ANTHER CULTURES OF A 10 × 10 DIALLEL CROSS OF WHEAT

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Abstract

Anther culture responsiveness, irradiation effect and reciprocal effect were evaluated on ten genotypes (V1-V10) and a 101 × 0 diallel cross. Gamma irradiation dose of 100 Gy was applied to seeds of parents and F_1 cross from which the donor plants were grown. Non-irradiated donor plants were also used for comparison. Anthers were plated on potato-2 callus induction medium and calli formed were transferred to MS medium supplemented with sucrose (3%), indolacetic acid (1.0 mg/L), kinetin (1.0 mg/L), inositol (100 mg/L) and solidified with agar (0.7%). Genotypes showed big differences for callus induction, plant regeneration and anther culturability rate. The most responsive materials were V2, V10 and V5 with 76.0, 27.4 and 10.8 green plants per 100 anthers respectively. No irradiation effect was found for the parents nor the F_1 crosses on the pooled data. Mean anther culture response of specific genotypes showed that irradiation significantly increased anther culturability rate of V3 from 0.1 to 27.6 green plants per 100 anthers. No reciprocal effect was observed.

1. INTRODUCTION

Wheat has been an important crop in feeding the population of Guatemala. Its improvement began in 1958 with close collaboration of CIMMYT and 26 varieties have been released since then. Recently, the anther culture and mutation induction techniques, with a great potential for improving varieties, were included in the wheat breeding program.

In the anther culture approach, the immature pollen grains form embryos or calli which become plants when transferred to regeneration medium. Most of the regenerated plants are sterile haploids but in some species like *Triticum* a spontaneous chromosome doubling occurs during callus development and plant regeneration.

The main advantage for the breeding programs is the time saved by obtaining homozygous plants from the F_1 hybrids. Besides, the selection efficiency increases due to visible expression of recessive alleles. Thus, smaller populations are needed as compared with the pedigree method.

The main problem in using this method is the low culturability of the anthers. One way to solve this is by selecting genotypes with high androgenetic response. However, it is necessary to develop or improve the procedures that produce haploidy in the important crops.

The first production of wheat haploid plants using anther culture was simultaneously reported in China and France. Since then, many studies have proven that the pathway from microspores to fertile plants is influenced by several factors as the genotype [1], the developmental stage of the microspore [2], as well as the physiological conditions of the donor plants and the culture medium.

The genotype is the most important factor affecting anther culture. Lazar *et al*. [1], in wheat, have estimated the genetic parameters, for the two principal characters, callus and

plantlet induction rates in a diallel analysis of five parental lines. They found that the total variation effect is mainly due to genetic factors with a preponderance of additive effects, but also with significant dominance (specific combining ability) and reciprocal effects. The results of the past years strongly suggest that the organization of the androgenetic process is under the control of alleles with quantitative effects, it is heritable and its level of expression can be enhanced by selection.

It is thus clear that success in the production of doubled haploid lines from the F_1 progenies will depend largely on the selection of the best responding genotypes with high specific combining ability.

Mutation induction has become an established tool in plant breeding to supplement existing germplasm variation and for improving cultivars in certain specific traits. Limitations arise mainly from the large mutagenized populations to be screened and from the unsatisfactory selection methods. Both limitations may be eased to some extent by advances in techniques of plant in-vitro culture [3].

When induced mutations and haploid production are combined, recessive alleles induced by a mutagen before or during the haploid stage will be homozygous in the doubled, diploid phase, and therefore phenotypically expressed [4].

Low-dose gamma irradiation of fresh explants can significantly improve regeneration from anther cultures in wheat and may stimulate a low frequency of regeneration in an otherwise non-responsive cultivar [5].

The aim of this study is to characterize the responsiveness of ten elite wheat genotypes to anther culture, the effect of gamma irradiation on their response and the reciprocal effect on 86 F_1 hybrid combinations from a 10 × 10 diallel cross. This should be of great help in selecting genotypes for breeding using the doubled haploid (DH) procedures.

2. MATERIALS AND METHODS

The Guatemalan variety ICTA Cumpale (V1), eight advanced lines from the crosses CMBW (V2), CM76694 (V3), CM90722 (V4), CM92909 (V5), CM76635 (V6, V7), CM113143 (V8), CM81812 (V9) introduced from CIMMYT, and an advanced line from the cross CG12334 (V10) were used by the National Wheat Program for a diallel cross. Ten seeds from each entry were taken for the study. Half of them were irradiated at a dose of 100 Gy with a ^{60}Co source at a dose rate of 7.17 Gy min^{-1}. All the plants were grown in 20 cm pots under natural light; the temperature varied between 18 and 28°C. Spikes were collected when the microspores were in the uninucleate stage and were subjected to a cold stress for 4 days at 4°C. Anthers were plated on potato 2 medium and kept in the dark at 25-27°C. After 4-6 weeks, calli were transferred to an MS regeneration medium with 1 mg/L indolacetic acid, 1 mg/L kinetin, 30 g/L sucrose and solidified with 7 g/L agar under 16 hrs photoperiod at 25-27°C.

Callus induction rate was considered as the number of calli formed per one hundred plated anthers. Plant regeneration rate was considered as the number of green plants regenerated per one hundred calli transferred and anther culturability as the number of green plants regenerated per one hundred plated anthers. The means for the ten genotypes as well as the 86 F_1 hybrids from the 10 × 10 diallel cross were compared.

3. RESULTS

The genotypes demonstrated a wide range of responses to anther culture and no significant effect of the irradiation treatment (Table I). In the non-irradiated controls, the callus induction rate varied from 1.0 to 146.7 with a mean value of 15.5 calli per 100 anthers. In the irradiated group, the callus induction rate varied from 0.6 to 60.0 with a mean value of 17.1 calli per 100 anthers. The plant regeneration rate varied from 1.6 to 128.6 with a mean value of 0.5 green plants regenerated per 100 calli transferred in the non-irradiated treatment and from 0.0 to 98.6 with a mean value of 0.4 green plants regenerated per 100 calli transferred in the irradiated treatment. Anther culturability rate also showed a wide range of responses among genotypes and no significant difference of the irradiation treatment. For the controls, the rate varied from 0.1 to 76.0 with a mean value of 0.1 green plants regenerated per 100 anthers and from 0.0 to 27.6 with a mean value of 0.1 green plants regenerated per 100 anthers for the irradiated group.

TABLE I. A COMPARISON OF ANTHER CULTURE RESPONSE OF THE IRRADIATED WHEAT GENOTYPES AND THEIR CONTROLS

Genotype	Plated anthers No.	Calli formed No.	Callus induction (%)	Calli transferred No.	Green plants No.	Plant regeneration (%)	Anther culturability (%)
Control							
V1	250	50	20.0	49	16	32.7	6.4
V2	150	220	146.7	220	114	51.8	76.0
V3	800	72	9.0	64	1	1.6	0.1
V4	700	7	1.0	7	9	128.6	1.3
V5	650	192	29.5	160	35	21.9	5.4
V6	250	44	17.6	40	27	67.5	10.8
V7	1250	90	7.2	79	69	87.3	5.5
V8	1050	44	4.2	6	6	100.0	0.6
V9	350	48	13.7	41	10	24.4	2.9
V10	350	120	34.3	120	96	80.0	27.4
Irradiated							
V1	300	55	18.3	53	39	73.6	13.0
V2	250	150	60.0	140	31	22.1	12.4
V3	250	80	32.0	70	69	98.6	27.6
V4	350	2	0.6	2	0	0.0	0.0
V5	400	55	13.8	45	11	24.4	2.8
V6	350	33	9.4	33	0	0.0	0.0
V7	350	41	11.7	41	21	51.2	6.0
V8	450	17	3.8	17	0	0.0	0.0
V9	200	4	2.0	2	0	0.0	0.0
V10	400	126	31.5	99	46	46.5	11.5

Based on anther culturability, 3 genotypes were classified as highly responsive, namely: V2, V10 and V6; 3 were classified as intermediate: V1, V7 and V5, while 4 were classified as poorly responsive, V9, V4, V8 and V3.

The same trend was observed for the F_1 hybrids but in this case, the range of the anther culture response was narrower than that of the ten parents (Table II). The callus induction rate when pooled by the same parent varied from 6.1 to 31.7 with a mean value of 18.3 calli per 100 anthers in the non-irradiated treatment. For the irradiated treatment, the range was

between 9.8 and 28.4 with a mean value of 18.0 calli per 100 anthers. Plant regeneration rate varied from 11.9 to 81.5 with a mean value of 0.5 green plants per 100 calli transferred for the non-irradiated treatment and from 22.1 to 62.4 with a mean value of 0.4 green plants per 100 calli transferred for the irradiated group. Anther culturability for the controls, when pooled by same parent, ranged from 1.8 to 18.4 with a mean value of 0.1 green plants per 100 anthers and from 2.2 to 11.6 with a mean value of 0.1 green plants per 100 anthers for the irradiated treatment.

Based on anther culturability, hybrids involving V2, V5 and V10 were classified as highly responsive, hybrids with V1, V3, V6, V7 and V9 were classified as intermediate and hybrids involving V8 and V4 were classified as poorly responsive.

TABLE II. MEAN CALLUS INDUCTION, PLANT REGENERATION AND ANTHER CULTURABILITY OF 86 F_1 HYBRIDS POOLED BY THE SAME PARENT

Parent	Plated anthers No.	Callus induction (%)	Plant regeneration (%)	Anther culturability (%)
Control				
V1	2850	15.4	44.8	6.4
V2	2500	27.0	70.8	18.0
V3	2050	15.6	50.2	6.0
V4	2600	6.1	81.5	4.2
V5	1750	31.7	53.4	14.8
V6	1300	10.8	77.5	7.2
V7	2100	22.2	44.1	8.4
V8	1800	16.1	11.9	1.8
V9	2050	12.3	50.2	5.9
V10	2100	26.7	55.9	14.3
Irradiated				
V1	2950	15.0	42.8	5.8
V2	2700	24.4	45.5	9.8
V3	2100	18.5	62.4	10.3
V4	2650	12.0	44.1	4.4
V5	1600	28.4	32.4	8.3
V6	1250	9.8	22.1	2.2
V7	1900	18.5	41.7	7.1
V8	1550	19.6	27.9	4.7
V9	2150	12.0	41.0	4.0
V10	2200	21.9	60.7	11.6

Anther culture responsiveness of the F_1 pooled as male and female parents did not show significant differences among genotypes (Table III). When pooled by female parents, the mean callus induction rate of the F_1's ranged from 9.1 to 30.1 calli per 100 anthers and from 7.2 to 29.5 when used as male parents. Plant regeneration rate varied from 19.7 to 58.7 green plants per 100 calli, when pooled by female parents and from 18.9 to 70.8 when pooled by the males. Anther culturability ranged from 3.2 to 13.7 green plants per 100 anthers when pooled by female parents and from 2.5 to 16.5 when pooled by the male parents.

Based on anther culturability rate, three genotypes were classified as highly responsive when used both as female or male parent: V2, V10 and V5.

TABLE III. MEAN CALLUS INDUCTION, PLANT REGENERATION AND ANTHER CULTURABILITY OF 86 F_1 HYBRIDS POOLED BY THE SAME FEMALE OR MALE PARENTS

Parent	Plated anthers No.	Callus induction (%)	Plant regeneration (%)	Anther culturability (%)
Used as female				
V1	5800	15.2	43.8	6.1
V2	5200	25.8	58.7	13.7
V3	4150	17.1	57.3	8.2
V4	5250	9.1	56.8	4.3
V5	3350	30.1	43.8	11.7
V6	2550	10.3	49.6	4.7
V7	4000	20.4	43.0	7.8
V8	3350	17.7	19.7	3.2
V9	4200	12.1	45.9	4.9
V10	4300	24.3	58.0	12.9
Used as male				
V1	4800	18.1	70.8	11.8
V2	3750	20.6	49.6	8.7
V3	5600	24.0	43.1	9.2
V4	5600	8.7	39.7	3.1
V5	4100	24.8	36.4	8.3
V6	3400	15.6	54.9	8.0
V7	3700	15.1	41.8	5.4
V8	3600	15.9	18.9	2.5
V9	3400	7.2	67.3	4.4
V10	4200	29.5	61.5	16.5

4. DISCUSSION

The genetic effect on anther culturability of wheat [1] was confirmed in this report. Big differences were found among the genotypes used. Three out of ten were classified as highly responsive, four as intermediate and three as poor. These results also confirm that wheat anther culture breeding depends largely on the selection of the best responding genotypes.

The overall analysis did not show any difference between non-irradiated and irradiated parents in the three traits studied (callus induction, plant regeneration and anther culturability rate). However, a significant difference was observed for the genotype V3, whose anther culturability rate rose from 0.1 to 27.6 green plants per 100 anthers when irradiated. Plant regeneration rate for this genotype was also raised from 1.6 to 98.6 and its callus induction, from 9.0 to 32.0 calli per 100 anthers, by irradiation.

These results may be due to the fact that the irradiation effect is dose-dependent and its extent varies with genotype [5]. In the same way, the overall analysis of the F_1 hybrids did not show any difference between them.

Reciprocal differences appeared not significant, suggesting no cytoplasmic effect on the anther culture response. Such reciprocal cross-specific responses of the F_1 progeny were previously shown by Bullock *et al*. [6]. Some slight differences observed on the mean anther culture response by pooling genotypes as male and female may be due to G × E interactions.

The information obtained in this study has practical applications in employing anther culture in wheat variety improvement in order to obtain large number of regenerated DH lines for evaluation and selection.

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ADVANCES IN THE USE OF MUTATION INDUCTION FOR GENETIC IMPROVEMENT OF BARLEY AND NATIVE GRAINS IN PERU

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Abstract

Barley seeds of two varieties were treated with several doses of gamma rays and sodium azide. Seeds of a quinoa (*Chenopodium*) variety were treated with three doses of gamma rays. Yield trials were conducted also for doubled haploid lines of barley derived from earlier mutagenic treatments. Some promising new barley mutant lines were identified in the yield trials. The results from the *Chenopodium* trials facilitate the determination of the optimum dose of gamma rays for the PRQ-22 variety.

1. INTRODUCTION

Barley and native grains are important crops in Peru, being basic foods in the high Andean region. The three million poor people who live in the rural highlands, are engaged in agricultural activities. Those crops grow under abiotic stresses (drought, frost, hail, infertile soils), biotic ones (diseases) and under a low farming technology. The main way to increase production is through breeding higher yielding varieties adapted to those conditions. The Universidad Nacional Agraria La Molina (UNALM) through its Cereal Research Program carries out in barley and native grains genetic improvement and agronomic trials with the support of the private industry, International Atomic Energy Agency and CIMMYT. The approaches used are germplasm introduction, germplasm collection, hybridization, induction of mutations, and doubled haploid production.

The objectives of the program are:

- To develop improved varieties with high yield, resistance or tolerance to biotic and abiotic factors, wide adaptation and good quality for different uses.
- To develop production technologies adapted to the different production areas.
- To expand the farming land through new varieties tolerant or resistant to frost, drought, hail and other limiting factors, thus, to be able to produce yield at the highest levels of the Peruvian Andes, mainly at Puno.
- To transfer the research results to agronomy students, agronomists and farmers.

2. RESULTS

2.1. Barley

2.1.1. Induction of mutations

This breeding method is used for improvement of some characteristics in commercial or traditional varieties. Two varieties were treated, UNA-80 (naked grain) and Yanamuclo (6 row) with gamma rays, 150, 250, 350 Gy.

The M_1 generation was grown at the La Molina location and was severely damaged by the weather. The material was harvested and the M_2 was to be planted on the highland in the growing season of 1998. Part of this material was randomly sampled for anther culture. From each variety and treatment 540 to 750 anthers were plated.

2.1.2. Yield trials of doubled haploid (DH) mutants

55 DH mutant lines were previously produced from M_1 plants of UNA-LA MOLINA 94 and L-2194 varieties after mutagenic treatment with N-methyl-N-nitrosourea and sodium azide. In addition, 31 DH lines derived from untreated plants of these two genotypes were included in the experiment. Four yield trials were conducted at La Molina, each containing 25 entries with two local checks. Randomized block design was used for the statistical analysis. Each trial was analyzed separately. The mean values for yield were compared using the LSD test. The coefficient of variation had high values in most of the trials. The yields were very low in general. These materials will be evaluated again at the highland locations.

Analysis of variance of yield trials at La Molina in 1997 of barley doubled haploid lines derived following induction of mutations and hybridization program showed that some of the lines gave significantly higher yields than the controls, or similar to them. Subsequent trials will ascertain this finding.

2.2. *Chenopodium* (quinoa)

2.2.1. Induction of mutations

The varieties used, breeding objectives and mutagenic treatments are shown in Table I.

TABLE I. THE VARIETIES TREATED, THE OBJECTIVES AND THE APPLIED MUTAGENIC TREATMENTS

Variety	Objective	Mutagenic agent	Dose
LM-89	White color of grain Lower saponin content	Gamma rays	50, 100 and 150 Gy
LM-89	White color of grain Lower saponin content	Sodium azide	1.0 and 2.0 mM
PRQ-22	Earliness Non-branching	Gamma rays	150, 250 and 350 Gy

2.2.2. Germination dynamics

Germination was assessed 4, 8 and 18 h after planting (Table II). After 8 and 18 h there were significant (5%) and highly significant (1%) differences among the treatments.

TABLE II. MEAN GERMINATION RATES OF QUINOA VARIETY PRQ-22 IRRADIATED WITH THREE DOSES OF GAMMA RAYS

	$\bar{x}$ Germination%, hours after planting		
Treatment	4	8	18
Check	7	90	100
150 Gy	1	39	91
250 Gy	5	55	94
350 Gy	4	50	91

2.2.3. Germination and survival

The experimental material was also sown in boxes under field conditions and the germination and survival were evaluated. Their mean values (Table III), decreased with increased gamma rays doses. Highly significant (1%) differences were observed for survival. Plants from the 350 Gy treatment did not develop true leaves; after approximately 10 days, the cotyledons turned yellowish and the plants died. The remaining seeds of these treatments will be planted under the highland conditions.

TABLE III. MEAN GERMINATION AND SURVIVAL RATES UNDER FIELD CONDITIONS OF QUINOA VARIETY PRQ-22 IRRADIATED WITH THREE DOSES OF GAMMA RAYS

Treatment	Germination %	Survival %
Check	80.25	78.75
150 Gy	70.50	67.75
250 Gy	63.0	62.25
350 Gy	66.0	2.75

TABLE IV. MEAN VALUES OF HYPOCOTYL AND ROOT LENGTH AND PLANT HEIGHT OF QUINOA VARIETY PRQ-22 ONE WEEK AFTER GAMMA RAYS IRRADIATION

Treatment	Hypocotyl length (cm)	Root length (cm)	Plant height (cm)
Check	3.53	3.33	69.50
150 Gy	3.85	2.88	68.25
250 Gy	3.78	3.33	51.75
350 Gy	3.60	2.78	-

2.2.4. Growth measurements

Hypocotyl and root growth were measured one week after planting and plant height after four weeks (Table IV). There were small differences between the treatments and the check for root and hypocotyl length. For plant height there was a slight difference between the check and the 150 Gy treatment and an increased difference with the 250 Gy one. The observed mean square values for these measurements did not show significant differences for hypocotyl and root length but there were significant differences for plant height.

IMPROVEMENT OF QUINOA AND BARLEY THROUGH INDUCED MUTATIONS AND BIOTECHNOLOGY

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Abstract

The main cropping problems in the Bolivian highlands are the long growing period of barley, high degree of environmental influence on the performance of quinoa, and low soil moisture at sowing time, leading to low germination rate and poor stands, and frost or chilling damages. The program aimed to establish protocols for induction of mutations with X rays and chemical mutagens (NaN_3, MNH, EMS) in quinoa, barley, native forage species and forest plants and to obtain mutant lines, especially in barley and quinoa; and to establish callus regeneration in quinoa and micropropagation of keñua (*Polilepis*). The project is still in its study stages, hence further evaluations are needed before firm conclusions are drawn.

1. INTRODUCTION

The main crop cultivation problems in the highlands of Bolivia are:

- The long vegetative growing period of barley that limits grain production.
- The high influence of the environment on phenotypic characteristics of quinoa.
- Low germination rate and seedling mortality due to low soil moisture during sowing time.
- Total or partial loss of the crops due to low temperatures.

2. OBJECTIVES OF THE PROGRAM

- To establish the protocols for induction of mutations with X rays and chemical mutagens (NaN_3, MNH, EMS) in quinoa, barley, native forage species and forest plants and to obtain mutant lines.
- Seed treatment protocol for barley and quinoa every year until desirable mutants are obtained.
- To establish the regeneration of callus of quinoa in order to induce mutations *in vitro*.
- To establish a protocol for micropropagation *in vitro* of keñua (*Polilepis*) as an alternative method of multiplication. This species offers an alternative for forestation of the highlands. However, only 1-3% of the seeds germinate, hence another approach should be found.

3. METHODLOGY

Dosimetry studies for mutation induction as a method of generation of genetic variability: X rays (120Kv and 50 mA) for 2.5, 5.0 and 7.5 min for quinoa and keñua and 7.5, 10 and 15 min for barley and wheat. Use of mutagenic chemicals NaN_3 in doses of 10^{-2}, 10^{-3} and 10^{-4} and EMS with concentration of 10^{-3} for 8, 12, 16 and 20 hours in the four species.

The calli were irradiated with X rays in the same doses employed in the seeds.

4. RESULTS

4.1. X ray treatments

The dosimetry studies with X rays in barley using 120 Kv and 50 mA with the varieties of barley IBTA 80 and Kolla indicate that there was no significant difference between the three treatments.

A reduction of the plant height of quinoa of 32 to 37% was observed. It is necessary to repeat the test since the data were very variable.

The X rays-irradiated calli of quinoa, cultivated in Murashige and Skoog media, could not be regenerated in order to obtain mutant plants of quinoa.

4.2. The evaluation of advanced lines of barley from Peru and Argentina

In agricultural year 1996-97 advanced mutant lines were evaluated together with their parental lines originating from Argentine (INTA, Ing. Alberto Prina). All plants developed with the same phenotypic characteristics, same height and vegetative cycle.

In 1997-98, 3 Argentine mutant lines and 9 mutant lines of variety Mutant Buena Vista, from the Cereals Program of UNALM-Peru, Ing. Luz Gomez Pando, were sown. This trial was lost due to drought and frosts.

4.3. Anther cultures for production of double haploid plants of barley and quinoa

The plantlets were sown on 5 different dates and under greenhouse conditions. It has not been possible to date to obtain plants from barley and quinoa for anther cultures *in vitro*; in other cases there was no response or callus formation, therefore it is necessary to continue this study.

4.4. Micropropagation of keñua

The seeds of keñua which were sown in *in vitro* culture to obtain aseptic plants did not germinate, therefore this procedure was discarded.

The results of phase I of testing different types of explants showed that there was excellent response from foliar explants, and very good response from the buds and shoot-tips and small branches.

In phase II, the culture procedures gave excellent regeneration (100%) and proliferation of buds. However, further studies are required.

In phase III it was found in a preliminary test that the concentrations of the auxins used were very low.

MUTATION BREEDING *IN VIVO* AND *IN VITRO* IN VEGETATIVELY PROPAGATED CROPS

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Abstract

Mutation breeding *in vivo* and/or *in vitro* in vegetatively propagated crops as well as somaclonal variation can be used in Brazil in several crops to increase the genetic variability in characteristics of high importance. This was the objective of this research using ornamentals, citrus and bananas. Somaclonal variants can also be useful in these crops, based on the preliminary results observed in banana (*Mycosphaerella musicola*); where a short plant variant was selected in Brazil and the mutant resistant to yellow sigatoka, selected in Venezuela, showed resistance also in Brazil. Despite the increase in genetic variability in M_1V_4 generation obtained after *in vitro* irradiation of meristems in banana, mutants resistant or tolerant to Fusarium were not selected, perhaps due to the limited number of plants evaluated. In citrus the first results from yield trials showed that following bud irradiation, it was possible to select plants of interest, e.g. mutants with a reduced number of seeds in the fruits. In ornamentals mutants induced by gamma rays in this project were released to the farmers. The results obtained in this research showed that biotechnology is a powerful tool that can be used in several ways in association with mutation breeding.

1. INTRODUCTION

Vegetatively propagated plants have high importance for the Brazilian economy. In Brazil, as in other countries, there are some difficulties concerning the application of the traditional plant breeding methods: a high degree of heterozygosity, polyploidy (in some crops), crossing barriers and complex inheritance [1]. This makes induced mutations an interesting tool to be used in breeding these crops, especially when possible to associate it with *in vitro* techniques [2]. This type of research has been carried out in Brazil using different crops and objectives [3]. Based on these aspects, this research was proposed in order to facilitate the development and/or use of in *vivo* or *in vitro* mutation breeding in crops like ornamentals, banana and citrus. In order to be able to carry out the work with the various crops and objectives, several official or private institutes involved in plant breeding using these crops cooperated in this research.

2. RESULTS

2.1. Mutation breeding in chrysanthemum

The cultivar Repin is one of the best in the Brazilian chrysanthemum market but it is limited by its light-pink flower color. Gamma rays were used to treat *in vitro* pedicel and axillary buds.

Pedicels irradiated with 8.0 Gy were cultured *in vitro* (2.0 mg/L of BAP; 0.5 mg/L IAA; pH 5.7; solidified with agar 7 g/L). The plants regenerated from adventitious bud were evaluated in the greenhouse for flower color. The results (Table I) showed that only solid mutants were obtained [4].

TABLE I. INDUCED *IN VITRO* MUTATIONS IN CHRYSANTHEMUM: MUTANTS OBTAINED AFTER PEDICEL GAMMA IRRADIATION

Dose (Gy)	Plants No.	Number of flower color mutants					
		Bronze	Champagne	Dark pink	Pale pink	Total[a] No.	Total %
0	105	0	0	0	0	0	--
8.0	690	29	8	4	4	45	6.5

[a]No chimerism observed: all solid mutants.

Plants containing 6 axillary buds were irradiated with 20 Gy of gamma rays. After applying the cutting back method several mutants for flower color were selected and two of them (white, named Cristiane and dark pink, named Ingrid) were included in yield trials. Due to the good results from this evaluation (Table II) these mutants were released as new cultivars.

The dark pink mutant was used for new mutagenic treatments with gamma rays and EMS (pedicels) 0.075 M, 105 min plus 15 min post washing. The evaluation of flower color mutants is in progress but most of them appear to be solid.

Plants with 6 axillary buds were treated with 20 Gy and the cutting back method was applied. Several mutants were obtained and the farmer selected 3 of them due to interesting color of the flowers. These mutants were included in yield trials. The results (Table III) showed that these mutants are of commercial interest and the farmer is multiplying them for commercialization. This can be one more case of a mutant obtained from a mutant as described by Broertjes et al. [5].

TABLE II. CHRYSANTHEMUM: MEAN PERFORMANCE OF TWO MUTANTS INDUCED *IN VIVO* IN REPIN

Cultivar	Plant cycle (days)	Flowers/plant No.	Plant ht.[a] (cm)	Fresh wt. 10 plants (g)	Flowers senescence (days)
Repin	94	7.2	123.0 a	615.83	10
Ingrid	94	7.2	119.9 a	542.50	10
Cristiane	91	7.0	98.5 b	535.42	10
CV%	-	38.7	3.3	17.7	-

[a]Significant differences at the 1% level

TABLE III. CHRYSANTHEMUM: MEAN PERFORMANCE OF THREE MUTANTS INDUCED *IN VIVO* IN INGRID.

Cultivar	Plant cycle (days)	Flowers/plant[a] No.	Plant ht.[a] (cm)	Fresh wt.[a] 10 plants (g)	Flowers senescence (days)
Ingrid	99	50.8 a	104.8 ab	0.782 ab	12
Tea rose	99	47.5 a	114.3 a	0.853 a	12
Vine	99	46.3 a	112.3 b	0.727 b	12
Bronze	99	66.0 b	94.8 c	0.877 b	12
CV%	-	2.47	5.11	12.57	-

[a]Significant differences at the 1% level.

In order to establish some aspects of methodology the frequencies of color mutants were evaluated in M_1V_2 branches obtained from 6 M_1V_1 axillary buds, after the irradiation of plants with 20 Gy of gamma rays. The results showed that there were no differences in the frequency of mutants obtained.

2.2. Mutation breeding in *Calathea louisae* and *Stromantha sanguinea*

As can be seen in detail in Tulmann Neto & Latado [6] and Latado & Tulmann Neto [4], the first step was to establish for both ornamentals their rhizomes' sensitivity to gamma rays (Tables IV and V); then irradiate to induce mutations using *in vivo* methodology (cutting back method). Doses of 25 and 35 Gy were used for Albertii cultivar (*Calathea*) and 30 and 40 Gy for Sanguinea cultivar (*Stromantha sanguinea*). After 6 multiplications, in order to ascertain the stability, the yellow variegated leaf mutant selected in Albertii and the gray rachis mutant selected from Sanguinea showed commercial interest. The grower is multiplying the mutants to be commercialized and exported to Europe under the names of Yellow Cais and Grey Cais, respectively.

TABLE IV. MEAN HEIGHT AND SURVIVAL IN THE M_1V_1 GENERATION OF *CALATHEA LOUISAE* CV. ALBERTII PLANTS OBTAINED FROM RHIZOMES IRRADIATED WITH GAMMA rayS, EVALUATED 93 DAYS AFTER PLANTING

Dose	$\bar{x}$ Plant ht.[a]		Survival[a]	
(Gy)	Cm	%	No.	%
0	7.5	100.0	50	100.0
10	8.1	108.0	49	98.0
20	6.5	86.7	50	100.0
30	5.0	66.7	30	60.0
40	4.8	64.0	3	6.0
50	-	-	-	-

[a]70 rhizomes/dose.

2.3. Mutation breeding in banana, Maçã cultivar and evaluation of somaclonal variants

The cultivar Maça (AAB group) is preferred by Brazilian consumers, but the farmers avoid growing it due to its high susceptibility to the soil-borne disease, Mal do Panamá, caused by *Fusarium oxysporum* fs. sp. *cubense*. Shoot tips from this cultivar were irradiated with

TABLE V. MEAN HEIGHT AND SURVIVAL IN THE M_1V_1 GENERATION OF *STROMANTHA SANGUINEA* CV. SANGUINEA OBTAINED FROM RHIZOMES IRRADIATED WITH GAMMA rayS, EVALUATED 93 DAYS AFTER PLANTING

Dose	$\bar{x}$ Plant ht.[a]		Survival[a]	
(Gy)	Cm	%	No.	%
0	5.7	100.0	61	100.0
10	6.9	121.0	38	62.3
20	6.2	108.8	38	62.3
30	5.6	98.2	36	59.0
40	4.7	82.5	27	44.3
50	4.0	70.2	6	9.8
60	-	-	-	-

[a]77 rhizomes/dose.

gamma rays (20, 35 and 40 Gy) and advanced *in vitro* till the M_1V_4 generation [7]. The plants obtained were evaluated under field conditions and 8 of them selected and included in a yield trial in infected soil. The results showed that no tolerant mutants were obtained and now the evaluation is continued in the greenhouse using infected soils.

Some of the problems in this type of research are the difficulties (costs, space in laboratory, need of special environmental conditions etc.) involved in obtaining large *in vitro* populations. Thus, in association with IAEA Seibersdorf Laboratory we started to develop methods to reduce the costs involved in the micropropagation of banana.

Also, we decided to include in the research an evaluation of a somaclonal variant, short plant stature, selected in the field from Nanicão cultivar (AAA group). This variant is maintaining the short stature and in the coming years the yield and fruit characteristics will be evaluated. Also under evaluation is a somaclonal variant resistant to yellow Sigatoka disease in Venezuela, obtained by Dr. Eva Garcia. This variant is resistant to this disease also under Brazilian conditions. This result shows the validity of somaclonal variants for plant breeding and also indicates that *in vitro* techniques, associated with mutation induction can be useful to obtain resistance to diseases.

2.4. Mutation breeding in citrus

Buds from the sweet orange Pera cultivar were irradiated with 40 Gy of gamma rays and 135 putative mutants were selected for certain agronomic characteristics such as: lower plant height, high yield, differences in fruit maturity and absence or lower number of seeds [3]. New clones were obtained by grafting buds from these selected plants. A total of 16 trials (5 replications/selected putative mutant) were established, in field conditions (Presidente Prudente town) in order to confirm the mutations' genetic stability and commercial value (Table VI). There will be a need of at least 4 more years to evaluate the commercial value of the selected plants. The first results obtained confirmed that some mutants showed good agronomic characteristics. One example can be observed in Tables VII, VIII and IX with the results of the evaluation of mutants selected for low number of seeds. The mutant 78 had a significant reduction in the number of seeds (Table VII) with very few alterations in other agronomic characteristics. The data from these tables can be compared with the selection carried out in the original cultivar, without irradiation (Tables X, XI and XII). Hence, it is possible to conclude that irradiation was responsible for the reduction in the number of seeds,

TABLE VI. ESTABLISHMENT OF 16 FIELD YIELD TRIALS DIVIDING THE 135 SELECTED PLANTS INTO 12 SUBGROUPS[a]

Subgroup		Trial No.	Putative mutants included, No.
1-	High yield (HY) of fruit	1	11
	" "	2	11
2-	Compact growing (CG)	3	8
3-	Lower number of seeds (LNS)	4	9
	" " "	5	9
4-	Late fruit maturity (LM)	6	11
5-	Semi-late fruit maturity (SLM)	7	7
6-	CG + LNS (CGLNS)	8	9
7-	" "	9	8
6-	CG + LNS + SLM (CGLNSSLM)	10	6
8-	SLM + LNS (SLMLNS)	11	12
	" "	12	12
9-	Lower citrus canker incidence (LICC)	13	13
10-	Morphological mutants (MM)[b]	14	7
11-	Other characteristics (OC)[c]	15	9
12-	Plants selected from controls (PSC)	16	9

[a]In each trial, Pera Commercial was included as control, trials in blocks, 5 replications from each selected mutant.
[b]Fruit skin, different leaf shape.
[c]Different fruit size, drought tolerance etc.

TABLE VII. EVALUATION OF FRUIT CHARACTERISTICS INCLUDING SOME MUTANTS SELECTED FOR LOW NUMBER OF SEEDS (LNS), COMPARED WITH PERA COMMERCIAL CULTIVAR (PCC) AS CONTROL; DATA FROM YIELD TRIAL NUMBER 3 (TABLE VI)[a]

Line	Fruit length (cm)	Fruit width (cm)	Length/width	No. seeds/fruit	Skin thickness (cm)	Weight (g)
PCC	**7.93**	**7.46**	**1.06**	**6.63**	**0.51**	**229.53**
37	7.50	7.10	1.05	2.70**	0.39**	202.13
81	7.53	7.03*	1.07	2.00**	0.46	195.00
29	7.33*	7.16	1.02	2.43**	0.41*	200.80
42	8.00	7.36	1.08	0.90**	0.53	222.46
82	7.60	7.23	1.05	3.53**	0.47	210.13
49	7.33*	7.00*	1.04	1.16**	0.43	189.06*
78	7.53	7.33	1.02	0.86**	0.42	218.20
5	7.80	7.23	1.07	1.53**	0.47	221.00
F	**1.64**	**1.08**	**1.63**	**18.47**	**1.73**	**1.13**
CV	**4.30**	**3.61**	**2.66**	**30.22**	**13.93**	**10.73**

[a]Differences from Pera Commercial Control (PCC): * = 5%, ** = 1%.

because in the original population the average minimum number of seeds per fruit in the selected plants was 3.56 (plant No. 68, Table X), as compared with 0.86 in the mutant 78 (Table VII).

TABLE VIII. EVALUATION OF PLANT CHARACTERISTICS INCLUDING SOME MUTANTS SELECTED FOR LOW NUMBER OF SEEDS (LNS), COMPARED WITH THE PERA COMMERCIAL CULTIVAR (PCC) AS CONTROL; DATA FROM YIELD TRIAL NUMBER 3 (TABLE VI)[a]

Line	Plant height (m)	Plant diameter (m)	Height/diameter
PCC	**2.26**	**1.91**	**1.17**
37	2.65	2.25*	1.17
81	2.43	2.16	1.12
29	2.66	2.27**	1.18
42	2.60	2.33**	1.11
82	2.26	2.11	1.09
49	2.28	2.14	1.07
78	2.56	2.37**	1.08
5	2.56	2.46**	1.04
F	**1.89**	**2.97**	**1.33**
CV%	**12.42**	**7.41**	**13.73**

[a]Differences from Pera Commercial Control (PCC): * = 5%, ** = 1%.

TABLE IX. EVALUATION OF FRUIT JUICE CHARACTERISTICS INCLUDING SOME MUTANTS SELECTED FOR LOW NUMBER OF SEEDS (LNS), COMPARED WITH THE PERA COMMERCIAL CULTIVAR (PCC) AS CONTROL; DATA FROM YIELD TRIAL NUMBER 3 (TABLE VI)[a].

Line	Brix	Acidity (%)	Ratio brix/acidity	Juice (%)
PCC	**8.03**	**0.62**	**13.03**	**44.47**
37	8.60	0.65	13.23	45.88
81	8.26	0.54	15.26*	44.32
29	8.20	0.61	13.30	46.43
42	8.53	0.50	16.90**	44.52
82	8.13	0.53	15.30*	45.20
49	7.73	0.57	13.40	46.14
78	8.00	0.59	13.40	50.88*
5	8.30	0.56	14.70	43.23
F	**1.26**	**1.25**	**3.38**	**1.24**
CV	**11.19**	**12.68**	**8.79**	**7.48**

[a]Differences from Pera Commercial Control (PCC): * = 5%, ** = 1%.

The first results obtained with use of RAPD for the analysis of these putative mutants showed the potential of this line of investigation. It will be continued using other methods, in cooperation with Instituto Agronômico de Campinas.

In order to facilitate future use of protoplasts combined with irradiation and mutation induction, research on the methodology to obtain regenerated plants from protoplasts was introduced. Despite several attempts, it was not possible before to obtain citrus plants regenerated from protoplasts in Brazil. 'Cleópatra' mandarin (*Citrus limonia* Osbeck) and 'Rangpur' lime (*Citrus reshni* Hort.) plants were regenerated from protoplasts of cell suspension. At first step, nucelar calli were cultured in a medium containing BAP and

maintained in growth regulator free medium. Protoplasts were isolated from embryogenic suspensions and plated at a rate of 2×10^5 protoplasts mL^{-1}, in agarose droplets. After somatic embryo germination, the plants were rooted and acclimated to the environment [8].

TABLE X. EVALUATION OF FRUIT CHARACTERISTICS INCLUDING PLANTS SELECTED FROM THE ORIGINAL, UNIRRADIATED PERA CULTIVAR CONTROL (PSC), COMPARED WITH PERA COMMERCIAL CULTIVAR (PCC); DATA FROM YIELD TRIAL NUMBER 3 (TABLE VI)[a]

Line	Fruit length (cm)	Fruit width (cm)	L/W	Seeds/fruit No.	Skin thickness (cm)	Weight (g)
PCC	**7.50**	**7.06**	**1.06**	**6.90**	**0.54**	**193.06**
1	7.46	7.03	1.06	5.76	0.50	196.16
97	7.50	7.06	1.06	5.36	0.42*	198.26
68	7.66	7.30	1.05	3.56**	0.42*	212.40
99	7.75	7.30	1.06	7.20	0.45	207.70
2	7.70	7.20	1.07	6.66	0.44	213.73
69	7.03*	6.70*	1.05	5.76	0.35**	162.30
3	7.33	7.03	1.04	6.20	0.44	195.20
F	**2.16**	**2.16**	**1.71**	**1.88**	**1.84**	**2.23**
CV	**3.53**	**3.10**	**1.72**	**23.78**	**15.97**	**9.48**

[a]Differences from Pera Commercial Control (PCC): * = 5%, ** = 1%.

TABLE XI. EVALUATION OF FRUIT CHARACTERISTICS INCLUDING PLANTS SELECTED FROM THE ORIGINAL UNIRRADIATED PERA CULTIVAR CONTROL (PSC), COMPARED WITH PERA COMMERCIAL CULTIVAR (PCC); DATA FROM YIELD TRIAL NUMBER 3 (TABLE VI)[a]

Line	Plant height (m)	Plant diameter (m)	Height/diameter
PCC	**2.41**	**1.95**	**1.23**
1	2.36	2.11	1.13
97	2.30	1.87	1.27
68	2.33	1.99	1.17
99	2.45	2.06	1.18
2	2.35	1.96	1.19
98	1.80	1.56	1.15
69	2.21	2.25	0.99*
3	2.53	2.28	1.11
F	**0.74**	**0.84**	**0.99**
	15.68	**18.21**	**12.02**

[a]Differences from Pera Commercial Control (PCC): * = 5%, ** = 1%.

The methodology to isolate and regenerate plants from protoplasts is now established and is being used to obtain somatic hybrids. For this, leaf protoplasts and protoplasts from embryogenic cell suspension were fused by PEG methods. Plants were recovered following protoplast fusion of two treatments (‘Cleopatra’ as the protoplast from embryogenic suspensions and ‘Rangpur’ as the leaf protoplast and the reciprocal cross). Among them,

eleven plants were confirmed to be allotetraploid somatic hybrids by molecular markers (RAPD) and isozymes markers analysis [8].

With the objective of obtaining seedless tangerine mutants, protoplasts of Murkote tangor were irradiated to establish suitable gamma rays treatment doses. The next step will be the irradiation with the dose chosen.

3. CONCLUSIONS

Mutation breeding in vegetatively propagated crops can be used in Brazil in several crops to increase the genetic variability in characteristics of high importance [9].

Biotechnology is a powerful tool that can be used in several ways in association with mutation breeding. Due to the need to use large populations in mutation breeding, there is an urgent need to develop methods that can reduce the costs when tissue culture technique is used.

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IN VITRO PROPAGATION OF AVOCADO (*Persea Drymifolia* Ness.)

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Abstract

In the past 20 years, reports on micropropagation and rooting *in vitro* of avocado shoots, with diverse origins and treatments, have been published. However, none of them reached the level required for large scale propagation of the species. It is considered that, in the first place, the micropropagation of avocado requires an efficient system of rooting. Therefore, a system to induce the rooting *in vitro* of avocado shoots, based on indole-3-butyric acid (IBA) pulses and some treatments based on thidiazuron (TDZ) was tested, using 40 explants per treatment. The treatments with TDZ did not succeed in rooting shoots. Some treatments with pulses of IBA induced the following rooting results: without growth regulators, 16%; 4,000 mg L^{-1} of IBA for 5 seconds, 8.3% rooted; 100 mg L^{-1} for 72 hours, 20%; 50 mg L^{-1} for 72 hours, 15.4%; 150 mg L^{-1} for 24 hours, 5% rooted. It is considered possible to improve these results by adjusting the range of IBA concentrations as well as in the time range of pulse applications. Finally, it is easier to establish *in vitro* explants derived from mature seeds or embryos germinated *in vitro*.

1. INTRODUCTION

According to the FAO, 75% of worldwide production of avocado in 1989 was in the American continents. Mexico produces 25% of the total amount, occupying first place worldwide in avocado production [1]. The avocado is one of the most important fruit bearing species in Mexico, evidenced by harvested surface areas of 99533 ha as well as by their production of 766404 tons in 1993 [2].

In Mexico, the main problems agriculture faces are lack of water for irrigation, salinity and high concentrations of calcium carbonates in the soil due to misuse of soil and irrigation water. At present, approximately 30% of the country´s five million irrigated hectares have different degrees and classes of drainage and/or salinity problems [3].

There are no well defined rootstocks in Mexico adapted to the diverse soil conditions where this crop is cultivated and where it has a potential. They are generally obtained from seeds that are collected from leftover fruits produced under open pollination in orchards or fields, or are obtained in the markets. Rarely are they produced from selected trees. The rootstock genetic nature can affect the scions with different intensity in some agronomic characteristics, such as vigor, productivity, quality of fruit, tolerance or susceptibility to pests and diseases, as well as the ability to develop in diverse soil conditions. Thus, a procedure for vegetative propagation of rootstocks and their selection is required.

The formation of the rootstock clones with interesting agronomic characteristics is hampered by the difficulty in inducing roots during vegetative propagation. Some less efficient rootstock vegetative propagation techniques than those desired have been developed and are used with a certain cost and labor factor by means of etiolation and other technical support [4, 5, 6, 7].

A highly useful tool in programs of genetic improvement, as in vegetative avocado propagation, is *in vitro* tissue culture in order to recover genotypes that might be lost under

natural conditions [1]. *In vitro* tissue culture is a plant propagation technique that has given good results in other species, for example *Cupressus*, *Ficus* and *Photinia*. But, on the other hand, the findings published on avocado in this regard, including those that report apparently acceptable results, do not facilitate commercial propagation of avocado by *in vitro* culture. The present study presents results of trials showing that it is necessary to adjust a set of factors until the establishment of a system that permits the cloning of avocado *in vitro*.

With these precedents, the objective of the present work was to establish a basic methodology of mass propagation *in vitro* of native avocado using different reported methodologies. Thus, to take advantage of available genotypes that tolerates marginal soil conditions.

2. MATERIALS AND METHODS

The current study took place in the Laboratory of Tissue Culture at the Institute of Agricultural Sciences of the University of Guanajuato, during 1994–1997, using vegetative material of the Mexican race (*Persea drymifolia* Ness.) and the medium of Murashige and Skoog (MS) [8]. Diverse growth regulators in different concentrations were tested utilizing different parts of the plant or explants taken from tender tree shoots germinated *in vitro* and greenhouse seed shoots.

2.1. Tender tree shoots

Using some avocado trees in orchards and fields close to the Institute, tender shoots, 6 to 7 cm long, were cut from semi-hard wood. They were subsequently washed in sterile containers with sterile distilled water containing 20% commercial sodium hypochlorite plus 0.1% of Tween 20, for 30 min with intermittent manual agitation. Then, the hypochlorite solution was poured out and substituted by sterile water with 2% Benlate for 30 min with intermittent manual agitation. The Benlate liquid was then poured off and once again 20% commercial sodium hypochlorite was added for a 10 min period. The hypochlorite was then poured off and the shoots were washed four times with sterile distilled water. After the last washing process, the bases of the shoots were cut, reducing their length to 4–5 cm, and they were placed in the culture medium.

The culture medium employed was MS [8], supplemented with 80 mg L^{-1} of adenine sulfate plus 2 mg L^{-1} of benzyladenine (BA). The explants were incubated in a growth chamber with a photoperiod of 16 hours light/8 hours of darkness; the daylight was given by fluorescent lamps, 35 µmol m^{-2} s^{-1}; at a temperature of 25 ± 2°C and they were maintained under these conditions for 30 days.

2.2. Mature embryo shoots

Shoots obtained from plants from the field generally present contamination problems. In order to obtain uncontaminated shoots for *in vitro* propagation, mature *in vitro* avocado seed embryos were germinated. For this, fruits that were washed with running water were used. Subsequently, the seed was extracted in a laminar air flow bench. The seeds were disinfected by the methodology described in 2.1 above. After disinfecting two types of explants were tested, one constituted of complete seed**s**, incubating them in flasks with a volumetric capacity of one liter and with 100 mL of medium MS. The second consisted of the utilization of the seed embryos. For this, the cotyledons of each seed were separated taking care not to harm the embryo and afterward, cutting the major part of the cotyledon, leaving approximately 1 to

1.5 cm^3 of the tissue with the embryo. That embryo was sown as complete seed in MS medium in 240 mL flasks containing 40 mL of MS medium. The culture medium of both types of explants was enriched with 0.3 mg L^{-1} of indolbutyric acid (IBA) and 3.0 mg L^{-1} of kinetin to accelerate germination. They were placed in the dark for two weeks at a temperature of 25 ± 2ºC.

Also, to test the shoots propagated in soil, avocado seeds were sown in sterile substrate under greenhouse conditions. The emerging stem was pruned to induce the proliferation of new shoots originating from the base of the stem. Before being cultivated *in vitro* these shoots were disinfected with the methodology described for tender tree shoots (2.1).

Of the shoots that were obtained from the seeds germinated in soil or *in vitro*, 3 to 4 cm segments were cut and given different treatments as shown in Table I. The shoots from treatments 2 to 18 described in Table I were planted in MS medium, supplemented with 0.3 mg L^{-1} of IBA and 3.0 mg L^{-1} of kinetin, establishing four explants per flask with 10 replications. Therefore, for every treatment 40 shoots were employed. Treatments 19 to 23 remained in the medium described in Table I for 30 days and from then on were subcultured in the same medium every 30 days. The evaluated variables were percentage of explants that form callus and percentage of explants with induced rooting.

TABLE I. TREATMENTS EMPLOYED TO INDUCE SHOOT ROOTING IN THE MEXICAN AVOCADO RACE CULTIVATED *IN VITRO*[a]

No.		No.	Treatments
1	**Control**	13	100 mg L^{-1} IBA in MS/24 hours
2	Alcohol 95%/5 sec	14	100 mg 1^{-1} IBA in MS/72 hours
3	6000 mg L^{-1} IBA in WES/5 sec	15	50 mg L^{-1} IBA in MS/12 hours
4	5000 mg L^{-1} IBA in WES/5 sec	16	50 mg L^{-1} IBA in MS/24 hours
5	4000 mg L^{-1} IBA in WES/5 sec	17	50 mg L^{-1} IBA in MS/72 hours
6	3000 mg L^{-1} IBA in WES/5 sec	18	150 mg L^{-1} IBA in MS/24 hours
7	2000 mg L^{-1} IBA in WES/5 sec	19	0.0001 mg L^{-1} Thidiazuron in MS 30 days
8	1000 mg L^{-1} IBA in WES/5 sec	20	0.001 mg L^{-1} Thidiazuron in MS 30 days
9	1000 mg L^{-1} IBA in WES/30 sec	21	0.01 mg L^{-1} Thidiazuron in MS 30 days
10	1000 mg L^{-1} IBA in WES/60 sec	22	0.1 mg L^{-1} Thidiazuron in MS 30 days
11	100 mg L^{-1} IBA in MS/3 hours	23	1.0 mg L^{-1} Thidiazuron in MS 30 days
12	100 mg L^{-1} IBA in MS/12 hours		

[a]WES = water: ethanol solution, 50%

In the same way, with shoots derived from germinated seeds *in vitro* a new experiment was conducted, applying CO_2 into the culture flask, by means of sodium bicarbonate, citric acid and distilled water. To this end, a volume of 180 mL was prepared with 20 mL MS medium. A small flask with distilled water was placed inside each flask containing MS medium. After sterilization and cooling, the small flasks containing the distilled water were held by the solidified MS medium. After disinfection in the laminar air flow bench, the shoots derived from germinated greenhouse seeds were planted, one per flask in the culture medium. Subsequently, a solution of 375 mg $NaHCO_3$ + 125 mg of citric acid was prepared, a solution that had previously been placed in an aluminum envelope and sterilized in an oven at 140°C. Upon contact with the water, the mixture began to bubble, releasing CO_2. The flask was covered quickly to prevent CO_2 from escaping. Five subcultures were made every 30 days using the same procedure. A total of 50 plants were treated, one per flask.

3. RESULTS AND DISCUSSION

3.1. Tender tree shoots

The establishment *in vitro* of explants originating from trees is difficult because contamination kills most of the explants. To overcome this problem, antibiotics were applied after the disinfection described above, employing in the culture medium three antibiotics: 25 mg L^{-1} of rifampicine, 25 mg L^{-1} of tetracycline and 1 mL L^{-1} of garamicine. These antibiotic treatments prevented the contamination of the culture media. However, it was not possible to induce root or shoot production, which was the main objective in these experiments. After more than four months, the explants produced only callus in the basal part, i.e. the part that was in touch with the culture medium.

Pliego-Alfaro and Bergh [1] mention that callus cultures can be established from almost any avocado explant. However, adventitious bud regeneration has not been observed so far. Probably the semi-hard wood of the tree shoots did not permit the initiation of other tissues. It has already been cited that adult avocado shoots proliferate poorly in tissue culture [9]. Also, Mora-Aviles [10], using microcuttings (1.5 cm) of Mexican race avocado trees, succeeded in promoting bud development and their elongation in a MS medium with several combinations of benzyladenine (0.5, 1.0 mg), indolbutyric acid and indolacetic acid (0.1, 0.5, 1.0 mg); however, roots were not induced in the explants.

Several researchers have reported success in the establishment *in vitro* of shoot proliferation and the subsequent rooting of the same [11, 12]. However, as the reported results show, these treatments are not sufficient to resolve the problems of the commercial multiplication of clones of agricultural interest. Future projects with this system should study, as Raviv and Reuveni [13] indicated, new and different growth regulators or substances affecting rooting of explants, not only auxins and sugars, which have been used up to now in solving the problem of propagation *in vitro* of avocado.

3.2. Mature embryo shoots

Germination commenced on the tenth day after the complete seeds were sown. In the case of the embryos, these initiated germination two or three days after being sown. Contamination with the utilization of complete seeds was 3%, and was absent in the case of the embryos. The percentage of embryo oxidation was 5%, it was not a problem in the case of the seeds. As observed in Table II, the percentage of germination of seeds and embryos was high. It was reached in a relatively short time, with low percentages of contamination of the culture medium and of oxidation of the explants. Oxidation was avoided by adding to each liter of culture medium antioxidant compounds, viz. 1.8 mg of thiosulfate and 6.0 mg of silver nitrate.

This procedure of using seeds or mature avocado embryos to obtain clean explants to try to carry out propagation by tissue culture, is acceptable. Germination is rapid and time can be gained in experimentation, however the costs are higher.

The main objective of culturing seeds or mature avocado embryos *in vitro* was the attainment of noncontaminated shoots with which to initiate the propagation process, including growth, multiplication and rooting of the new shoots. It was considered necessary to induce the rooting of the shoots that served as explants for their subsequent growth with the formation of new leaves and internodes and from then on to propagate them using segments

of the elongated shoots. The effects of the rooting treatments listed in Table I can be observed in Table III. It can be seen that none of the treatments with thidiazuron led to the formation of callus or to rooting. On the other hand, in the treatments with IBA callus was formed in most cases, and four of those treatments plus the control induced rooting.

TABLE II. CULTURING *IN VITRO* OF MEXICAN RACE MATURE AVOCADO EMBRYOS

Criteria	Mean
Germination of complete seeds	94.0%
Germination of embryos	99.0%
Days to seed germination	10 days
Days to embryo germination	3 days
Contamination rate (in seeds)	3.0%
Oxidation rate (of embryos)	5.0%
Length of the radicle 15 days after germination	2.5 cm
Length of the radicle 30 days after germination	3.5 cm
Length of the stem 30 days after germination	2.5 cm
Plants with secondary shoots 15 days after germination	60.0%

TABLE III. FORMATION OF CALLUS AND ROOTS IN AVOCADO SHOOTS OF THE MEXICAN RACE WITH GROWTH REGULATORS' TREATMENTS LISTED IN TABLE 1

Treatment No.	Callus diameter (cm)	With callus (%)	With roots (%)	Treatment No.	Callus diameter (cm)	With callus (%)	With roots (%)
1	0.46	67.00	16.00	13	0.73	60.00	0.00
2	1.08	50.00	0.00	14	0.32	43.00	20.00
3	1.28	100.00	0.00	15	0.50	20.00	0.00
4	0.69	58.00	0.00	16	0.78	40.00	0.00
5	1.19	92.00	8.30	17	0.27	7.00	15.40
6	0.55	33.00	0.00	18	0.92	46.00	5.00
7	0.68	67.00	0.00	19	0.00	0.00	0.00
8	0.65	17.00	0.00	20	0.00	0.00	0.00
9	1.02	42.00	0.00	21	0.00	0.00	0.00
10	0.50	59.00	0.00	22	0.00	0.00	0.00
11	0.00	00.00	0.00	23	0.00	0.00	0.00
12	0.67	90.00	0.00				

As can be seen, the treatments with IBA did not involve continuous application of growth regulators, as reported previously [12, 14, 15, 16, 17]. There, avocado explants were cultured *in vitro* with concentrations of IBA that ranged from 1 to 10 mg L^{-1} in the medium, and several of them achieved rooting. In this study treating the explants in pulses, gave positive results. In doses of 4,000 mg L^{-1} in rooting difficult plant cuttings, such as *Cupressus* [18], IBA pulses with doses of 3,000 and 4,000 mg L^{-1} [5], gave rooting of avocado cuttings. Furthermore, in *Photinia,* which is a difficult rooting plant, IBA pulses of 10 to 100 mg L^{-1} to explants cultured *in vitro* induced rooting in microcuttings [19].

Various species respond differently to different treatments of growth regulators. Treatments based on pulses to the microcuttings of avocado confirm the difficulty of establishing a satisfactory system for the rooting of avocado. Thus, in the control (no growth

regulators) rooting of 16% of microcuttings was obtained (Table III), confirming, at least partially, the results obtained by Pliego-Alfaro [20], of 100% rooting in shoots derived from germinated seeds in *in vitro* solid medium culture, without growth regulators. On the other hand, with a pulse of IBA of 100 mg L^{-1} for 72 hours, 20% of the explants in treatment No. 14 rooted (Table III); yet, this rate cannot be recommended for commercial propagation of the species. Furthermore, treatment No. 17 with a pulse of 50 mg L^{-1} for 72 hours induced 15.4% of the explants to form roots (Table III). Treatment No. 5 with a pulse of 4,000 mg L^{-1} of IBA induced 8.3% rooting, and treatment No. 18 with a pulse of 150 mg L^{-1} of IBA induced a 5% of rooting. The rest of the treatments did not induce any rooting (Table III). This can indicate that more work is required, with a tighter dose range, around the concentrations of treatments Nos. 14 and 17, before a higher rate of avocado microcuttings rooting can be reached, without neglecting the possible reasons for the rooting achieved in the control, as Pliego-Alfaro [20] reported. Surely, the juvenility of the explants should be stressed.

The experience of the authors trying to micropropagate avocado has proved that during the *in vitro* culture of the explants not only has the development and the formation of roots been inhibited, but also the shoots in culture did not elongate. Therefore, the objective of the last experiment was to determine the effect of the application of CO_2 upon the development of the cultured explants. The results obtained from this experiment were: 1) The explants in treatment grew slowly (in approximately 6 months they doubled their length from 2.5 to 5 cm), but even so this was more than other shoots had elongated in other tested treatments. 2) The development of the shoots was more in length than in thickness. 3) None of the 46 cultured shoots which survived for 6 months formed roots. 4) The stem portion and leaves grown *in vitro* in the treatment with CO_2 always showed a more juvenile appearance than the original part.

The response of the shoots may be explained by three different physiological interrelated processes [21]: 1) Injuries and mechanical damages to the plant tissues increase ethylene production. 2) In the majority of plant species, ethylene retards stem and root elongation. 3) Concentrations of 5 to 10% of CO_2 inhibit many of the effects of the ethylene, above all because it reduces ethylene production. The progress in this experiment promises to be part of the solution of *in vitro* propagation of avocado. This problem has not been resolved and continued investment for this purpose is needed. As an example, the California Avocado Society in their 1996–1997 Production Research Program, included within 15 selected programs to be financed by industry through the California Avocado Commission, research on avocado rootstock development by somatic hybridization and genetic engineering [22].

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