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INDUCED MUTATION IN TROPICAL FRUIT TREES

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FOREWORD

Despite the tremendous efforts devoted to fighting poverty and ensuring food security for all, humankind still faces a serious challenge. This situation is currently exacerbated by the huge increase in food prices along with a significant decrease in food crop productivity in many developing countries. The ultimate challenge now is to understand that alleviation of poverty in the world requires efforts from all the parties involved: governments, international organizations, scientists, breeders and farmers, and should also include concerns about climate change and variability.

Over the past 40 years, the Agency, through the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, has assisted Member States in increasing food crop productivity and enhancing the livelihood of people around the world. Many success stories can be found in Asia, Africa and Latin America, as well as in developed countries including Europe, Japan and the USA. This continuing effort is supported by scientists who, under the Agency's coordinated research projects (CRPs), work to expand knowledge of the mechanisms and the benefits of induced mutagenesis together with efficiency enhancing bio/molecular technologies such as *in vitro* and molecular characterization techniques.

This book reports on the results obtained in inducing mutations in selected fruit trees, important for human dietary needs and a healthy lifestyle. Fourteen countries participated in the CRP entitled "Improvement of Tropical and Subtropical Fruit Trees through Induced Mutations and Biotechnology". The original and most advanced results are presented here with the hope that this publication will assist scientists using induced mutation to improve fruit trees.

The Agency acknowledges the assistance of R. Sangwan (France) who edited and reformatted the various contributions. The IAEA officers responsible for this publication were S. Mohan Jain and M. M. Spencer of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

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PREFACE

Tropical and subtropical fruits are grown mainly for nutrition, food security depending on the fruit type, agro-food industries, and income generation. There is a great commercial potential for tropical fruits in international markets. For example, mango, banana, citrus, litchi, papaya, and guava are major fruits consumed fresh or canned or as pulp or puree. The production, cultivation and maintenance of tree species provide highly sustainable production systems that conserve soils, microenvironments and biodiversity. The fruits are consumed fresh, as fresh and processed juices, beverages, as processed and dried fruits, and as other fresh and processed raw materials for use in the food processing industries. Fruits are also targets for major supermarket outlets in the developed and developing countries and this has led to a strong focus on the quality and food safety targets, which are of increasing economic importance. Fruits and nuts are a valuable source of good nutrition, e.g. rich in vitamins, sugars, and aroma and flavour compounds.

In tropical and subtropical regions, there are not enough resources available for research and development programmes to develop fruits, especially genetic improvement and exploitation for socioeconomic benefits and agro-based industries. As a result, fruit improvement programs are lagging. In addition, the neglect of horticultural crops, human population growth, rapid industrialization and deforestation are leading to the loss of valuable germplasm for the genetic improvement of fruits. The longer these problems remain unresolved, the more germplasm will be depleted, which could lead to a point of greater dependence on imports, destruction of local farming and agro-based industries, malnutrition, and loss of exports. In order to sustain fruit production, germplasm collection, conservation and utilization are of paramount importance for the genetic improvement of fruits using conventional and modern methods such as biotechnology, molecular markers and mutagenesis. Post-harvest storage losses and poor transportation have an adverse impact on total fruit production and lead to economic losses. The European and North American markets have a huge potential for tropical fruit imports. These markets have strict quality control and require high phytosanitary conditions of imported fruits. Plant breeders should develop new fruit cultivars with traits such as disease resistance/tolerance, improved quality (such as nutrition, processing or fibre quality) and longer shelf-life. Moreover, the development of a fruit based industry would generate more employment in the rural area.

Genetic improvement of tropical and subtropical fruits is essential for increasing fruit production. The major problems facing fruit tree breeding are long juvenile periods, unavailability of suitable germplasm, and large tree size. In many fruit crops such as avocado, mango, litchi and others, breeding by controlled crosses is hampered due to delayed flowering, or unsuccessful fruit setting due to abortive embryos or massive fruit drop. For most fruit tree species, the popular/desired varieties/cultivars have a range of agronomic and horticultural issues such as propagation, yield, appearance, quality, disease and pest control, abiotic stress and poor shelf-life. Diseases caused by *Fusarium, Phytophthora* and *Colletotrichum* combined with insects and pests account for significant losses in fruit production or to trees. The exploitation of genetic variation, natural or induced, assists greatly in the genetic improvement of fruit trees. Deforestation and other factors have restricted the availability of suitable natural germplasm for fruit breeding.

Induced mutations are highly effective in enhancing natural genetic resources, and have significantly assisted in developing improved fruit cultivars. Mutations can be induced by physical and chemical mutagens. Important agronomic traits improved by induced mutations in some major fruits include disease resistance (in Japanese pear and peach), seedlessness (in

citrus, guava), reduced height (in papaya, pomegranate), and earliness (in banana, apricot, jujube, plum, apple). The selection of an appropriate technology is very important for mutation induction and the selection of mutants. Advances in plant tissue culture and plant molecular biology can be integrated with conventional techniques in generating new mutations. The relevant plant tissue culture techniques used in the improvement of fruit trees include somatic embryogenesis, somaclonal variation, micropropagation and micro-grafting, cryopreservation of embryogenic cell cultures, in vitro selection, and somatic hybridization. Mostly tropical and subtropical fruit trees are recalcitrant in large scale plant production through somatic embryogenesis mainly due to the low germination rate of somatic embryos. However, the in vitro mutagenesis approach is suitable for vegetatively propagated crops because large numbers of propagules can be used for mutation induction in a small space, and several cycles of subculture are carried out in a short period of time to dissociate chimeras and increase the plant population of mutants for selection and evaluation. For some traits, the selection of mutants is done in a short period of time by exerting the selection pressure on irradiated in vitro cultures, and mutant plants can be regenerated and multiplied in large numbers by micropropagation.

The FAO/IAEA funded CRP entitled 'Improvement of Tropical and Subtropical Fruit Trees through Induced Mutations and Biotechnology' was initiated in 2000 and concluded in 2005. Its goal was to address major constraints in tropical and subtropical fruits such as mango, citrus, guava, cashew, avocado, papaya, litchi, annona, jujube, carambola, pitanga and jaboticaba. The first Research Coordination Meeting for this CRP with the objective of generating and characterizing radiation induced and natural genetic diversity in tropical and subtropical fruit trees for improving nutrition balance, food security, and enhancing economic status of the growers in Member States was held in Vienna in September 2000. This book describes the results of different projects within this CRP dealing with mutation induction in tropical and subtropical fruits. It includes chapters on different fruits and describes efficiency, enhancing biotechnologies such as: plant tissue culture and regeneration, mutation induction, mutants selection and evaluation. As there are hardly any academic references on this subject, this book is expected to assist researchers, students and industry working in the field of genetic improvement of tropical and subtropical fruits. There is great potential in improving tropical and subtropical fruit crops using gamma irradiation together with plant tissue culture, *in vitro* mutagenesis and selection, molecular markers and conventional propagation methods. Somatic embryogenesis could be an ideal system for the selection and multiplication of mutants, saving time and money in dissociating chimeras since the embryogenic structures originate from a single cell.

CONTENTS

SUMMARY

"*The IAEA serves as the global focal point for nuclear cooperation, mobilizing peaceful applications of nuclear science and technology for critical needs in developing countries, including fighting hunger, disease, poverty and pollution of the environment and thereby contributing to the sustainable development goals of its Member States*" this exert from the IAEA's constitution guides the work of all staff members and scientific partners in their common search for a real socio-economic development.

The efforts of the Joint FAO/IAEA Division together with renowned scientists and breeders throughout the globe have led to the development of improved cultivars adapted to specific needs and made a major contribution in many regions and/or countries in the world [1]. Plant breeding and in particular mutation breeding is based on the fine art of selecting the "one" genotype, which can bring solution to a given situation, such as tolerance and/or resistance to: drought, high salinity content, diseases, and pests. Mutation induction is used to increase the chances of creating a favourable and permanent genetic change with possible positive impact in crop improvement.

The best agronomically interesting mutants known to date are the dwarf mutants of wheat and rice with height reductions and considerable yield increase under high fertilization inputs, which led to the "green revolution" in cereal production in the mid-1960s [2]. Most mutations have been induced at random, either spontaneously or through the use of experimental agents such as chemicals mutagens: sodium diazide and ethyl methane sulphonate (EMS) or through physical ionizing radiations. More recently researchers have used disruption of genes through the insertion of a DNA fragment, referred to as *insertional mutagenesis* to induce mutations in plants [3]. Furthermore, using a combination of mutation induction, genetic and molecular characterization techniques (marker assisted selection and T-DNA tagging) scientists have been able to perform the identification and cloning of agronomically important mutated genes involved in several favourable mutations [3].

The application of these techniques and close collaboration with the Joint FAO/IAEA Division has allowed the development of several thousands of interesting mutants in crop plants [4]. Recently, the Joint Division has updated this list, which contains many varieties of cereals, pulses, oilseeds, fibre crops and ornamentals that have been developed using induced mutation among which about 50% were released mainly during the last two decades [2]. These mutants with desirable characteristics were either directly released for cultivation or used in hybridisation programmes with other mutants and/or varieties to develop elite genotypes/varieties, and have made significant contribution to boost the production of several crops including barley, rice, wheat and mungbean, and other so-called "orphan crops" including bambara groundnut, amaranthus, and cowpea, which readily benefit from local traditional know-how and acceptability [5].

To implement the right selection the breeder has to identify one or a couple of traits such as size, number and shape of leaves, root proliferation type, plant height, timing of flowering, structure and colour of flowers, and/or number, size and shape of seeds. These traits in a given plant at a given time could translate into higher yield, tolerance and/or resistance to selected stresses. It is not an easy task, but the 21 participants to the present CRP D23026 on: "*Identification and pyramiding of mutated genes: novel approaches for improving crop tolerance to salinity and drought"* initiated in 2004 (Bulgaria, China, Cuba, Egypt, Ghana, India, Indonesia, Israel, Italy, Pakistan, Thailand, Tunisia, Turkey, USA, Vietnam and one CGIAR - IRRI in the Philippines) convincingly embarked on this extraordinary adventure of

attempting tropical fruit trees improvement using mutation induction and other biotechnologies.

Tropical fruit trees are important multi-purpose crop species which not only supplement but also improve the quality of daily food intakes to the small farm holders while also providing fodder, fuel, timber and even medicine. It appears that the major constraint to the effective use of these fruit crops is access to improved varieties. The projects developed under this CRP will focus on a group of species with relevance to the country and/or probable general impact to other parts of the world namely: Mango, Guava, Tangerine Pummelo, Shamouti orange, Satsuma, Avocado, Papaya, Litchi varieties, Pitanga and Jaboticaba. The objectives of the CRP were stated as follow:

- To generate and characterize radiation induced and natural genetic diversity in tropical and subtropical fruit trees for improving nutrition balance, food security, and enhancing economic status of growers in Member States.
- To overcome major constraints in plant regeneration by tissue culture for large-scale multiplication of desirable induced mutants in order to sustain natural and induced fruit tree biodiversity leading to an improved economic viability of growers and nutrition component of their diets.
- To assess the impact of induced mutants on fruit yield and quality components, depending on the fruit tree life cycle, under the field conditions.
- To assess the root stocks of induced mutants, especially those tolerant to abiotic and biotic stresses, for grafting and their impact on yield.

All agronomically important mutants isolated from these collections will be supplied to Members Sates and further on to the farmers and assist Member States address issues like food security together with nutritional balance and enhanced economic status and livelihood for their populations.

Thus, the papers presented in this book highlight increasing cross-cutting techniques through the use of plant tissue culture, irradiation-induced mutation, molecular markers technology, isolation and characterization of mutants. The compilation of this volume has demanded an active participation of a number of tropical fruit tree specialists. The formidable task of correspondence with authors of the chapters in this volume and the splendid cooperation among participants in the preparation of the manuscripts have led to very extensive and well documented chapters in accordance with the guidelines provided by IAEA publications. As editors, we would like to acknowledge our thanks to P. Lagoda for discussions, suggestions and critical reading of the manuscript.

The book is divided in three parts; the first chapter will describe the study and results obtained while using mutation induction together with plant tissue culture and molecular markers to identify new mutants in mango, citrus, and avocado trees for disease resistance and fruit quality.

The second chapter reports on research conducted for the improvement of some exotic fruit trees which may be less known but nevertheless are of great socio-economic value in numerous developing countries; papaya, guava, several litchi varieties, carambola, cherimoya, pitanga and jaboticaba.

In the third chapter a few innovative protocols and/or technologies are presented, which describe new approaches for improving and genetically characterizing fruit tree genotypes and/ or mutant lines.

All manuscripts were reviewed by the competent reviewers and revised accordingly.

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CHAPTER I

In vitro induced mutation techniques applied to improve some major tropical fruit trees

Recovery of mango plants with antrachnose resistance following mutation induction and selection *in vitro* with the culture filtrate of *Colletotrichum gloesporoides* Penz

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Abstract. Embryogenic mango cultures of three cultivars on semi solid medium were irradiated at 100 Gy: monoembryonic 'Tommy Atkins' and 'Keitt' and polyembryonic 'Hindi be Sennara'. Two weeks after irradiation, cultures were inoculated into liquid maintenance medium containing 10% (v/v) culture filtrate of *Colletotrichum gloeosporioides* Penz. Following two weeks of exposure to culture filtrate, the embryogenic cultures were sub-cultured onto semi solid maintenance medium. Living pro-embryonic masses were manually separated from necrotic tissue four weeks later and were transferred onto semi solid maintenance medium. Somatic embryos were recovered and their shoots have been rescued by *ex vitro* grafting. Field plantings were established in early 2005; however, the results are inconclusive at the time of writing.

1. Introduction

The mango (*Mangifera indica* L.) is one of the best fruit crops of the world and its annual production is exceeded only by *Musa* (banana and plantain), citrus, grape and apple [1]. It is the most important fruit crop of Asia. The species is highly heterogeneous and is a polyploid (2n=4x=40). The primary breeding objectives for this crop species include: 1) tree size and shape; 2) regular bearing; 3) fruit quality; and 4) resistance to various diseases, but particularly anthracnose.

Anthracnose, caused by the pathogen *Colletotrichum gloeosporioides* Penz, is considered to be the major production problem of mango in the humid subtropics and tropics [2]. The disease affects flowers, leaves and fruit, and only limited resistance has been recognized within the species. Certain of the so-called "Florida" cultivars have been demonstrated to have moderate levels of resistance to anthracnose; however, in many growing areas, the resistance appears to be minimal. Current control measures include weekly applications of systemic fungicides from the time of flowering until harvesting, and increasingly this practice has been regarded as unsustainable. Infections on immature fruit are generally quiescent, but as the fruit ripen, disease lesions enlarge rapidly, resulting in substantial spoilage in the field, en route to markets and in the marketplace. It has been estimated that in many mango-producing countries more than 50% of fruit is lost. Anthracnose is therefore considered to be the major post harvest problem of mango fruit.

Conventional plant breeding has had relatively little impact on mango improvement for many reasons: a long juvenile period (c 7 years), consumer resistance to change, polyembryony and polyploidy, the very low frequency of fruit set following controlled pollinations etc. [3]. Most mango cultivars are derived from dooryard seedling selections, and their superior qualities have been maintained by vegetative propagation. In this manner, the unique gene combinations that confer superior phenotypes have been maintained. Polyembryonic mangoes, which are considered to have evolved under tropical conditions in Southeast Asia, are seed-propagated; whereas, the monoembryonic mangoes, which originated in the subtropics, are generally propagated by grafting onto seedling rootstocks. It is believed that

the most valuable monoembryonic mango selections of India are derived from selections made in the 16th century and were vegetatively propagated since that time.

Modern breeding mango programmes in Israel, India, South Africa, Brazil and Australia have released a few cultivars. However, with the exception of Malika, none of these has found acceptance in the marketplace due to strong consumer resistance. Various biotechnologies are beginning to be adapted to mango to address the most important breeding goals, e.g. gene mapping and marker assisted selection [4], genetic transformation [5] and *in vitro* mutation induction and selection.

The genetic engineering strategies would enable the alteration of an existing cultivar for one (or more) horticultural trait, thereby assuring the genetic integrity of the cultivar. The application of somatic cell genetics to vegetatively propagated tree species is dependent on the ability to regenerate plants from single cells of elite mature phase selections. Somatic embryogenesis of mango has been described [4, 6, 7]. The procedure involves the induction of embryogenic cultures from the nucellus, an integument of maternal origin within immature ovules and seeds. The procedure involves the recovery of embryogenic cultures from the explanted nucellus on defined medium [8]. Conditions for optimizing maintenance [8, 9], control of maturation of somatic embryos from embryogenic cultures [4, 10, 11, 12, 13] and germination of somatic embryos have been described.

Culture filtrates produced by pathogenic fungi and bacteria can be utilized not only to select for resistance to the pathogen *in vitro* [14], but also to induce the host resistance response. Litz *et al.,* [15] observed that *C. gloeosporiodes* culture filtrate can be used as a selective agent with mango suspension cultures in maintenance medium formulation. Somatic embryos were recovered from embryogenic cells and proembryonic masses (PEMs) that survived exposure to the culture filtrate, and regenerants appeared to show resistance to inoculation with the pathogen. Jayasankar *et al.,* [16] characterized the *in vitro* effects of *C. gloeosporiodes* phytotoxin (s) that had been purified according to established protocols [17, 18] and crude culture filtrate of *C. gloeosporiodes* on the mortality and growth of 'Hindi be Sennara' and 'Carabao' embryogenic cultures. The LD50 values were established for the effect of culture filtrate and phytotoxin on embryogenic cultures and the growth curves of challenged cultures. Using the same mango cultivars [19], embryogenic cultures were 1) exposed continuously for 4 cycles of challenge/selection/re-growth or 2) challenged for one, two, three and four complete cycles with the purified and partially purified culture filtrate of *C. gloeosporiodes*. At the end of each cycle, surviving embryogenic cultures were cloned and either re-challenged or sub-cultured onto somatic embryo maturation medium.

Three successive challenges with either crude filtrate or purified phytotoxin caused the expression of anti fungal genes *in vitro*, which was measured by co-culturing challenged material with a virulent strain of the pathogen [19]. Co-culture of the pathogen with resistant cultures resulted in the suppression of fungal growth, and the anti-fungal properties of the embryogenic cultures increased with each cycle of challenge and selection. Enhanced production of extra cellular chitinase and β-1,3-glucanase from selected, anti fungal cultures were observed. A newly expressed chitinase isozyme was observed at 45 kDa with polyembryonic 'Hindi be Sennara' and at 25 kDa with polyembryonic 'Carabao', relative to the controls. There was stable expression of the anti-fungal nature of resistant lines in suspension and in somatic embryos for >2 years after selection. Several RAPD markers were associated with selected anti-fungal cultures [20]. There was no variation in RAPD markers of the unchallenged controls with respect to the parent trees, demonstrating that exposure to either

the phytotoxin or culture filtrate is essential for anti-fungal expression. These results demonstrated that embryogenic cultures are genetically stable, and the induced variation does not result from somaclonal variation. Furthermore, the phytotoxins themselves are highly mutagenic.

Because relatively long term exposure of embryogenic cultures to *C. gloeosporioides* culture filtrate resulted in substantial genetic variation as indicated by extensive changes in RAPD banding patterns, embryogenic cultures were exposed to ionizing irradiation in the current study, which was followed by a single challenge with culture filtrate. In this way, exposure to culture filtrate was minimized. Plants were regenerated from embryogenic cultures that had been selected for resistance to the culture filtrate.

2. Materials and methods

2.1. Establishment of embryogenic cultures

Embryogenic cultures were induced from the nucellus of immature mango seeds ca. 40 days after pollination. The nucellus was excised from surface-disinfected 'Hindi be Sennara' (polyembryonic), 'Keitt' (monoembryonic) and 'Tommy Atkins' (monoembryonic) fruit under sterile conditions, and explanted onto induction medium in sterile plastic disposable Petri dishes. Induction medium was composed of B5 [21] major salts [without $(NH_4)_2SO_4$], MS [22] minor salts and organic components, 400 mg/L glutamine, 60 g/L sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 g/L gellan gum. Cultures were incubated in darkness at 25^{o}C.

Embryogenic cultures were maintained as suspension cultures in liquid induction medium in 125 ml Erlenmeyer flasks (40 ml medium per flask) on a rotary shaker (110 rpm), and sub-cultured into fresh medium at 2-week intervals. Cultures were incubated in semi darkness at 25^{o}C.

2.2. Irradiation of embryogenic cultures and determination of LD50

Embryogenic cultures consisting of PEMs were plated on semi solid maintenance medium. Each Petri dish was inoculated with 6 clumped PEMs, each of which formed 1 cm diameter cluster. After one week, the cultures were exposed to different levels of γ irradiation from a ^{60}Cobalt source: 0, 50, 100, 150 and 200 Gy. There were six plates for each treatment and the control.

After one month, the LD50 was determined by counting the number of living (white) and dead (brown) PEMs in each cluster and determining the percentage survival.

2.3. In vitro selection with C. gloeosporioides culture filtrate

Liquid maintenance medium was prepared with 10% (v/v) culture filtrate of *C. gloeosporioides* that had been grown for 2 weeks in BBB/AAA medium. This level was chosen based upon earlier studies with this pathogen. Two weeks after irradiation at 100 Gy approximately 200 mg of PEMs were inoculated into 40 ml medium containing *C. gloeosporioides* culture filtrate in 250 ml Erlenmeyer flasks: 'Tommy Atkins', 'Hindi be Sennara' and 'Keitt'. The flasks were maintained at 110 rpm for one week at 25^{o}C in semi darkness. At the end of this period, the cultures were decanted into a sterile Petri dish, and

observed under a dissecting microscope. Living (white) PEMs were aseptically removed with a sterile spatula and were transferred onto semi solid maintenance medium (without culture filtrate). Somatic embryo development was initiated after approximately 3 months.

2.4. Somatic embryo development

Embryogenic cultures were transferred to Petri dishes containing semi solid medium consisting of B5 major salts (complete), MS minors salts and organic components, 1 mg/L benzyl adenine (BA), 400 mg/L glutamine, 60 mg/L sucrose and 6 mg/L gellan gum. Following the appearance of heart stage somatic embryos, they were transferred onto similar medium (without BA) containing 40 mg/L sucrose, 20% (v/v) filter-sterilized coconut water, 400 mg/L glutamine and 2 g/L gellan gum. Cultures were maintained in darkness at 25^{o}C.

2.5. Plant regeneration

Under conditions described above, somatic embryos developed to maturity and germinated. Although various treatments have been discussed for enhancing the recovery of plants from somatic embryos, the conversion rate has been low. Recently, it has been possible to effectively rescue all shoots that emerge from somatic embryos of avocado either by micro-grafting or by *ex vitro* grafting [23]. The latter technique (cleft grafting) is more successful, and is being utilized to rescue all plants that develop from mango somatic embryos.

Mango seeds of monoembryonic ‘Haden’ and ‘Tommy Atkins’ were germinated in potting mixture in the greenhouse. A V-shaped cleft was made on the stem of each seedling. Shoots ca 0.5 cm long were excised from plantlets derived from somatic embryos, and the base of each shoot was trimmed to fit the V-shaped cleft in the rootstock. The scion was inserted into the V-shaped cleft, and secured with Parafilm. Graft unions were generally secure after 3 weeks.

3. Results

3.1. Embryogenic responses of mango cultivars

3.1.1. Embryogenic suspension cultures

Embryogenic cultures of ‘Hindi be Sennara’, ‘Keitt’ and ‘Tommy Atkins’ in suspension had similar morphologies, and consisted of PEMs and small pro-embryonic cell aggregates. Suspension cultures of ‘Keitt’ on the other hand, consisted entirely of pro-embryonic cells and small aggregates of proembryonic cells. ‘The growth curves of embryogenic suspension cultures of ‘Tommy Atkins’ and ‘Hindi be Sennara’ were comparable. The growth curves of ‘Hindi be Sennara’ and ‘Keitt’ indicated that these cultures proliferated more rapidly than the other cultivars. Although ‘Hindi be Sennara’ suspension culture growth appeared to be more rapid than ‘Keitt’, the difference was not significant.

3.1.2. Somatic embryo development

Early cotyledonary somatic embryos of ‘Hindi be Sennara’ and ‘Tommy Atkins’ developed either in liquid or solid maturation medium; however, ‘Keitt’ responded differently. Cotyledonary somatic embryos of ‘Keitt’ were unable to develop in liquid maturation

medium, and embryogenic cultures continued to proliferate rapidly on semi solid maturation medium, even in the absence of 2,4-D. Cotyledonary 'Keitt' somatic embryos generally could not develop to maturity due to hyperhydricity and browning of the cultures. 'Keitt' was considered unsuited for subsequent manipulations because 1) it was difficult to screen living from necrotic tissue; and 2) the frequency of recovery of mature somatic embryos was very low.

3.1.3. Determination of the LD50

The response of embryogenic mango cultures to irradiation was cultivar-specific, and appeared to be related to the growth responses of embryogenic suspension cultures. The LD50 for 'Tommy Atkins' and was approximately 100 Gy; whereas, the LD50 for 'Keitt' was approximately 130 Gy. The growth curves of suspension cultures of 'Hindi be Sennara' and 'Tommy Atkins' were similar; however, the LD50 of the two cultivars were different. It was impossible to determine the LD50 for 'Hindi be Sennara' within the dose range of 0-200 Gy. The LD50 for 'Keitt' was approximately 130 Gy. An explanation for the 'Hindi be Sennara' response is not certain; however, the rate of proliferation of 'Hindi be Sennara' embryogenic suspension cultures was somewhat greater than the other cultivars, and this may have masked the response to irradiation. For practical purposes, we adopted the same irradiation dose for each cultivar, i.e., 100 Gy.

3.1.4. Regeneration of plants from irradiated embryogenic cultures

We made no attempt to regenerate 'Keitt' from irradiated embryogenic cultures due to the deficiencies of its *in vitro* responses (see section 4.1.2.). With respect to 'Hindi be Sennara' and 'Tommy Atkins', only the best quality somatic embryos were selected for maturation and germination. These somatic embryos were defined as being bipolar and having a hard white, opaque appearance. The frequency of appearance of somatic embryos of this type has ranged from 10 to 30% of the total number of somatic embryo regenerated, and this appears to be cultivar-specific: 30% of 'Hindi be Sennara' and 10% for 'Tommy Atkins' somatic embryos. There was no difference in recovery of good quality somatic embryos between the irradiation treatment and the control for each cultivar. Virtually all of the selected good quality somatic embryo germinated and formed plantlets *in vitro*; however, the survival of these plantlets in soil has been poor. *Ex vitro* cleft grafting of somatic embryo-derived shoots has enabled the rescue of all *in vitro* regenerants (Figure 1).

Plants derived from irradiated cultures of each of 'Hindi be Sennara' and 'Tommy Atkins' have been established *ex vitro*. Approximately, the same number of control plants has been established. All of this material was transferred in a greenhouse at the time of writing.

The goal of this study is to identify resistance to anthracnose, which affects fruits, flowers, leaves and stems. However, we have expanded the objective of this study, and are interested in the effects of irradiation and selection on root traits as well. Since the rescue of regenerants is dependent on *ex vitro* grafting, the root system is of a different genetic background. Therefore, we have begun to air layer the scions in order to retrieve self-rooted plants.

Self-rooted plants begun to be transferred to the field in April-May, 2005. Thereafter, were be monitored regularly for anthracnose symptoms on foliage. No challenge inoculate was applied to the test plants because different tissues and different stages of development are generally most susceptible to specific strains of the pathogen. We did not anticipate that flowering will

occur until the end of the second year. Thereafter, monitoring included symptom expression on flowers and fruit. Differences in growth rate of individual lines of different cultivars were also monitored.

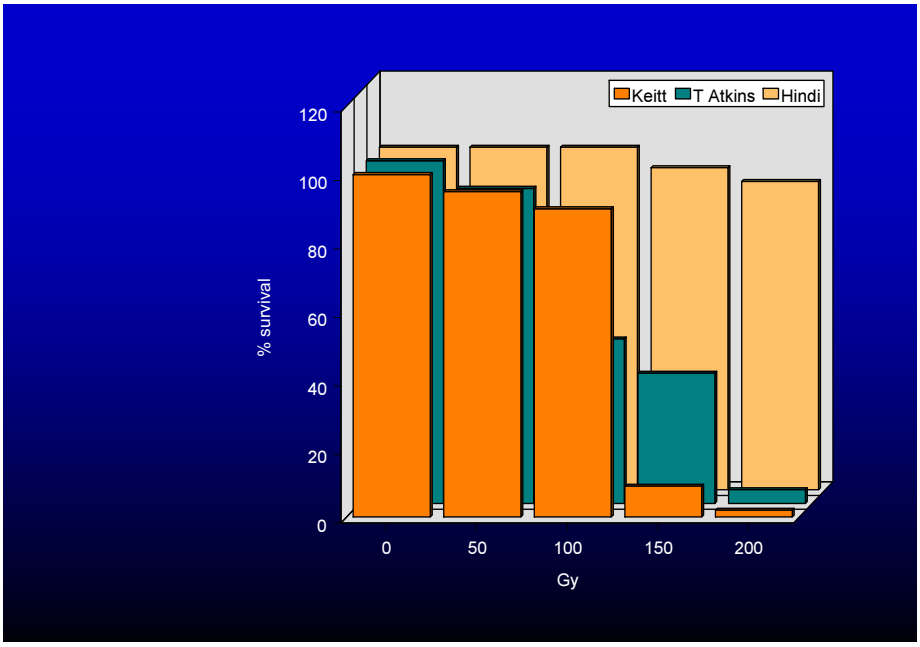

Figure 1. Survival of 'Keitt', 'Tommy Atkins' and 'Hindi' mango embryogenic cultures following irradiation at 0, 50, 100, 150 and 200 Gray.

4. Discussion and conclusions

Since improvement of perennial fruit tree species by conventional means is difficult, a common strategy has been replacement of an outstanding cultivar with a similar but less satisfactory selection. Genetic engineering strategies have great potential for improving this heterogeneous group of plants, because they can alter one or more traits without otherwise changing the cultivar. Although mutation induction of embryogenic cultures followed by imposition of selection for a specific trait theoretically is as efficient as genetic transformation, this strategy must rely upon a selection agent whose action at the cell level is equal to its action at the whole plant level. At this time, there are few efficacious agents.

In vitro selection of plants for resistance to a disease such as anthracnose theoretically is possible [14, 16, 19, 20]. On the other hand, recent studies with grapevine have demonstrated that anthracnose-resistant selections that were selected *in vitro* lose resistance in the field after a few years (personal communication, Dennis J. Gray, University of Florida, MFREC, Apopka). Therefore, vigorous screening and assessment in the field over a few years is essential.

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Radiosensitivity and *in vitro* studies of *Citrus suhuiensis*

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Abstract. Radiosensitivity tests and *in vitro* studies were carried out on two varieties of *Citrus suhuiensis*, 'limau madu' and 'limau langkat'. Fresh seeds were desiccated for periods of 1, 2, 3 and 4 days and irradiated with gamma ray doses of 50, 100, 150, 200 and 250Gy. Shoot tips were also irradiated with gamma ray doses of 5, 10, 15, 20 and 25Gy. Results showed that lower moisture content seeds were more resistant to irradiation dose. It was determined that the LD50 of the 'limau madu' seeds was 200Gy at 25.48% moisture content, while the LD50 for 'limau langkat' seeds was 50Gy at 44.97% moisture content. A test on the DNA content of irradiated and non-irradiated samples using flow cytometry methods proved that irradiation had affected the DNA content (C-value) of the plant cells. Generally the 2C DNA content (pg) increases with increasing dose of radiation. Though low doses of radiation (50-100 Gy) resulted in a prominent effect on 2C DNA content, a few plants showed another 4C DNA peak with various seed desiccation levels. In inducing multiple shoots from apical shoot tips, it was found that MS medium with the addition of 2.5mgL^{-1} BAP was the optimal medium, where one explant was able to produce a mean of 13.5 adventitious shoots. Further results showed that MS medium with the addition of 0.6mgL^{-1} GA_3 was the best medium for shoot elongation. In inducing root formation, 0.2mgL^{-1} NAA in MS medium proved to be the best medium. MT medium with the addition of growth regulators at different concentrations were tested on mature and immature embryos for the production of somatic embryos. It was found that immature embryos cultured on MT medium with 500mgL^{-1} malt extract, 0.5- 1.0mgL^{-1} 2, 4-D and 0.5 1.0mgL^{-1} BAP could produce proembryonic callus, which progressed to develop viable somatic embryos.

1. Introduction

Mutation induction has a high potential for bringing about genetic changes and improvements [1] and has contributed considerably to plant breeding. The number of mutant varieties officially released and recorded in the FAO/IAEA Mutant Varieties Database before the end of 2000 was 2252 among which many fruit trees [2]. Examples include mutants such as russet-free fruit in apple, seedless *Citrus*, disease resistance in Japanese pear and compactness in sweet cherry [3].

The responsiveness of cells to physical and chemical mutations is influenced, to a varying degree, by numerous biological, environmental and chemical factors. These factors modify the effectiveness of mutagens in cells. Moisture content was shown to be one of these factors. Low moisture content seeds have been shown to improve the tolerance of the seeds to radiation [4]. The effects of gamma irradiation on *Citrus* seeds have been previously studied, particularly in Shamouti orange (*Citrus sinensis*) seeds [5] and Satsuma (*Citrus unshiu*) [6]. A radiation dose of 0-120Gy was used on Shamouti, while 0-100Gy was used on Satsuma.

In vitro culture techniques offer a new breeding strategy that may lead to new desirable varieties [7, 8, 9]. *In vitro* techniques also have an enormous potential of broadening the scope

of mutation breeding by accelerating the process and thus leading to more economical outputs [10]. Spiegel-Roy and Kochba [11] used *in vitro* ovular callus of *Citrus sinensis* in their experiment with irradiation by gamma rays of 8-16kR. Other scientists who have used *in vitro* derived plant materials include De Guzman [12]; *in vitro* shoot tips of *Musa* spp. and Broertjes [13]; *in vitro* leaflet blades of *Solanum tuberosum* explants.

In *Citrus* tissue cultures, somatic embryogenesis was induced using MT medium [14] enriched with growth regulators at specific concentrations. Growth regulators commonly used include auxin, cytokinin, gibberellic acid and abscissic acid. Besides these, malt extract and casein hydrolysate are also widely used to induce somatic embryogenesis in *Citrus* cultures as well as coconut milk [15]. A few examples of research on *Citrus* somatic embryogenesis are shown by Bulton and Kochba [16], Speigel Roy and Vardi [17], Tisserat *et al.,* [18] and Wann [19].

The aim of our study was to determine the moisture content and radiosensitivity of *C. suhuiensis* seeds towards gamma radiation, as a first step in *Citrus* mutation breeding programme. The radiosensitivity of *in vitro* shoot tips was tested, too. The study also involved multiple shoot induction and somatic embryogenesis development. The DNA content of plants derived from irradiated seeds was also determined, as this may be an indicator for chromosomal aberrations.

2. Materials and methods

2.1. Radiosensitivity

2.1.1. Seed irradiation

Experiments were carried out to determine the effect of seed moisture content on the radiosensitivity of *C. suhuiensis* using two different varieties, 'limau madu' and 'limau langkat', collected from field-grown trees in orchards in Jerangau, Terengganu Provinces. Fresh seeds were desiccated in an air-conditioned room (22± 1°C and 67± 2% RH) for the periods of 1, 2, 3 and 4 days. After each desiccation period, seeds were irradiated with doses of 50, 100, 150, 200 and 250Gy. Both desiccation durations and irradiation dosages were based on preliminary tests implemented earlier on *Citrus* seeds [4, 5, 6, 7]. Fresh non-irradiated seeds were used as controls. Treated and control seeds were germinated in sand filled containers in the green house. Plant height and other morphological characteristics of the seedlings such as leaf shape and colour of the leaves were recorded. All irradiation work was performed at the Malaysian Institute of Nuclear Technology Research (MINT) in Dengkil, Selangor. All samples were irradiated with ^{60}Co at 17.7 Gy per minute.

2.1.2. In vitro derived shoot tips irradiation

For the shoot tip irradiation experiment, adventitious shoots of *C. suhuiensis* cv limau madu generated *in vitro* were used. The shoot tips were irradiated with gamma rays of 5, 10, 15, 20, and 25Gy doses. This range of doses was selected based on *in vitro* shoot irradiation done by several other authors [20, 21, 22, 23]. Irradiated shoot tips were then transferred to basal a Murashige and Skoog [24] as regeneration medium. Different parameters such as survival rates, regeneration capabilities, growth rate and morphological characteristics were observed and recorded.

2.2. *In vitro* micropropagation

Mature seeds of 'limau madu' and 'limau langkat' were surface sterilized by rinsing in 80% alcohol for 2 minutes followed by a treatment with a 20% Clorox solution (5.25% sodium hypochlorite) to which were added a few drops of Tween 20 placed on a rotary shaker for 20 minutes. Seeds were then finally rinsed with sterile distilled water 3 to 4 times and plated in a sterile Petri dish lined with filter paper. The test of the sterilized seeds was removed using forceps and seeds were then cultured on MS [24] basal medium.

2.2.1. Multiple shoots induction

Shoot tips obtained from *in vitro* germinated seedlings were used for the induction of multiple shoots. Various concentrations of Benzylaminopurine (BAP) were tested in order to obtain the optimal growth medium concentration.

Shoot tips were cultured on the following media:

a. MS medium with an addition of:
   2.5, 3.0, 3.5, 4.0 and 4.5 mg l^{-1} BAP
b. MS medium without plant growth regulators was used as control

2.2.2. Shoot elongation

Research on shoot elongation was carried out using adventitious shoots induced from *in vitro* shoots tips (2.2.1). Various concentrations of Gibberrelic acid (GA_3) were tested to obtain the optimal growth medium concentration required for the elongation of irradiated shoot tips.

Adventitious shoots of 'limau madu' were cultured on the following media:

(a) MS medium with an addition of:
    0.2, 0.4, 0.6, 0.8 and 1.0mg/l GA_3
(b) MS medium without plant growth regulators was used as control

2.2.3. Root induction

An experiment on the induction of roots from adventitious shoots was carried out using various concentrations of α-naphthalene acetic acid (NAA). Shoots of 'limau madu' were cultured on the following media:

(a) MS medium with an addition of:
    0.2, 0.4, 0.6, 0.8 and 1.0mgL^{-1} NAA
(b) MS medium without plant growth regulators was used as control

Media used in these two experiments were based on previous *in vitro* experiments on related species [25, 26].

2.2.4. Somatic embryogenesis

Zygotic embryos were excised from surface sterilized immature and mature seeds using a stereo-microscope. Only the largest embryonic axes were dissected from the poly-embryonic seeds. Media used in these two experiments are based on previous *in vitro* experiments done on *Citrus* species [25, 26, 27, 28, 29, 30, 31].

Embryos were cultured on the following media:

(a) MT + 500mgL^{-1} malt extract (ME) with a combination of: 0.5mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP; 0.5mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP; 1.0mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP and 1.0mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP.
(b) MT + 500mgL^{-1} ME was used as the control

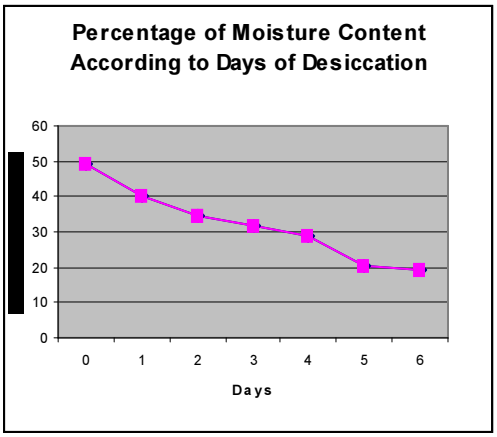
3. Results and discussion

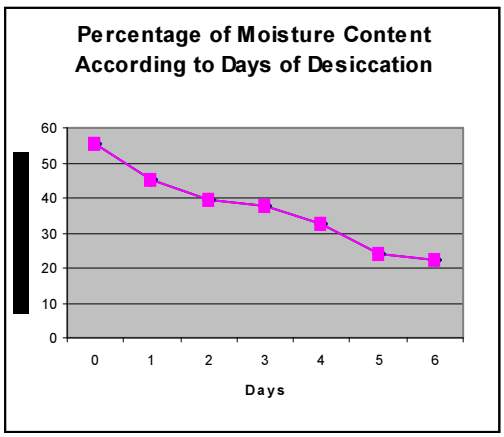
3.1. Radiosensitivity

3.1.1. Seed irradiation

Results presented in Figure 1 showed that after 6 days of desiccation the moisture content fell to 20% in both varieties.

If at higher water content (43.82%) the increase of irradiation dose resulted in a drastic reduction of the germination rates (down to 0%), at lower water content the increase of irradiation dose had a lesser effect. In fact, at 25.48% some seeds still germinated (20%). This implies that irradiation is, in fact, affected by the moisture content of the seeds (Table I). The differences in sensitivity due to moisture content and radiation dosage and their interactions were found to be statistically significant ($P<0.05$). While the controls started to germinate within the second week of planting, irradiated seeds only germinated after the third week. Seedlings produced by irradiated seeds were shorter compared to the controls (Table II). Differences in the height of shoots produced between irradiated and non irradiated seeds could be due to an effect of radiation on the growth of the shoots, or it could also be due to the late germination of the radiated seeds. The late germination of the irradiated seeds can also in itself be the effect of radiation, as the rate of germination decreases with the increase in radiation dosage. Some morphological differences in the leaves were also observed (Figure 2).





(a) (b)

Figure 1. Effects of desiccation on moisture content of seeds of (a) 'limau madu' and (b) 'limau langkat'.

Figure 2. Examples of morphological differences in leaf shape of seedlings from irradiated seeds.

TABLE I. EFFECT OF DESICCATION AND RADIATION ON GERMINATION PERCENTAGE OF 'LIMAU MADU' SEEDS

Percentage of Germination							
% Moisture content	Radiation dosage (Gy)						Mean
	0	50	100	150	200	250	
43.82	96	66	38	0	0	0	55.15[a]
29.32	91	50	32	19	13	7	55.52[a]
26.24	87	77	78	31	29	15	47.24[b]
25.64	85	89	92	69	31	18	35.63[c]
25.48	85	79	76	74	52	20	28.80[d]
Mean	72.73[a]	60.21[b]	54.00[c]	35.75[d]	26.52[e]	17.61[f]	

Note: Means with the same letter in the same row or column are not significantly different (Duncan's Multiple Range Test)

Table III shows the effect of irradiation on the germination percentage of 'limau langkat' seeds. In general, the effect on the germination is similar to that observed in the limau madu seeds. The 'limau langkat' seeds appeared to have a lower tolerance to irradiation, and this might be due to the higher moisture content in 'limau langkat' seeds. There is a significant difference in the percentage of germination among the seeds irradiated with the different doses, even though the different moisture contents of the seeds did not differ significantly.

As shown in Table IV there is a significant difference in the height of seedlings that germinated from seeds radiated with 150, 200 and 250 Gy, compared to the controls and seeds irradiated with low doses; 50 and 100Gy. The seedling height was significantly reduced with increasing irradiation dose.

It was determined that the LD50 of the 'limau madu' seeds was 200Gy at 25.48% moisture content, while the LD50 for 'limau langkat' seeds was 50Gy at 44.97% moisture content.

Although it may seem that lower moisture content would provide better results, it has been shown that the viability of citrus seeds of the same size (*C. reticulata*) significantly decreased at moisture contents of below 25% [20]. An earlier test [31] on these seeds showed that there was no survival at the dosage of 600Gy with germination percentage of 1.5 starting at the dose of 400Gy. As a conclusion the dose 400 Gy may be considered as the lethal for *Citrus suhuensis* seeds.

TABLE II. EFFECT OF DESICCATION AND RADIATION ON THE HEIGHT OF 'LIMAU MADU' SEEDLINGS

Mean Height (cm)							
% Moisture content	Radiation dosage (Gy)						Mean
	0	50	100	150	200	250	
43.82	8.70	10.68	10.05	0	0	0	9.47^{a}
29.32	9.75	8.90	9.45	10.38	9.75	7.35	8.76^{b}
26.24	10.08	10.83	8.80	6.88	7.68	7.88	8.69^{b}
25.64	10.30	10.43	10.53	9.30	6.88	5.15	9.26^{a}
25.48	10.85	9.85	10.70	10.20	9.3	7.80	4.90^{c}
Mean	9.94^{a}	10.14^{a}	9.91^{a}	7.35^{b}	6.34^{c}	5.64^{d}	

Note: Means with the same letter in the same row or column are not significantly different (Duncan's Multiple Range Test)

TABLE III. EFFECT OF DESICCATION AND RADIATION ON THE GERMINATION PERCENTAGE OF 'LIMAU LANGKAT' SEEDS

Percentage of Germination							
% Moisture content	Radiation dosage (Gy)						Mean
	0	50	100	150	200	250	
55.15	35	33	30	2	0	0	8.33^{c}
44.97	58	53	47	31	20	0	8.83^{c}
39.63	43	22	19	18	15	10	13.21^{b}
37.91	40	34	34	17	12	11	16.00^{a}
32.32	34	31	30	26	23	17	16.08^{a}
Mean	22.20^{a}	18.05^{b}	15.80^{c}	9.65^{d}	6.25^{e}	3.00^{f}	

Note: Means with the same letter in the same row or column are not significantly different (Duncan's Multiple Range Test)

Further observations were carried out on the shoot growth of 'limau madu' plants derived from irradiated seeds after about one year of age. All plants derived from desiccated seeds (2-3 days) and subjected to low-medium doses of radiation (50-150 Gy), seemed to have normal shoot growth in terms of plant height, number of branches, shape and colour of the leaves. Nonetheless, a few plants developed shorter internodes, curled leaves or necrotic spots on their leaves. Irrespective of the dose of radiation, 3 days of seed desiccation prior to irradiation yielded normal plants and the differences in plant height and number of branches were significant ($P \leq 0.05$) when compared with control plants, and those that received 2 days of desiccation. Seed desiccation for 4 days coupled with high dosage of irradiation (200-250 Gy) produced plants with short internodes (rosette-like) but with green normal leaves (Figure 3) except for one plant that showed curled leaves.

TABLE IV. EFFECT OF DESICCATION AND RADIATION ON THE HEIGHT OF 'LIMAU LANGKAT' SEEDLINGS

Mean Height (cm)							
% Moisture content	Radiation dosage (Gy)						Mean
	0	50	100	150	200	250	
55.15	6.8	7.5	9.0	7.5	0	0	9.47[a]
44.97	8.2	7.7	6.5	5.5	7.0	0	8.76[b]
39.63	7.0	5.8	7.2	6.6	7.5	6.8	8.69[b]
37.91	7.0	5.8	10.0	8.5	6.6	7.2	9.26[a]
32.32	7.5	7.5	8.5	7.6	8.0	5.6	4.90[c]
Mean	9.94[a]	10.14[a]	9.91[a]	7.35[b]	6.34[c]	5.64[d]	

Note: Means with the same letter in the same row or column are not significantly different (Duncan's Multiple Range Test)

3.1.2. In vitro shoot tips irradiation

For shoot tips irradiation, although the percentage of survival was high (58%) even at 25Gy (Table V), it was observed that most of the surviving shoots did not develop properly. Shoots were either stunted, or devoid of chlorophyll (i.e. albinos). However, the controls and shoots exposed to lower dose (5-10Gy) of gamma rays showed continuous development and growth. Mutagens have been known to delay the onset of cell divisions and slow down the growth of shoots [32 33]. Further investigation is needed for a clear understanding on the effects of gamma irradiation on *in vitro* shoot tips.

3.2. Citrus organogenesis in vitro

3.2.1. Multiple shoots induction

Results from Table VI show that shoot tips of 'limau madu' responded best when treated with 3.5mgL^{-1} BAP on MS medium. Shoots obtained from this experiment were later used for shoot tip irradiation and regeneration purposes.

BAP is a cytokinin that has been proven to stimulate the formation of adventitious buds [34, 35]. In our experiments it also appeared that an increase of BAP in the medium increased the number of adventitious shoot formed, but concentrations of BAP exceeding 3.5mg l^{-1} induced callus formation and thus decreased the number of adventitious shoots. The response of explants to BAP is in agreement with earlier studies on multiple shoot induction from various explants and species [25, 36, 37, 38, 39]. Although the MS medium with 3.5mg l^{-1} BAP allowed the development of most adventitious shoot from a single explant, it was difficult to isolate the shoots as they were compact and almost joined together. This made the task of isolating the individual shoots (as how they would be used in irradiation) more tedious.

Therefore, it was decided that MS medium with 2.5mg l^{-1} BAP was the optimum medium to use to obtain suitable shoots for mutation breeding purposes.

TABLE V. SURVIVAL RATE OF SHOOT TIPS OF CITRUS SUHUIENSIS CV 'LIMAU MADU' AFTER IRRADIATION

Treatment (Gy)	Mean% of survival
0	100
5	77.5
10	77.25
15	67.75
20	66.25
25	58.0

TABLE VI. MEAN NUMBER OF 'LIMAU MADU' MULTIPLE SHOOTS PRODUCED BY SHOOT TIPS CULTURED ON MS MEDIUM SUPPLEMENTED WITH VARIOUS CONCENTRATIONS OF BAP

Treatment	Mean no. of shoots
MS	1.00^{e}
MS + 2.5mgL^{-1} BAP	13.50^{c}
MS + 3.0mgL^{-1} BAP	22.50^{b}
MS + 3.5mgL^{-1} BAP	26.25^{a}
MS + 4.0mgL^{-1} BAP	9.50^{d}
MS + 4.5mgL^{-1} BAP	3.50^{e}

Note: Means with the same letter are not significantly different (Duncan's Multiple Range Test).

3.2.2. Shoot elongation

There is an increase in shoot elongation with the increase in GA_3 concentration (Table VII) although there is no significant difference in the results of shoot elongation in medium with 0.4, 0.6, 0.8 and 1.0mg l^{-1} GA_3. This result is similar to that of an earlier experiment conducted with Carrizo citrange, where the addition of 1.0mg l^{-1} GA_3 to MS medium enhanced stem elongation of the shoots [25]. The mean value however shows that 0.6mg l^{-1} is the best concentration of GA_3 for shoot elongation in 'limau madu'.

Other than shoot elongation, shoots cultured on MS medium with the addition of 0.2mg l^{-1} GA_3 also showed root formation, as previously described in other studies [40, 41, 42].

TABLE VII. MEAN ELONGATION OF 'LIMAU MADU' SHOOTS ON MS WITH GA_3 MEDIUM (FOUR WEEKS IN CULTURE)

GA_3 (mgL^{-1})	Mean elongation of shoot (cm)
0	0.21^a
0.2	0.26^a
0.4	0.53^b
0.6	0.69^b
0.8	0.64^b
1.0	0.51^b

Note: Means with the same letter in the same column are not significantly different (Duncan's Multiple Range Test).

3.2.3. Root induction

Among the concentrations of NAA tested, it was found that the highest percentage of explants that formed roots were cultured in MS medium with 0.2 mg l^{-1} NAA (Table VIII). Although not all explants formed roots, almost all explants had some swelling at the base of the shoots.

TABLE VIII. EFFECT OF NAA INDUCING ROOT FROM 'LIMAU MADU' SHOOTS

NAA_3 (mg l^{-1})	Percentage explants that produced roots
0	0.0^d
0.2	77.5^a
0.4	57.5^b
0.6	60.0^b
0.8	30.0^c
1.0	10.0^d

Note: Means with the same letter in the same column are not significantly different (Duncan's Multiple Range Test).

3.2.4. Somatic embryogenesis

In general, most of the mature embryos of 'limau madu' cultured produced callus except for those cultured on MT [14] plus ME media with a combination of 0.5mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP (Table IX). For limau langkat, lower callus formation was observed (Table X). The formation of callus was seen as early as the first week. The callus produced in the 'limau langkat' and 'limau madu' cultures were creamy white in colour and had a friable texture. After two subcultures for about four weeks all calli formed did not seem to develop further. The proliferation stopped and the calli turned brownish in colour and dry.

There was no change in the appearance of the cultures even after transfer to basal MT medium. It was also observed that the calli had turned quite hard and were clumped together, unlike the previous friable states. This observation was seen both in 'limau madu' and 'limau langkat' cultures.

TABLE IX. PERCENTAGE OF 'LIMAU MADU' EMBRYOS THAT PRODUCED CALLUS

Treatment	% of embryos that formed callus
MT + ME	0^{b}
MT + ME + 0.5mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	56.25^{a}
MT + ME + 0.5mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	6.25^{b}
MT + ME + 1.0mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	68.75^{a}
MT + ME + 1.0mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	62.50^{a}

Note: Means with the same letter are not significantly different (Duncan's Multiple Range Test)

TABLE X. PERCENTAGE OF 'LIMAU LANGKAT' EMBRYOS THAT PRODUCED CALLUS

Treatment	% of embryos that formed callus
MT + ME	0^{a}
MT + ME + 0.5mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	31.25^{ab}
MT + ME + 0.5mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	6.25^{b}
MT + ME + 1.0mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	43.75^{a}
MT + ME + 1.0mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	6.25^{b}

Means with the same letter are not significantly different (Duncan's Multiple Range Test)

In cultures where there was no callus growth, the embryos germinated into plantlets. These embryos germinating on a medium with 1.0mg l^{-1} of 2, 4–D tended to develop long roots and stunted shoots, while embryos germinating on media with 1.0mg l^{-1} of BAP tended to have well-developed shoots and stunted roots. However, embryos with the equal combination of BAP and 2, 4 – D germinated into normal plantlets.

When immature embryos were cultured on MT plus ME media with combination of 2,4 – D and BAP, almost all the embryos cultured formed pro-embryonic masses with the exception of those cultured on basal medium (Table XI, Table XII). Pro-embryogenic masses proliferation was quite slow, as it was only seen on the second week of culturing. After eight weeks of culture globular embryos were observed (Figure 4A).

TABLE XI. PERCENTAGE OF 'LIMAU MADU' IMMATURE EMBRYOS THAT PRODUCED PRO-EMBRYONIC MASSES

Treatment	% of embryos that formed pro-embryonic masses
MT + ME	0.00^{b}
MT + ME + 0.5mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	31.25^{a}
MT + ME + 0.5mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	12.50^{b}
MT + ME + 1.0mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	18.75^{a}
MT + ME + 1.0mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	20.83^{a}

Note: Means with the same letter are not significantly different (Duncan's Multiple Range Test)

TABLE XII. PERCENTAGE OF 'LIMAU LANGKAT' IMMATURE EMBRYOS THAT PRODUCED PROEMBRYONIC MASSES

Treatment	% of embryos that formed proembryonic masses
MT + ME	0^{b}
MT + ME + 0.5mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	56.25^{a}
MT + ME + 0.5mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	6.25^{b}
MT + ME + 1.0mgL^{-1} 2,4-D + 0.5mgL^{-1} BAP	68.75^{a}
MT + ME + 1.0mgL^{-1} 2,4-D + 1.0mgL^{-1} BAP	62.50^{a}

Note: Means with the same letter are not significantly different (Duncan's Multiple Range Test)

After this stage, the embryos started to develop quite rapidly. Within two weeks the embryos had changed from globular to heart (Figure 4B) and torpedo shape. At this stage embryos were isolated and cultured on MS basal medium for further development (Figure 4C). After a week of culture embryos started to produce roots and also showed signs of shoot formation (Figure 4D).

In an attempt to determine the appropriate stage for embryo transfer to growth regulator free medium, cultures of different stages (globular, heart and torpedo) were transferred to basal MT medium and regular observations performed. Results showed that the globular and heart shaped embryos did not develop further and the globular embryos turned brownish in color with loss of moisture. However, the torpedo shaped embryos started to develop further to formed root as well as shoots upon transferred to the basal medium.

(A) *(B)*

Figure 3. Seedling from irradiated seed (A) and control (B).

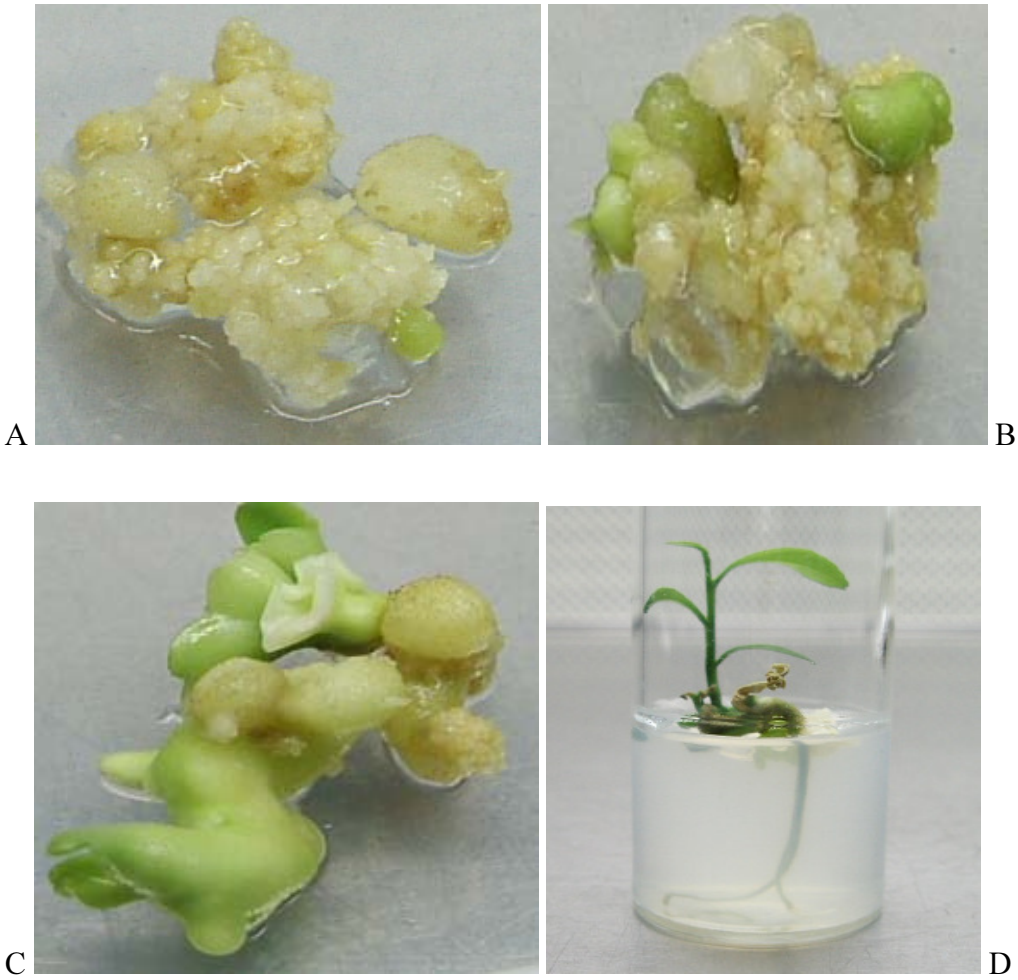


Figure 4. Formation of somatic embryos from immature embryos of 'limau madu'.

4. Conclusion

Desiccated seeds showed a higher percentage of germination after irradiation, compared to seeds that were not desiccated. Seeds with a longer period of desiccation also showed better results. This confirms that moisture content plays a role the efficiency radiation and therefore on the sensitivity of the seeds towards radiation. Seeds exposed to radiation up to 250Gy germinated, even though at a comparatively low rate (20%). It was determined that the LD50 of the 'limau madu' seeds was 200Gy at 25.48% moisture content, while the LD50 for 'limau langkat' seeds was 50Gy at 44.97% moisture content. Differences in the height of shoots produced from irradiated and non radiated seeds were observed and this could be an effect of radiation on the growth of the shoots, or alternatively it could also be due to the late germination of the irradiated seeds. Multiple shoot formation was obtained on MS medium supplemented with 2.5 mg/L BAP and shoot elongation and rooting were successfully obtained. Somatic embryogenesis was observed when immature embryos were used as explants and MT medium was used together with 500mgL^{-1} malt extract, 0.5- 1.0mgL^{-1} 2, 4-D and 0.5 –1.0mgL^{-1} BAP.

The project has shown the potential of using induced mutation on seeds and *in vitro* shoot tips for the improvement of *Citrus suhuiensis*. The *in vitro* techniques for the establishment of

culture materials through adventitious/ multiple shoots and somatic embryogenesis have been developed.

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Development of seedless fruits mutants in citrus including tangerine (*C. reticulata*) and pummelo (*C. grandis*) through induced mutations and biotechnology

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Abstract. The development of seedless fruit mutants in citrus, including Tangerine (*C. reticulata*) and Pummelo (*C. grandis*), through induced mutation and biotechnology was studied at the Gamma Irradiation Service and Nuclear Technology Center, Pichit and Phare Horticultural Research Center for 4 years (August 2000 to September 2004). The results showed successful induction of mutants with gamma irradiation using both chronic and acute procedures for pot plants, scions and *in vitro* plantlets of tangerine (*Citrus reticulata* var. Shogun and Sai Nam Puaeng) and pummelo (*Citrus grandis* viz. Kao Thong Dee). MS medium with 2 mgL^{-1} of BA was found to be the most suitable medium for shoot proliferation. The seedlings were sub-cultured at least 4 times, and then they were treated with acute and chronic irradiation. Shoot induction from M_1V_0 to M_1V_4 generation was performed in basic MS medium with 2 mgL^{-1} added BA. Rooting was induced in the M_1V_4 in half-strength MS enriched with BA 2 mgL^{-1}. Later, the shoots were excised and grafted on mature plants or the plantlets directly transferred in the field and later the fruits from mature trees were evaluated for seedlessness in M_1V_4 at Pichit and Phare Horticultural Research Center.

1. Introduction

Tangerine (*C. reliculata*) and pummelo (*C. grandis*) are regarded as economically important species of citrus in Thailand. According to a 1998 DOAE report [1], the areas planted with tangerine account for 178,898 rai (≈ 28,623 ha) and for pummelo it is about 209,580 rai (≈33,532 ha). The total market value for tangerine accounts for about 5.5 billion Baht/year, (≈ US $ 170 Millions) whereas 2.1 Billions Baht/year (≈ US $ 64 Millions) for pummelo. Major diseases such as *Phytophthora infection*, Greening and Tristeza, which cause reduction of yield and decrease the fruit quality, are among the challenges currently addressed by the research community in Thailand.

Improvement of quality, such as seedlessness of fruits, is another desired character, which may be investigated as it is one of the easiest changes usually observed in mutation breeding programmes. Various physical mutagens such as X-ray, ^{60}Co produced gamma ray, fast

neutrons, laser beam, electron beam and ion beam etc, have been used in mutation breeding of horticultural crops. Gamma rays produced by ^{60}Co source are currently mostly used and appears more effective than other mutagens in the efficiency of mutation induction [2].

Espino et al., [4] mentioned that the use of mutation breeding techniques on various fruit crops including Calamondin (*Citrus mandurensis Lour.*) and Pummelo (*Citrus grandis*) has been performed with the primary aim of inducing, selecting and evaluating beneficial mutations and utilizing them for the development of improved varieties by a range of gamma ray doses (0 - 500 Gy). The authors reported that irradiation caused noticeable delay in shoot and root growth, inhibition on germination, leaf deformities and streaking (chlorophyll deficiency). In a citrus mutation breeding programs using gamma rays and seedless fruit cultivars were develop from budwoods irradiated with doses ranging from 30 to 75 Gy [4]. Pummelos (*Citrus grandis* [*C. maxima*]), mandarins (*C. reticulala*) and navel oranges (*C. sinensis*) were particularly sensitive to high levels of irradiation. Irradiation derived valencia oranges and grapefruit (*C. paradisi*) exhibited a higher frequency of branches with seedless fruits. Out of 2400 irradiated grafted trees 13 produced branches with seedless fruit including a seedless Nova tangelo mandarin (Nova SL). Tulman-Neto et al., [5] reported on buds irradiated with a 4 kR dose the cutting-back method was applied to V_1M_1 branches derived from irradiated scions. The first 6 buds from these branches were used to obtain the M_IV_2 generation. Buds from the M_IV_2 branches were grafted again onto new rootstocks in order to obtain the M_IV_3. Selection was then performed and 217 among the surviving plants were selected. These plants differed from the controls in such traits as canopy height, number of seeds/fruit, yield, fruit shape and leaf morphology. On the basis of a higher frequency of morphological changes in the leaves and fruits, it appeared that the fifth and sixth buds along the M_IV_1 branch should be used when applying this methodology. Division of plants into quadrants for selection was effective since, despite the cutting back method, the plants still showed chimaeras. The selected clones were evaluated in the field to confirm the genetic stability of the changes.

Cheng Ming et al., [6] reported that pollen viability and seed numbers in fruits were evaluated in 938 unit branches of 74 trees of *Citrus grandis* [*C. maxima*] cv. Shatian treated with ^{60}Co, fast neutrons and lasers. Eight few-seed fruited branches (counting between 20.1 and 40.0 seeds per fruit) and twelve fewer-seed branches (less than 20 seeds/fruit) were obtained. The same work reported that laser and ^{60}Co treatments are more effective at inducing few or fewer seeded fruits and increase the occurrence of pollen abortion patterns (abortion rate >45%) than neutrons.

In the present investigation, improvement of commercial tangerine (*C. reticulata viz*. Shogun or Sai Nam Puaeng variety) and pummelo (*C. grandis viz.* Kao Thong Dee variety) for seedlessness was performed through induced mutations with gamma rays.

2. Material and methods

2.1. In vitro gamma irradiation induced mutation techniques

Surface sterilized seeds were plated on Petri dishes containing Murashige and Skoog medium [7] for germination. Then the cotyledonary nodes of the germinated seedlings from both tangerine and pummelo were transferred onto MS medium enriched with BA 1-2 mg ml^{-1}. This first culture was followed by 4 subsequent sub-cultures every 15 days for shoot multiplication.

The targeted shoots were then treated either with:

(1) Acute irradiation at different doses (4-8Krd) for a short period of time or
(2) Chronic irradiation with low doses gamma rays (0.2 Gy/Hr) for long periods of times.

2.2. Mutation induction with gamma irradiation of grafted scions

Different radiation experiments were performed: i) Acute irradiation of scions of tangerine viz. Shogun or Sai Nam Puaeng and pummelo *viz*. Kao Thong Dee at different doses (4-8 krad), and ii) Chronic irradiation of tangerine pot plants *viz*. Shogun or Sai Nam Pueng and pummelo pot plants *viz*. Kao Thong Dee at different doses (0.05-0.15 Gy/hr). The scions which were treated by acute irradiation were grafted onto mature plants to produce new shoots. Those new shoots were excised and re-grafted onto selected mature rootstock plants several times up to the M_1V_4 generation. Then the morphological characters were recorded in comparison with the control for traits such as seedlessness of the fruits at maturity stage.

Plants treated by chronic irradiation were directly planted in the field and morphological characters of M_1V_1, and subsequent grafted generations were recorded and compared with the control. In the other method, the scions or buds from the treated plants were used to graft or bud with another mature plant until M_1V_3 and then new shoots from M_1V_3 will be M_1V_4. Then, these will be evaluated compared with the control.

3. Results and discussion

3.1. Germination

The germination rates of seeds from pummelo (Kao Thong Dee variety) and tangerine (Shogun variety, Sai Nam Puaeng and Number One variety) were recorded at 75.71%, 9.09% respectively, and at 1.2% for Kao Thong Dee, Sai Nam Puaeng and Shogun varieties. Cotyledonary nodes from germinated seeds were cultured in MS with 2, 4, 6, 8, and 10 mgL^{-1} of BA. MS medium with 2 mgL^{-1} of BA appeared as the most suitable medium for shoot multiplication. These explants were sub-cultured at least 4 times and treated with acute and chronic irradiation. Shoot induction from M_1V_0 generation to M_1V_4 were observed on basic MS with BA 2 mg/1. Root induction in the M_1V_4 was done in half-strength MS with BA 2 mgL^{-1} and then grafted onto mature plants or directly planted in the field and evaluated for seedlessness.

3.2. Radiation induced mutation

3.1.1. Pummelo (Citrus grandis) var. Kao Thong Dee

Chronic irradiation with doses different doses: 0, 4, 8, 12, 16, 20 and 24 Krad was applied to *in vitro* derived plantlets growing in MS medium enriched with BA (2 mg/L). After 24 months of sub-culture, those irradiated shoots were submitted to a final sub-culture in order to proceed to homogeneous M_1V_4 generation. The average umber of healthy plants and the percentage of surviving scions were recorded both in M_1V_2 and M_1V_3. A second group of sub-cultured plantlets were grown in a MS medium with 2 mg/L BA and submitted to acute irradiated shoots with doses of 0, 4, 8, 12, 16, 20 and 24 Krad. The average healthy plant index and the percentage of surviving scions were recorded (Table I. and Figure 1).

After 24 months, shoots derived from acute irradiation with doses of 0, 4, 6 and 8 krad were sub-cultured to proceed to M_1V_2-M_1V_4 generations. The average healthy plant index and percentage of surviving scions and shoots (M_1V_4) were recorded (Table 2, Figure 2). Later, these acute irradiated plants will be planted in the field of Pichit Horticultural Research Center for seedlessness evaluation in the M_1V_4 generation.

Figure 1. Pummelo var. Kao Thong Dee submitted to chronic irradiation and sub-cultured to M_1V_4 generation.

Figure 2. Pummelo var. Kao Thong Dee submitted to acute irradiation.

TABLE I. PLANT HEALTHY INDEX AND PERCENTAGE OF SURVIVING SCIONS (M_1V_1 - M_1V_3) OF PUMMELO VAR. KAO THONG DEE PLANTLETS WHICH WERE SUBMITTED TO CHRONIC IRRADIATION

	Dose (Krad)	M_1V_1			M_1V_2			M_1V_3		
		No. of scions	Average		No. of scions	Average		No. of scions	Average	
			Healthy P. index	Surviving (%)		Healthy P. index	Surviving (%)		Healthy P. index	Surviving (%)
First group										
1	0	-	-	-	-	-	-	-	-	-
2	4	-	-	-	10	6	100.00	30	7	100.00
3	8	-	-	-	24	6	100.00	2	7	100.00
4	12	-	-	-	2	7	100.00	-	-	-
5	16	-	-	-	14	7	100.00	-	-	-
6	20	-	-	-	6	7	100.00	-	-	-
7	24	-	-	-	-	-	-	-	-	-
Second Group										
1	0	-	-	-	-	-	-	-	-	-
2	4	14	7.00	100.00	-	-	-	-	-	-
3	8	26	7.00	100.00	-	-	-	-	-	-
4	12	-	-	-	-	-	-	-	-	-
5	16	6	6.00	100.00	-	-	-	-	-	-
6	20	32	7.00	100.00	-	-	-	-	-	-
7	24	-	-	-	-	-	-	-	-	-

Plant Healthy Index: 1= death, 2 = unusual symptom 76-100%, 3 = unusual symptom 51-75%, 4 = unusual symptom 26-50%, 5 = unusual symptom 1-25%, 6 = healthy, 7 = very healthy

TABLE II. HEALTHY PLANT INDEX AND PERCENTAGE OF SURVIVING SCIONS (M_1V_2) AND SHOOTS (M_1V_2, M_1V_4) OF PUMMELO VAR. KAO THONG DEE PLANTLETS WHICH WERE SUBMITTED TO ACUTE IRRADIATION

Treatment	Dose (K rad)	Scions			Shoots					
		M_1V_2			M_1V_2			M_1V_4		
		No. of scions	Average		No. of shoots	Average		No. of shoots	Average	
			Healthy P. index	Surviving (%)		Healthy P. index	Surviving (%)		Healthy P. index	Surviving (%)
First Group										
1	0	-	-	-	-	-	-	-	-	-
2	4	-	-	-	-	-	-	6	5	100
3	6	-	-	-	-	-	-	14	6	100
4	8	-	-	-	-	-	-	-	-	-
Second Group										
1	0	-	-	-	-	-	-	-	-	-
2	4	28	6	100.00	40	6	100	-	-	-
3	6	-	-	-	-	-	-	-	-	-
4	8	-	-	-	-	-	-	-	-	-

Notes: Plant Healthy Index: 1= death, 2 = unusual symptom 76-100%, 3 = unusual symptom 51-75%, 4 = unusual symptom 26-50%, 5 = unusual symptom 1-25%, 6 = healthy, 7= very healthy

*3.2.2. Tangerine (*C. reticulata) *var. Shogun, Sai Nam Puaeng and Number One*

Seeds from different sub-cultivars of tangerine varieties: Shogun, Sai Nam Puaeng and Number One were treated either with acute irradiation or with chronic irradiation with doses varying from 0, 4, 8, 12, 16, 20, 24 krad and 0, 4, 6, 8 krad, respectively. (After several months of sub-cultures the M_1V_4 generations were grafted onto mature root-stock or directly transferred in the field. Data were recorded: average healthy plant index and percentage of surviving shoots.

All putative mutants from acute and chronic irradiation of scions, pot plants and plants from *in vitro* mutation techniques were evaluated for seedlessness of the fruits in the M_1V_4 generation.

4. Conclusions

Under this research project improvement of quality, such as seedlessness of fruits for Tangerine (*C. reliculata*) and pummelo (*C. grandis*), which contribute ain a noticeable manner to the income generated by agriculture in Thailand was studied using induced mutation and *in vitro* techniques. The radiation was performed in two sets of experiments: i) Acute irradiation of scions of tangerine viz. Shogun or Sai Nam Puaeng and pummelo *viz*. Kao Thong Dee at different doses (4-8 krad), and ii) Chronic irradiation of tangerine pot plants *viz*. Shogun or Sai Nam Pueng and pummelo pot plants *viz*. Kao Thong Dee at different doses (0.05-0.15 Gy/hr). The scions and/or in vitro derived buds from the treated plants were sub-sequently re-grafted on mature plant until M_1V_4 for stabilisation of the putative. The optimal media were determined and the protocol carefully described. Some of these irradiated plants were evaluated and confirmed as producing seedless fruits in the field at Pichit and Phare Horticultural Research Center.

Figure 3. Tangerine var. Shogun # 4 submitted to chronic irradiation.

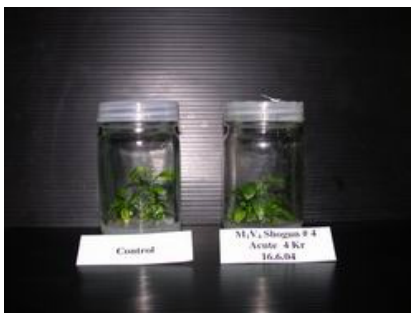

Figure 4. Tangerine var. Shogun # 4 submitted to acute irradiation.

Figure 5. Pummelo var. Kao Thong Dee planted in the field at Pichit Horticultural Research Center

Figure 6. Tangerine var. Shogun derived from shoots submitted to chronic irradiation at the dose of 35.911 Krad.

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Mutation induction for improving of tangerine in Iran

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Abstract. Genetic variation is an essential component of crop breeding. Induced mutations are highly effective in enhancing natural variability of genetic resources, and have been instrumental in developing improved cultivars of crops including fruits crops. Recent advances in biotechnological techniques have shown a great potential as efficient methodologies for vegetative micropropagation, screening techniques and genetic characterization including mutation induction. On this basis, a local, well- adapted and widely-consumed Clementine cultivar was selected for introduction into a mutation induction programme in order to reduce the excessive seeds in the fruits. A physical mutagen (γ-ray) was applied at doses of 35, 40 and 45 Gy on selected seedling apical buds. Irradiated buds were grafted onto sour orange root stocks (M_1V_1) and chimeras disassociated by further vegetative propagation. Finally M_1V_3 plants were transferred to the field and after the production of fruits, selection for the desired fruit types was undertaken. The results showed that the radiation treatment was able to produce mutant genotypes with seedless fruits, early and late ripening and cold tolerance.

1. Introduction

Since 1930, about 130 citrus cultivars of oranges, tangerines, sweet lemons, sour lemons, grapefruits and other hybrid lines have been introduced in Iran. Out of these introductions, and after preliminary cultivar comparisons in different research experiment stations, a number of selected citrus cultivars have been distributed in three regions of the country including the Caspian Sea region (Gillian & Mazandaran), the central region (Kerman, Fars, Kermanshah, Ilam, Kohkilouyieh, Bouyer Ahmad and Khorasan provinces) and finally along the Persian Gulf and Oman Sea (Khoozestan, Boushehr, Hormozgan and Sistan Balouchestan provinces). The total area of citrus fruits cultivated amounts to 235000 hectares, of which 92% is productive and 8% is non-productive (juvenile trees). Mazandaran province ranks first in the list with 37%-of the total cultivated area. Fars, Jiroft, Kahnooj, Hormozgan and Kerman areas respectively have 20.7, 14.8, 13.2 and 5.2% of the area. The rest, which is about 8.7%, is spread into other provinces (Table I).

2. National production

Tangerine ranks third in total citrus production in Iran. Tangerine varieties cultivated in Iran include, unchiu, younesi, page, oneco, kino and clementine. Clementine has been introduced into Iran along with unchiu. It originates from Algeria; and can be cultivated in the Caspian, Jiroft and other regions with a moderate climate. In Table I the cultivated area (productive & non-productive), production and yield performance data are given. Tangerine potentials for production, especially for export, can be considered to be well above its present status.

Clementine variety is well adapted to both the climate and other biotic and abiotic stresses in the north of Iran and has a constant high productivity. The induction of mutation of one or a few characters through the use of physical mutagens has proved to be an efficient tool for enhancing the frequency of spontaneous mutations [4, 5]. According to a literature review, somatic mutations have contributed to the development of new varieties in many fruit tree species.

TABLE I. CULTIVATED AREAS, PRODUCTION AND YIELD PER HA IN CITRUS FRUIT IN DIFFERENT PROVINCES [1]

Province	*Cultivation (ha)*		*Yield (Kg/Ha)*	*Production (ton)*
	Productive	*non-productive*		
Ailam	25	5	24	5086.02
Boshehr	641	2202	18027	8187.61
Jiroft &Kahnoj	2841	31784	516051	16236.17
Khrasan	17	49	386	7841.46
Khozestan	182	4715	47831	10145.01
Sistan &Balochestan	493	1957	12235	6251.92
Fars	3168	44503	957149	21507.52
Kerman	401	11098	96297	8676.97
Kermanshah	190	23	135	5869.57
Golestan	204	1899	29694	15632.85
Kohkiloyeh &Boierahmad	111	621	11784	18968.13
Gylan	307	7330	105098	14337.91
Mazandaran	3897	80377	1433612	17836.18
Hormozgan	7822	28434	483201	16994.07
Total	20298	214996	3711522	-

The most frequently detected mutations include; a reduction in plant height (dwarfness or semi-dwarfness) and the initiation of compact types (due to changes in the plant architecture, an increase or decrease in lateral branching or changes in branch position), changes in the flowering and ripening period, an increase in pollen sterility and changes to fruit size, shape or the number of seeds. The aim of this research proposal was to induce seedless/ less seeded Clementine from the presently seeded type through the use of physical mutagenesis.

3. Materials and methods

3.1. Shoot- tip grafting

In vitro grafting of shoot- tips has been widely used to obtain virus-free plants. Citrus plants obtained by this technique do not possess thorns or juvenile characters. Therefore, shoot- tip grafting can be used as a procedure for the production of true – to- type, virus-free, non-juvenile plants.

Seeds of Ponsiruse (which has trifoliate leaves and serves as a morphological marker for the identification of adventitious buds produced by the root stock) were sterilized for 10 min in 0.7% sodium hypochlorite solution and rinsed three times with sterile distilled water. After removing the seed coats, seeds were cultured on Murashige and Skoog medium [2] solidified

with agar. Seeds were incubated for two weeks in continual darkness and at 26 ±1ºC of temperature [3].

Within 8 to 15 days, numerous buds sprouted. Shoots shorter than 3 cm long were then used as source of shoot tips. They were stripped of larger leaves, cut to about 1 cm long, surface-sterilized by immersion in a 0.25% sodium hypochlorite solution containing 0.1% tween-20 wetting agent for a few minutes and then rinsed three times with sterile distilled water.

3.2. Grafting

Two week- old Ponsiruse seedlings were decapitated under aseptic conditions, leaving about 15 mm of each epicotyl. The roots were also cut to a length of 40-60 mm, and the cotyledons removed. A vertical incision, 1mm long, was made in the stem, starting at the point of decapitation, followed by a transversal incision of at the 1-2mm long bottom. These cuts were made through the cortex to the cambium, and the flaps created by these incisions were lifted slightly to expose the cortex. A shoot tip was then placed on the vascular ring in the opened flaps of the incision at the top of the decapitated epicotyl. The grafted plants were subsequently placed on paper bridges over MS liquid medium supplemented with 75g/l sucrose and maintained at a constant temperature of 26±1ºC in a 16hr daily photoperiod of 1000 Lux illumination (Figure 1.A-B,C).

Thirty days after grafting, approximately 80% of the grafted seedling wounds showed advanced recovery. After five to six weeks, the successfully - grafted shoot- tips produced 1 to 4 young leaves, but only 45% of the original number of grafted seedlings survived.

During scion growth a number of buds emerged from the root stock. Taking into consideration that Ponsirus leaves are trifoliate and Clementine leaves are unaffiliated it was therefore possible to expedite scion growth by cutting off adventitious Ponsirus buds like (Figure 1.E, F).

When the root and scion length reached 7 to 8 cm respectively, and primary leaflets showed suitable growth, plantlets were removed from the culture medium and following a thorough rinsed with water, planted into sand filled pots. The experiments were watered every week using MS medium, containing only macro and micro-salts. Three weeks later fully developed plants were transferred into bigger pots containing sand, clay and animal manure in 1:1:2 proportions (Figure 1 F).

3.3. In vitro mutation induction

Bacterial and virus free tangerine scions were irradiated with gamma ray ($^{60}C0$) using doses of 0, 35, 40, and 45Gy. Irradiated scions were then grafted onto sour orange rootstocks (600 individuals for each dose). At the end of the growing season, M_1V_1 shoots were cut back at the second basal bud and three to eight buds were then individually re-grafted on separate rootstocks.

To isolate induced mutants exhibiting chlorophyll abnormalities the same procedure was repeated on the original rootstocks with two – bud scions the following year. In M_1V_2 and M_1V_3 a number of morphological mutations affecting, leaf size, shape, and reduced internodes length were detected and recorded. M_1V_3 plants were later on transferred to the field (Figure 1G) and, after fruit production, an evaluation for fruit shape and colour, number of seeds per fruit, cold resistance and earliness was performed.

4. Results and discussion

Analysis of variance indicated that the grafting success in the two hormonal pre-treatment had significant differences at the 1% level (Table II). WE may conclude that an application of 2,4-D as a hormone pre-treatment produced successful grafting in more than 17% of the grafts while, in the case of the BAP pre-treatment, only 8% successful grafts were produced.

Decreasing the sucrose concentration to 7% in the medium compared to 8.5%, 10%, and 11.5% had also significant positive effects on the success rate of grafting. Considering the interaction effect of hormonal pre-treatment and sucrose concentration, it was determined that the 2, 4-D pre-treatment with a 7% sucrose concentration produced the best results.

TABLE II. OVERALL RESULTS OF THE PERCENTAGE OF SUCCESSFUL GRAFTING OF HORMONAL PRE-TREATMENT AND SUCROSE CONCENTRATION, ON THE BASIS OF ANALYSIS OF VARIANCE

Sources of variance	Degrees of freedom	Mean percentage of successful grafting
Sucrose conc.	3	79.2572**
Pre-treatment	1	4.477**
Sucrose conc. × Pre-treatment	3	46.610**
Error	16	73.36**

** Significant difference at 1% level

M_1V_3 plant after three years in the field have reached to 80% of productivity. 2000 trees were evaluated in the main field for the following traits: number of seeds in fruits, earliness, late maturity, cold tolerance and other favourable traits.

As shown in Table III and Figures 9 and 10, six (6) mutated seedless trees were obtained, all deriving from a 40Gy of Gamma ray irradiation assay. We also identified 13 cold tolerant trees. This trait is very important for the citrus production areas of the country, since it often happens that the temperature drop below zero. Out of these 13 trees, seven trees were resulted from irradiation with 35 Gy and six from irradiation with 40 Gy Gamma rays dose. There were significant differences in the maturation dates; two trees were early maturing, eight mid maturing and four late maturing trees. These latter traits are likely to be very well accepted by farmers, since this should allow having fresh fruits for a longer period of time and therefore present better marketing opportunities.

TABLE III. ANALYSIS OF FRUIT QUALITY IN THE FIRST YEAR OF PRODUCTION

Dose	Early maturity	Mid maturity	Late maturity	Cold resistant	Seed less
35Gy	2	8	4	7	-
40Gy	-	15	3	6	6
45Gy	-	1	1	-	-

The Total Soluble Solids (TSS) scale for all selected trees was determined and determined between 9 to10, except for one of the late maturing trees, which had a 6.8 value. The TSS of Clementine varies from 8.5 to 10 in the region. These findings need to be confirmed in the future, prior to release and commercialization.

The seedlessness trait was observed the following year, confirming the stability of the mutation for that specific trait (Figure 2).

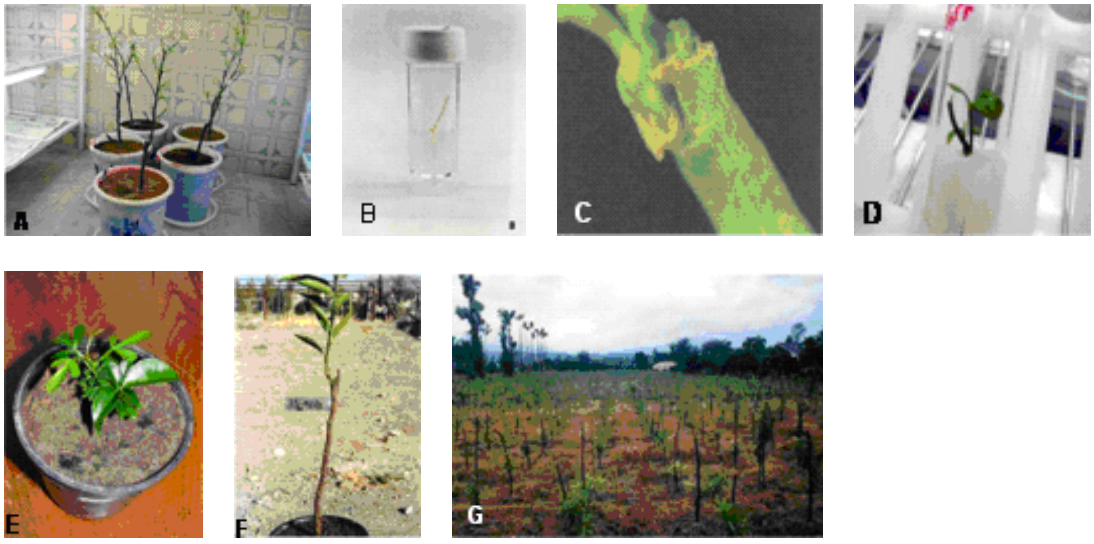

Figure 1: Infected citrus plants growing in pots in the green house were completely defoliated by hand and placed in a warm green house, or in a growth chamber.

Figure 2: Seedless tangerines compared to the parental type (A, B, C), late maturing tree (D).

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Somatic embryogenesis and embryo culture coupled with gamma irradiation for generating avocado (*Persea americana* Miller) mutants in the Philippines

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Abstract. Plant regeneration through somatic embryogenesis from immature zygotic embryos and embryo cultures from mature fruits were achieved in select avocado accession 'Semil' and other seedling trees in the Philippines. Embryogenic cultures were induced from immature zygotic embryos of eight (8) avocado genotypes using either SE1 medium (MS + 30 g/l sucrose + 5 mg/l 2, 4-D + 0.5 mg/l BAP) or SE2 medium (MS + 30 g/l sucrose + 0.1 mg/l picloram). Embryogenic cultures of 2 genotypes namely 'Semil' and 'Mainit' developed into somatic embryos after repeated subcultures in SE2, SE3 (MS + 30 g/l sucrose + 0.1 mg/l TDZ + 0.5 mg/l GA_3) and SE4 (MS + 30 g/l sucrose + 2 mg/l BAP + 1 mg/l IBA) media. Plant/shoot regeneration from 'Semil' somatic embryos was recorded in 3 trials at 16.3, 23.0 and 20.7%, and was affected by culture age, light treatment and media used. R4 regeneration medium (B5 macro salts + MS minor salts and vitamins + 60 g/l sucrose + 400 g/l glu + 2 mg/l BAP + 4.5 g/l Phytagel was found to be the best. Gamma irradiation (10 to 30 Gy) of embryogenic cultures of 'Semil' resulted in reduced proliferation and formation of cotyledonary stage somatic embryos. However, shoot regeneration from somatic embryos from gamma-irradiated cultures was comparable or even higher (17.8 to 26.9%) as compared to the control (18.3%). Over 200 somatic embryo-derived putative variant/mutant lines from tissue culture and gamma irradiation experiments are being maintained as shoot cultures. Due to slow growth and other related problems, micrografting and *in vitro* rooting were used to rescue and ensure the greenhouse establishment of putative mutant shoots, and fast-track mutant confirmation by genetic analysis. Preliminary genetic analyses by SSR revealed that (a) the 3 asexually propagated 'Semil' mother trees are genetically similar, and (b) mutations marked by the generation of a new allele (band) at the SSR locus was evident among the somatic embryo-derived regenerants from non-irradiated embryogenic cultures. Mass screening for mutations will be done on all successfully potted out regenerants. A second protocol involving embryo culture and gamma irradiation was also done. Mature embryos of 9 genotypes were cultured using liquid MS basal medium with 30 g/l sucrose and 2 mg/l BAP, with 100% germination. Germinating zygotic embryos of 'San Felix' were also gamma irradiated and the LD_{50} between 20 to 30 Gy was established. M_1V_4 shoots were generated from gamma-irradiated seedlings after subsequent micropropagation cycles. This is the first successful application of tissue culture and gamma irradiation technologies towards the improvement of a woody perennial fruit crop in the Philippines.

1. Introduction

Avocado (*Persea americana* Miller) is a widely cultivated fruit introduction in the Philippines although it remains as a backyard crop far below the ranks of mango, banana and pineapple. It is the most nutritious of all fruits [1]. Among the nutritive strengths are high calorie content,

high concentrations of vitamins A, C, E and Bs and magnesium, high levels of protein and fat, mostly of the mono-unsaturated oleic acid [2]. Despite its promise in the food industry, there is no formal avocado breeding in this country due to the lack of commercial-scale culture. At present, all eight recommended avocado varieties are products of selection [3-5]. Local and worldwide production is beset by a lack of good rootstocks to withstand *Phytophthora* root rot devastation. Resistance has been found to be present in subgenus *Eriodaphne* but is conspicuously absent in species of the genus *Persea* where avocado belongs. Worse, the sub-generic barrier is unbridgeable by either grafting or conventional hybridization [6].

Plant biotechnology can supplement avocado breeding by generating new variability through mutation breeding, somaclonal variation and genetic engineering, all unobtainable by using conventional approaches. Its success depends highly on the efficiency of the *in vitro* plant regeneration system. In general, woody perennials are more difficult in tissue culture as compared to annuals and other succulent crops. In avocado, plant regeneration is achieved through somatic embryogenesis from immature zygotic embryos [7-10] and embryo culture [11]. However, these systems are far from being routine and are often genotype-dependent. To date, low frequency of plantlet recovery has persisted in avocado somatic embryos. In the Philippines, only the variety 'RCF Purple' has been tissue cultured and regeneration from somatic embryos was also low [12]. Hence, improved plant regeneration systems via somatic embryogenesis and embryo culture are needed as efficient tools for non-conventional genetic improvement. Efficient regeneration systems can generate novel tissue culture-induced somaclonal variants [13] in avocado.

Mutation breeding by ionizing radiation such as gamma and x-rays has been the most popular and effective strategy to induce and select for an added desirable trait to an already improved variety of fruit trees without upsetting their outstanding traits. This is not possible using conventional breeding. Fifty of the nearly 2000 cultivars derived from mutation induction are fruits belonging to more than 20 different species [14]. Among these mutants are apple with changed skin color [15]), disease resistant Japanese pear [16], seedless grapefruit with red flesh [17] and spineless pineapple [18]. Other mutant characters usually include compact tree with spreading habit, early flowering or maturity, and fruit color [19].

Tissue cultures further enhance the effectiveness of mutant induction in crops. Large populations can undergo mutagenic treatments by targeting few cells or tissues of wide variety of explants where plants actually develop followed by aseptic propagation cycles of subculture aimed at separating mutated from non-mutated sectors. Two banana (*Musa acuminata* Colla) cultivars 'Klue Hom Thong KUI' and 'Novaria' are products of *in vitro* mutation [14]. With the recent breakthroughs in plant regeneration protocols of woody fruit crops, *in vitro* mutation can be better exploited. To date, there are limited reports on the radiosensitivity in terms of survival, proliferation and subsequent regeneration of cultured zygotic embryos and embryogenic cultures of avocado, more so in other tropical fruit crops.

In this report, two regeneration systems of avocado via somatic embryogenesis and embryo culture are described and used in combination with gamma irradiation as a strategy for inducing genetic variability in select avocado trees in the Philippines.

2. Materials and methods

2.1. Plant materials and preparation of explants

Avocado trees known for their good fruit characteristics from the provinces of Laguna and Batangas were selected as sources of immature and mature fruits for use in somatic embryo induction and embryo culture, respectively. Each tree was labeled according to the location or farm where they were grown, with the exception of asexually propagated accession "Semil" planted at the IPB Fruit Orchard at Mainit Bay, Laguna and the 'RCF Purple' at IPB Nursery. A total of 1,107 immature fruits (1.5 to 4 cm in length) were collected during the March to April fruit setting seasons for four consecutive years (2002 to 2005). In 2002, 'Semil', 'Mainit', 'San Felix' and 'Batangas' were used while in 2003 only 'Semil', 'UPCO 1', 'UPCO 2' and 'Kanluran' were collected. In 2004, 'Mainit' 'Semil' and 'San Felix' were again used while in 2005 only 'RCF Purple' and 'Mainit' were tested. Young fruits were surface disinfested with serial immersion in 70% ethyl alcohol (3 min) and 50% commercial bleach (2.5% active ingredient, a.i.) (20 min), and rinsed with sterile distilled water prior to embryo excision. Young seeds were extracted aseptically, cut into quarters each serving as explants and cultured *in vitro* to induce embryogenic cultures. These embryogenic cultures were used to study the factors affecting the induction of somatic embryogenesis and the subsequent regeneration from somatic embryos (SEs), and to determine radiosensitivities of two avocado genotypes when exposed to gamma rays.

For embryo culture, 9 genotypes namely 'San Felix 1', 'San Felix 2', 'Calauan', 'UPCO 1', 'RCF Purple', 'Victoria', 'Mainit', 'Semil', and 'Los Baños' were used. Mature seeds were surface sterilized in the same manner as the immature fruits. The embryos together with a 5 mm x 5 mm section of one cotyledon were excised and used as explants for embryo culture leading to shoot regeneration, and in gamma irradiation experiments.

2.2. Culture media and incubation

Tissue culture media used in embryo culture, somatic embryogenesis induction, SE maturation and subsequent plant regeneration were all based on MS [20] and B5 [21] basal media. Different media formulations specific for embryo culture (EC), somatic embryogenesis (SE1 to SE4), maturation (M) and regeneration (R1 to R5) from SEs, and shoot multiplication (SM1 and 2) are listed in Table I. All growth regulators were sourced from freshly prepared 200 mg/l stock solutions. All media were adjusted to pH 5.8 before addition of agar (0.6% w/v, HiMedia, India) unless otherwise specified, dispensed into 4.5 cm x 10.2 cm 'Ampicin' bottles in 10-ml aliquots and sterilized at 121 C for 20 min. All cultures were placed in an air-conditioned room (27 ± 2 C) provided with either continuous 24-hour light or dark conditions.

2.3. Induction of somatic embryogenesis from embryogenic cultures

The young seeds (Figure 1a) were cultured to induce callus and embryogenic cultures in 2 published media, namely SE1 consisting of MS salts and vitamins supplemented with 30 g/l sucrose, 5 mg/l 2, 4-diclorophenoxy acetic acid (2, 4-D) and 0.5 mg/l BAP [12] and SE2 composed of MS basal medium with 30 g/l sucrose and 0.1 mg/l picloram [7]. Explants forming callus and embryogenic masses at the initial culture (SC0) were subcultured onto fresh media of the same composition (SC1) to allow the selection of embryogenic cultures with good proliferation rates (Figure 1b). To promote somatic embryogenesis, embryogenic cultures were subcultured onto 3 media namely, 1) SE2, 2) SE3 = MS + 30 g/l sucrose,

0.1 mg/l thidiazuron (TDZ) and 0.5 mg/l gibberellic acid (GA_3), 3) SE4 = MS + 30 g/l sucrose, 2 mg/l BAP + 1 mg/l indole-3-butyric acid (IBA). Regular subcultures were done every 4 weeks. All SEs at cotyledonary stage (Figure 1c) were transferred to maturation medium (M) consisting of B5 salts and MS vitamins, 50 g/l sucrose, 100 ml/l coconut water (CW) and solidified with 4.5% gellan gum 'Phytagel' (Sigma, USA). All cultures were incubated under continuous dark condition with maturation lasting for 6 to 8 weeks.

2.4. Plant regeneration from somatic embryos

After maturation, all SEs were randomly transferred to the 5 regeneration media (i.e. R1 to R5), which were reported for plant/shoot regeneration in various crops (Table I). These media included R1 and R2 = MS medium + 5 and 10 mg/l abscisic acid (ABA), respectively [12]. R3 = B5 macro salts, MS minor salts and vitamins + 400 mg/l glutamine (glu) + 100 ml/l CW + 4.5% Phytagel [22]. R4 = B5 macro salts, MS minor salts and vitamins + 400 mg/l glu + 2 mg/l BAP + 4.5% Phytagel [23]. R5 = MS macro salts, B5 minor salts and vitamins + 60 g/l maltose [24]. R1 to R4 had 60 g L^{-1} sucrose as carbon source. This experiment was done in 3 trials based on the age of embryogenic cultures where the SEs originated. Trials 1 and 2 had SEs from 8-month-old embryogenic cultures while trial 3 used 13-month-old embryogenic cultures. For each trial, 3 cycles of subculture in regeneration media each lasting for 4 weeks were made. The first cycle (SC1) was done using 5 respective media (R1 to R5) which lasted for 4 weeks under continuous light while the 2 subsequent cycles (SC2 and SC3) used only R4 and/or R4- (i.e. R4 without BAP) media under dark followed by light, and light conditions. This was done to assess the effects of extended culturing on the regeneration efficiency of SEs. All non regenerating SEs which remained green or whitish green from previous cycles were used in the subsequent cycles (ex. SC2 and SC3). Following this scheme, a total of 1,258 SEs were used in this plant/shoot regeneration experiment.

2.5. Embryo culture and direct shoot regeneration from lateral buds of germinating zygotic embryos

Excised embryos from mature seeds were cultured in liquid EC medium consisting of MS medium with 30 mg/l sucrose and 2 mg/l BAP to allow germination. Percentage shoot regeneration and the number of shoots produced per embryo were obtained 8 weeks after culture. Subsequent shoot proliferation from elongated shoots was done by shoot tip and nodal cuttings cultured in agar-solidified SM1 medium composed of B5 basal medium with 30 g/l sucrose and 1 mg/l BAP (Table I).

2.6. Gamma irradiation of embryo and embryogenic cultures

2.6.1. Embryo cultures

Five-day-old germinating embryo cultures of avocado 'San Felix' were transferred into empty sterile bottles lined with filter paper and subjected to increasing doses of gamma rays (0, 2.5, 5, 10, 20, 30, 40 and 50 Gy) at the Philippine Nuclear Research Institute (PNRI) in Diliman, Quezon City, Metro Manila. This experiment involved 7 embryos per treatment and had three replicates. The irradiated embryos together with the non-irradiated control were subcultured back to fresh EC medium a day after irradiation for shoot recovery and regeneration. One week later, percentages of embryos with green shoots and embryos with emerging shoot and root were obtained. Percentage of embryos with elongated shoots (ready for nodal cutting) and the number of shoots induced were recorded 13 weeks after irradiation.

TABLE I. LIST OF MEDIA FORMULATED FOR EMBRYO CULTURE, SOMATIC EMBRYOGENESIS, AND REGENERATION FROM SOMATIC EMBRYOS (SES), AND SHOOT MULTIPLICATION OF AVOCADO REGENERANTS

Culture stage/activity	Media code	Media composition	Reference
Embryo culture	EC	MS salts and vitamins + 30 g/l suc + 2 mg/l BAP	Avenido et al., 2003
Induction of SEs	SE1	MS salts and vitamins + 30 g/l suc + 5 mg/l 2,4-D + 0. mg/l BAP	Raviv et al. 1998
	SE2	MS salts and vitamins + 30 g/l suc + 0.1 mg/l picloram	Pliego-Alfaro and Murashige 1987
	SE3	MS salts and vitamins + 30 g/l suc + 0.1 mg/l TDZ 0.5 mg/l GA_3	Mendoza 1999
	SE4	MS salts and vitamins + 30 g/l suc + 2 mg/l BAP + mg/l IBA	Mendoza 1999
Maturation of SEs	M	B5 salts and MS vitamins + 50 g/l suc + 100 ml/l CW 4.5% gellan gum 'Phytagel'	Avenido 2005 (pers.comm.)
Regeneration from SEs	R1	MS salts and vitamins + 60 g/l suc + 5 mg/l ABA + 5% agar	Raviv et al., 1998
	R2	MS salts and vitamins + 60 g/l suc + 10 mg/l ABA + 5% agar	Raviv et al., 1998
	R3	B5 macro salts + MS minor salts and vitamins + 60 g suc + 400 mg/l glu + 100 ml/l CW + 2.5 g/l Gelrite	Pateña et al., 2002
	R4	B5 macro salts + MS minor salts and vitamins + 60 g/l suc + 400 mg/l glu + 2 mg/l BAP + 4.5 g/l Phytagel	Dewald et al., 1989
	R4-	B5 macro salts + MS minor salts and vitamins + 60 g/l suc + 400 mg/l glu + 4.5 g/l Phytagel	Modified from Dewald et al., 1989
	R5	B5 macro salts + MS minor salts and vitamins + 60 g/l maltose + 400 mg/l glu + 5% agar	Parrot et al. 1994
Shoot multiplication/ maintenance	SM1	B5 salts and vitamins + 30 g/l suc + 1 mg/l BAP	Avenido et al., 2003
	SM2	B5 salts and vitamins + 30 g/l suc + 1 mg/l BAP + 1 mg/l GA_3	Avenido 2005 (unpublished)

All media, unless otherwise specified, were solidified using 4.5 g/l HiMedia agar (India).

2.6.2. Embryogenic cultures

Newly subcultured (5 days from subculture) embryogenic cultures of avocado 'Semil' and 'Mainit' in 3 media namely SE2, SE3 and SE4 were exposed to increasing doses of gamma rays (0, 10, 20 and 30 Gy) at the PNRI. Ten pieces of 6-month-old embryogenic cultures (approximately 8 mm x 8 mm) were used per treatment and this experiment had 3 replications. All cultures were transferred to fresh media of the same composition one day after irradiation for re-growth, proliferation and advance to torpedo and cotyledonary stages. Percentages re-growth (as defined by more than 50% increase in the original explant size) and advance to torpedo/cotyledonary stages of SEs were obtained 3 weeks later. All proliferating embryogenic cultures were subcultured using the respective SE2, SE3 and SE4 media until

SC4 when sufficient cotyledonary embryos were ready for maturation phase (i.e. 13 months from initiation). Procedures and media used for maturation and plant regeneration followed that of non-irradiated cultures.

2.7. Micropropagation and micrografting of somatic embryo-derived shoot regenerants

All SE-derived shoot regenerants (both non-irradiated and gamma-irradiated) were micropropagated by shoot tip and nodal cuttings in agar-solidified SM2 medium composed of B5 basal medium with 30 g/l sucrose and 1 mg/l each of BAP and GA_3 (Table I). Due to the typical slow growth observed among these regenerants compounded by serious problems of unwanted callusing and contamination, a micrografting technique was adapted from the technique of Raharjo et al. [25]. The present mirografting technique uses both shoot cultures and *in vitro* germinated seedlings of 'Mainit' as 'rootstocks'. 'Mainit' is the lone avocado seedling tree survivor in a *Phytophthora* root rot-infested field in Mainit, Bay, Laguna. This is now being done to rescue putative mutant shoots from SEs by micrografting onto bigger shoots or germinating seedlings of 'Mainit'.

2.8. Statistical analyses

Tissue culture data on the effects of media and gamma rays (percentages and actual counts), when possible, were analyzed statistically using SAS System. Arc-sign-transformed data were analyzed by ANOVA procedure and means of significant parameters were separated by LSD ($P=0.05$).

2.9. Genetic analysis among avocado mother plants and regenerants by SSR

While waiting for the growth of SE-derived putative mutants, three (3) asexually propagated (grafted) trees of avocado cv. 'Semil' and the seedling tree of 'Mainit' were sampled for genetic analysis. These trees served as embryo sources for the induction of SEs, which eventually produced tissue culture regenerants.

2.9.1. DNA materials

At approximately 3 grams per sampling, leaf flashes of each tree were harvested and used as the starting materials for DNA extraction. For each tree, at least three (3) independent samplings were obtained. The procedure for the isolation and preparation of genomic DNA was based on the standard CTAB DNA extraction procedure [26].

For the quantification of the DNA samples, equal volumes of the samples were electrophoresced on an agarose gel together with DNA concentration markers. The concentrations of the DNA samples were determined based on their band intensities, in comparison with the concentration markers, after staining the gel with ethidium bromide. A working stock of 10 ng/uL was prepared for each DNA sample for use in subsequent molecular analysis. To prevent degradation, both the original and working stocks of the DNA samples were stored at -20 C.

2.9.2. DNA extraction from leaf samples of somatic embryo-derived regenerants

Preliminary analysis was done on a limited number of shoot regenerants of 'Semil' that had sufficient leaf samples for DNA micro-extraction. The protocol for DNA micro-extraction

was as described by Fulton et al. [27], with some modifications. DNA yield was then determined by agarose gel quantification as described above.

From embryo no. 11, seven (7) non-irradiated regenerants from either batch of embryogenic culture (8 and 13 months old) were initially sampled. The following numbers of irradiated regenerants were available for leaf sampling: 0 Gy = 9 regenerants; 10 Gy = 9; and 20 Gy treatment = 12 regenerants. However, due to very limited *in vitro* leaf materials, quantifiable DNA samples were successfully extracted and processed only from five (5) and four (4) non-irradiated and gamma-irradiated tissue culture regenerants, respectively.

2.9.3. Microsatellite markers

A total of 24 microsatellite (SSR) markers were identified from the integrated genetic linkage map of avocado [28]. The SSR markers were selected to represent each linkage group of the avocado genome. A standard quality of the SSR primer pairs were then synthesized through the commercial custom service of the Invitrogen, Inc. USA.

2.9.4. PCR analysis

The polymerase chain reaction (PCR) analysis of the 'Mainit' and 'Semil' avocado mother trees was performed following the conditions previously optimized for SSR analysis in avocado [28]. At a final DNA concentration of 30 ng per 10 µL total reaction, the PCR consisted of 1U Taq polymerase, 0.15 µM of 3'- and 5'- end primers, 0.1 mM of each nucleotide, 1.5 mM MgCl2, and l x PCR buffer (10 mM Tris-HCL pH 8.3, 50 mM KCl). The reaction was performed on a MJ Research PTC-100 thermocycler (MJ Research, Watertown, Mass.) with the following temperature profile: initial denaturation of 94 C for 30 sec; 32 cycles of 95 C for 15 sec, 45 to 50 C (depending on primer pair % G-C content) for 25 sec, and 68 C for 25 sec; and a final amplification extension of 68 C for 2 min.

For the resolution and analysis of SSR fingerprints, the PCR products were separated by electrophoresis on 5% denaturing polyacrylamide gel (PAGE) and detected following the established non-radioactive silver staining protocol in the laboratory [29].

2.9.5. Data analysis

Different alleles for each SSR were observed and scored among the avocado DNA samples. As an initial estimate of genetic diversity or relatedness, average allele frequencies were compared among the avocado mother trees based on the number of SSR loci surveyed.

3. Results and discussions

3.1. Shoot regeneration from embryo cultures of avocado and subsequent micropropagation

Zygotic embryos from 255 ripe fruits of nine (9) avocado genotypes namely 'Calauan', 'San Felix 1', 'San Felix 2', 'UPCO 1', 'RCF Purple', 'Victoria', 'Mainit', 'Semil' and 'Los Baños' were cultured in EC medium consisting of MS salts with 30 g/l sucrose and 2 mg/l BAP. In the four-year experimental period, all genotypes exhibited 100% germination and shoot formation within 2 weeks from culture. This proved that embryo culture is a fast and efficient regeneration system suitable for diverse avocado genotypes. Average number of shoots produced per embryo ranged from 1.3 to 4.2 with an average of 2.0 shoots for the 9

genotypes tested (Table II). Cutting the main shoot and subculturing the original explant onto fresh EC medium (SC1) resulted in the production of additional (1 to 7) shoots per explant. Succeeding micropropagation of nodal cuttings and shoot tips from these seedlings was achieved best in MS medium with 30 g/l sucrose and 1 to 2 mg/l BAP. In terms of embryo germination and shoot regeneration across genotypes, this embryo culture system is therefore an ideal tool for genetic manipulation by gamma irradiation.

TABLE II. *IN VITRO* GERMINATION AND SHOOT PRODUCTION OF EMBRYO CULTURED MATURE ZYGOTIC EMBRYOS OF NINE AVOCADO GENOTYPES COLLECTED FROM 2002 TO 2005 FRUITING SEASON USING LIQUID MS MEDIUM WITH 30 G L^{-1} SUGAR AND 1 MG L^{-1} BAP

Genotype/variety	Fruiting year	No. of zygotic embryos cultured	Per cent germination	Ave. no. of shoots per embryo*
'Calauan'	2002	56	100	1.7 ± 1.2
'San Felix 1'	2002	8	100	4.2 ± 2.5
'San Felix 2'	2002	12	100	2.2 ± 1.1
'UPCO 1'	2003	22	100	2.3 ± 0.6
'RCF Purple'	2003	5	100	1.6 ± 0.6
'Victoria'	2003	3	100	2.0
'Mainit'	2004	40	100	1.5 ± 1.0
'Mainit'	2005	31	100	1.3 ± 0.6
'Semil'	2004	56	100	1.5 ± 0.8
'Los Baños'	2005	22	100	2.1 ±1.8
Total/ave.		255	100	2.0 ± 1.0

*Observed eight weeks after culture

3.2. Improved plant regeneration in avocado by somatic embryogenesis

3.2.1. Callus and embryogenic culture induction

The study on the initiation of callus and somatic embryogenesis from immature seeds of 8 Philippine avocado genotypes involved a total of 1,107 immature fruits collected in 4 consecutive fruiting years (2002 to 2005). The callus and embryogenic culture induction responses of these genotypes were tested using 2 published media namely SE1 consisting of MS basal medium + 5 mg/l 2, 4-D and 0.5 mg/l BAP [12] and SE2 consisting of MS basal medium with 0.1 mg/l picloram [7]. Tables III and IV summarize the callus and embryogenic culture formation in 'Semil' and 7 other avocado genotypes tested, respectively. SE2 medium promoted higher responses as compared to SE1 in almost all genotypes tested. Witjaksono and Litz [9-10] used a similar induction medium with that of SE2 medium except for the use of B5 basal medium instead of MS.

Initially, a mixture of organogenic and non-organogenic callus together with embryogenic masses was observed among the responding primary explants at SC0. The embryogenic masses were selected and subcultured separately from the second subculture (SC2) onwards.

Average embryogenic culture formation in 'Semil' over the 4 fruit setting years ranged from 2.6 to 9.5% and 3 to 27.8% in SE1 and SE2 media, respectively, (Table III). Likewise, higher average embryogenic culture formation among the 7 other genotypes was obtained in SE2 (4.2 to 40%) as compared in SE1 (2.1 to 10%) during 2004 to 2005 seasons. In the 2002 to 2003 seasons, 5 genotypes induced 6.7 to 20.4% embryogenic cultures in SE2 medium and none in SE1 medium (Table IV). Significant variations in the embryogenic culture formation may be attributed to the variable ages of fruits collected with only the fruit size used as the basis for selection... This can be improved by using fruits based on days after pollination which could lead to a consistently higher embryogenic culture formation for different genotypes. The inherent genotypic differences in response to the tissue culture media and incubation conditions used also contributed to this observed variation.

TABLE III. PERCENTAGE CALLUS (C) AND EMBRYOGENIC CULTURE (EC) FORMATION FROM IMMATURE ZYGOTIC EMBRYOS OF AVOCADO 'SEMIL' CULTURED IN TWO PUBLISHED MEDIA NAMELY SE1 = MS MEDIUM + 30 G/L SUCROSE + 2 MG/L 2,4-D [12] AND SE2 = B5 MEDIUM + 30 G/L SUCROSE + 0.1 MG/L PICLORAM [7] DURING 2002 TO 2005 FRUITING SEASONS

Fruiting Year	Callus (C) formation		Embryogenic culture (EC) formation	
	SE1 medium	SE2 medium	SE1 medium	SE2 medium
2002	44.0 (37/84)	70.1 (56/79)	9.5 (8/84)	27.8 (22/79)
2003	50.0 (14/28)	64.5 (20/31)	7.1 (2/28)	6.4 (2/31)
2004	59.6 (59/99)	43.0 (43/100)	9.1 (9/99)	3.0 (3/100)
2005	34.2 (13/38)	78.9 (30/38)	2.6 (1/38)	21.0 (8/38)
Ave.	47.0 ± 10.6	64.1 ± 15.2	7.1 ± 3.2	14.6 ± 11.8

3.2.2. Induction, maintenance and maturation of somatic embryos

The embryogenic cultures of 'Mainit' and 'Semil' initiated in April 2002 were transferred onto 3 media, namely, 1) SE2; 2) SE3 (MS + 0.1 mg/l TDZ and 0.5 mg/l GA_3; 3) SE4 (MS + 2 mg/l BAP + 1 mg/l IBA) for induction of somatic embryogenesis. Embryogenic cultures of 'Semil' proliferated profusely and produced different stages of somatic embryos from globular, heart, torpedo and cotyledonary stages after undergoing regular subcultures (i.e. every 3 to 4 weeks) on the 3 media. On the other hand, few and sporadic cotyledonary stage SEs of 'Mainit' were obtained even after prolonged subculture cycles in the 3 media. In an earlier report, SE3 and SE4 media were found effective in somatic embryogenesis induction of another Philippine avocado cultivar 'RCF Purple' [12, 30].

SEs of 'Semil' at cotyledonary stage that are opaque and white often with shiny surface were considered ready for the maturation phase (Figure 1 d-e). This was done by transferring SEs onto maturation medium consisting of B5-MS basal medium with 60 g/l sucrose and 100 ml/l CW, and incubating them for 6 to 8 weeks under dark condition.

3.2.3. Plant or shoot regeneration from somatic embryos

Regeneration of shoots and rooted plantlets from SEs was observed at varying frequencies 8 weeks after subculture onto the regeneration media. After the initial subculture (SC1), shoot

and plant regeneration were observed only from R4 and R5 media for trial 1 (1.2%), R2 and R5 media for trial 2 (0.8%) and in all 5 media except R2 in trial 3 (4.0%) (Table V). SEs in trials 1 and 2 were both derived from 8-month-old embryogenic cultures. However, the slightly higher percentage regeneration in trial 1 as compared to trial 2 can be attributed to the immediate exposure to light after the SEs were transferred to the regeneration media. In contrast, SEs in trial 2 was kept under extended dark for 4 more weeks after subculture onto regeneration media and were shifted only to light incubation 4 weeks later. SEs in trial 3, being from the older (13-month-old) embryogenic cultures, not only showed the highest average regeneration (4.0%) at SC1 but was also obtained in 4 out of 5 media tested. This higher regeneration over trial 1 can be attributed to better physiological maturity among the SEs derived from the older embryogenic cultures. Moreover, R4 medium originally for mango [23] was consistently the best regeneration medium for SEs with 3.2% and 15.1% induced to regenerate into shoots and rooted plants in trials 1 and 3, respectively.

TABLE IV. PERCENTAGE CALLUS (C) AND EMBRYOGENIC CULTURE (EC) FORMATION FROM IMMATURE ZYGOTIC EMBRYOS OF SEVEN (7) AVOCADO GENOTYPES CULTURED IN TWO PUBLISHED MEDIA NAMELY SE1 = MS MEDIUM + 30 G/L SUCROSE + 2 MG/L 2,4-D [12] AND SE2 = B5 MEDIUM + 30 G/L SUCROSE + 0.1 MG/L PICLORAM [7] DURING 2002 TO 2005 FRUITING SEASONS

Fruiting year	Genotype	Callus (C) formation		Embryogenic culture (EC) formation	
		SE1 medium	SE2 medium	SE1 medium	SE2 medium
2002	'Mainit'	27.0 (10/37)	63.4 (26/41)	0	14.6 (6/41)
	'San Felix'	17.2 (5/29)	41.4 (12/29)	0	13.8 (4/29)
	'Batangas'	34.9 (15/43)	20.4 (9/44)	0	20.4 (9/44)
2003	'UPCO 1'	36.4 (4/11)	91.7 (11/12)	0	16.7 (2/12)
	'UPCO 2'	84.6 (11/13)	80.0 (12/15)	0	6.7 (1/15)
	'Kanluran'	33.3 (12/36)	47.5 (19/40)	0	0
2004	'Mainit'	26.1 (6/23)	30.0 (6/20)	0	0
	'San Felix'	38.3 (18/47)	43.8 (21/48)	2.1 (1/47)	4.2 (2/48)
2005	'RCF Purple'	60.0 (12/20)	90.0 (18/20)	10.0 (2/20)	40.0 (8/20)
	'Mainit'	29.3 (12/41)	41.5 (17/41)	7.3 (3/41)	12.2 (5/41)
Ave.		38.7 ± 19.6	55.0 ± 25.0	1.9 ± 3.6	12.9 ± 11.8

Subsequent subcultures of the non-regenerating SEs onto R4/R4- media resulted in significantly higher percentage plant and shoot regeneration among the SEs (Table 6). In SC2, the average percent regeneration for trials 1 to 3 at 5.2, 16.1 and 9.1% were over 3-, 19- and 1-fold increase, respectively, from that of SC1. The higher regeneration in trial 3 as compared to trial 1 is again expected if age and maturity of SEs are to be considered. However, the higher regeneration in trial 2 over that of trial 1 (both 8-month-old) may be attributed to a more synchronized embryo development, possibly due to an extended dark incubation during SC1. The final subculture (SC3) resulted in still significant increases in regeneration compared to that of SC1 but generally much lower from those of SC2 except among the

younger SEs of trial 1 with the highest response (9.9%). SEs from trials 2 and 3 had an average of 6.0 and 7.6% plant/shoot regeneration at SC3.

TABLE V. EFFECTS OF CULTURE AGE, MEDIA AND LIGHT TREATMENT ON PERCENTAGE PLANT/SHOOT REGENERATION FROM SOMATIC EMBRYOS (SES) OF AVOCADO 'SEMIL' 8 WEEKS AFTER TRANSFER TO REGENERATION MEDIA

Trial no./ culture age/ light treatment	Regeneration medium	Total no. of SEs used (SC1)	% Regeneration		
			Shoot	Shoot and root	Total
1 8-mo.-old cultures; 16-h light for 8 weeks	R1	64	0	0	0
	R2	62	0	0	0
	R3	52	0	0	0
	R4	62	3.2	0	3.2
	R5	38	0	2.6	2.6
	Ave.				1.2
2 8-mo.-old cultures; dark→light	R1	117	0	0	0
	R2	113	0	1.8	1.8
	R3	103	0	0	0
	R4	85	0	0	0
	R5	85	1.2	1.2	2.4
	Ave.				0.8
3 8-mo.-old cultures; 16-h light for 8 weeks	R1	94	1.1	0	1.1
	R2	100	0	0	0
	R3	91	2.2	0	2.2
	R4	86	11.6	3.5	15.1
	R5	106	0.9	0.9	1.8
	Ave.				4.0

Table VI also shows the total percentage regeneration after 3 subculture cycles at 16.3% (trial 1), 23% (trial 2) and 20.7% (trial 3). These results proved that the extended dark treatment in trial 2 induced the highest number of SEs to form plantlets/shoots. Such treatment prolonged the maturation phase, possibly resulting in better embryo development which eventually increased regeneration efficiency over time. Moreover, this regeneration was kept comparable for SEs derived from 8-month and 13-month-old embryogenic cultures of 'Semil' avocado.

A total of 189 plantlet and shoots were regenerated from 'Semil' SEs (Figure 2). Some SE-derived lines were lost due to the typical slow growth and poor vigor. These were compounded by problems associated with long-term micropropagation such as callusing and loss through bacterial or fungal contamination. Over 100 shoots are now being micropropagated or micrografted to avocado rootstocks (Figure 1f) to ensure greenhouse establishment. All shoot regenerants are now being readied for transfer to soil. Screening for tissue culture-induced variations using molecular markers can be completed once all regenerants are successfully established in soil.

TABLE VI. EFFECTS OF CULTURE AGE, LIGHT INCUBATION AND SUBCULTURE CYCLES ON PERCENTAGE PLANT/SHOOT REGENERATION FROM SOMATIC EMBRYOS OF AVOCADO "SEMIL

Trial no./ culture age/ light	Regene-ration medium	No. of SEs used (SC1)	% Rege-neration	No. of SEs used (SC2)	% Rege-neration	No. of SEs used (SC3)	% Rege-neration	% Total rege-neration
A	R4	62	3.2	91	7.7	38	13.2	
8-month old	R4-		-	73	2.8	30	6.6	
light	Ave.		1.2		5.2		9.9	16.3
B	R4	85	0.8	206	16.1	116	6.0	
8-month old dark→light	Ave.		0.8		16.1		6.0	23.0
C	R4	86	15.1	152	15.7	82	2.4	
13-month old	R4-		-	159	2.5	78	12.8	
light	Ave.		4.0		9.1		7.6	20.7

3.3. Effects of gamma irradiation on avocado tissue cultures

3.3.1. Shoot regeneration from germinating embryo cultures

The radiosensitivities of embryo cultures of avocado 'San Felix', as gauged from germination and seedling growth, were determined after exposure to increasing doses of gamma rays (i.e. 2.5, 5, 10, 20, 30 40 and 50 Gy). At 7 days post irradiation, the percentages embryos with green shoots, and embryos with emerging shoot and root did not differ statistically with the 100% response of the non-irradiated control (Table VII). Exposure to increasing doses resulted in significant reduction in shoot lengths in a manner consistent at 7, 14 and 30 days post irradiation. Average shoot lengths of non-irradiated embryos at 7, 14 and 30 days after culture were 11.7, 18.5 and 29.2 mm, respectively. Reduction in shoot lengths by as much as 43%, 38% and 51% over the control was observed at 7 days (6.6 mm), 14 days (11.4 mm) and 30 days (14.4 mm), respectively, in 20 Gy. However, shoots of embryos exposed to lower doses (i.e. 2.5, 5.0 and 10 Gy) were longer but statistically comparable with that of control (Table VIII). On the contrary, root lengths as a parameter taken 7 and 14 days after irradiation did not show any effect among the irradiated, and between the control and the treated embryos (data not shown).

Subsequent growth of shoot cultures 90 days after irradiation was consistent with the early effects of gamma rays on shoot lengths of germinating embryo cultures (Table 7). Percentage embryos with elongated shoots (i.e. ready for micropropagation) at lower doses (2.5 to 30 Gy) ranged from 83.4 to 89.7% and were statistically comparable. The non-irradiated control had 100% of embryos with elongated shoots. As for the average number of shoots regenerated, 2.5 and 5 Gy had 4.5 and 3.7 shoots, respectively, which were comparable with that of control with 3.2 shoots produced. Shoot production at higher doses ranged only from 1.2 to 2.8 shoots and was statistically lower from the control (3.2 shoots) and at lower doses (i.e. 2.5 to 5 Gy).

TABLE VII. EFFECTS OF GAMMA IRRADIATION ON PERCENTAGES OF EMBRYOS WITH GREEN SHOOT, SHOOT-ROOT EMERGENCE, SHOOT ELONGATION AND AVERAGE NUMBER OF SHOOTS INDUCED FROM MATURE ZYGOTIC EMBRYOS OF AVOCADO 'SAN FELIX' WHEN CULTURED IN LIQUID MS MEDIUM WITH 2 MG/L BAP

Treatment/dose (Gy)	% Embryos with green shoots after 7 days	% Embryos with shoot and root after 7 days	% Embryos with elongated shoots after 30 days	Ave. no. of shoots induced after 90 days
0	100.0a	80.9a	100.0a	3.2ab
2.5	100.0a	95.7a	84.9ab	4.5a
5	100.0a	90.5a	89.7ab	3.7ab
10	100.0a	80.9a	83.4b	2.8bc
20	100.0a	90.5a	70.5bc	2.8bc
30	100.0a	85.7a	88.9ab	2.3bcd
40	100.0a	85.7a	31.0cd	1.3cd
50	85.7a	85.7a	14.3d	1.2d

Means within a column followed by different letters are significantly different by LSD (*P=0.05*)

All shoots obtained from gamma-irradiated embryos including the control were propagated *in vitro* using SM1 medium to induce new shoots from lateral buds. These shoots were again micropropagated until the 4th generation of shoots (M_1V_4) was obtained for rooting. This new system for mutation induction breeding is the most promising strategy for obtaining new variability or improved horticultural traits particularly in the apomictic fruit species such as lanzones (*Lansium domesticum* L.) and mangosteen (*Garcinia mangostana* L.).

TABLE VIII. EFFECTS OF GAMMA IRRADIATION ON THE AVERAGE SHOOT LENGTHS OF GERMINATING MATURE ZYGOTIC EMBRYOS OF AVOCADO 'SAN FELIX' CULTURED IN LIQUID MS MEDIUM WITH 2 MG/L BAP

Treatment/dose (Gy)	Average shoot lengths in cultured zygotic embryos after		
	7 days	14 days	30 days
0	11.7ab	18.5a	29.2a
2.5	12.2a	17.4a	25.3ab
5	13.2a	19.5a	25.9ab
10	13.5a	19.8a	30.1a
20	6.6bc	11.4b	14.4b
30	5.9c	8.8b	13.1bc
40	5.5c	6.5b	9.0bcd
50	5.2c	7.2b	7.7d

Means within a column followed by different letters are significantly different by LSD (*P=0.05*)

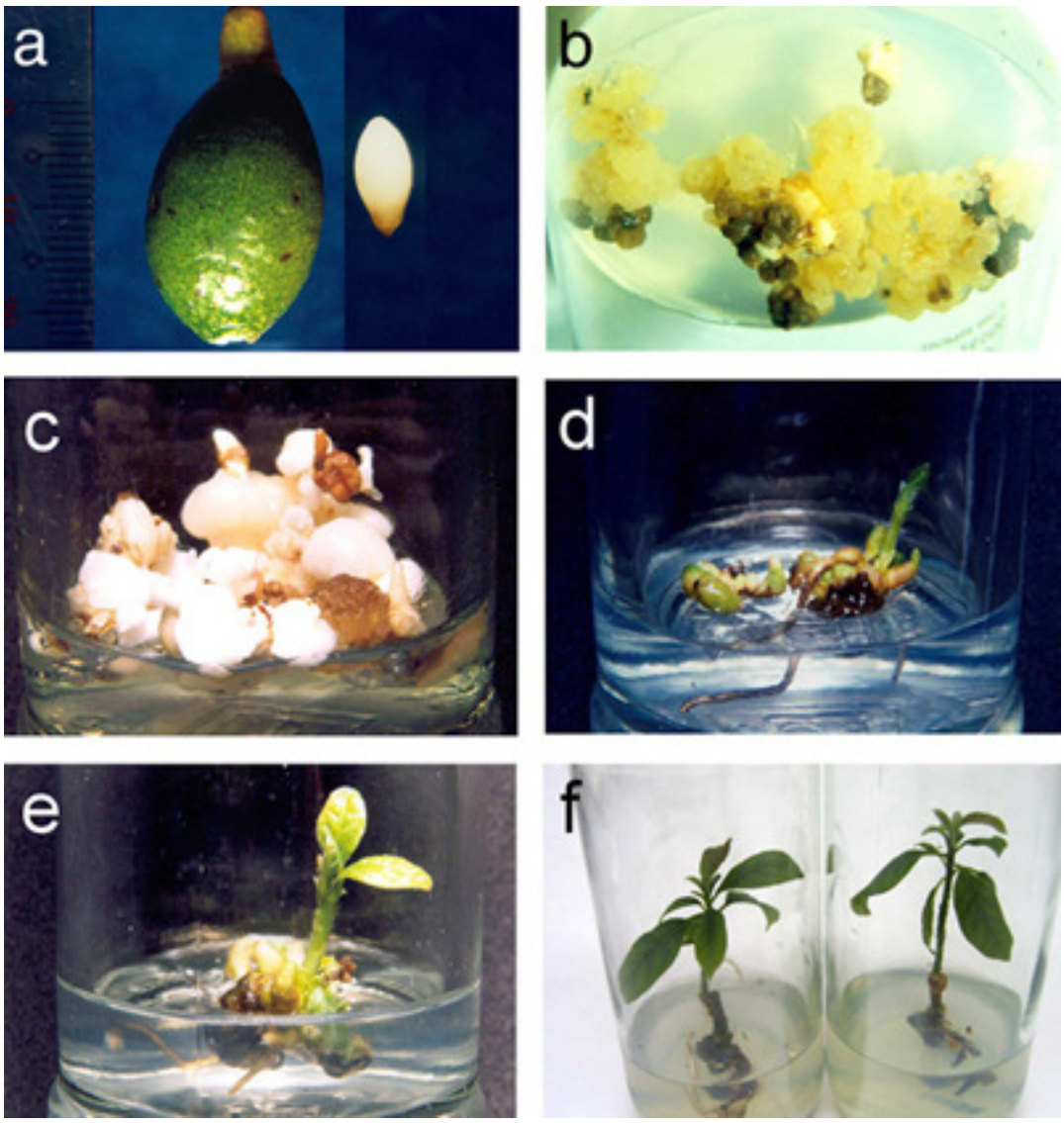

Figure 1. Plantlet/shoot regeneration system from somatic embryos of avocado 'Semil' and the subsequent rescue by micrografting technique. a) Immature fruit and excised zygotic embryo as explant, b) Proliferating embryogenic cultures, c) Cotyledonary stage somatic embryos ready for maturation phase, d-e) Plantlet regeneration from somatic embryos, f) Successfully micrografted regenerants as putative avocado mutant lines.

3.3.2. Proliferation of gamma-irradiated embryogenic cultures

Embryogenic cultures of avocado proliferate fast and regenerate through SEs, which are believed to be of single cell origin and are therefore ideal targets for genetic manipulation. A technique of gamma-irradiating embryogenic cultures of avocado or other fruits for that matter is therefore a potent way of producing useful mutants. This means less risk of obtaining chimaeric plants and a higher probability for mutated cells to express the mutation in the phenotype [31]. In this study, embryogenic cultures of avocado 'Semil' and 'Mainit' were exposed to increasing doses (0, 10, 20 and 30 Gy) of gamma rays. Three weeks after irradiation per cent re-growth as defined by more than 50% increase or proliferation in original explant size was obtained in SE2, SE3 and SE4 media.

Results indicated that percentage re-growth was significantly affected by the dose of gamma rays applied and the media used (Table IX). These effects were consistent for both 'Semil'

and ‘Mainit’ avocado. Per cent re-growth in 10 Gy was higher at 66.7% (actual enhancement) but was comparable with the non-irradiated control with 56.4%. On the other hand, more than 50% reduction in re-growth as compared with the control was observed in 20 Gy at 26.1%. In a related study using avocado cell suspensions, Witjaksono and Litz [32] identified 25 Gy as the dose wherein 50% of the samples proliferated further (proliferation dose 50 = PD_{50}) after irradiation treatment. Moreover, the 8.4% re-growth in 30 Gy was found to be the lowest. Among the 3 media used, SE4 induced significantly higher per cent re-growth of 54% as compared to SE2 and SE3 with 39.2% and 25.0%, respectively.

In this same experiment, the development of advance stages (i.e. torpedo and cotyledonary) of SEs was also observed from the irradiated cultures (Table X). Highly significant effects were obtained due to dose, genotype and their interaction. Significant effects were also noted for dose x media interaction. Percent embryogenic cultures forming advance stages of SEs were significantly lower at 13.3%, 8.3% and 1.7% in 10, 20 and 30 Gy, respectively, as compared with the non-irradiated control with 20% response. Similar to re-growth, 50% reduction in this parameter as compared with the non-irradiated control was observed near 20 Gy. With regards to the media used, highest response was observed in SE2 (16.2%) followed by SE4 (7.9%) and least in SE3 (8.3%). However, no significant difference among the 3 media was observed.

TABLE IX. EFFECTS OF GAMMA RAYS ON PERCENTAGE PROLIFERATION OR RE-GROWTH OF EMBRYOGENIC CULTURES OF AVOCADO ‘SEMIL’ AND ‘MAINIT’ 3 WEEKS AFTER SUBCULTURE IN 3 MEDIA FOR SOMATIC EMBRYOGENESIS

Treatment/dose (Gy)	Genotype	% Re-growth and proliferation in 3 media for somatic embryogenesis			
		SE2	SE3	SE4	Ave.
0	‘Mainit’	53.3	65.0	50.0	56.1
	‘Semil’	60.0	80.0	30.0	56.7
					56.4a
10	‘Mainit’	40.0	83.3	53.3	58.9
	‘Semil’	80.0	90.0	53.3	74.4
					66.7a
20	‘Mainit’	16.7	20.0	3.3	13.3
	‘Semil’	56.7	50.0	10.0	38.9
					8.4c
30	‘Mainit’	3.3	20.0	0	7.8
	‘Semil’	3.3	23.3	0	8.9
Ave.		39.2b	54.0a	25.0b	

SE2= B5 basal medium + 0.1 mg L^{-1} picloram; SE3 = MS + 0.1 mg L^{-1} TDZ and 0.5 mg L^{-1} GA_3; SE4 = MS + 2 mg L^{-1} BAP + 1 mg L^{-1} BAP - Means within a column followed by different letters are significantly different by LSD (*P=0.05*).

3.3.3. Plant/shoot regeneration from resulting somatic embryos

Proliferating embryogenic cultures of 'Semil' after gamma irradiation were subcultured until SC4 (6 months from initiation) in SE2, SE3 and SE4 media to allow formation of sufficient cotyledonary stage SEs. The same protocols and media for the non-irradiated materials were used for maturation and regeneration. Table XI presents regeneration obtained from SEs derived from gamma-irradiated embryogenic cultures. Plantlet/shoot regeneration during the first subculture (SC1) ranged from 4.2 to 10.7% with an average of 6.5%. At SC2 the regeneration response ranged from 4.9% (20 Gy) to 11.1% (10 Gy) with an average of 8.2%. During the final subculture (SC3), percent regeneration ranged from 5.2% (10 Gy) to 6.7% (0 and 30 Gy) with an average of 6.2%. Total percent regeneration from SEs exposed to 10, 20 and 30 Gy was comparable at 20.5%, 17.8% and 26.9%. Non-irradiated control had 18.3% regeneration response. Total average regeneration obtained after 3 subculture cycles was 20.9%. These are comparable to that of non-irradiated embryogenic cultures of 'Semil' reported earlier. Despite the negative effects of gamma irradiation on percent re-growth and advance to cotyledonary stage, there seemed to be no direct negative effect on the regeneration efficiency from the resulting SEs produced from repeated subcultures of gamma-irradiated embryogenic cultures.

TABLE X. EFFECTS OF GAMMA RAYS OF % COTYLEDONARY STAGE SOMATIC EMBRYO FORMATION IN AVOCADO 'SEMIL' AND 'MAINIT' 3 WEEKS AFTER SUBCULTURE IN 3 MEDIA FOR SOMATIC EMBRYOGENESIS

Treatment/ dose (Gy)	Genotype	% Re-growth and proliferation in 3 media for somatic embryogenesis			
		SE2	SE3	SE4	Ave.
0	'Mainit'	6.7	6.7	0	4.4
	'Semil'	56.7	26.7	23.3	35.6
					20.0a
10	'Mainit'	0	0	3.3	1.1
	'Semil'	30.0	20.0	26.7	25.6
					13.3ab
20	'Mainit'	0	0	0	0
	'Semil'	36.7	3.3	10.0	16.7
					8.3b
30	'Mainit'	0	0	0	0
	'Semil'	0	6.7	3.3	3.3
Ave.		16.2a	7.9a	8.3a	1.7c

SE2= B5 basal medium + 0.1 mg/l picloram; SE3 = MS + 0.1 mg/l TDZ and 0.5 mg/l GA_3; SE4 = MS + 2 mg/l BAP + 1 mg/l BAP; Means within a column followed by different letters are significantly different by LSD (*P=0.05*)

There were over 200 plantlet and shoot regenerants from 'Semil' avocado obtained from this regeneration experiment (Figure 2). However, due to the reduced vigor in many regenerants, including the problems associated with long-term shoot cultures, only 82 putative mutant

lines are maintained. These are now being micropropagated followed by micrografting or *in vitro* rooting for greenhouse establishment and genetic analysis to select induced mutants by molecular markers.

TABLE XI. PERCENTAGE PLANT/SHOOT REGENERATION FROM SOMATIC EMBRYOS DERIVED FROM GAMMA-IRRADIATED EMBRYOGENIC CULTURES OF AVOCADO 'SEMIL' IN DIFFERENT REGENERATION MEDIA AND SUBCULTURE CYCLES

Gamma ray dose (Gy)	% Plant/shoot regeneration from somatic embryos			
	SC1 (R1 to R5 media)	SC2 (R4 medium)	SC3 (R4 and R4- media)	Total
0	4.3	7.3	6.7	18.3
10	4.2	11.1	5.2	20.5
20	6.8	4.9	6.1	17.8
30	10.7	9.5	6.7	26.9
Ave.	6.5	8.2	6.2	20.9

3.4. Genetic analysis using molecular markers

3.4.1. Screening for informative SSR markers among avocado mother trees used as sources of explants

Out of the 24 SSR primer pairs, only two (2) failed to amplify distinct SSR fragments among the avocado mother trees. A total of 43 alleles (approx. 2 alleles/SSR) were resolved from the 22 avocado SSR loci (Table XII). Ten SSR loci (24 alleles) are unlinked based on the integrated genetic linkage map of avocado [28]. With one locus per linkage group (LG), the SSR loci are mapped on linkage group numbers 2-5, and 7-12 (Table XII). The three asexually propagated 'Semil' mother trees used as embryo source for embryogenic culture induction are genetically similar based on allele frequency and size.

Based on unlinked SSR loci, the average allele frequency of 'Mainit' differed from 'Semil' by 0.17 or 17% (Table XII). DNA polymorphism between the two avocado cultivars at these loci was 70.8%.

TABLE XII. ALLELE FREQUENCIES OF AVOCADO MOTHER TREES ‘MAINIT’ AND ‘SEMIL’ AT 16 SSR LOCI

Avocado SSR	Alleles (size in base pairs)	Allele frequency			
		‘Mainit’	‘Semil’ 1	‘Semil’ 2	‘Semil’ 3
A1	76, 69	1.0	0.5	0.5	0.5
A2	97, 82, 74	0.7	0.7	0.7	0.7
A3	58, 49, 41	0.7	0.7	0.7	0.7
A4	124	1.0	1.0	1.0	1.0
A5	44, 33	0.5	1.0	1.0	1.0
A6	71	1.0	1.0	1.0	1.0
A7	120	1.0	1.0	1.0	1.0
A8	71	1.0	1.0	1.0	1.0
A9*	153	1.0	1.0	1.0	1.0
A10*	142	1.0	1.0	1.0	1.0
A11	128	1.0	1.0	1.0	1.0
A12*	153	1.0	1.0	1.0	1.0
A13*	64, 54	0.5	1.0	1.0	1.0
A14*	115, 97, 74	0.7	0.3	0.3	0.3
A15	69	1.0	1.0	1.0	1.0
A16*	158, 182	1.0	0.5	0.5	0.5
A17*	542, 108, 93	0.7	0.3	0.3	0.3
A18*	294, 215	1.0	0.0	0.0	0.0
A19*	261, 240, 197, 154, 141	0.4	0.8	0.8	0.8
A20	94, 88	1.0	0.5	0.5	0.5
A21	97	1.0	1.0	1.0	1.0
A22*	257, 250, 222, 203	0.8	0.5	0.5	0.5
Ave. in all 22 loci		0.86	0.76	0.76	0.76
*Ave. in unlinked loci **		*0.81*	*0.64*	*0.64*	*0.64*

*Unlinked SSR loci based on the integrated genetic linkage map of avocado [28]. With one locus per linkage group (LG), the SSR loci are mapped on linkage group numbers 2-5, and 7-12.

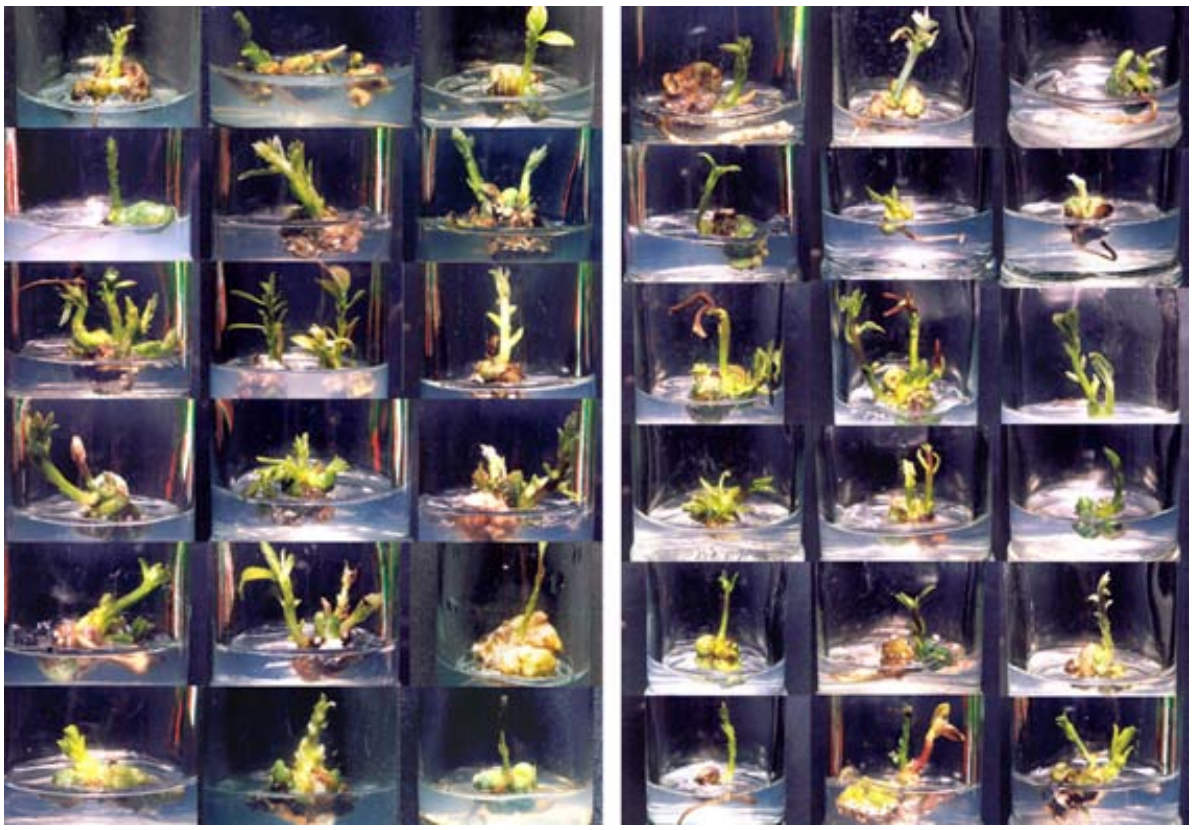

Figure 2. Samples of somatic embryo-derived regenerants from non-irradiated (Left) and gamma-irradiated (Right) embryogenic cultures of avocado 'Semil' which are now being micropropagated/micrografted for greenhouse establishment and genetic analysis.

3.4.2. Preliminary SSR mutation screening among the somatic embryo-derived regenerants of 'Semil' embryogenic cultures

Based on the relative shift of allele frequency among the regenerants from different embryogenic cultures in comparison with 'Semil' mother trees, *in vitro* mutation was noted in six (6) out of 10 microsatellite loci (Table XIII). The frequency of allele mutation at these loci ranged from 0.3 to 0.7, with the highest frequency observed at A12 SSR locus.

In vitro mutation was evident among the regenerants from non-irradiated embryogenic cultures, irrespective of embryogenic culture age (Table XIII). However, data were not conclusive because of the very few sample (DNA) number of *in vitro* cultures that represented the different mutation treatments. In most cases, mutation was marked as the generation of a new allele (band) at the SSR locus. Among the tissue culture regenerants screened, 213a (non-irradiated, 13-month-old embryogenic culture) and 60b (non-irradiated, 8-month-old embryogenic culture) were the apparent mutant regenerants. The regenerants had both an average allele mutation frequency of 0.27 at 10 SSR loci (Table XIII). Interestingly, the microsatellite allele frequency of 38d did not change. However, most of its alleles shifted/changed in bp-size (Table XIII). Regenerant 48.2 had also a shifted allele at A14 locus but without a relative change in allele frequency. As induced by gamma ray irradiation, deletion of alleles were noted at A12 (*m12.1* allele) and A16 (*m16* allele) SSR loci in regenerants 106 (20 Gy) and 232 (10 Gy), respectively (Figure 4; Table XIII).

TABLE XIII. ALLELE FREQUENCIES OF 'SEMIL' MOTHER TREES AND TISSUE CULTURE REGENERANTS, WITH AND WITHOUT GAMMA-IRRADIATION TREATMENT, AT REPRESENTATIVE 10 MICROSATELLITE (SSR) LOCI

Avocado SSRs	Alleles	'Semil' (allele freq)	Frequency of allele change/mutation[a]						
			Non-irradiated. (13-mo.)			Non-irradiated (8- and 13-mo)		Irrad. 10 Gy	Irrad. 20 Gy
			38d	48.2	213a	60b	65b	232	106
A1	69, *m1*	0.5	0.0	0.0	0.5	0.5	0.0	0.5	0.0
A4	124, *m4*	0.5	0.0	0.5	0.5	0.5	0.5	0.5	0.5
A8	71	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A9	*m9,* 153	0.5	-	0.0	0.5	0.5	0.0	0.0	0.0
A10	142	1.0	-	0.0	0.0	0.0	0.0	0.0	0.0
A12	*m12*,15 *m12.1*	0.3	-	0.4	0.7	0.7	0.4	0.4	0.0
A13	64, 54	1.0	-	0.5	0.5	0.5	0.5	-	0.5
A14	115,*m14*	0.5	-	0.0	0.0	0.0	0.0	0.0	0.0
A15	69	1.0	-	0.0	0.0	0.0	0.0	-	0.0
A16	182,158 & *m16*	1.0	-	0.0	0.0	0.0	0.0	0.3	0.0
Ave.		0.73	0.00	0.14	0.27	0.27	0.14	0.21	0.10

[a] Allele mutation = change of allele frequency at the locus in comparison with the explant
- = missing data; score needs verification or no DNA sample

Micrografting on top of *in vitro* root induction among the regenerated shoots is currently being done to rescue somatic embryo-derived regenerants and fast-track growth of all these materials generated following tissue culture and gamma irradiation. Mass screening for mutations using the established molecular markers can be done as soon as the micrografted and rooted putative mutant lines are established in soil. With the promising results in the preliminary genetic analysis on limited regenerants, conclusive evidences on the extent and kind of mutations induced by tissue cultures, gamma irradiation and their interaction effects can be established in avocado for the first time. More importantly, the potential of *in vitro* mutation as a non-conventional breeding strategy can be evaluated, and when relevant, applied towards the improvement of other important and promising tropical fruit crops.

Efforts are now focused on the establishment of putative mutant avocado lines in soil for genetic analyses, and the first ever greenhouse and field evaluation of a woody perennial fruit species derived from tissue culture and gamma irradiation technologies. The generation of potentially useful new information and the confirmation of more confirmed mutants or variants are expected to boost the combined use of plant biotechnology and gamma irradiation as a strategy for strengthening various fruit crops' breeding programs in the Philippines.

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Combining zygotic embryo culture and mutation induction to improve salinity tolerance in avocado (*Persea americana* Mill)

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Abstract. Mutation induction and biotechnological techniques are some of the current approaches used in plant breeding. In the present work radiation-induced mutation followed by *in vitro* culture of zygotic embryos and high osmotic pressure selection methods to improve salt tolerance in avocado are investigated. The *in vitro* germination, rooting, bud multiplication and plantlet acclimatization of Cuban avocado varieties were recorded. The germination rates of whole embryos *in vitro* were found to be higher when using mature rather than immature embryos. Almost 80% of the whole embryos derived plantlets produced were successfully acclimatized under greenhouse conditions. An *in vitro* propagation method for avocado breeding purposes was optimized and documented. However, *in vitro* multiplication results indicated the need to improve bud multiplication methods in avocado. The survival rates of gamma rays irradiated and salt pressured avocado embryos were also investigated. Both mutagenic (LD_{50} = 27-28 Gy) and selective (LD_{20} = 157 mM of NaCl) doses were established. A procedure combining zygotic embryo culture and mutation induction was used to obtain. Putative mutant lines derived from salt tolerant rootstocks were developed. Putative M_1V_3 progenies were planted in the field for segregation analysis. An avocado gene bank was established under the same study. Therefore this methodology appears as an alternative to traditional breeding methods, particularly for improving agronomic characteristics such as salt tolerance in avocado.

1. Introduction

Avocado is an important tree crop originating from Central America whose fruits have been incorporated into the dietary culture of many countries of the world. In spite of its wide acceptance, soil-borne diseases such as *Phytophthora* root-rot and abiotic stress such as salinity have limited the intensification of its production [1, 2, 3]. Limitations to avocado breeding and production are, among others, the long juvenile period, the large area required for cultivation and the extensive natural cross-pollination [4]. In Cuba, breeding efforts have been limited to the selection of desirable genotypes and their *ex situ* conservation for future use in genetic crosses.

Mutation induction techniques are alternative breeding methods, which have been widely used for genetic improvement of major crops, ornamentals and eventually perennial fruit crops [5]. However, studies aimed at improving avocado using mutation induction technique are very scarce. Collaborative efforts were made by the Salvador Sánchez Colin CICTAMEX Foundation and the Institute for Nuclear Research (ININ) of Mexico in order to obtain dwarf and improved architectural genotypes. These studies demonstrated the usefulness of mutation induction to modify plant architecture, vegetative growth, flowering, fruit setting and certain changes in the fruiting behaviour of avocado [6, 7, 8].

In addition to mutation induction, biotechnological techniques appear as important approaches to improve avocado because versatile micropropagation and regeneration systems are now available [9, 10, 11, 12, 13, 14]. It has been also indicated [15] that the combined use of mutation induction and biotechnological techniques is a more effective approach for breeding purposes in plants because it could be a way for selection optimization, shortening breeding schemes and therefore, diminishing costs of breeding efforts. Thus, a FAO/IAEA Coordinated Research Project aimed at improving fruit crops by mutation induction and biotechnology was established in 2004 [16].

We recently initiated a genetic breeding program with the main objective of improving tolerance to biotic and abiotic stresses in Cuban avocado varieties by combining the use of mutation induction and other supportive biotechnology techniques [17, 18]. Some results obtained through from this program are presented as a first effort to create the basis of avocado improvement in Cuba. The work was aimed at: *i)* developing an effective plant micropropagation system of Cuban avocado varieties by of zygotic embryos, *ii)* developing radiosensitivity curves for zygotic embryos of rootstock and commercial cultivars and establish the efficient mutagenic doses, *iii)* developing salt toxicity screening protocol for zygotic embryos of rootstock, and *iv)* establishing *in vitro* methods for selection of mutant lines with salt tolerance in avocado and build the corresponding gene bank.

2. Materials and methods

2.1. Zygotic embryo culture

The fruits were obtained from open-pollinated trees of Duke, Hass, Suardía Estación, Catalina and Jaruco No. 1 varieties located at the Cuban avocado germplasm, Alquizar station, Tropical Fruit Research Institute (IIFT). Genotypes were selected on the basis of their relevance for breeding purpose in Cuba. Duke-7 variety is used as rootstock for *ex situ* conservation and production in Cuba; and the remaining varieties are the most important cultivars in Cuba.

Zygotic embryo cultures were developed as indicated previously [17]. Seeds of different developmental stages extracted from 4 - 43 week-old fruits were used. An embryo was considered as mature when it was extracted from ripe fruits, this criterion was actually genotype dependant. Seeds were dipped into 90% (v/v) ethanol and rapidly flamed for surface sterilization. Aseptic seeds were divided in half and the plumule-radicle axes together with 1 cm-thick sections of cotyledon excised and plated into tubes containing the adequate nutrient medium.

In all experiments, zygotic embryos were placed on filter paper bridges into glass tubes containing 5 ml of MS salt medium [19] diluted to half strength (½ MS) and supplemented with 100 mg/L of i-inositol, 30000 mg/L of sucrose, activated charcoal at 1g/l, pH 5.7 ± 0.1; except for multiplication experiments where 0.5 mg/L of benzylaminopurine (BAP) and gibberellic acid (GA_3) were also added. Four week-old plantlets were transferred to glass pots containing 10 ml of fresh medium without hormones and grown for eight more weeks with a one subculture. Cultures were grown in a climate controlled room with a relative humidity of 60%, temperature of 25 ± 2^{o}C and light intensity of 2500 lx provided by Chiyoda lux fluorescent lamps and measured using a Yu116 Luxometer (Russia). A 16-hour light photoperiod was used.

Three month-old plantlets were transferred to pots containing a mixture of soil, organic matter and charcoal breeze at a v/v ratio of 1: 1: 0.4 for acclimatization before transfer to standard greenhouse conditions. During acclimatization, plants were covered with transparent nylon for two weeks and watered three times per week. The first watering was made using half strength MS salt medium. This step proved to be critical during material the screening for adaptation to salinity stress.

2.2. Radiosensitivity curves

Glass tubes containing mature zygotic embryos of Duke-7 and Hass varieties were irradiated in a dose range between 15 and 50 Gy. Then, the embryos were immediately transferred to new tubes with fresh medium. Irradiation was conducted in a Russian PX-γ-30M ^{60}Co irradiator at 35ºC. The rate dose values ranged between 38-46 Gy/min, estimated by a Fricke dosimeter.

The germination percentages of zygotic embryos were used as criteria to determine variability of sensitivity to gamma rays [18]. This indicator was calculated for each treatment (efficient radiation dose) as the ration: germinated embryos/total number of embryos. At least three independent experiments were developed with a minimum of 40 embryos per each treatment. Embryo survival data were computed for polynomial fit analysis according to the Origin-PC package (Microcal Software, Inc.).

2.3. Salt toxicity curves in rootstock

To establish salt toxicity curves, zygotic embryos of rootstock Duke-7 were placed on filter paper bridges into glass tubes containing 5 ml of MS salt medium supplemented with 100 mg/L of i-inositol, 30g/L of sucrose, activated charcoal 1g/L, the pH was adjusted at 5.7 ± 0.1; and different NaCl concentrations between 25 and 250 mM prepared. The germination percentages of whole embryos were also used as criteria to determine variation of sensitivity to salinity in eight week-old zygotic embryo cultures of the different varieties. This indicator was calculated as previously indicated for radio sensibility curves. At least four independent experiments were performed with a minimum of 40 embryos per treatment. Embryo survival data were computed for polynomial fit analysis according to the Origin-PC package.

2.4. Establishment of in vitro selective methods and gene bank development

On the basis of the mutagenic dose (< LD_{50} value) and selective NaCl concentration (LD_{20} value) calculated from corresponding survival curves (see results), a combined procedure was implemented in order to develop avocado mutant lines with putative improved tolerance to salinity. Control treatments were also included in order to corroborate results obtained in the survival curves. The percentage of entire sprouts for each treatment was estimated as previously indicated. Plantlets were evaluated visually for each treatment and anomalies such as leaf deformation, atrophy and abnormal pigmentation were recorded. Four week-old healthy plantlets were transferred to glass pots containing 10 ml of fresh medium and grown for eight more weeks before transfer to pots for acclimatization. Cultures were grown in a climate controlled room as indicated above.

3. Results and discussion

3.1. Zygotic embryo culture

The *in vitro* response of cultivated zygotic embryos is shown in Table I. For all genotypes, the percentage of germinated entire embryos was higher using mature than immature embryos, in accordance with a previous study [20]. 16 - 34% of the immature embryos did not germinate, while this percentage ranged between 2 and 7% for mature embryos. Nearly 80% of the plantlets obtained by embryo culture techniques have been adapted to greenhouse conditions (Figure 1).

TABLE I. *IN VITRO* RESPONSE OF AVOCADO ZYGOTIC EMBRYOS CULTIVATED IN ½ MS MEDIUM

	Varieties				
	Duke-7	Hass	Suardia Estación	Catalina	Jaruco No. 1
Immature embryos					
Number of cultivated embryos	70	51	50	49	65
Percentage of germinated entire embryos	44	38	40	45	33
Percentage of non-germinated embryos	16	34	29	23	31
Percentage of adapted plantlets[1]	83	80	83	85	86
Percentage of germinated incomplete embryos	26	22	28	38	33
Percentage of contaminated cultures	14	6	5	4	3
Mature embryos					
Number of cultivated embryos	203	99	55	81	63
Percentage of germinated entire embryos	71	71	83	81	80
Percentage of non-germinated embryos	2	5	8	6	7
Percentage of adapted plantlets[1]	80	84	81	89	80
Percentage of germinated incomplete embryos	26	12	4	9	10
Percentage of contaminated cultures	1	12	5	4	3

(1) Based on number of germinated entire embryos

According to the literature, germination rates of *in vitro* propagated avocado depend on the genotype employed, the type of explant, the level of salts in the medium and the hormone concentration in the medium. It was found that *in vitro* propagated embryos of Fuerte variety in ½ MS medium supplemented with 0.5 mg/ml of BAP showed a germination percentage between 4 and 66% depending on embryo maturity [21]. Rodríguez *et al.,* [20], using identical experimental conditions, observed that embryo germination of Hass, Suardia Estación and Catalina varieties were near to 30%. However, when the medium was supplemented with 0.5 mg/ml of BAP and GA_3 this rate was found to increase up to 46%.

Zygotic embryo culture was limited by the number of germinated incomplete embryos and by contamination. The percentage of germinated incomplete embryos per genotype was always higher when immature embryos were used, except for Duke-7 variety, whose mature embryos

were of small size and therefore too difficult to manipulate. In immature embryos, the plumule-radicle axis was easily broken when it was excised from cotyledons. This was a serious limitation to the experimental procedure.

On the other hand, culture contamination rates were genotype dependant on and ranged between 1 and 14%. In fact, it has been shown that *in vitro* contamination could be high when axillary buds are used as explants in avocado micropropagation [22, 23]. In these studies, fungicide application and strict control of humidity during culture were necessary for the control of contamination. Our results indicated that the application of ethanol at 95% as disinfecting agent was effective to ensure the production of sterile buds from zygotic embryos.

In order to establish a useful micropropagation method, the *in vitro* multiplication rates of three avocado genotypes were studied (Table II). Multiple bud induction appeared to be closely genotypes dependant. Duke-7 variety showed a lower multiplication response. In general, the average number of induced buds was lower for mature (ranging between 2 and 4) than for immature cultivated embryos (ranging between 3 and 6), in accordance with a previous study [20]. Plantlets derived from the subculture of those buds showed poor rooting and adaptability capacities.

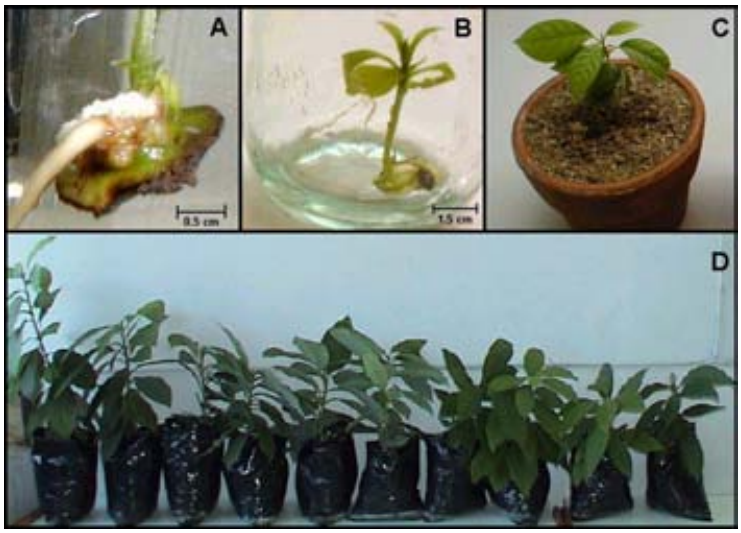

Figure 1. A-B, Avocado embryos cultivated on 1/2 MS salt medium supplemented with 0.1 g/l of i-inositol, 30 g/l of sucrose, activated charcoal 1g/l. C, Three month-old plantlets of Duke-7 variety adapted on organic substrate. D, Six month-old plantlets of Duke-7 variety adapted to greenhouse conditions as indicated in Materials and Methods.

TABLE II. EFFECT OF THE HORMONES BAP AND GA_3 AT 0.5 MG/L ON THE *IN VITRO* MULTIPLICATION RESPONSE OF CULTIVATED ZYGOTIC EMBRYOS

	Varieties		
	Duke-7	Hass	Suardia Estación
Immature embryos			
Percentage of culture with multiple buds	31	46	55
Number of buds per cultivated embryo[1]	3,3 ± 0,1	6,0 ± 0,3	6,3 ± 0,4
Mature embryos			
Percentage of culture with multiple buds	80	83	80
Number of buds per cultivated embryo[1]	2,0 ± 0,1	4,1 ± 0,2	3,2 ± 0,3

(1) Average values and standard errors are presented.

Skene and Barlass [21] have shown that mature embryos cultivated in ½ MS medium supplemented with 0.5 mg/ml of BAP produced between 4-5 buds per embryo. Multiplication rate of cultivated axillary buds in MS medium supplemented with 0.65 mg/ml of BAP was between 2.3 and 3.7 [24, 25]. This index was 3 when buds were cultivated in Woody Plant Medium supplemented without hormones, but it was zero in the presence of 1 mg/l of BAP [22]. Biasi *et al.,* [26] also demonstrated a linear dependence between the concentration of cytokinin and the multiplication rate of cultivated axillary buds in a range between 0 and 4 mg/ml. However, BAP or kinetin concentration equal to or higher than 4 mg/ml produced tissue vitrification, as per previous studies [24, 27]. All these studies, including our data, provide evidence that the practical utility of these methods is still limited and could be further optimized.

3.2. Radiosensitivity curves of mature zygotic embryos

Radio sensibility curves for the Duke-7 and Hass varieties are shown in Figure 2. Inhibition of the fraction of germinated entire embryo depended on the radiation dose, according to the equation $Ln(x) = a + b_1x + b_2x^2$, where Ln (x) is the logarithm of the fraction of germinated entire embryo, x is the radiation dose; and a, b_1 and b_2 are the equation parameters (Table III). The fit of the experimental data to the theoretical model was equal to 0.95 and 0.96 for Duke-7 and Hass radio sensibility curves, respectively. In contrast to the performance observed in radio sensibility curves based on the fraction of germinated entire embryo, the variation of the other plantlets morphological descriptors, as indicated in material and methods, was not correlated with an increased radiation dose (data not shown), which demonstrates their inadequacy as indicators of the toxicity produced by radiation. Interestingly, other qualitative indicators such as leaf and root anomalies, atrophy and chlorophyll-deficient sprout and albinism were observed at doses higher than LD_{50} values. However, quantification of these indicators was not possible because the percentage germination of entire embryos dramatically decreased for doses higher than LD_{50}.

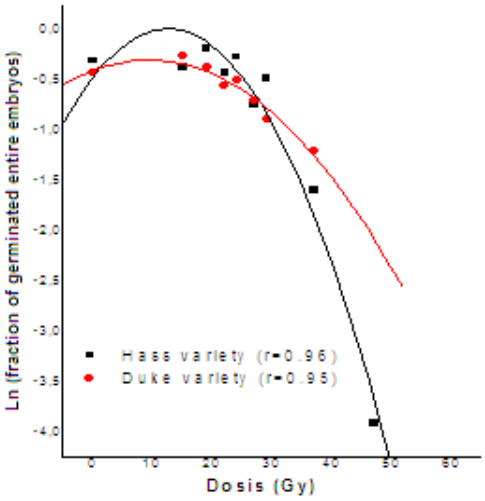

Figure 2. Effect of gamma rays on survival of avocado embryos of Duke-7 and Hass varieties

TABLE III. FIT AND EQUATION PARAMETERS OBTAINED FROM POLYNOMIAL ANALYSIS

		Equation parameters		
Varieties	R-Square	a	b_1	B_2
Duke-7	0.95 *	-0.3943	0.0166	0.0009
Hass	0.96 *	-0.4711	0.0775	0.0004

(*) Significant for $p < 0.0001$

Based on the equations parameters, lethal media dose (LD_{50}) values, here defined as the dose at which the 50% of the fraction of germinated entire embryos is obtained, were calculated to be 28 and 27 Gy for Duke-7 and Hass varieties, respectively. Doses values lower than LD_{50} were not toxic for both varieties and did not significantly change the variety performance in culture (Table IV). *In vitro* germination, rooting and contamination levels of cultured zygotic embryos were very similar for non-irradiated and irradiated embryos. This result suggested a similar sensibility to gamma rays for both varieties, however; doses higher than LD_{50} showed that the Hass variety was more susceptible to lethal effects of radiation.

TABLE IV. *IN VITRO* RESPONSE OF IRRADIATED[1] AND NON-IRRADIATED EMBRYOS OF THE DUKE-7 AND HASS VARIETIES CULTIVATED IN ½ MS MEDIUM

	Non-irradiated embryos		Irradiated embryos	
	Duke-7	Hass	Duke-7	Hass
Number of cultivated mature embryos	203	99	698	317
Percentage of germinated entire embryos	71	71	52	76
Percentage of non-germinated embryos	2	5	7	3
Percentage of germinated incomplete embryos	26	12	35	9
Percentage of contaminated cultures	1	12	6	12

(1) Considering only irradiated embryos at doses lower than LD_{50} values

Sánchez-Colín et al., [6] have previously established the LD_{50} values between 20 and 40 Gy for avocado ecotypes based on the loss of grafting capacity of irradiated avocado scions. Using the same criteria, an LD_{50} value of 30 Gy for Hass variety has been also reported [28]. The results obtained here with the Hass variety suggested that zygotic embryos are slightly more sensitive to gamma rays than scions, maybe due to differences of radio sensibility for both tissue types and higher moisture content in embryos than in scions. The effect of moisture content on radio sensibility of avocado varieties has been previously demonstrated [7].

3.3. Salt toxicity curves in rootstock

The Duke-7 zygotic embryos cultures under salinity conditions were characterized by a significant increase in anomalies such as embryos with multiple buds, plantlets with abnormal leaf development (vining growth, abnormal-pigmentation and deformations), somatic embryogenesis and secondary and aerial roots (Figure 3).

Saline concentration higher than 150 mM NaCl was found to be very toxic to Duke-7 embryos and no anomalies were observed because of the limited number of zygotic embryos that survived these stress conditions. The number of embryos with multiple buds was duplicated with respect to the control (absence of NaCl) at saline concentrations of 25 and 50 mM, while the number of plantlets with abnormal leaf development tripled those obtained in the control treatment (Table V). The "vining" leaf was the most frequent abnormality observed in this study. Interestingly, secondary or aerial roots were observed only in saline treatments. Somatic embryogenesis was not a significant event under these conditions and was not quantified.

TABLE V. *IN VITRO* RESPONSE OF DUKE-7 ZYGOTIC EMBRYOS CULTIVATED UNDER SALINITY CONDITIONS

	NaCl (mM)						
	0	25	50	100	150	200	250
Number of cultivated embryos	79	79	73	65	52	50	55
Percentage of germinated entire embryos	87	70	59	52	15	-	-
Percentage of zygotic embryos with multiple buds [1]	7	14	13	6	-	-	-
Percentage of plantlets with leaf abnormal development [2]	7	19	21	11	-	-	-
Percentage of plantlets with secondary or aerial roots [1]	0	12	25	7	-	-	-

(1) Calculated respect to the number of germinated entire embryos; (2) although different leaf anomalies were observed, only the climbing leafs were considered to calculate percentage values.

A survival curve for the Duke-7 variety is shown in Figure 4. Inhibition of the fraction of germinated entire embryos depended on the saline concentration, according to the equation $Ln(x) = a + b\,x$, where $Ln(x)$ is the logarithm of fraction of germinated entire embryos, x is the NaCl concentration (mM); and $a = -0.0865$ and $b = -0.0108$ being the intercept in Y and scope, respectively. The fit of experimental data and theoretical model was equal to 0.96 ($p < 0.008$). Based on the equation parameters, lethal media dose (LD_{50}) values were calculated as 56 mM of NaCl for the Duke-7 variety. Doses values higher than LD_{50} were clearly toxic for

Duke-7 zygotic embryos. A concentration of 157 mM NaCl corresponding to LD_{20} value was selected as a selective saline dose for the breeding procedure (see below) because this value was highly toxic for Duke-7 embryos.

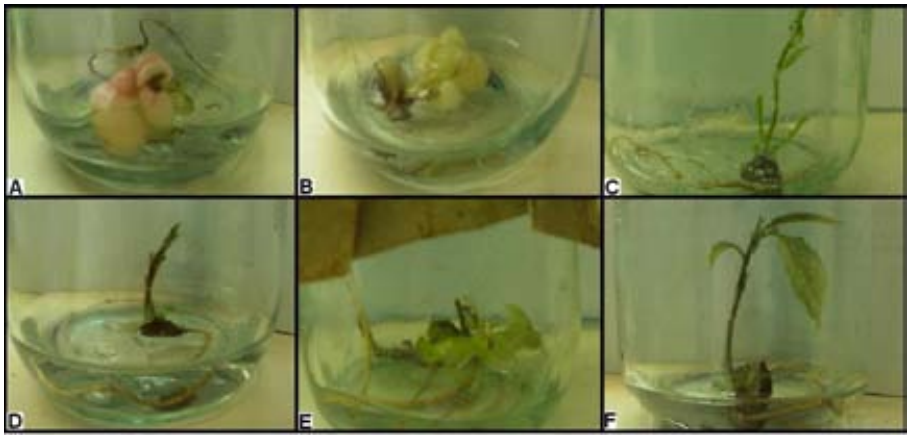

Figure 3. Plantlet anomalies induced by salinity condition in culture. (A and B) deformed leafs and abnormal pigmentation, (C) multiple sprouts, (D) climbing leaf, (E) secondary and aerial roots and (F) healthy plantlet.

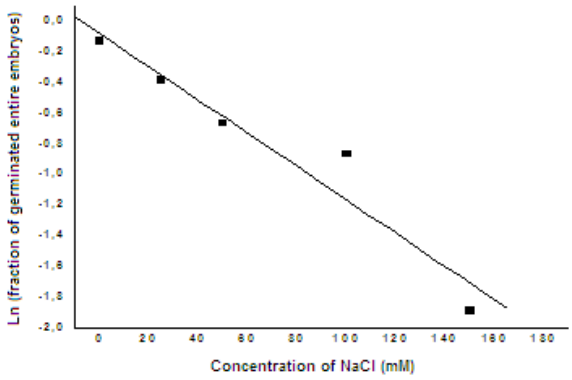

Figure 4. Effect of saline concentration on survival of avocado embryos of Duke-7 variety.

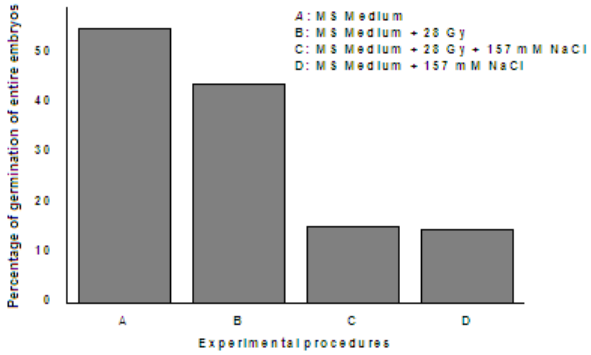

Figure 5. In vitro response of Duke-7 zygotic embryos in different treatments

3.4. Combining mutagenic and selective saline doses in the same approach

The treatments were as follow: A) Culture of zygotic embryos in MS salt medium for four weeks for plantlet induction, B) Culture of zygotic embryos irradiated at mutagenic dose in MS salt medium for four weeks for plantlet induction, C) Culture of zygotic embryos irradiated at mutagenic dose in MS salt medium supplemented with 150 mM of NaCl for four weeks for plantlet induction, D) Culture of zygotic embryos in MS salt medium supplemented with 150 mM of NaCl for four weeks for plantlet induction. Embryos were sub-cultured in fresh medium and grown during eight weeks and later acclimatization and growing under greenhouse conditions was done as indicated in materials and methods.

The usefulness of the culture of zygotic embryo method for selecting mutant lines from Duke-7 rootstock tolerant to salinity conditions was evaluated as shown in the procedure C (Figure 5) by comparison with control treatments (procedures A, B and D). In the procedure C, the mature zygotic embryos irradiated at mutagenic dose (28 Gy of gamma rays) were cultured in MS salt medium supplemented with 150 mM of NaCl during twelve weeks. Those plantlets that survived and developed healthily under these conditions were considered as putative resistant mutants for salinity. The percentage germination of entire embryos obtained in procedures C and D was very similar; however the number of "healthy plantlets" recovered from C was eventually higher than from D. These "healthy plantlets" were adapted under greenhouse conditions and the putative M_1V_3 mutant lines were planted in field conditions for later segregation analysis (Figure 6). Some of this material has shown a delay in development and semi-dwarfness with respect to the non-irradiated control, however, with respect to salinity, it will be necessary to evaluate these characters in segregating populations.

Figure 6. Juvenile putative mutant trees under greenhouse (A) and field (B) conditions.

4. Conclusions

In the present study, zygotic embryo culture combined with mutation induction was implemented in a breeding experiment, with the aim of investigating improvement of salinity resistance in rootstock avocado varieties. This methodology appears as excellent an alternative to conventional breeding methods for the improvement of important characteristics, where *in vitro* selection methods could be applied. It has been indicated earlier [15, 16] that the use of *in vitro* techniques such as anther/microspore culture, shoot organogenesis and somatic embryogenesis can overcome some of the limitations in the application of mutation techniques, such as the lack of effective mutant screening techniques and the unrealistically large, but necessary, size of the mutated population, calculated on the basis of an expected frequency of mutation for a desired character. Here, putative M_1V_3 mutant lines were planted under field conditions for later segregation analysis for salinity tolerance. Thus a mutant gene bank was established. However, the putative tolerance could be evaluated in the M_2 progenies by both using *in vitro* selective procedures indicated in the present work, and by mutant field performance under severe salinity conditions. Effective multiplication methods still need to be developed for avocado. Although an *in vitro* micropropagation method has been reported in avocado [24], its potential usefulness for breeding purposes needs to be evaluated. Somatic embryogenesis could be an interesting alternative for this purpose [14] since it avoids chimera development when mutation induction is used. However, according to our results this requires further research efforts.

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Regeneration from irradiated avocado (*Persea americana* Mill.) embryogenic cultures

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Abstract. Somatic embryogenesis was induced from zygotic embryos excised from immature avocado fruit from selected genotypes grown in the highlands of Cisarua, West Bogor, Indonesia. The pro-embryonic masses developed first on semi-solid medium and were then transferred to liquid cultures for proliferation. The embryogenic masses were then irradiated at 9, 18 and 35 Gy using a ^{60}Co irradiation source. 3 sub-cultures in liquid medium ensured adequate proliferation prior to transfer to fresh development medium. After 1-3 months, somatic embryos with more than 0.5 cm in diameter were transferred to a germination medium, while the smaller somatic embryos (<0.5 cm in diameter) were sub-cultured one more time for additional growth. After 1-2 months on germination medium, plantlets were transferred individually to new medium.

1. Introduction

Biotechnology and induced mutation are valuable tools for enhancing genetic variability of vegetatively propagated crops, including avocado, which has not benefited much from conventional breeding. Induced mutation was used to develop the rootstock variety 'D9' from irradiated 'Duke' and has a high level of tolerance of root-rot disease [1]. Irradiation of budwood has resulted in variability with respect to plant stature, flowering time, fruit-setting, etc [2]. Irradiation of embryogenic cultures may be more efficient for recovery of mutants than budwood irradiation. A protocol for avocado somatic embryogenesis has been reported by several authors [3, 4, 5]. The protocol for irradiation of avocado embryogenic cultures and recovery of the plantlets from irradiated cultures has also been described [6, 7, 8]. Plants from irradiated cultures have been regenerated (Litz and Raharjo, 2004, personal communication). In this paper we describe somatic embryogenesis of Indonesian avocado genotypes, and recovery of plantlets from irradiated and non-irradiated embryogenic cultures.

2. Materials and methods

2.1. Embryogenic avocado cultures from the highlands

Embryogenic cultures were induced according to standard protocols [3, 4, 5]. Fruitlets were harvested from trees in dooryards in the district of Cisarua (800-1100 m above sea level), West Java. Trees bearing many fruitlets were used. The fruitlets 0.3-0.6 cm long were cleaned of flower parts., disinfested with 15% commercial bleach, rinsed twice with sterile distilled water and dissected longitudinally in a laminar air flow hood. The ovule halves were plated on 10 mL B5P induction medium (Table I) in glass bottles, capped with aluminum foil and secured with rubber bands. They were maintained under diffuse light in plastic boxes. Secondary SEs or PEMs appeared after 2 weeks. The secondary SEs were subcultured onto MSP maintenance medium (Table I) until rapidly growing secondary SEs and/or PEMs were obtained. The responding explants were scored as follows: the donor trees were represented

by the first number and the responding explants were given a second number in sequence of the appearance of secondary SEs or PEMs. The PEMs were subcultured on MSP maintenance medium [4, 5] in Petri dishes sealed with Saran Wrap and maintained in plastic boxes under diffuse light at 25°C.

Suspension cultures were initiated according to [5], using 0.5-2 g PEMs from 8-15 day old cultures on MSP medium. Liquid maintenance medium was modified MSP medium (MS3: 1NP) [5]; 35 ml medium were dispensed into 100 ml Erlenmeyer flasks. Rapidly growing suspension cultures consisting of PEMs and/or SEs were subcultured biweekly. The cultures were grown on a gyratory shaker at 120 rpm at 25°C under diffuse light. After 2-3 subcultures, the PEMs/SEs were irradiated.

2.2. Irradiation of embryogenic cultures

The PEMs/SEs from 2-week-old suspensions of 1-3 month old cultures were decanted and spread over MSP semi-solid medium overlaid with sterilized filter paper [8] and sealed with Saran Wrap. There were 5 embryogenic culture lines. The cultures were irradiated from a ^{60}Co irradiation source: 9, 18 and 36 Gy, which represented proliferation doses (PD) of 87.5, 75 and 50%. The irradiated cultures with inoculums of 0.5-1 g were proliferated for 1-3 passages in MS3:1P medium depending on the rate of proliferation [8]. Irradiated cultures were transferred to embryo development medium.

2.3. Somatic embryo development

SEs/PEMs suspension cultures (2-4 flasks of each irradiation dose of each embryogenic lines) were transferred to SE development medium [5, 7]. The PEMs/SEs were transferred into a Petri dish lined with 4-6 layers of sterile tissue paper for 2-3h to allow them air-dry. Air-dried PEMs/SEs were transferred to SED medium in bottles capped with plastic stoppers, sealed with Saran Wrap and maintained in plastic boxes under diffuse light at 25°C. After 1-2 months approximately 0.5 cm diameter opaque SEs were transferred to SEG medium, while the smaller SEs (<0.5 cm diameter.) were subcultured into SE enlargement (SEE) medium. There were 3-5 SEs in each vessel. SEE medium has the same composition as SED medium except that the gelling agent was reduced to 3 g L^{-1}. After 2 months of culture, SEs in SEE medium developed shoots, and were not transferred to germination medium.

2.4. Somatic Embryo Germination

SEs of ≥0.5 cm in diameter from SED medium were transferred to SEG medium (Table I). There were 3-5 SEs per vessel. The cultures were maintained in darkness at 25°C until shoots emerged. SEs with more than 1 cm in diameter and without shoots were dissected and the shoot meristem region transferred to fresh SEG medium.

2.5. Plantlet Regeneration

SEs with shoots of more than 0.5 cm long from SED or SEE medium were transferred individually into test tubes containing 25 ml avocado shoot proliferation (ASP) medium (Table I), containing 0.1 mg/l BA or 0.1 mg/l IBA or a combination of 0.1 mg/l BA and 0.1 mg/l NAA. The cultures were capped with vented plastic film (Suncap^{R}, Sigma) and maintained under light (90-110 µmol m^{-2} s^{-1} with a photoperiod of 16/8 h) at 25°C. SEs were transferred to fresh medium every 6 weeks. Multiple shoots >1 cm long were excised and sub-

cultured to the same medium but with 0.1 mg L^{-1} BA and 0.1 mg L^{-1} NAA (ASE medium) for elongation.

TABLE I. MEDIA COMPOSITION FOR AVOCADO SOMATIC EMBRYOGENESIS

Components	B5P[a]	MSP[b]	LM[c]	SED[d]	SEE[e]	SEG[f]	ASP[g]	ASE[h]
Major Salts (mg l^{-1})								
NH_4NO_3	0	1650	1200	1650	1650	800	800	800
KNO_3	2527.5	1900	3030	1900	1900	2200	2200	2200
$(NH_4)_2SO_4$	134	0	0	0	0	0	0	0
$CaCl_2$ $2H_2O$	150	440	440	440	440	440	440	440
$MgSO_4$ $7H_2O$	246.5	370	370	370	370	370	370	370
KH_2PO_4	0	170	170	170	170	170	170	170
NaH_2PO_4	150	0	0	0	0	0	0	0
MS Minor Salts (mg l^{-1}l)								
KI	0.830	0.830	0.830	0.830	0.830	0.830	0.830	0.830
H_3BO_3	6.200	6.200	6.200	6.200	6.200	6.200	6.200	6.200
$MnSO_4$ H_2O	16.900	16.900	16.900	16.900	16.900	16.900	16.900	16.900
$ZnSO_4$ $7H_2O$	8.600	8.600	8.600	8.600	8.600	8.600	8.600	8.600
Na_2MoO_4 $2H_2O$	0.250	0.250	0.250	0.250	0.250	0.250	0.250	0.250
$CuSO_4$ $5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CoCl_2$ $6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Organic Addenda (mg l^{-1})								
Thiamine HCl	4	4	4	4	4	4	4	4
myo-Inositol	100	100	100	100	100	100	100	100
Sucrose	30000	30000	30000	45000	45000	30000	30000	30000
Liquid Coconut Endospem[i] (%)	0	0	0	10	10	0	0	0
Plant Growth Regulator (mg l^{-1})								
Picloram	0.1	0.1	0.1	0	0	0	0	
NAA	0	0	0	0	0	0	0	0.1
BA	0	0	0	0	0	1	1	0.1
GA_3	0	0	0	0	0	10	0	0
Inert Support (g l^{-1})								
TC Agar	8	8	0	6–7	6–7	0	8	8
Gelrite	0	0	0	0	0	3	0	0

[a]B5 [9] induction medium.; [b] Maintenance medium; [c] Liquid Maintenance medium; [d] SE Development medium; [e] SE Enlargement medium; [f] SE Germination medium; [g] Shoot Proliferation medium; [h] Shoot Elongation medium; [I] Filter sterilized medium.

3. Results and discussion

3.1. Induction and maintenance of embryogenic avocado cultures

Embryogenic cultures consisting of either secondary SEs or PEMs were induced from globular and translucent embryos (Figure 1A, B). The induction frequency of 16 avocado genotypes was 5-52%. Some cultures consisted of PEMs (Figure 1C). Other cultures initially consisted of slowly proliferating secondary SEs, which became prolific after 4-6 subcultures.

Rapidly proliferating PEMs were subcultured on MSP medium, and were used to initiate liquid cultures after 8-14 days. The SEs/PEMs of 5 embryogenic lines (Figure 1D) were irradiated and maintained in suspension for 1-3 subcultures.

TABLE II. EMBRYOGENIC RESPONSE ON INDUCTION MEDIUM AFTER 2 MONTHS

Genotype	No. Explants	No. explants Contaminated	No. Embryogenic Explants	% Induction
10	135	37	37	37.8
11	90	30	19	31.7
14	85	39	25	54.3
15	70	17	4	7.6
16	75	17	15	25.9
17	90	21	20	29.0
18	72	24	7	14.6
19	69	13	10	17.9
20	90	26	13	20.3
21	90	26	6	9.4
22	62	22	2	5.0
23	70	12	14	24.1
24	70	32	3	7.9
25	75	34	14	34.2
26	75	35	21	52.5
27	62	21	19	46.4

3.2. Somatic embryo development from irradiated and non-irradiated cultures

After 1-3 months on SED medium, more than 4,000 SEs from 5 different embryogenic lines and different irradiation levels had developed. Opaque SEs over 0.5 cm in diameter. (Fig 1E) were transferred to SEG medium, while the smaller SEs (less than 0.5 cm in diameter.) were transferred to SEE medium for enlargement. The small SEs failed to enlarge, and became necrotic or dedifferentiated on SEE medium.

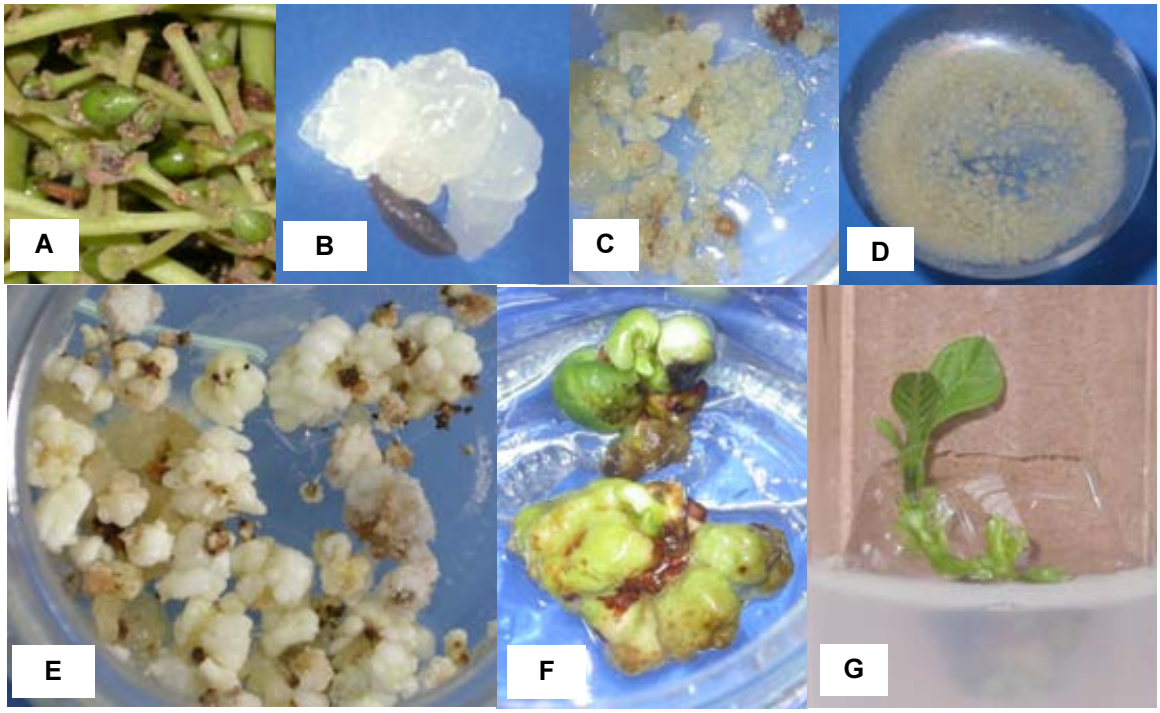

Figure 1. Somatic embryogenesis. A. fruitlet, B. responding explant forming secondary SEs, C. PEMs, D. suspension cultures, E. SEs, F. SE shoot, G. plantlet.

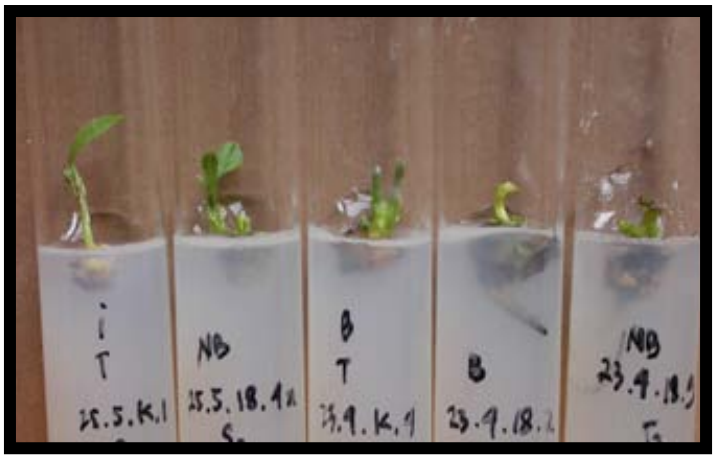

Figure 2. Plantlet regeneration from SEs from irradiated and non-irradiated avocado cultures. 25.5 indicates tree no 25 and the 5th ovule forming embryonic cultures, K= control, 18 = 18 Gy, the last number is the genotype number.

TABLE III. SUMMARY OF THE NUMBER OF CULTURES AND SES THAT DEVELOPED ON SED AFTER 3 MONTHS

Embryogenic line	Irradiation level (Gy)	No. of culture	total SE
23.4	0	59	354
	18	56	262
	35	20	68
25.5	0	56	1456
	9	38	76
	18	99	1683
	35	42	120
27.3	9	20	60
	18	63	189
	35	21	42
10.2	0	15	195
	18	18	90
	35	11	22
22.1	0	43	86
	9	16	64
	18	6	0
	35	5	5
total		609	4772

3.3. Somatic embryo enlargement and germination

After 2-8 weeks, shoots with bract-like structures emerged in some SEs (Fig 1F) The SEs were yellow but were green if exposed to light. On SEE medium, they enlarged and some developed shoots regardless of the size. The percentage of shoot formation from the embryogenic lines was low, i.e., 2.5% (Table IV).

3.4. Plantlet recovery

SEs with shoots >0.5 cm were transferred individually to tubes. The growth of these SEs was variable (Figure 2) multiple shoots with or without scaly leaves, rosette internodes with scaly leaves, elongated and thin stems, fast and slow growing, etc. Rapidly growing shoots were subcultured on the same medium for multiplication. After several subcultures, the growth of shoots was slow and callus formed at the bases of shoots. Shoot-developing SEs and individual shoots were transferred to fresh ASE medium (Table I), for shoot and leaf development. Nine regenerants had shoots that multiplied.

Somatic embryogenesis of avocado is routine and followed existing protocols [3,4]. Embryogenic cultures were established from local West Indian or West Indian x Guatemalan hybrids. The induction of embryogenic cultures [3] was high and several prolific cultures were established either on semisolid or in liquid medium. These cultures were irradiated [8]. Several thousand SEs were obtained and shoots were recovered. Shoot development in enlargement medium without GA_3 occurred even with small SEs (<0.5 cm diameter.). Overall, the rate of shoot development from SEs was low, i.e., 2.5%, whereas 36% of SEs of 'Fuerte' developed shoots [8]. This low rate can be overcome by screening and selecting genotypes that regenerate at high frequency.

TABLE IV. SHOOT DEVELOPMENT FROM SES ON SEE AND SEG, AFTER 1-3 MONTHS

Embryogenic line	Gy	SEG Medium			SEE Medium			Total shoots
		No. culture	Total SEs	No. SE with shoot	No. cultures	Total SEs	No SE with shoot	
23.4	0	22	66	5	41	164	3	8
	18	96	192	11	14	70	2	13
	35	19	38	1	10	30	0	1
25.5	0	1	3	0	172	860	4	4
	9	0	0	0	5	30	0	0
	18	117	498	2	87	609	5	7
	35	0	0	0	0	0	0	0
27.3	9	10	30	4	5	0	1	5
	18	37	74	4	18	7	4	8
	35	36	72	15	3	10	5	20
10.2	0	4	0	0	17	85	1	1
	9	0	0	0	0	0	0	0
	18	4	12	2	4	24	2	2
	35	4	8	0	3	6	1	1
22.1	0	0	0	0	11	22	0	0
	9	6	18	2	2	16	0	2
	18	0	0	0	0	0	0	0
	35	0	0	0	1	1	0	0
Total Number (percentage)		356	1011	46 (4.5%)	393	1934	28 (1.4%)	74 (2.5%)

TABLE V. THE NUMBER OF REGENERANTS AND SHOOTS FOR EACH SE FROM DIFFERENT EMBRYOGENIC LINES 7 MONTHS AFTER IRRADIATION WITH ^{60}CO-γ RAY AT DIFFERENT LEVELS

Line	Gy	No. regenerants	No. shoots/ regenerant [1]
23.4	0	8	12 (1), 7 (1), 4 (1), 2 (1), 1 (4)
	18	13	9 (1), 3 (2), 1 (10)
25.5	0	5	1 (5)
	18	8	2 (2), 1 (6)
27.3	9	4	7 (1)
	18	8	2 (2), 1 (6)
	35	19	35 (1), 7 (1), 2 (2), 1 (15)
10.2	0	2	2 (1), 1 (1)
	18	4	5 (1), 1 (3)
22.1	9	2	1 (2)
	35	1	1 (1)
Total regenerants		74	

[1]Number of regenerants in parentheses

The variability of SEs that developed shoots may be due to the size of the SE, genotype and their interaction with culture medium and mutagenesis. Altering the plant growth regulator

composition of the medium improved the growth of slow growing shoots and rosettes. SE shoots were rooted using an established protocol [10]. Plants were not recovered, although shoots from SEs can be rescued by micrografting [11]. Raharjo et al. [12] have induced roots from micrografted scions by air layering.

The efficacy of mutation induction using the embryogenic pathway for producing genetic variability remains uncertain since no plantlets have been established in soil. In vitro morphological differences were probably caused by culture conditions.

4. Conclusion

The protocol of somatic embryogenesis and irradiation of in vitro cultures was effective for producing plantlets and shoots from plant materials. The large number of regenerated plantlets and shoots could provide enough materials for morphological and molecular analysis of the mutants. The regenerated plantlet may need to be micrografted to ensure their survival.

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CHAPTER II

Improvement of exotic tropical fruit trees through radiation induced mutation

Radiation-induced mutation breeding of papaya

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Abstract. Irradiation-induced mutation breeding of papaya commenced at the Malaysian Agricultural Research and Development Institute (MARDI) in August, 2000. This research was initiated under a Coordinated Research Project (CRP - D23023) with assistance from the International Atomic Energy Agency (IAEA). In the preliminary dosimetry study, seeds from two local papaya varieties, Sekaki and Eksotika were irradiated, either as dry seeds or as pre-soaked seeds (soaked overnight in water and surface-dried) with radiation doses ranging from 0 to 300 Gy. 100 Gy dose was lethal for all wet pre-soaked seeds while dry seed did not show loss of viability, even at 300 Gy. From the growth data it was estimated that dose of 525 Gy reduced shoot elongation by 50%, and this dose was recommended for mass irradiation of dry seeds. For wet, pre-soaked seeds results indicated that 42.5 Gy was the optimal dose for mass irradiation. At this dose, both seeds germination and seedlings growth were reduced by 50%. In a massive irradiation experiment 2,000 Eksotika seeds were irradiated at 42.5 Gy (pre-soaked) and another 2,000 at 525 Gy (dry). In the M_2 population, numerous physiological defects were observed, including stem splitting, leaf variegation and puckering, and crinkled dwarfs. In the M_3 population, a wide variability was recorded for a number of traits. M_3 seedlings derived from pre-soaked seeds irradiated a low 42.5 Gy dose presented a high number of plants that were shorter and more vigorous in leaf development compared to those irradiated at 525 Gy and to non-irradiated control seedlings. The distribution patterns of M_3 progenies for nine quantitative field characters showed great variation, often exceeding the limits of the control population. There appears to be good prospects in improving Eksotika papaya especially in the development of dwarf trees with lower fruit bearing stature, higher total soluble solids in fruits and larger fruit size. Several M_2 and M_3 putative mutants also showed very good resistance to malformed top disease. However, no resistance to papaya ringspot virus disease was found in the 1920 M_2 seedlings that were inoculated.

1. Introduction

Papaya (*Carica papaya* L.) is one of the most important export fruits in Malaysia, grossing annual revenue of US$ 28 million in 2002. There are two major constraints faced by the papaya industry which are papaya ring spot virus (PRSV) disease and the rapid deterioration of the fruit after harvest. At the Malaysian Agricultural Research and Development Institute (MARDI), conventional breeding for tolerance to PRSV resulted in the selection of four elite lines [1]. The 'Rainbow' variety with PRSV resistance developed by the University of Hawaii in 1998 was the world's first commercial transgenic papaya. Following this success, South East Asian countries in the Papaya Biotechnology Network coordinated by the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) have embarked on the development of transgenic papayas with coat-protein mediated vector to create resistance to PRSV as well as delayed fruit ripening using the anti-sense ACC-oxidase gene [2].

Development of new papaya varieties with improved performance and disease resistance using irradiation is another approach that can be explored. Irradiation-induced mutation is increasingly used as a complementary tool in plant breeding. It is most appropriate for improving one or two easily identifiable traits in an otherwise well accepted breeding line or commercial variety. Worldwide, more than 1,500 mutant cultivars of crop plants and ornamentals have been released to growers in the past 30 years [3, 4]. Eight percent of agricultural land in China, for example, is planted with mutants of rice, wheat, corn and cotton. In Malaysia, mutation breeding covered a range of crops including rice, banana,

soybean, mungbean, groundnut, rubber tree, langsat, mangosteen, some legume cover crops and ornamental plants. Several rice mutant varieties such as Muda 2 (short stature), Mahsuri mutants (improved quality) and Manik 817 (short stature glutinous rice) have been released [5]. For fruit trees, a new variety of local banana, Novaria with early fruiting and improved bunch characteristics has been developed using gamma irradiation of *in vitro* cultured Grand Nain banana [6]. There have been no reports, however, on the use of irradiation for improving papaya cultivars particularly in developing disease resistance to ring spot virus.

The objectives of the project were to generate variability in papaya variety Eksotika by gamma irradiation and to screen for variants that have resistance to ring spot virus disease and delayed fruit ripening characteristics. It also aimed at the evaluation, description and documentation of induced mutants with traits of horticultural interest and the promotion of genetic conservation program for such mutant them in germplasm for breeding purpose and exchange of crop genetic materials.

This article reports the progress of the research with a focus on the sensitivity of papaya seeds to irradiation and the variation observed M_2 and M_3 populations. The potential of using irradiation for the production of useful mutants and improve varieties in papaya is also discussed.

2. Materials and methods

Irradiation of the seed was carried out at the Malaysian Institute for Nuclear Technology Research (MINT). Seeds were irradiated in Gamma Chamber GC4000A (10kCi) with low dose irradiation between 10 – 100 Gy, while for higher dose between 300 – 1000 Gy the seeds were irradiated in the Shepperd & Associates Model 109-68 Irradiator (24 kCi). In all cases the radiation source was a ^{60}Cobalt.

2.1. Radiosensitivity tests

The first phase of the project was to determine the sensitivity (‘half-kill` or LD 50) of papaya seeds to various doses of gamma-irradiation. The seeds Sekaki and Eksotika have been dried and stored at 10°C for 3 months. Two treatments were imposed on these seeds, i.e. dry and wet, pre-soaked (soaked for 24 hours and surfaced dried). The irradiation doses used were 0, 10, 25, 35, 75, 100, 125, 150, 200, 250 and 300 Gy. Each batch of seeds for irradiation included 100 seeds. After irradiation, seeds were germinated in sand beds for evaluation of their viability.

In the second experiment, 100 dry Eksotika seeds were irradiated with doses of 0, 300, 400, 500, 500, 700, 800, 900 and 1000 Gy for determination of the LD50.

In the third experiment, wet Eksotika seeds (soaked for 24 hours and surface dried) were used for irradiation at doses of 0, 30, 35, 40, 45, 50, 55, 60, 65 and 70 Gy for determining the dose for LD50 based on the first approximation from results of an earlier experiment of about 60 Gy.

2.2. Mass irradiation of Eksotika seeds

Mass irradiation of Eksotika dry seeds at 525 Gy (recorded as D525 Gy) was carried and Eksotika wet seeds at 42.5 Gy (W42.5 Gy). These doses were recommended following the

results of sensitivity tests carried earlier by Chan *et al*., [7]. Each batch consisted of 4,000 seeds. Two hundred and fifty seedlings were raised out of each irradiated batch.

2.3. Evaluation of M_2 and M_3 *populations*

M_2 seeds collected from 500 progenies (250 each from treatments W42.5 Gy and D525 Gy) were sown and after eight weeks of culture the seedlings were planted in the fields. The planting distance was spaced 1.8 m between plants and 2.1 m between rows. The experimental plot was irrigated with a drip system.

M_3 seeds were obtained by self-pollinating M_2 hermaphrodite trees .This was done by placing a small wax envelope over unopened flowers. About 100 M_3 seeds per batch from the D525 Gy treatment and 125 seeds per batch from W42.5 Gy treatment were selected. The M_3 seeds of each treatment were bulked and a random sample of seeds was drawn. Five hundred seedlings from each treatment were raised. At six weeks after sowing, the leaf length and the height to the first cotyledon (indicating dwarf stature) were measured for 200 seedlings each of W42.5 Gy and D525 Gy treatments compared with 50 control seedlings.

1,000 M_3 plants; 500 each from W42.5 Gy and D525 Gy treatments were planted in the field for evaluation of morphological changes and fruit ripening characteristics. Fifty control trees were planted for comparison in field evaluations, 200 trees from each irradiation treatment 42.5 Gy and 525 Gy and 50 trees from controls were measured for documentation of the distribution in M_3 populations for nine pre-selected characters. At the flowering stage, data on days to appearance of first flower, flowering height (distance of flowering node to ground) and the sex of flower were recorded. At one year from seed sowing, data on tree height, stem diameter, fruit number and tolerance to malformed top disease (ranking from symptomless '0' to most severe '9') were taken. Five mature fruits from each tree were harvested to record the mean fruit weight, flesh colour and total soluble solids % (TSS). The latter was recorded with a hand refractometer (0 -25 ^{0} Brix).The yield of each tree was computed from the product of fruit number and the mean fruit weight.

2.4. Screening M_2 *seedlings for PRSV tolerance*

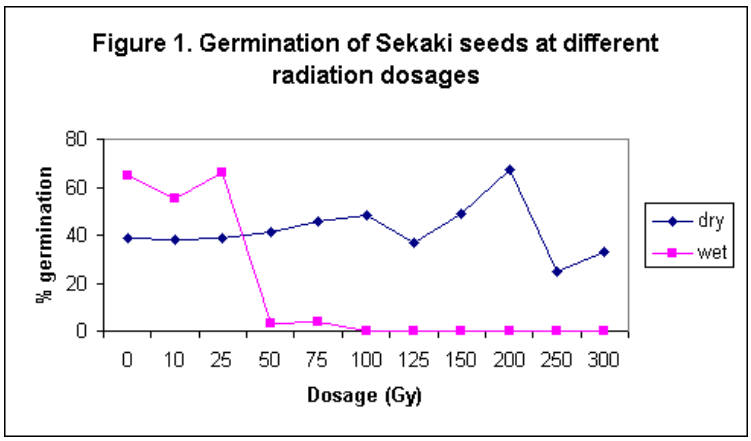
Two groups of 960 irradiated seedlings derived from seeds irradiated with 42.5 Gy and 525 Gy together with 60 control seedlings were planted in seedling trays. The seedlings were inoculated at the 8th week after planting at the 12-leaf stage. Two newly matured leaves were inoculated by gently rubbing the top surface of the leaves with the index finger dipped in the inoculums. The inoculums were prepared by grinding fresh, infected leaves in 0.01M phosphate buffer at pH 7.0 containing 0.25% DIECA (sodium diethyldithiocarbamate), carborundum, and mesh 600 then suspended in the buffer before inoculation.

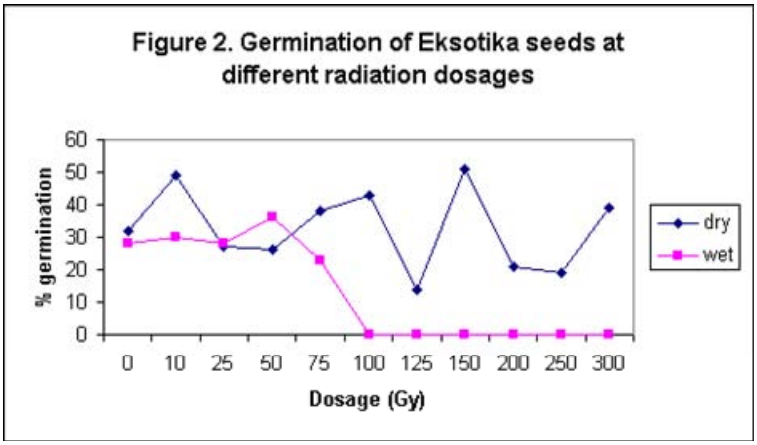
Plant disease severity was scored from 0 (disease-free) to 5 (terminal shoot yellowing with leaf mottling and mosaic and also small water-soaked spots on petioles and stem) to 9 (severe infection with plants near death). Infected plants were removed while symptomless ones cutback for re-growth and the new shoots re-inoculated. This process was repeated twice to reduce the risk of selecting 'escapes' from the disease. This method has been successful for selecting elite PRSV tolerant papaya lines at MARDI [8].

3. Results and discussion

3.1. Radiosensitivity tests

From the results shown in Figure 1 (for Sekaki) and Figure 2 (for Eksotika), wet pre-soaked seeds appear to have a higher sensitivity to irradiation than dry seeds, and Wet Eksotika seeds appeared to have a higher tolerance to irradiation dose than Sekaki, maintaining good germination up to 100 Gy irradiation dose while wet Sekaki seed were almost non-viable after a dose of more than 50 Gy. No conclusion could be drawn, however for dry papaya seeds because good germination was still observed at the highest dose of 300 Gy. Another experiment using higher doses is necessary to determine the 'half-kill' (LD50) for dry papaya seeds. The LD50 or 'half-kill' of seeds was about 650 Gy.





Figs. 1 and 2. Water soaked seeds show higher sensitivitiy to irradiation and the number of viable seedlings decreases quite drastically as compared to dry seeds in both varieties.

The growth of Eksotika seedlings was apparently not affected at 300 Gy, but then the plants exhibited a fairly linear decline in growth. At 700 Gy all seedlings showed stunting and crinkled leaves and very few survived transfer to the field.

For mass irradiation of dry Eksotika seeds the previously determined LD50 of 650 Gy could not be used because while half of the seeds germinated, the seedlings were too weak to survive transplanting. The dose for mass irradiation will be estimated instead from the dose that will cause 50% growth reduction (or GR50). From the results on growth, it could be observed that the decline in plant height was from 37.5 mm to 12.5 mm when dose was increased from 0 to 700 Gy. The height of control plants was 37.5 mm, so a 50% reduction

would be 18.75 mm, therefore 525 Gy could be the dose rate for mass irradiation of dry Eksotika seeds in future.

A gradual decline in plant height and leaf dimensions was apparent when increasing doses from 30 to 50 Gy. Growth data from 55 to 70 Gy were not presented, because of inadequate seedlings for data measurement. The LD50 for germination was 42.5 Gy and 50% reduction in plant height was also observed at this dose. Therefore, 42.5 Gy appears to be the suitable dose for mass irradiation of wet, pre-soaked Eksotika seeds.

3.2. Evaluation of M_2 and M_3 populations

3.2.1. Greenhouse experiments

Seedlings of the M_2 population, especially those harvested from the high dose irradiation of D525 Gy showed quite extensive morphological damages including stem splitting (17.1%), leaf variegation (14.3%), crinkled dwarfisms (13.9%) and leaf puckering (9.9%) [8]. In an evaluation of fruit bearing trees in the field, M_2 progenies did not show phenotypic differences in fruit characters except for one hermaphrodite tree, which bore fruits with distinct striations like a small melon The double-stem characteristic of some seedlings was expressed also in mature trees and both stems appeared to bear equal amounts of fruit, which might be used when screening for yield increase. About 25% of the population were male sterile and did not bear any fruit. Leaf variegation and leaf puckering characteristics observed in seedling stage were not expressed in mature trees.

The distribution of height for irradiated seedlings, regardless of treatments, was skewed towards the lower end, with a stronger effect with W42.5 Gy treatment. This implies that dwarf-stature progenies might be obtained in M_3 population compared with non-irradiated control seedlings.
The effects of irradiation on M_3 seedlings height and vigor, as reported by Chan and Ong, [1] showed that in general, the M_3 populations were taller and more vigorous, particularly in the W42.5 Gy treatment. In the D525 Gy treatment, the negative effect of higher irradiation dose was evident with more seedlings showing very stunted growth.

3.2.2. Field characters

There seems to be little prospect in selecting for improved earliness in flowering for the M_3 population. For ‘flowering height’ which measures the height of the first-formed flower from ground level, the two irradiated treatments and the control showed similar medians at just over 90 cm. However, these putative mutants may be useful for getting short bearing stature and ease of fruit harvest. The general pattern for yield distribution is rather similar for irradiated and non-irradiated control, suggesting that irradiation may not be effective for increasing yield.

For total soluble solids % in fruits, the distribution of irradiated populations exceeded the upper limits of 15.5% of the control, reaching as high as 16.5%. This is rather unusual for Eksotika papaya whose TSS% range is between 13-15%. There is good prospect for increasing TSS% from selection in the M_2 population. For flesh colour shown in Table I, the control population was very stable with all the trees bearing fruits with orange-red flesh. However, for irradiation-derived trees, a fairly high number of unfavourable yellow-orange types had surfaced. Furthermore, it is very surprising to find the emergence of yellow flesh types, ranging from 0.4% for 525 Gy treatments to 1.0% for 42.5 Gy treatments. Yellow flesh

is controlled by a dominant gene and irradiation appears to have caused a rare mutation from the normal recessive red gene.

TABLE I. FLESH COLOUR SEGREGATION IN M_2 POPULATIONS

	Orange-red		Yellow-orange		Yellow		Total
	Number	%	Number	%	Number	%	
42.5 Gy	333	84.9	55	14.1	4	1.0	392
575 Gy	280	97.5	6	2.1	1	0.4	287
Control	50	100.0	0	0.0	0	0.0	50

Eksotika papayas segregate usually as 2 hermaphrodites: 1 female in a normal populations and this is indicated in the control (Table II). In the case of irradiation-derived M_3 populations, a small number of male trees emerged. Irradiation had caused the hermaphrodite gene M^H to mutate into the male gene M. The change was more significant in populations irradiated at the higher 525 Gy dose.

TABLE II. SEX SEGREGATION IN M_2 POPULATIONS

	Hermaphrodite		Female		Male		Total
	Number	%	Number	%	Number	%	
42.5 Gy	257	64.1	143	35.7	1	0.2	401
575 Gy	229	65.1	119	33.8	4	1.1	352
Control	35	70.0	15	30.0	0	0.0	50

Malformed Top Disease (MTD), caused by *Cladosporium*-thrip complex, is very damaging on young foliage of Eksotika papaya. The majority of trees in the three populations succumbed to this disease. For the control, there were no trees having a disease rating better than 4, but several trees from the irradiated populations had resistant ratings of 0-1. This shows a very good prospect in selecting mutants in the M_3 population for resistance to MTD.

3.3. Screening M_3 seedlings for PRSV tolerance

The results of the screening are shown in Table III. In the first group of plants, a high number of seedlings did not show symptoms and these were cutback, and the plants allowed to develop new shoots, which were re-inoculated on the emerging, This process was repeated 3 times to eliminate possible escapes until the 4^{th} inoculation when only 2 seedlings did not show any symptom. These were then planted in field for the production of M_4 seeds.

TABLE III. RESULTS OF SCREENING OF M_2 POPULATIONS FOR PRSV RESISTANCE

	Number and % of surviving symptomless plants									
	Pre-inoculation		1st inoculation		2nd inoculation		3rd inoculation		4th inoculatio	
Batch 1	No.	%	No.	%	No.	%	No.	%	No	%
42.5 Gy	480	100	109	22.7	48	10.0	8	1.7	2	0.4*
575 Gy	480	100	348	72.5	82	17.1	5	1.0	0	0.0
Control	56	100	45	80.3	6	10.7	0	0.0	0	0.0
Batch 2										
42.5 Gy	480	100	7	1.5	0	0.0				
575 Gy	480	100	36	7.5	0	0.0				
Control	60	100	0	0.0	0	0.0				

* The 2 plants were field planted, but subsequently succumbed to PRSV.

In the second group of plants, there were fewer escapes, indicating probably an improvement in the inoculation technique. On the second inoculation, there were no surviving plants. Finally, from a total of 1920 irradiation-derived M_3 seedlings screened, only 2 were selected. However, when these 2 were planted in the field and allowed to produce M_4 seeds, they also succumbed to PRSV under field pressure. Irradiation, therefore, did not seem to be a successful for developing PRSV resistance in Eksotika papaya.

4. Conclusions

Doses of 42.5 Gy for wet, pre-soaked Eksotika seed and 525 Gy for dry seed appeared to be optimal for mass irradiation. Many changes in the M_2 were injury-related to the irradiation process. Wider variability was generally observed in irradiation-derived M_2 populations for many traits. Therefore, there are good prospects in improving Eksotika papaya especially in developing dwarf trees with lower fruit bearing stature, higher total soluble solids in fruits and very importantly, resistance to malformed top disease. However, no PRSV resistant selections were made from screening 1,920 M_3 seedlings. M_4 seeds have been obtained by self-pollination of selected mutants with superior traits. These are currently planted for further evaluation and selection of improved mutants.

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Guava (*Psidium guajava* L) improvement using *in vivo* and *in vitro* induced mutagenesis

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Abstract. *In-vitro* mutagenesis followed by micropropagation via axillary bud proliferation in shoot tips of guava (*Psidium guajava* L.) cultivar Safeda was carried out. Shoot tips were irradiated with 15 to 90Gy gamma rays radiations using ^{60}Co gamma cell source and cultured on MS medium containing 3.0% sucrose, BAP and glutamine. Shoots proliferation was observed 7 weeks after culture initiation. Higher shoot proliferation rates were recorded on MS medium supplemented with 1.0 mgL^{-1} BAP and 250 mgL^{-1} glutamine. Rooting was observed on half-strength MS medium supplemented with IAA and IBA. Radio sensitivity test was assessed by determining the percentage of shoot tips survival and shoot proliferation rates. The LD50 (The dose at which 50% of the population killed) was observed on 45 Gy. The doses above 75 Gy were found to be lethal to all explants. Similarly, seeds and bud woods of guava Cv. Safeda were exposed to gamma rays at 0.05 to 0.3 kGy and 20 to 100 Gy respectively using ^{60}Co gamma source. The LD50 for seeds was determined at 0.19 kGy, and 0.3 kGy was found to be lethal. Seeds irradiated with low doses germinated earlier as compared to those which received higher doses of irradiation. The LD50 for surviving bud woods grafted on the rootstocks in the nursery was determined at 60 Gy. There was constant and continuous reduction in the survival rates of buds as the doses increased. The bud woods of guava Cv. Safeda were more radiosensitive than seeds of the same cultivar. The most favourable doses were established between 0.15 and 0.2 KGy and led to mutations such as: highest number of fruits (40), highest fruit size (59mm) and highest fruit weight of (128.38g). The highest number of seeds (300) and 100 seed-weight (2.29g) were recorded at 0.05KGy while the lowest number of seeds (167) and the lowest 100 seed-weight of 1.56g was observed in the 0.30KGy treatments.

1. Introduction

Guava (*Psidium guajava* L) is one of the most important fruit crops of the Indo-Pakistan subcontinent and its importance is increasing due to its characteristics of high nutritional value, bi-annual fruit bearing, and affordable market prices for the general population. However, in Pakistan guava is usually propagated by seeds [1, 2]. Natural cross pollination, up to 35% common in guava cultivars [3, 4, 5] is responsible for the variability observed. In order to grow guava as a commercial fruit crop, there is an urgent need for the development of improved and demand-oriented cultivars, as well as for easy methods for vegetative propagation because it is one of the few horticultural crops, which unfortunately does not lend themselves to asexual propagation methods [6, 7].

Micropropagation coupled with induced mutations has been described as a suitable technique to improve vegetatively propagated crops [8, 9,10]. The impact of mutation techniques for the improvement of fruit trees has been reported worldwide [11]. In many vegetatively propagated crops, mutation induction in combination with *in vitro* culture may be the only effective method applicable for their improvement [12, 13].

Guava cultivar Safeda is highly appreciated for excellent quality fruits in the Chat region of Pakistan but the commercialization is still not fully developed due to the high number of seeds. On the other hand, the cultivar Safeda has a large tree size (about 6 meters high); therefore there is a need to modify the plant architecture to a compact or semi-dwarf type of growth. Compact or dwarf plant character not only permits more trees to be grown per unit area but also facilitates cultural practices such as fruit picking and pesticides spraying, because of the time and manpower involved as well as the high risks of fruit damages. The cultivar Safeda was therefore selected for a mutation breeding research programme to develop mutants for the industrial market on the basis of the good fruit quality traits.

As a first step in a comprehensive mutation breeding strategy, the present study was carried out to investigate the sensitivity of *in vitro* guava shoots, seeds and bud woods to gamma ray irradiation. The successful outcome of a mutation depends on the effective induction of mutations as well as a reliable recognition and recovery of the desired mutant plants. The choice of the proper mutagenic agent based on the efficiency, the safety issues on handling, the dose and the treatment conditions are important factors to be considered. But consideration must also be given to some morphological and/or physiological characteristics of the treated plant materials such as: the vegetative stage of the plant or the explants used the sensitivity of the plant species to the effects of the mutagenic agents and the possible genotypic differences in sensitivity to the mutagenic treatments.

The research work on the radiation effects in guava shoots, seeds and bud woods has not been carried out in Pakistan and it is reported for the first time in the present studies.

Some drawbacks in popular guava cultivars grown in Pakistan as given in Table I could be alleviated through the generation of induced mutations. On the other hand induced mutations as a mean of cultivar improvement in guava, has a special appeal to solve the technical problems in emasculation and pollination encountered during crossing and other unpredictable results observed [14]. Induced mutations could be also very useful to generate efficient genetic variability for early bearing, short stature and seedlessness (or low number of seeds) traits in guava. The objective of the present research was to induce *in vivo* and *in vitro* mutations in seeds, bud-woods and shoot tips of guava for the development of early bearing, dwarfness and reduced seed number guava mutants.

2. Materials and methods

2.1. In vitro mutagenesis

Shoot tips of guava cv. Safeda were obtained from a mature bearing plant in the orchard of NIFA Campus. The samples were wrapped in a cloth and placed in a brass jar and irradiated at 15 to 90 Gy of gamma rays with 15 Gy intervals, using ^{60}Co gamma source. Immediately after irradiation these shoot tips were brought to the laboratory and treated with 2 drops of Zip as a detergent, then washed with tap water for 30 minutes. The material was placed on a shaker for 30-40 minutes in 0.5% PVP solution. The explants were then briefly rinsed with 70% ethanol. An additional surface sterilization of these shoots was carried out with 0.05% of Mercuric distilled water solution under laminar flow and plated onto MS basal medium as described by Murashige and Skoog [15] supplemented with different concentrations of BAP and glutamine, 3% sucrose and solidified with 0.8% plant agar was used for culturing the shoot tips.

TABLE I. SOME DRAWBACKS IN POPULAR GUAVA CULTIVARS GROWN IN PAKISTAN, WHICH COULD BE ALLEVIATED THROUGH INDUCED MUTATION

Cultivar	Drawback
Safeda	(1) Large no. of seed in fruit
	(2) Very short shelf life
	(3) Large tree size.
	(4) Late bearing /maturity
Seedless	(1) Shy bearer
	(2) Irregular shaped
	(3) Too vigorous
	(4) Small size fruit size.
Pear-shaped	(1) Tree very tall with heavy branching.
	(2) Irregular fruit shaped
	(3) Large no. of seed
Red fleshed	(1) Very short shelf life
	(2) Large no. of seed
	(3) More acidic as compared to white fleshed.

Cultures were kept in a growth chamber at 25±2°C under a 16-h photoperiod with a light intensity of 2500 Lux to initiate growth. The experiment was arranged in a randomized complete block design with three replications per treatment, each with 20 explants. Radiation responses were evaluated in terms of explants survival and shoot proliferation after 7 weeks of culture. Surviving shoot tips were transferred to fresh medium, and sub-cultured to M_1V_2 and subsequently up to M_1V_4. Rooting was induced on half-strength MS medium supplemented with IAA and IBA.

2.2. In vivo mutagenesis

2.2.1. Irradiation of seeds

Healthy, well-ripened and uniformly sized fruits of guava Cv. Safeda were collected from a young bearing tree in the NIFA Campus in August, 2000. Seeds were extracted and kept in the open air to allow proper dessication until the moisture content reached 10-12%.Uniform seeds were subjected to 0.05, 0.10, 0.15, 0.20, 0.25, and 0.3kGy of gamma rays using ^{60}Co gamma source. The treated seeds, along with the control were sown in pots containing a mixture of silt and soil at 2:1 (v/v) ratio in a glass house. The pots including 40 seeds per pot were arranged in a randomized block design in 3 replicates. The ambient temperature in the glass house was 35-40 during daytime and 25-30°C at night. The seedling emergence was counted continuously after three weeks of sowing. The LD50 was calculated on the basis of percent of germination of the seeds. Seeds collected from early bearing M_1 plants were sown to produce M_2 generation in a semi-permanent orchard and were evaluated for fruit characteristics.

2.2.2. Irradiation of bud woods

Bud sticks of about 12 cm of length from the new flushes including 5-7 buds were selected from a mature bearing guava Cv. Safeda. These shoots were defoliated, and terminal apex was excised 15 days prior to budding. Axillary buds were then removed from the bud sticks individually and immediately kept in a brass jar for irradiation. Dose levels were 20, 40, 60, 80 and 100 Gy gamma rays ^{60}Co gamma cell source, for 60 buds per treatment. The treated buds along with controls were immediately grafted on one year old guava rootstocks in a net house with 20 buds per replication in each treatment, and the experiments replicated three times. All the buds were tightly covered with a transparent polythene sheet to avoid desiccation of the buds. Grafted buds were continuously observed and survival data recorded after 6 weeks of budding and green colour was considered as sign of success while a blackish colour was recorded as failure of the grafting. The portion above the grafted buds was removed after the buds successfully restart growing. Data on the percentages of survival of bud woods were recorded and LD50 determined for the bud wood experiment.

3. Results and discussion

3.1. In vitro mutagenesis

Radio sensitivity showed that lethality increases as the radiation dose increased and doses above 75Gy gamma rays were lethal to the explants. The LD50 found in the present experiment was 45Gy (Figure 1). The results of the present investigation clearly show that shoot tip explants can be subjected to *in vitro* induced mutations for creating genetic variability. Some plantlets with variegated leaves and short internodes in 45Gy doses have been found (Figure 2).

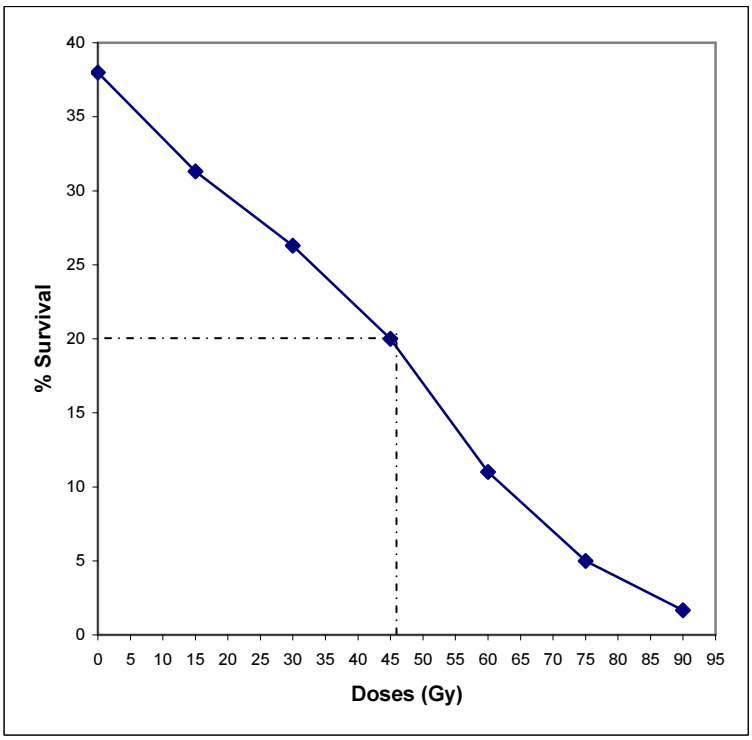

Figure 1. Effect of different doses of gamma rays on survival percentage of the shoot tips in guava Cv. Safeda.

Figure 2. Variegated leaves of in vitro plantlets after irradiation of shoot tip.

Shoot proliferation rates varied with the different concentrations of BAP and glutamine (Table II). Generally, the number of shoots developed into plantlets increased with an increasing concentration of BAP combined with glutamine. These results are in conformity with the results reported by Osei-Kofi et al., [16] stating that, up to a certain limit, high cytokinin favors buds and shoots formation. The highest number of shoots (43) developed into plantlets in MS medium supplemented with 1.0 mgL^{-1} BAP combined with 250 mgL^{-1} glutamine (Figure 3). Shoot tips cultured on MS medium without growth hormone produced 13 plantlets. The number of shoots developed into plantlets increased with the increasing concentration of BAP and glutamine. From the foregoing observation it can be concluded that BAP and glutamine are a suitable growth hormone combination for the proliferation of shoots on guava explant. The proliferation rate was optimum at 1mgL^{-1} of BAP and 250 mgL^{-1} glutamine. The superiority of BAP over other cytokinin for multiple shoot formation in fruit plants has also been reported by other investigators, including Jaiswal and Amin [11].

TABLE II. EFFECT OF DIFFERENT CONCENTRATIONS AND COMBINATIONS OF GROWTH HORMONES ON DEVELOPMENT OF GUAVA SHOOTS WHEN SUPPLEMENTED TO MS MEDIUM

Media concentration	No. of shoot cultured	No. of shoot developed into plantlets	Av. No. of shoot/per culture
MS control		13 d	1.8
MS+ 0.50 mgL^{-1} BAP	60	25 bcd	2.0
MS+ 1mgL^{-1} BAP	60	31 abc	2.7
MS+ 0.50 mgL^{-1} BAP+ 250 mgL^{-1} glutamine	60	35 ab	2.9
MS + 1 mgL^{-1} BAP + 250 mgL^{-1} glutamine	60	43 a	1.5

Means of the same category followed by different letters are statistically different at 5% level of significance, using LSD test.

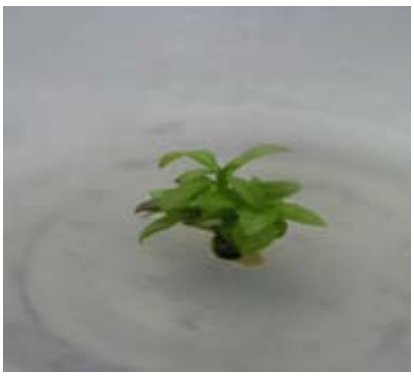

Figure 3. In vitro regeneration of guava shoot tip.

Rooting of plantlets was achieved using half-strength MS medium supplemented with IAA and IBA. The highest numbers of rooted plants (46) were observed in half-strength MS medium supplemented with 2.5mgL^{-1} IAA and 2.5mgL^{-1} IBA (Table III and Figure 4). Rahman et al., [17] also obtained highest percentages of rooting when they combined 0.2mgL^{-1} IBA with 0.2mgL^{-1} NAA. No rooting was observed in hormone free medium.

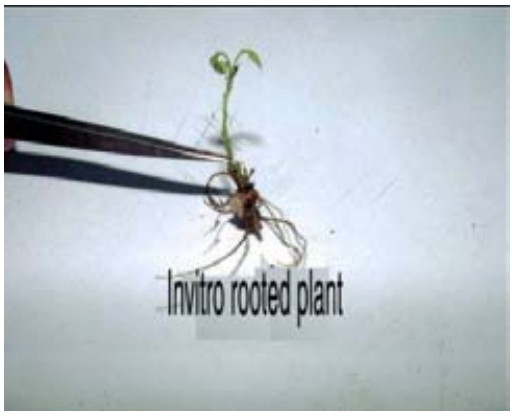

Figure 4. In vitro rooted plantlet of guava.

The *in vitro* mutagenised plantlets were successfully established in small plastic pots with 1:1 (v/v) mixture of garden soil and compost after washing the roots thoroughly to remove any remains of the medium (Figure 5). In the first week of transfer, the potted plantlets were covered with glass beakers to maintain high humidity.

Figure 5. Guava plant after acclimatization.

TABLE III. EFFECT OF DIFFERENT CONCENTRATIONS OF AUXINS ON ROOTING OF GUAVA CV. SAFEDA PLANTLETS WHEN CULTURED ON MS MEDIUM

Media concentration	No. of plantlets cultured	No. of plants fail to root	No. of plants rooted	Av. No. of roots/ plantlet
½ MS (Control)	60	60	0 d	0
½ MS + IBA 2 mgL^{-1}	60	36	24 c	2.4
½ MS + IBA 2.5 mgL^{-1}	60	32	28 bc	3.4
½ MS + IAA 2 mgL^{-1}	60	39	21 c	2.3
½ MS + IAA 2.5 mgL^{-1}	60	26	34 b	2.6
½ MS + IAA 2 mgL^{-1} + IBA 2 mgL^{-1}	60	24	36 b	3.13
½ MS + IAA 2.5 mgL^{-1} + IBA 2. mgL^{-1}	60	14	46 a	3.8

Means of the same category followed by different letters are statistically different at 5% level of significance, using LSD test.

3.2. In vivo mutagenesis

3.2.1. Effect of irradiation doses on bud wood

The effect of different doses of gamma rays on the survival percentage of bud woods is shown in Figure 6. The LD50 for buds survival was exactly 60 Gy. There was a gradual decrease in survival percentages as compared to the control, but after 40 Gy doses the survival rate significantly decreased, with 100 Gy causing 86.67% lethality.

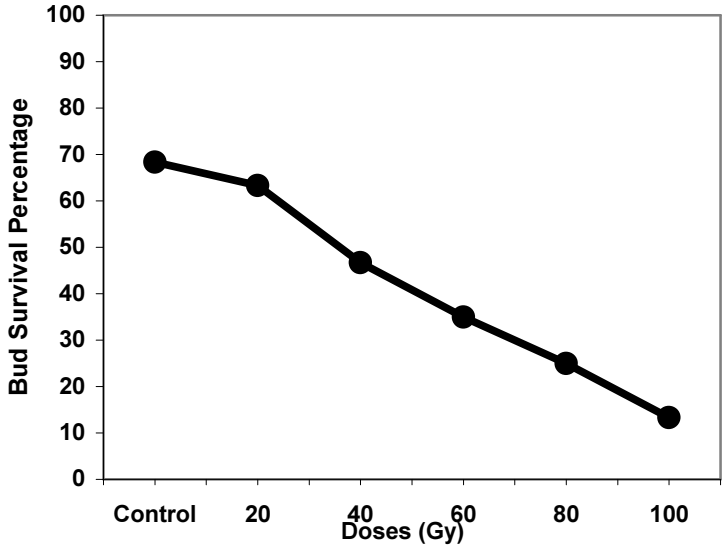

Figure 6. Effect of different doses of gamma rays on the buds survival percentage of guava cv. safeda budded on the root stocks.

3.2.2. Effect of irradiation doses on seed germination rates

The effect of different doses of gamma rays on the percent germination of guava seeds is shown in Figure 7. The germination percentage decreased with the increase in radiation doses and a significant reduction of 79.2 and 80.7% respectively were noted at 0.25 and 0.30 KGy respectively. The LD50 found in our experiment for guava seeds was 0.19KGy. The sensitivity effect of different doses of gamma rays on guava seedlings in our experiment is shown in Figure 8. About 86.6% lethality of guava seeds was observed at dose 0.3 kGy.

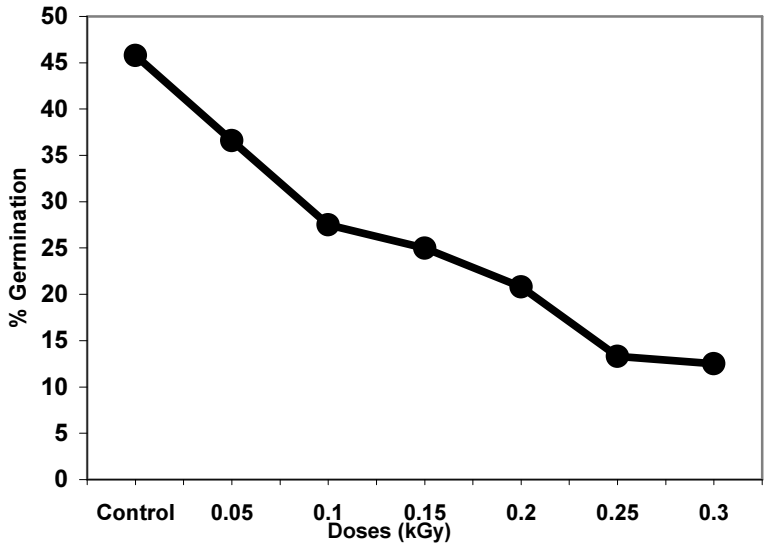

Figure 7. Effect of different doses of gamma rays on the % Germination of guava seeds cv. safeda.

Figure 8. Irradiation sensitivity of guava seedlings (A-control, B- 0.05, C-0.1, D-0.15, E-0.2,F-0.25,G-0.3KGy).

3.2.3. Effect of gamma rays on the fruit characters of M_2 generation

The fruit characteristics data recorded in M_2 generation are presented in Table IV and described as follows.

TABLE IV EFFECT OF DIFFERENT DOSES OF GAMMA RAYS ON THE CHARACTERISTICS OF FRUIT IN M_2 GENERATION OF GUAVA

Doses KGy	No. of fruits/plant	Fruits size/ Circumference (mm)	Fruit weight (g)	No. of seeds/fruit	Total seed weight (g)
0.05	16f	61a	65.23e	300a	2.29a
0.1	28 c	51c	80.23d	270b	2.01b
0.15	40a	54b	95.59c	254c	1.74c
0.2	32b	59a	128.38a	240d	2.29a
0.25	24d	49cd	101.95b	183e	1.77bc
0.30	20e	46d	56.23f	167f	1.56c
LSD (0.05)	3.570	3.018	4.915	5.636	0.248

Means of the same category followed by different letters are statistically different at 5% level of significance, using LSD test.

(a) Number of fruits per plant: The number of fruits per plant was significantly affected by increasing doses of gamma rays. The number of fruits per plant increased with the increase in radiation dose up to 0.20KGy and then decreased from 0.25 KGy. The highest number of fruits (40) was recorded in the plants treated with 0.15 KGy (Figure 9). In this experiment some mutants have produced clusters of fruits i.e. two or more fruits on one peduncle (Figure 10) while the lowest numbers of fruits were found in the plants treated with 0.30 KGy.

Figure 9. A heavy bearing guava mutant in a Semi permanent orchard.

Figure 10. Cluster fruiting in M_2 Plants of guava.

(b) Fruit size: The size of fruit was significantly affected by different doses of gamma rays. Some deformed and segmented types of fruits have also found in 0.20KGy treatment (Figure 11). The highest fruit circumference was 61 mm recorded in the treatment of 0.05KGy while the lowest fruit circumference at 46 mm was recorded in 0.30KGy treatment. There was a gradual decrease in fruit size with increase in radiation dose.

(c) Fruit weight: The fruit weight was significantly affected by different doses of gamma rays. The fruit weight increased up to 0.20KGy treatments and then decreased with an increase in radiation dose. The highest fruit weight 128.38g was recorded in 0.20 KGy treatments while the lowest weight of 56.23 g was recorded in 0.3 KGy treatments.

(d) Number of seeds per fruit: The number of seeds per fruit was also significantly affected by different doses of gamma rays. The highest number of seeds (300) was recorded in the 0.05 KGy (Figure 12 B) while the lowest number of seeds (167) was recorded in the 0.30 KGy treatments (Figure 12 A). There was a gradual decrease in the number of seeds per fruit as radiation dose was increased.

(e) Total seed weight: The total seed weight per fruit was significantly affected by different doses of gamma rays. The highest seed weight of 2.29g was recorded in the control while the lowest seed weight of 1.56g was in the 0.30 KGy treatments.

Figure 11. Segmented and deformed shaped M_2 fruits.

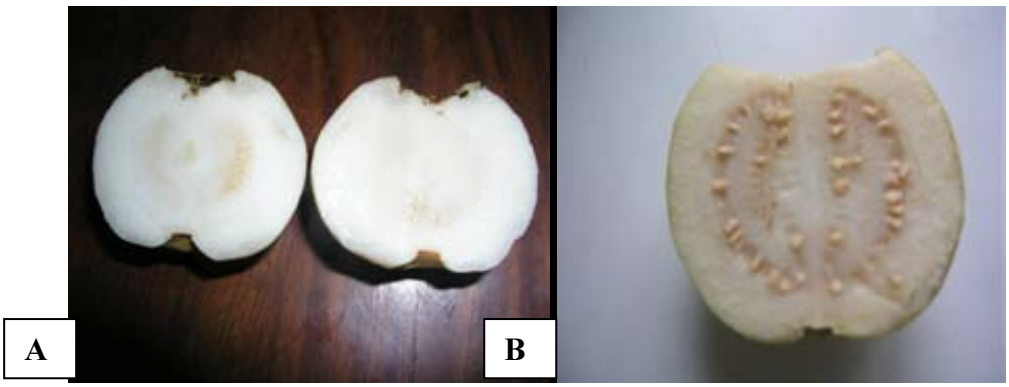

Figure 12. (A) Low Seeded Fruits (B) Multiple Seeds.

Hearn [11] carried out research on the development of seedless grafted fruit cultivars through bud wood irradiation. He found several of the plants from the 3 Kr treatments had fruit with reduced seed content, but none was commercially seedless and had 9 or less seeds in any fruit. Sattar et al., [18] also related the seedlessness in Kinnow to the higher level of pollen and ovule sterility to induced mutations. This view point is supported by the work of Spiegel-Roy [19] who recorded lowered pollen viability and increased ovule sterility in the seedless Citrus mutants.

Ikeda [13] recorded the fruit weight in several induced apple sports. He compared the various induced sports fruit weight with Fuji as a control. The fruit weight was decreased in all the induced sports as compared to the control. The studies of Ikeda are in line with our results in guava. We also obtained the highest fruit weight in the control fruits plants while those treated with different doses of gamma rays have reduced fruit size as well as fruit weight.
Sattar et al., [18] studied the fruit size of the mutant Kinnow and its successive generations which were not significantly different from that of the parent Kinnow. Donini et al., [8] also reported some minor changes in fruit trees due to induced mutations. Spiegel-Roy [19] reported induced mutations of commercial significance including highly coloured seedless grape fruit, seedless Minneola tangelo, and differences in size and colour in apple.

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Induction of mutation in Jujube (*Zizyphus jujuba* Mill) using tissue culture combined with ^{60}Coγ-RAY irradiation

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Abstract. *In vivo* and *in vitro* mutagenesis techniques were assayed to explore effects of irradiation in jujube (*Ziziphus jujuba* Mill) improvement. ^{60}Co γ-ray irradiated seeds and shoot tips of a land race of jujube originating in Shangdong province of China were micropropagated up to M_1V_4 generation on MS basal medium containing 2 mg/L BA and 0.4 mg/L IBA. The rooting MS medium contained 1 mg/L BA and 0.6 mg/L IAA, ZEA 1 mg/L, 2, 4-D 0.5 mg/L, and NAA 0.5 mg/L in different combinations. Adventitious buds were also produced from irradiated calli derived from leaf and hypocotyl fragments and the elongated adventitious buds rooted *in vitro* prior to green house transfer. Different doses (20 to 900Gy) were tested for *in vitro* explants as well as the jujube kernels irradiation. Six types of leaf shape and seven types of fruit shape mutations were observed and different ripening characters and growth habits were recorded in the orchard on putatively mutated mature trees. Even though there is a need for confirmation and molecular characterization, these mutations may be considered as a new and powerful way for jujube improvement in order to develop genotypes with promising value added traits.

1. Introduction

The jujube (*Ziziphus jujuba* Mill), which is native to China, and includes more than 400 cultivars has been cultivated for more than 4,000 years. The fruits of jujube are edible and have also been used for medicinal purposes for millennia, i.e., as an herbal tea to release sore throat. This fruit tree is also known for withstanding a wide range of temperature variations and being able to strive in many types of soils, from sandy well-drained soils to heavy soils with high salinity or high alkalinity. Some cultivars of the jujube with inferior fruit quality are being used in water and soil preservation programmes across China or as ornamental miniature garden plants.

Radiation induced mutation has been used in crop breeding programs for decades, and many new plant varieties have been reported in the IAEA database mutant germplasm (http://adminonline.iaea.org/InfoCatalogue/Public/04001.htm).

In tropical and subtropical fruit trees, mutation breeding has been used to create useful genotypes or varieties with better yield and improved fruit quality using *in vitro* explants and/or *in vivo* plant material such as seeds. One of the first coordinated research project (CRP) initiated in the early 1990s by the FAO/IAEA Joint Division focussed on "Cellular biology and biotechnology including mutation techniques of creating new useful tropical and subtropic fruit genotypes for quality improvement". The selected crops included banana (*Musa* spp), papaya (*Carica papaya* L.), mango (*Mangifera indica* Linn), guava (*Psidium guajava* L.), mandarin (*Citrus reticulata*), pear (*Prunus* spp.), peach (*Amygdalus persica* L.), litchi (*Litchi chinesis*) and jujube (*Zizyphus* spp.).

Under the present CRP (D23023) initiated in 2004 on "Improvement of Tropical and Subtropical Fruit Trees through Induced Mutation and Biotechnology", we used seed irradiation combined with plant tissue culture approach to make use of somaclonal variation in an attempt to enhance genetic variability and select for improved lines. Micropropagation and micro-grafting on local jujube varieties were performed, which increased noticeably the rates of recovery of healthy mutant genotypes and select for best performing ones with valuable agronomic traits [1, 2, 3, 4, 5, 6, 7].

2. Materials and methods

2.1. In vivo radiation

Seeds of a land race of jujube (*Ziziphus jujuba* Mill) were kindly provided by the Fruit Research Institute of Shangdong Province. The jujube kernels irradiated at different ^{60}Coγ-ray doses (300, 400 and 500 Gy) were surface sterilized in 70% ethanol for 20 sec and transferred to a 0.1% mercuric chloride solution for 8 min with occasional vigorous shaking. After a thorough rinse in sterile distilled water, the seeds were plated on MS basal medium for germination and cultured under constant temperature of 25 °C with a 16: 8 h photoperiod and a light intensity of 3000 lx for germination. The seedlings were cultured on the MS medium for 7- 10 days until the cotyledons were fully expanded and radicles elongated. For plant material increase some seedlings were cut into fragments including two leaves and used as explants for axillary bud induction on MS basal medium containing 2 mg/L BA and 0.4 mg/L IBA. The shoots obtained were sub-cultured onto freshly made medium every 30 days at least 4 times during the entire experimentation period and data recorded.

The media used were as follow:

- **A**, MS basal medium containing BA 2 mg/L and IBA 0.4 mg/L;
- **B**, MS basal medium containing BA 2 mg/L and NAA 0.4 mg/L; **C**, MS basal medium containing BA 2 mg/L and IAA 0.4 mg/L.

2.2. In vitro irradiation

In a second experiment, cotyledon, leaf, hypocotyl and shoot tips from non-irradiated seedlings were cut into fragments and used as initial plant material for micropropagation and the subsequent adventitious shoots tips irradiated. The initial fragments were surface sterilized in 70% alcohol for 20 s followed by an immersion in a 0.1% mercuric chloride solution for 8 min, and finally rinsed 3 times with sterile distilled water. These leaf and cotyledon fragments were cut into 2mm x 2mm pieces and 3 mm long strips respectively before being plated onto MS medium containing BAP 2mg/L for two weeks at 25°C under a photoperiod of 16h/8 and an intensity of 3000lx and allowed to produce organogenic calli. These fragments were sub-cultured on the same shoot propagation medium as stated in the previous experiment 4 times, namely from M_1V_1 to M_1V_4 generation.

Several MS media were prepared using different phyto-hormone concentrations and/or combinations:

- The medium for inducing callus from leaf explant was MS medium containing ZEA 1mg/L, NAA 0.5mg/L and 2, 4-D 0.5 mg/L.

- The media for inducing shoots from leaf callus are listed in Table I.

- The medium for shoot rooting was MS medium containing IBA 1mg/ L and IAA 0.6mg/L.
- The media for inducing adventitious bud from stem explants had three series: A., B and C.
  - ✓ A series was MS+ BAP combined with IBA. BAP concentration was 0.5, 1.0, 2.0 and 4.0 mg/L, respectively, and IBA concentration was 0.2, 0.4 and 0.8 mg/L, respectively. There were 12 media.
  - ✓ B series was MS+ BAP combined with NAA. BAP concentration was 0.5, 1.0, 2.0 and 4.0 mg/L, respectively, and NAA concentration was 0.1, 0.5, 1.0 and 2.0 mg/L, respectively. There were 16 media.
  - ✓ C series was MS + IAA combined with NAA. IAA concentration was 0.2, 0.5, and 1.0 mg/L, respectively, and NAA concentration was 0.2, 0.5 and 1.0 mg/L, respectively.

TABLE I. GROWTH HORMONE COMPOSITION IN MEDIA FOR CALLI CULTURE

Medium	ZEA（mg/L^{-1}）	NAA（mgL^{-1}）	BA（mgL^{-1}）
A_1	0.5	0.5	
A_2	1	0.5	
A_3	2	0.5	
A_4	3	0.5	
B_1	0.2	0.2	2.0
B_2	0.2	0.5	2.0
B_3	0.2	1.0	2.0
B_4	0.2	2.0	2.0
C_1	0.5		0.2
C_2	1		0.2
C_3	2		0.2
C_4	3		0.2

3. Results and discussion

3.1. In vivo irradiation

As shown in Figure1, the germination rate of kernels irradiated at 50 ~ 450 Gy doses varied from 96% to 80%, and for kernels irradiated at 500 ~ 900 Gy doses from 78% to 68%.The germination curve showed that 250 ~ 350 Gy irradiation doses could be used to induce mutation. The survival rate was 94%~ 90% for seedlings derived from kernels irradiated at 50 ~ 250 Gy doses after 90 days culture. However, above 300 Gy irradiation dose, the survival rate of seedlings decreased with the increasing doses. The seedlings derived from kernels irradiated at above 550 Gy dose did not survive pass 90 days of culture. The irradiation dose recognized as causing noticeable morphological mutations was established between 150~500 Gy. Mosaic leaf and irregular incision on leaves mutations appeared at the irradiation dose of 150 to 450 Gy. However these two mutated characters disappeared in the newly formed leaf after 90 days of culture. Dichotomy mutation, which is a twining of the apical bud, later

developing into two twigs appeared at irradiation dose of 350~400 Gy. Similarly a few short stalk mutation appeared at 400 ~ 500 Gy irradiation dose (Table II).

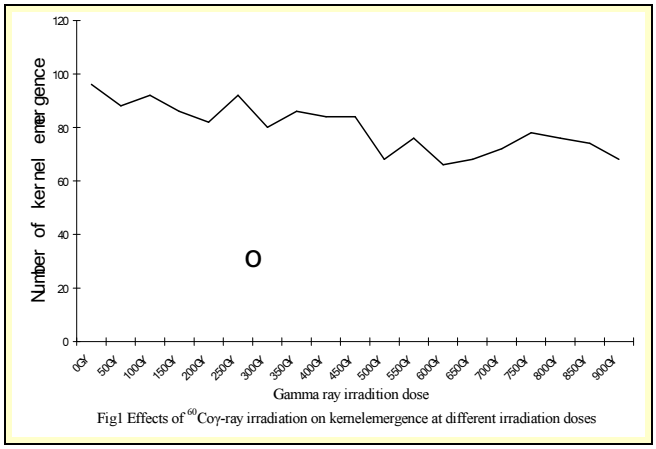

Figure 1. Effects of ^{60}Co γ-ray irradiation doses on germination rate of jujube kernels.

TABLE II. EVALUATION OF DERIVING SEEDLINGS FROM IRRADIATED KERNELS

Dose (Gy)	Number of surviving seedlings			Mutant phenotypes			
	30	60	90	Mosaic leaf	Irregular incision leaf	Double tips	Short stalk
CK	92	92	92				
50	90	90	90				
100	94	94	94				
150	94	94	94	10	1		
200	90	90	90	10	4		
250	90	90	90	18	5		
300	86	86	64	12	4	10	
350	84	84	54	2	2	4	4
400	78	62	36	4	8		20
450	64	38	28				10
500	64	16	10				
550	20	0	0				
600	12	0	0				
650	6	0	0				
700	4	0	0				
750	0	0	0				
800	0	0	0				
850	0	0	0				
900	0	0	0				

Seeds irradiated at 400 and 500 Gy doses did not germinate into viable seedlings. But seeds irradiated at 300 Gy irradiation dose developed into 123 plants. These plants were grown and

among them 6 plants germinated later i.e., more than 15 days after the control seeds, 5 plants showed a dwarf stature, 3 plants produced multiple twigs, and 2 plants produced chimeric leaves.

Through shoot micropropagation 120 M_1V_1 shoots were obtained, which in turn produced in M_1V_2, then M1V3 generations and in M_1V_4 5847 shoots, were scored. These 5847 shoots were transferred to MS rooting medium as described previously and finally 3098 entire plantlets were maintained in culture (A to G) in jujube M_1V_4 mutants as compared with the non-irradiated control (CK). The plantlets were later transplanted into pots in the green house prior to transfer to the orchard. Most of these jujube plants have since flowered and fruited.

Results collected from this experiment indicated that ZEA combined with NAA (A series) was effective in inducing shoots. Especially the MS medium containing ZEA 1.0 mg/L and NAA 0.5 mg/L was the most efficient. The frequency of shoot induction reached 36%, and 81 shoots were induced from 100 calli, but this result was observed after 12 months of culture with one subculture every two months. Media containing ZEA combined with BA (C series) could induce shoots, but the frequency of shoot induction was very low, while those containing ZEA combined with NAA and BAP (B series) failed to induce any shoot.

Among the M_1V_4 plants, many mutations mainly in leaf shape and size, fruit shape and size and ripening characters were observed sometimes as transient phenotypes. Figure 2 and Table III show the different leaf shapes and length observed during the course of this experiment.

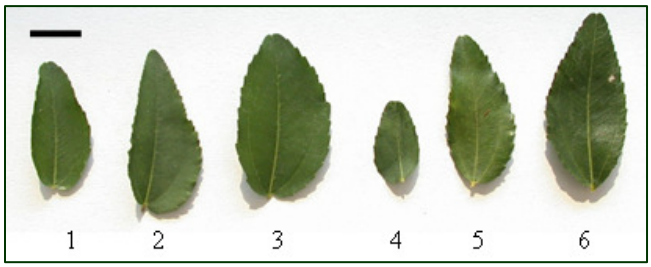

Figure 2. Six types of leaf shape in jujube M1V4 mutants. The black bar indicates 1 cm ovate type, and number 4 was near ovate, while the other types took oval shape at the bottom and were acute at the leaf tip. The leaf size variation quite was significant (Table 1).

TABLE III. LEAF SIZE AND LEAF FRESH WEIGHT OF THE SIX TYPES OF LEAF SHAPE IN JUJUBE M_1V_4 PUTATIVE MUTANTS

Leaf type	Leaf length * (mm)	Leaf width (mm)	L/W ratio	Leaf fresh weight (mg/leaf)
1	2.32±0.05	1.26±0.07	1.8	1.10±0.1
2	2.98±0.04	1.43±0.1	2.1	1.30±0.1
3	2.83±0.07	1.74±0.2	1.6	4.80±0.2
4	1.44±0.04	0.89±0.1	1.6	0.16±0.02
5	2.70±0.07	1.32±0.1	2.0	1.25±0.1
6	3.31±0.06	1.70±0.2	1.9	2.58±0.1

* Data were the average ± SD of 20 leaves. The difference in leaf length, leaf width and leaf fresh weight among the six leaf types was significant at $P<0.001$ according to the result of variance analysis.

Seven types of fruit shape were observed in the M_1V_4 mutants. All the fruits were drupe, varying from round to oval (Figure 3). Fruit diameters also varied from 0.9 cm to 2.1 cm, in comparison non-irradiated jujube fruits varied from 1.2 to 1.3 cm in diameter.

Besides the mutation in leaf and fruit shape differences in fruit ripening timing were observed. In fact all the eight types of fruit shown if Figure 3 were picked on the same day, but here are obvious differences in the ripening time. Type B and F got early ripening, type A, E, G were ripening, and type C, D and the control fruit were still green.

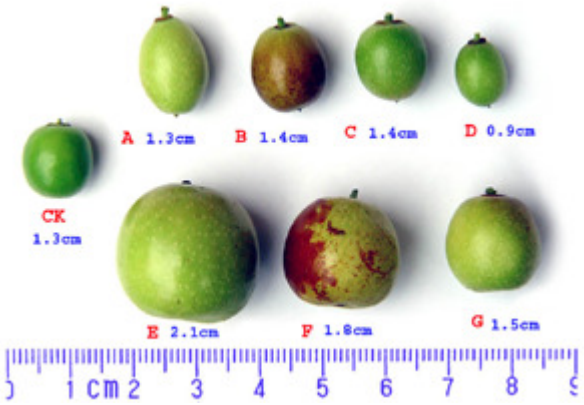

Figure 3. Seven types of fruit shape observed.

Growth habit of these jujube mutants were also different, some trees grew to about 1.5 meter tall with a crown diameter of 1.5 meter after 3 years. While, some trees seemed to retain a dwarfing habit and grew to only about 50 cm tall with a crown diameter of only 50 cm. This type of putative mutant jujube tree could have good potential as potted plants.

3.2. In vitro irradiation

In the early stage of this project a number of seedlings were cut 1.5 cm below their cotyledons and surface sterilized, transferred onto the MS medium containing IBA 0.4 mg/L. These explants were cultured for 40 days, and irradiated at 20 Gy dose. Subsequently the explants were cut into fragments including 3 nodes, which were sub-cultured on a medium with the same composition as above mentioned. These explants were later rooted and transferred into pots, prior to being transplanted in open field. The surviving 571plants were carefully observed and the putatively mutated characters recorded as follow:

- leave narrowing in one plant,
- leave yellowing in one plant,
- multiple twigs (without main stem, but more than two twigs growing on the basal part of stem) in 2 plants,
- early blooming (10 days earlier than normal)in one plant,
- later blooming (more than 10 days later than normal) in one plant, and
- one plant bearing sweeter fruits.

Figure 4 shows 45 days-old *in vitro* derived plantlets irradiated at doses varying from 5 to 30 Gy, which were able to survive with no visible damaging effects of irradiation. However, when irradiated at 35 Gy dose, one-fifth of the plantlets died; at 40 Gy dose one-fourth died;

and at 45 Gy dose about half of the plantlets died. Therefore, 25 ~ 40 Gy irradiation dose was determined as being suitable for inducing the mutations on jujube *in vitro* derived plantlets.

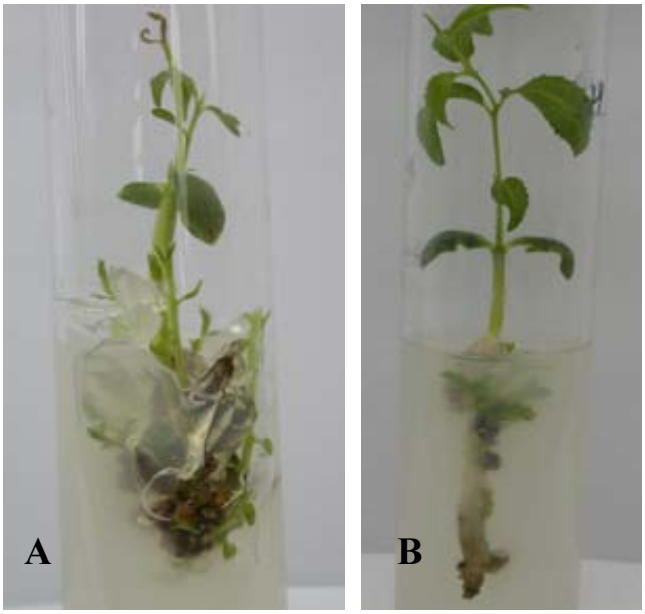

Figure 4. Clustering shoots regenerated indirectly from radicle calli (A) and small shoots regenerated directly from the swelling radicle of jujube seedling. Jujube seedlings were cultured onto MS medium containing 2 mg/L BA and 0.4 mg/L IBA. The photos were taken 27 days after the geminated seedlings were transferred to this medium.

The survival curve (Figure 5 A) show that the 30- day-old plantlets irradiated at a dose of 5, 10, 15 or 20 Gy were able to survive completely with no apparent effect of irradiation. Above 25 Gy dose, the rate of seedling survival decreased with increasing irradiation dose. In this case 25~35 Gy dose irradiation was determined as inducing jujube plantlets mutation. And 45-day-old (15 days plus 30 days) test-tube seedlings irradiated at a dose of 5, 10, 15, 20, 25 or 30 Gy were able to survive completely, and the irradiation had no effect on seedling survival; one-fifth seedling, died when irradiated at 35 Gy dose; one-fourth seedlings died when irradiated at 40 Gy dose; about half of seedlings died when irradiated at 45 Gy dose (Figure 5 B). Therefore, 25 ~ 40 Gy irradiation dose could be used to induce the mutation of jujube test-tube seedlings.

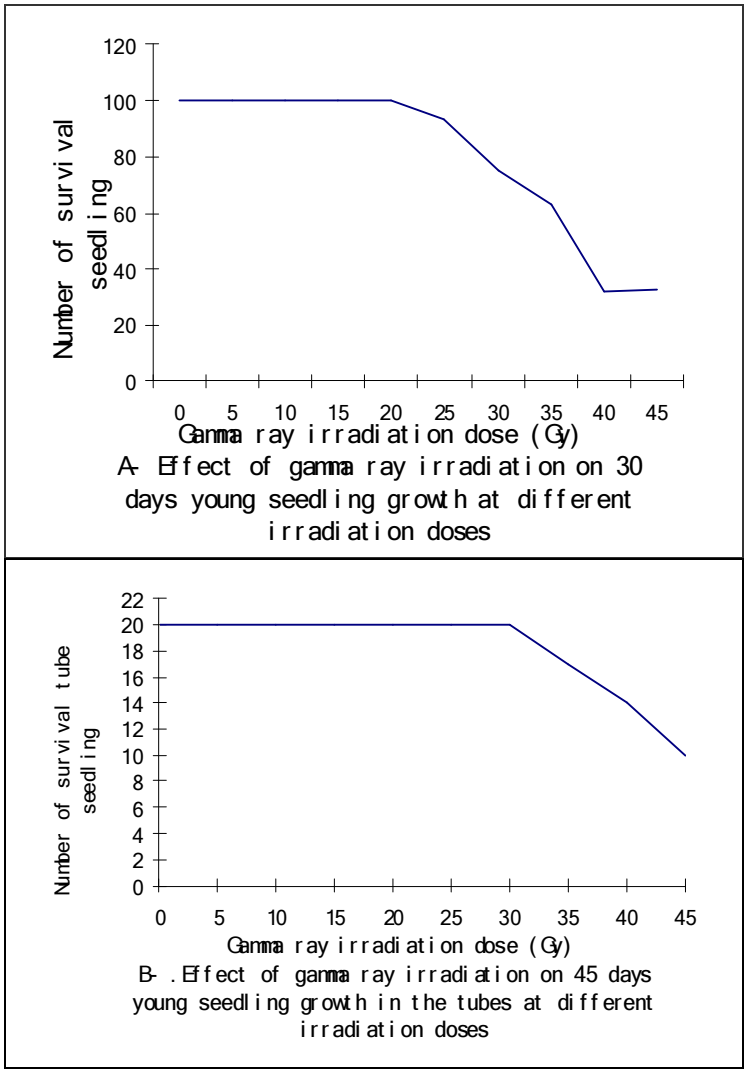

Figure 5. Effect of ^{60}Co γ-ray irradiation at different doses on growth of 45-day-old test-tube seedlings (A-30days; B-45 days old cultures).

Effect of ^{60}Co γ-ray irradiation dose on growth and shoot differentiation of jujube leaf calli. The results (Table IV and V) indicate that the growth of jujube leaf calli irradiated at 9 or 12 Gy dose was inhibited remarkably [10]

TABLE IV. EFFECT OF DIFFERENT GAMMA RAY IRRADIATION DOSAGE ON CALLUS GROWTH

Dose (Gy)	Average weight of 20 calli (mg)
0	1.824
3	1.873
6	1.975
9	1.442
12	1.171

TABLE V. RESPONSE OF SHOOT DIFFERENTIATION TO ^{60}COγ- RAY IRRADIATION AT DIFFERENT DOSES

^{60}Coγ-ray dose (Gy)	Callus number of shoot differentiation /100 calli	Shoot number /100 calli
0	28	50
3	30	51
6	15	19
9	0	0
12	0	0

4. Discussion and conclusions

The germination of jujube seeds is inhibited remarkably when jujube seeds are irradiated with ^{60}Co γ-ray, but irradiating jujube kernels can improve their germination, and the effective irradiation dose to induce mutation is 250 ~ 350 Gy. The ^{60}Co γ-ray irradiated 30~45-day-old seedlings from seeds or test-tube seedlings can be induced to mutate and the effective dose is 25~35 Gy and 25~40 Gy, respectively. By evaluating the plants from the seeds and from test-tube seedlings which were all irradiated with^{60}Co γ-ray we have found that the irradiation can efficiently induce mutation of some plant characters, but the mutant characters appearing are largely variable. Mutant characters, such as narrow leaf, yellow leaf and multiple-twig appeared in the two kinds of plants. Irradiating test-tube germinating seedlings may lead to genotypes with different blooming times, and also different quality of the fruits, which may be sweeter.

According to the results observed during these experiments the plant tissue culture combined with ^{60}Coγ-ray irradiation treatment is an effective way to obtain mutant plants. However, it is necessary to master the following key techniques for making the way practical: (1) developing a large number of tissue-cultured explants as initial plant material; and (2) selecting an effective dose of ^{60}Coγ-ray irradiation with no damaging effects.

The above experiments are only the preliminary research on jujube mutation breeding. Some mutant characters may not be displayed because of a small number of test samples; besides, the analysis and evaluation on the occurrence frequency of various mutant characters remain to be further studied. The number of irradiated samples needed to improve some character of the certain variety, the amount of funds and the genetic stability of a mutant plant also need to further observe and analyses.

The success of inducing shoots from the radicles demonstrated led to unforeseen results but could be compared to observation made in tissue culture of sweet potato where adventitious roots formed from the stem nodes and later developed into viable explants [8, 9, 10, 11]. The role of exogenous hormones in shoot differentiation was also confirmed under this project and may lead to successful embryogenesis programme in Jujube

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Mutation breeding in South Africa 2003–2004

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Abstract. Four commercial citrus cultivars have been produced from conventional citrus mutation programme in South Africa. Three hundred and fifty citrus mutant lines obtained from sectoral chimeras using *in vitro* techniques were included in the citrus evaluation programme and useful mutations of particularly difficult-to-breed cultivars is continuing. Gamma irradiation response curves (Co^{60}) as well as the LD50 levels were determined for Citrus, Litchi, Guava, Cherimoya, Pitanga, Jaboticaba and Carambola. Grafting and propagation techniques for all species, and in particular, those where no information exists, were established. Citrus, Litchi, Guava, Cherimoya, Pitanga, Jaboticaba and Carambola are all characterised by having long juvenile phases making conventional breeding programmes slow and costly. Mutation breeding is being used in an attempt to overcome this problem and produce material which can be used in the screening of advantageous genes in subsequent breeding programmes.

1. Introduction

In order to improve sales on international markets, it is extremely important for growers to provide high quality fruit. Biotechnology including mutation induction and in vitro techniques appears as an important tool for the improvement of any conventional breeding programme. Regarding citrus fruits, in general, seedlessness is a highly desirable trait that can be readily obtained by exploiting irradiation breeding techniques. Such a trait would be very useful in creating marketable cultivars from progeny derived from conventional breeding programmes. Spontaneous mutation, although of low occurrence, is an important source of genetic variation in the form of chimeric fruit or fruit sector chimeras, which may express various new traits. Characteristics other than seedlessness are more effectively harnessed by direct selection of mutations in the form of sectoral chimeras from fruit. This eliminates the extremely low probability of success in conventional breeding for specific traits. Furthermore, direct selection of such traits compared with other mutation breeding strategies (e.g. irradiation technology) precludes the establishment of extensive plantings for evaluation of *potential* advantageous traits. In co-operation with pack houses country-wide, essentially the entire season's crop can be evaluated for useful traits.

By using *in vitro* ovule rescue from these chimeric fruit showing excellent characteristics including: rind and/or flesh colour, rind and/or flesh texture, disease and/or pest resistance, early or late maturation, this genetic variation could be captured — particularly for varieties difficult to breed using conventional methods.

The major problems restricting further expansion of the litchi market include short harvest season, high number of seeds per fruit and short shelf life. Tackling these improvements, using conventional breeding, is hindered by the long juvenile phase (often over eight years). Irradiation is currently being used in an attempt to circumvent this problem. It is envisaged that plants derived from this programme could be used for the identification of advantageous genes for future breeding programmes.

The project also aimed to breed and select improved cultivars for the South African guava industry that are especially suited to the fresh market but also acceptable to the processor. These cultivars should, in addition, be resistant to Guava Wilt Disease (GWD).

Mutation breeding on alternate crops was also undertaken during this comprehensive project with the aim of conducting feasibility studies on potential crops, with a view to the possible commercialization of the crop in South Africa. A number of crops not grown commercially in South Africa possess the potential to be developed on a small scale. Some of these fruit are ideal for introduction into resources poor farmers' programmes and are suitable for intercropping: Carambola – *Averrhoa carambola,* Cherimoya – *Annona cherimola,* Pitanga – *Eugenia uniflora, and* Jaboticaba – *Myricaria cauliflora.* To successfully develop these crops the following aspects must receive attention: clonal propagation, introduction of seedlessness (pitanga and Jaboticaba), improved shelf-life and improved fruit set (*Annona* spp). As mutation has not been undertaken on any of these four species previously all of these initial studies are preliminary investigations into the possibility of using this form of breeding as a beneficial tool in the development of alternate crops in southern Africa. It is envisaged that in *Annona* species irradiation could lead to a mutation that will result in a smaller tree size and less vigorous vegetative growth and in the Pitanga and Jaboticaba a mutation could lead to seedlessness or a smaller seed. The possibility also exists that the juvenile stage of the Jaboticaba could be reduced. This is currently the largest drawback to the commercial production of Jaboticaba as trees take between 7 and 14 years to come into bearing.

2. Materials and methods

2.1. Citrus varieties

The main attributes identified and selected for, included horticultural traits which affect fruit marketability on both local and international markets. During the past seasons, fruit sector chimeras with potential for disease and other physiological disorder resistance or tolerance have also been selected. Some of the traits selected are:

(a) Horticultural traits:
- Improved rind and flesh color
- Improved rind and flesh texture
- Increased Brix levels
- Improved TSS: Acid ratios
- Thin albedo, thin rind, ease of peeling, improved fruit size (usually a branch or branchlet mutation rather than a chimera)
- Altered maturity dates (extension of harvest season).

(b) Disease resistance:
- Citrus greening disease resistance
- Citrus blackspot resistance

(c) Physiological disorder resistance:
- Rind splitting resistance.

2.1.1. In vitro ovule rescue technique

Fruit were describes and the juice brix determined. Chimeric sectors were excised from the fruit, and the ovules carefully removed from the fruit, and each set of ovules from each fruit was meticulously kept separate during the sterilisation process. Sterilisation in mesh filters was carried out by first washing in 70% [v/v] ethanol for 30 seconds followed by a transfer to a 1% NaOCl [v/v] solution for 3 minutes. The ovules were finally washed twice in a sterile distilled water bath. Ovules were cultured, 3-5 ovules per culture bottle containing 10ml

germination medium (Figures 1, 2, 3). The germination medium comprised of Murashige and Skoog [1] nutrients, Murashige and Tucker [2] vitamins, 50 $g.l^{-1}$ sucrose, 1.5 $g.l^{-1}$ malt extract and 3 $g.l^{-1}$ Gelrite.

Figure 1. Typical early ripening sectorial chimera used for ovule rescue and plant regeneration.

Figure 2. Typical undeveloped ovules used for embryo regeneration.

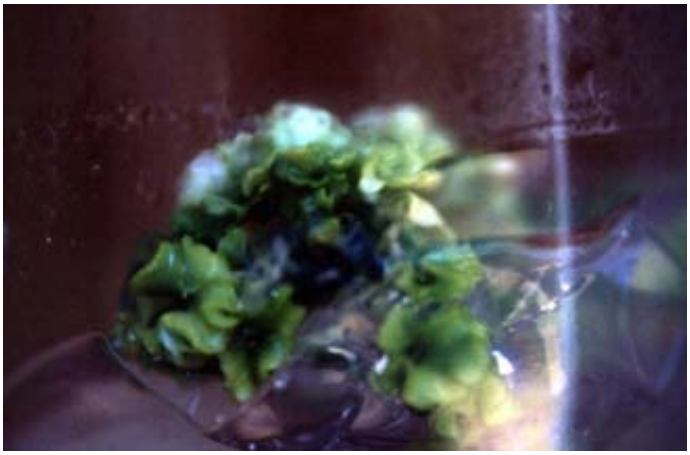

Figure 3. Embryo developments from ovules.

2.1.2. Embryo development

Embryos developed from the ovule from 4 weeks after culture initiation, depending on the cultivar and the size of the ovules. Once embryos were visible, they were then transferred to medium supplemented with 1 $mg.l^{-1}$ GA_3. Recalcitrant embryos were later transferred to the same medium containing a higher concentration, i.e. 5 $mg.l^{-1}$ of GA_3 as growth regulators.

2.1.3. Plant growth and maintenance

Plants obtained from chimeric sectors are maintained on medium for 1-2 months depending on the size of the plant and are then micro micro-grafted directly onto rootstocks for evaluation purposes. This reduces the time from tissue culture to field evaluation drastically. Currently it appeared that extremely chlorophyll-poor shoots were obtained from extremely red Cara Cara Navel sectors and these were not able to survive the harsh ambient conditions. Shoots already submitted for evaluation are now in a 'forcing house' which should yield fruit this season.

2.1.4. Mutation induction

Prior to 2002 budwood were irradiated at the South African Nuclear facility (Pelindaba), this unit closed down during 2002 and a similar source at the Nelspruit Private Hospital was used during the past season. Irradiation of budwood was conducted at the Oncology Department of the Nelspruit Private Hospital using Cobalt 60 units in order to artificially induce mutations. A Siemens Linear Accelerator with energy of 6MV (mega volt) photons was used for irradiation purposes.

100 mm Bud sticks with 4-6 buds per stick were exposed to acute Gamma Ray irradiation (30-75 Gray) and subsequently top-worked onto field rootstocks, or budded onto nursery rootstocks.

Evaluation blocks containing irradiated material were established at the ARC-ITSC experimental farms at Malelane in Mpumalanga, and at Addo in the Eastern Cape. At Malelane, blocks I1, A4, F8 G4, G5, G6 and G8 contain trees topworked or grafted with irradiated material, while blocks B9, C7, C9, E2, F3, H4, H8, J9, J10, K2 and K11 at Addo contain similar irradiated material. Most of these orchards have a variety of cultivars, which is ideal for promoting cross-pollination and facilitates the determination of stable seedlessness.

2.2. Litchi varieties

Following the determination of the LD50 value for litchi budwood, various litchi cultivars were subjected to 20 and 30 Gray of acute gamma irradiation. Budwood of the following cultivars were irradiated on 31st of October 2002 at doses of 20 and 30gy respectively: Kwai May Pink, McLeans Red, HLH Mauritius, Brewster and Fay Zee Siu. The scions were whip grafted onto McLeans Red seedlings produced in 6 litre polyethylene bags filled with composted bark.

Seed from the following litchi cultivars were irradiated at the Oncology Department of the Nelspruit on the 8th of January 2003 at 20, 30 and 40Gy respectively: Haak Yip, McLeans Red Brewster, Garnet, Kwai May Pink, Kaimana, HLH Mauritius and Wai Chee.

2.3. Guava varieties

Guava wilt resistant tissue culture plants were exposed to X-ray radiation at 3, 6, 12, 24 and 48 Gy (40 plants per dose and per selection). After X-ray exposure the plants were cut into nodal segments and placed on nodal multiplication medium (Table I).

TABLE I. GUAVA NODAL MULTIPLICATION MEDIUM

Murashige and Skoog [1] salts,	($mg.l^{-1}$)
Inositol	100.0
thiamine-HCl	13.5
nicotinic acid	4.9
pyridoxine-HCl	1.2
Biotin	0.2
folic acid	0.9
L-cysteine-HCl	14.5
Glycine	3.8
D-Ca-pantothenate	2.4
Riboflavin	3.8
choline chloride	1.4
ascorbic acid	1.8
benzyleaminopurine	2.0
Phytagel® (Sigma)	2500.0
Sucrose	30000.0
pH	5.7

Plants were cultured at 25-28°C, with a daily photoperiod of 16 hours (32 µmol m^{-2} s^{-1}).

The resultant plants were sub-cultured eight times at five-week intervals. Approximately 2.5 cm long i*n vitro* derived shoots were dipped into a 50% aqueous solution of ethanol containing 500 $mg.l^{-1}$ 3-indolebutyric acid. Shoots were rooted in a mist bed in seedling trays containing a 1:1 (v/v) mixture of composted bark and river sand. After three months the rooted plants were transferred to 4l polyethylene bags containing composted bark. Six hundred and seventy plants have been established in the field for evaluation.

Tissue culture plants derived from mature TSG2 cultivars were irradiated *in vitro* with acute gamma radiation at 0, 37.5, 50.0, 62.5 and 75.O Gy (derived from a cobalt 60 (Co^{60}) source located at the Atomic Energy Board at Pelindaba near Pretoria). Twenty five plants were irradiated for each treatment. The procedures described for the propagation of X-ray irradiated plants was followed for the subsequent *in vitro* propagation of the plants. The plants were sub-cultured seven times in tissue culture to bulk up plant material, and reduce the incidence of chimeras. Four hundred and eighty plants were hardened off, and established in the field (Figures 4, 5, 6).

Figure 4. Germinating embryos.

Figure 5. Technique of micro-micrografting which enables very small (1 to 2mm of diameter) citrus shoots to be hardened-off to ambient conditions thereby limiting loss of novel genetic material.

Figure 6. Examples of attributes selected from sectoral chimeras.

A: Improved rind colour and texture; B: Altered maturity date; C/D: Improved flesh colour E: Improved juice colour; F: Improved rind colour; G: Improved rind thickness; H: Potential greening tolerance/resistance

2.4. Mutation breeding on alternatives crops

Graft wood from carambola, cherimoya, pitanga and Jaboticaba were subjected to 0, 25.0, 37.5, 50.0, 62.5 and 75 Gy of acute gamma radiation derived from a cobalt 60 (Co60) source located at the Atomic Energy Board at Pelindaba near Pretoria. Fifty pieces of budwood per specie were irradiated at each irradiation level. The scions were whip grafted onto seedlings produced in 6 l polyethylene bags filed with composted bark on the 17/10/200. Scion survival was evaluated on the 05/02/2001.

3. Results and discussion

3.1. Citrus

At present, mutants in the evaluation programme have not yielded fruit. *In vitro* ovule rescue was conducted using the following cultivars during the 2002/2003 season: Navel, Palmer Navel, Cara Cara Navel, Star Ruby Grapefruit, Valencia, Delta Valencia, Midknight Valencia, Early Valencia, Late Valencia, Turkey Valencia, Clementine, Clementine Nules, and Temple.

For most of these cultivars the following attributes were selected for:

- Improved rind and flesh colour and texture
- Increased Brix levels
- Altered maturity dates (extension of harvest season)
- Possible Citrus greening disease resistance
- Possible disease resistance

Registered citrus cultivars derived from the mutation breeding programme are listed in Table II. Mutants that are currently been evaluated for commercialization are listed in Table III.

TABLE II. MUTANT CULTIVARS REGISTERED

Group	Cultivar	Irradiation (Gy)	Fruit			Seed
			Colour	Texture	Quality	
Manderin	Nova	30	-	-	-	Seedless
Lemon	Eureka	60	-	-	-	Seedless
Grape fruit	Henderson	60	Improved	-	-	-
Grape fruit	Henderson	60		Improved	Reduced bitter flavor, high Brix, low acid	-

Results from the mutation breeding programme demonstrate that induced mutations serve as a valuable supplement to conventional breeding procedures for the production of new citrus cultivars. Genetic variability masked by dominant genes could be revealed, while some disadvantages of hybridization (e.g. long juvenile phase) could be circumvented. Compared to the relative high cost of conventional crossbreeding, mutation breeding is an economical and time saving tool to be utilized in a cultivar improvement strategy.

A combination of conventional breeding, mutation breeding and biotechnological procedures can provide a means of producing new, seedless cultivars with a wider range of colour, quality and time of maturity.

The survival rates of irradiated scions were determined on the 19th December 2002. A LD_{50} value of approximately 36 Gy was determined for litchis during the previous season. The survival percentages from this season indicated that even lower doses should be used as most of the budwood did not survive. It may be advantageous to select an irradiation dose not higher than 20Gy in order to obtain an acceptable survival rate. The irradiated scions will be cut back in early spring in order to force the development of axillary buds. This process will be repeated three times in the nursery or field to reduce the incidence of chimeras. The plant will be incorporated in the ARC-ITSC litchi seedling trails, which form part of the litchi improvement program. At this point no fruit have been evaluated as the trees are still too small to yield fruit.

TABLE III. MUTANTS IDENTIFIED THAT DEMONSTRATE COMMERCIAL POTENTIAL

Group	Cultivar	Improvement	Comment
Mandarins			
	Murcot	Low seeded-seedless	Variable
	Clementine SRA 63	Seedless + improved fruit size	Re-valuation
	Clementine SRA 84	Improved fruit size	Re-valuation
	C18	Large seedless fruit, easy-peel, imp internal colour	
	C17	Low seeded high quality fruit	
	C27	Large fruit ave. seed content	
	Nova C1	Fruit early,	Re-valuation
	Nova C2	Improved pigmentation	Re-valuation
	B17 C17	Seedless-lowseeded	Re-valuation
	Afoure A34	Seedless	Re-valuation
Shaddocks			
	Pomelit A3	Seedless, thin rind, even segments, good flavour	Possible commercialisation
Valencias			
	Delta B42	Soft rag, good internal quality	Conformation
	Johnstone Valencia	Seedless-lowseeded	Re-evaluation
Lemons			
	Eureka A16	Seedless, elongated	Shoot tip grafting
	Limoneria A41J27	Elongated for 2 seasons	Re-evaluation
	Lisbon S19	Seedless	Re-evaluation
	Lisbon H40- & J10	Elongated fruit	
Navels			
	Kirkwood Red	Improved colour pigmentation	Chimeras
	Lane late A/7/19	Thin rinds	
Pummelos			
	Pomelit E12	Improved colour	Top worked

3.2. Guava

Exposure of plants to 40 and 50 Gy of Gamma radiations resulted in the death of 48 and 52% of the population respectively. The first fruit derived from these trees has been evaluated for seedlessness and other advantageous characteristics for two seasons. Some of the trees have exhibited large fruit in the 2002 season; however these results should be interpreted with caution, and could be attributed to the age of the plants and the rejuvenating effect of tissue culture. Apart from increase in fruit size, none of the fruit evaluated have exhibited any advantageous characteristics. Evaluation is currently focussed on testing the population for nematode resistance. Nematode control measurements were suspended in mid 2002, and have resulted in the loss of 90% of the population. Nematode resistance of the remaining population will be assessed in 2005 season.

Two weeks after irradiation none of the plants exhibited any necrotic spots, lesions or discolorations. After tree weeks in culture, plans irradiated at 75 Gy began to exhibit necrotic spots, which were followed by total necrosis of most of the irradiated plants by the sixth week. Six weeks after exposure to gamma irradiation an LD50 was extrapolated to 20 Gy (Figure 7). Similarly shoot and node production where reduced by 50% at 21 and 20 Gy respectively (Figures 8 and 9). Plants surviving gamma irradiation at 37.5 Gy were sub-cultured onto fresh guava multiplication medium (Table IV). Eight percent of the plants from the original population exhibited sufficient growth to warrant subculture after five weeks. These results indicate that lower Gamma ray irradiation doses should have been used in this experiment. The plants were established in the field in October 2003, and the first fruit will be evaluated from these trees in 2005.

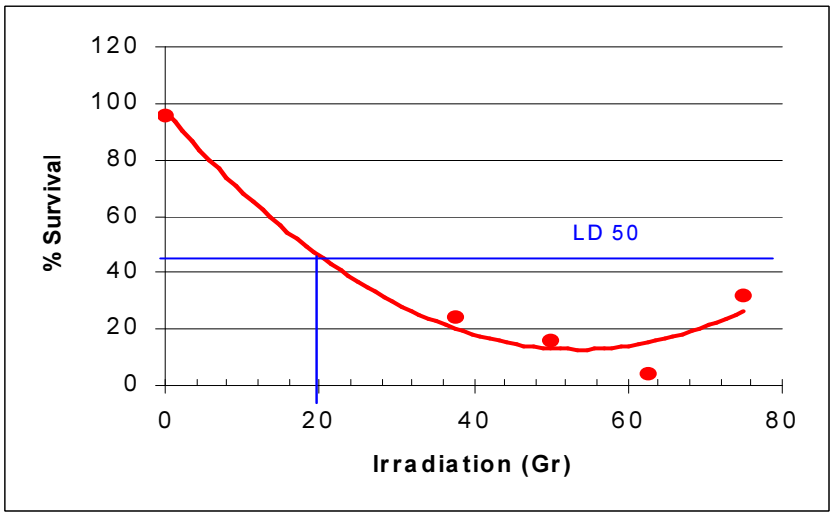

Figure 7. Acute gamma irradiation survival curve for in vitro guava plants.

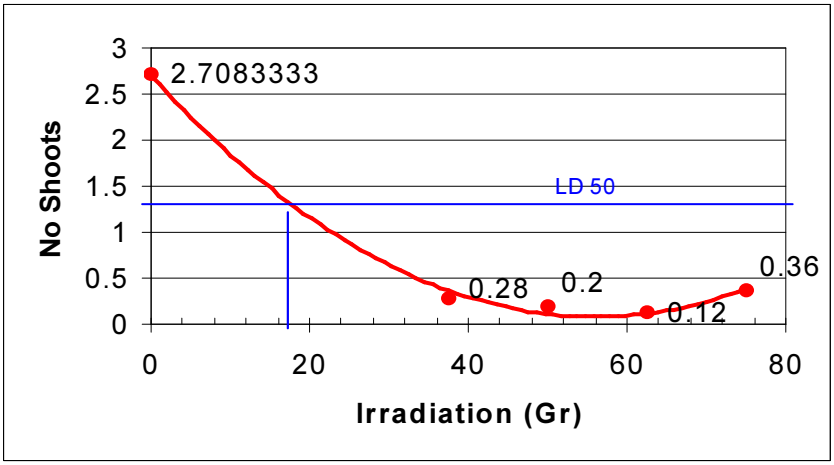

Figure 8. Effect of Gamma irradiation dose on shoot production of in vitro guava plants.

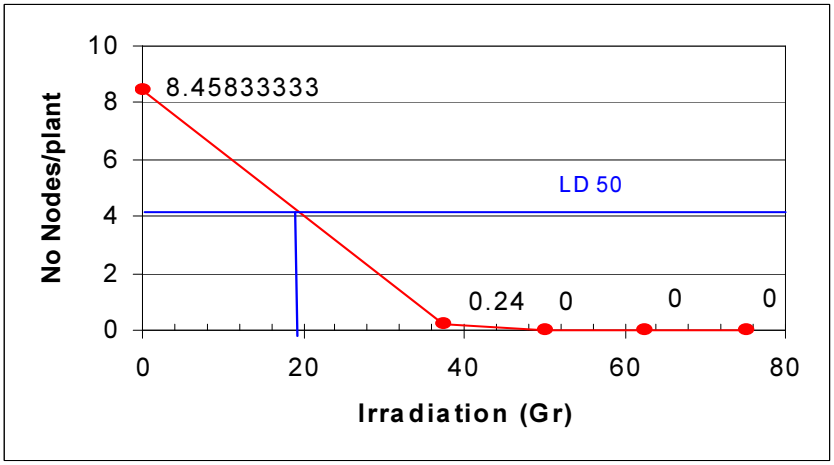

Figure 9. Effect of Gamma irradiation dose on node production of in vitro guava plants.

3.3. Other species

LD50 Values of 30, 30, 45 and 7.9 Gy were recorded for Carambola, Cherimoya, Pitanga and Jaboticaba respectively (Figures 10, 11, 12, 13). The accuracy of the values obtained for Pitanga should be used with caution as a very low graft success rate was obtained. The major reason for the low graft take was probably do to graft wood been harvested too late in the season. Flower formation was well advanced when graft wood was obtained and this process would have reduced the reserves within the graft wood. Trees from these radiation treatments were planted in the orchards during September 2001. Survival rates of trees were generally poor. The trees planted and irradiation doses are contained in Table IV.

TABLE IV. NUMBER OF IRRADIATED TREES PLANTED INTO ORCHARDS FOR EVALUATION

Species	No planted	Irradiation dose
Pitanga	2	25 gray
	6	37.5 gray
Jaboticaba	2	25 gray
	2	50 gray
	2	75 gray
Annona	14	25 gray
Carambola	10	25 gray
	11	37.5 gray

All these trees will be cut back during September and the second vegetative flush evaluated. On-going evaluation of the trees will be undertaken in the orchard.

Further irradiation was conducted on carambola, jaboticaba and annona during February 2002. Carambola trees were cut back in the orchards and were top-worked with the 360 graftwood that were subjected to irradiation. A very poor take percent was obtained.

It is evident from results above that top-working of carambola was unsuccessful. This was however, not due to irradiation but rather to technique of top-working as this has not previously been undertaken on carambola.

Two hundred Jaboticaba trees were top-worked with irradiated budwood and 50 as control. Sixty-four grafts with irradiated graftwood were successful and 36 of the control. It would thus appear that Jaboticaba is very sensitive to irradiation and a lower Gy should be used in further trials.

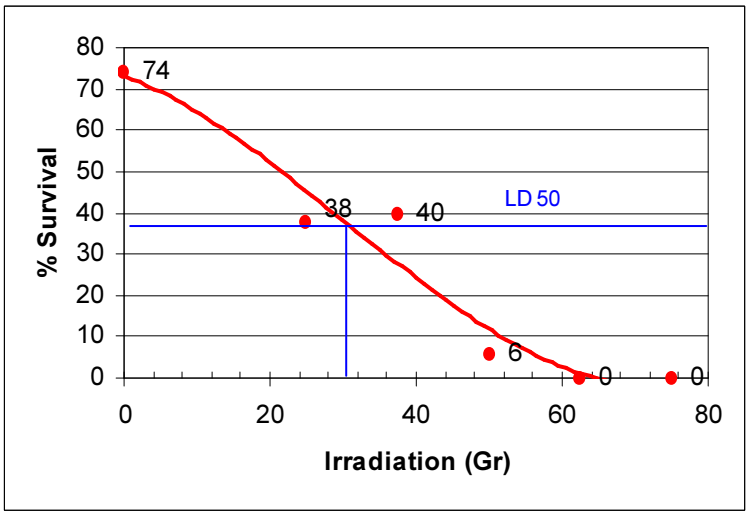

Figure 10. Gamma irradiation response curve for carambola.

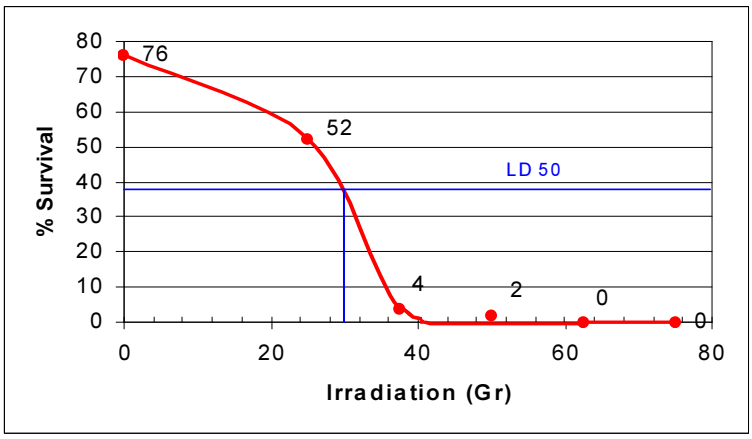

Figure 11. Gamma irradiation response curve for cherimoya.

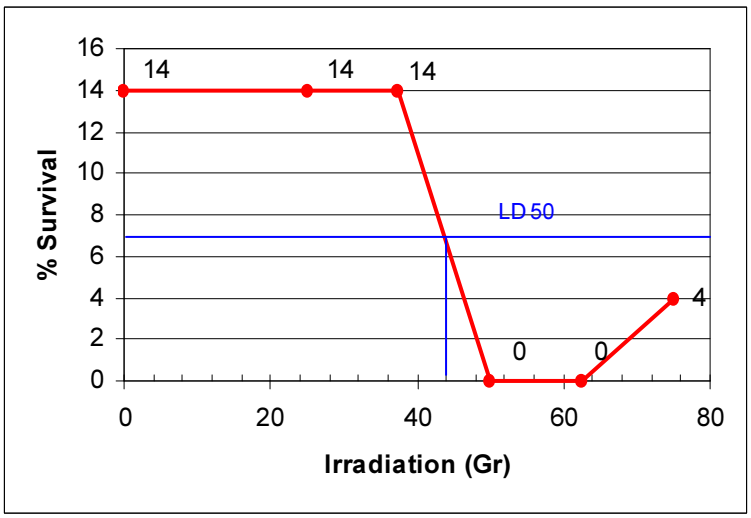

Figure 12. Gamma irradiation response curve for pitanga.

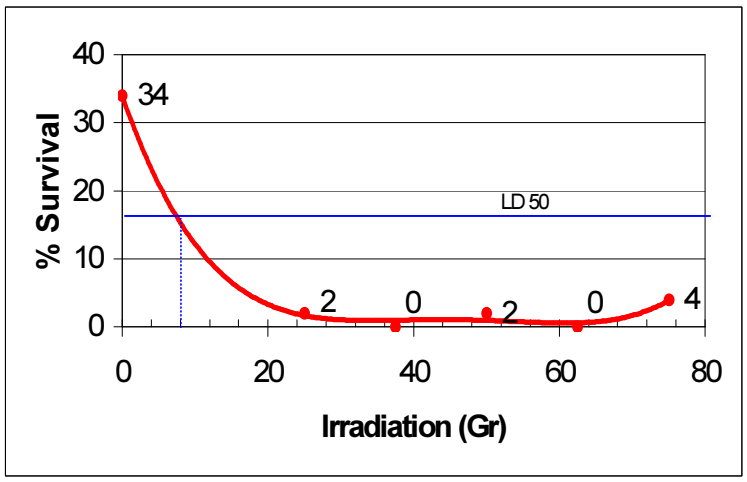

Figure 13. Gamma irradiation response curve for Jaboticaba.

4. Conclusions

Irradiation doses of vegetative material and seed of litchis will be adapted according to results obtained during the past season. Higher numbers of different cultivars will be irradiated for establishment in orchards of the litchi improvement program.

Annona budwood was irradiated at 35 Gy. Two hundred graftwood was irradiated and grafted on seedling rootstocks and 50 controls were grafted. Grafting success was very poor. Grafting time was probably incorrect and grafting should be done during September. These trials will be repeated during September 2002.

Pitanga budwood was irradiated during September 2002 and grafted onto seedling rootstocks. No rootstocks were available and had to be propagated before irradiation could be undertaken.

The surviving trees were planted out in orchards during September 2001. Further irradiation was carried out on graftwood of cherimoya, Jaboticaba and carambola. Jaboticaba budwood were subjected to 10 Gy, carambola and cherimoya to 35 Gy gamma radiations. The carambola budwood was top worked onto trees cutback in the orchard and Jaboticaba and cherimoya were grafted onto seedling rootstocks in the nursery. Controls were grafted and top worked with graftwood that was not irradiated.

Results obtained previously showed that pitanga appeared to be the most resistant to gamma irradiation with an LD50 of 45 Gy, Annona and pitanga exhibited an LD50 of 30 Gy. Jaboticaba exhibited a very low tolerance to gamma irradiation with an LD50 of 7.9 Gy.

Irradiated trees were planted in the orchard at Nelspruit.

- Forty-five irradiated pitanga seedling trees and two grafted trees were planted in an orchard.
- Only 14 Annona trees survived and have been planted in the orchard. The poor survival was due mainly to poor quality budwood and these trials have been repeated during February 2004. The budwood was subjected to irradiation doses of 25 Gy.
- Fifteen irradiated Jaboticaba trees were planted. (Irradiation dose 0f 10 Gy).
- Eighteen irradiated carambola trees were planted (35Gy). These trees are currently flowering and will be evaluated when the fruit matures.
- Irradiated pitanga seedling trees have started bearing fruit but no seedlessness or reduction in seed size was observed. Fruit yield was high but fruit colour and size does not compare favorably with superior selections.
- All trees that have been planted in the orchard will be evaluated when they start bearing.
- During the previous season carambola trees were top worked and poor results were obtained due to problems with top working. The method of top working has however been refined and a percentage take of 80% obtained. Carambola budwood was irradiated at 35 Gy and trees have been topworked in the orchard. Percentage take appears promising and trees will be monitored and evaluated. Annona budwood will be irradiated after harvesting and trees will be topworked in the orchard.

Initial problems that were encountered were mainly due to grafting techniques that had not previously been utilized on these crops. The techniques have now been refined and progress appears to be good. Further irradiation of budwood will be undertaken to increase the number of irradiated trees as grafting and top working techniques are now effective.

Optimal irradiation levels, irradiation response curves and LD50 values were established for guava, carambola, pitanga, litchi, annona, Jaboticaba and citrus and will facilitate the further breeding of these crops. However, since the trials conducted were relatively small, more meaningful results in terms of mutation breeding of these species will be obtained if the programme is expanded to include larger numbers of plants. Field trials to determine irradiation effects have commenced. With regard to the use of citrus sectoral chimeras, several lines (over 300 to date) were included in the Agricultural Research Council's evaluation programme. The identification and harnessing of useful mutations using *in vitro* technology is being run concurrently with the citrus harvest season in South Africa. The mutation breeding programme will be expanded if additional funding can be procured.

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CHAPTER III

"Technology packages" for crop improvement using mutation induction and biotechnology

"Technology packages" for crop improvement using mutation induction and biotechnology

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Abstract. "Technology packages" in crop breeding may be defined as sets of various technologies used in synergy in order to achieve specific research goals. The tremendous advances in cell, molecular, atomic and even nano-technology afford scientists with powerful tools for exploring the Living Kingdom to the benefit of Human Kind. Mutation induction is undoubtedly one of these technologies, which has resulted in tremendous changes in the way genetics and derived genomics can be applied for crop improvement. Classical breeding approaches associated with agronomy and biotechnologies allow less than 2% of the population of industrialized countries to produce ample food to satisfy their national needs. The rapid expansion in science-based knowledge, relating to genetics, genomics, exploitation of biodiversity and induced mutations now has the potential to bridge the gap from research to application in agriculture in developing countries. In a timescale of decades some important changes could be efficiently implemented. In addition to the current goals of plant breeding new ones such as the following are emerging continuously: a demand for more meat in global diets - requiring serious increase of primary production; the demand for bioenergy crops other than just fuel woods mean that there is a new range of target plants for domestication and improvement where minimal or no classical breeding has been applied so far. When considering the major technological developments together with their potential in crop improvement for developing countries, there is a great need of clear and wise assessment of their usefulness and applicability. Various approaches such as DNA, markers, TILLING, high-throughput sequencing and reverse genetics are applicable to breeding programmes in developing countries. In the present chapter, all approaches considered appropriate are assessed on the basis of their advantages and constraints.

1. Introduction

Much of the plant breeding process involves the selection of new combinations of genetic characteristics, which are already present in the available varieties. However, in addition it is often desirable to introduce new traits that are present neither in current crop germplasm nor in related wild species (like biotic or abiotic stress resistance genes, disease resistance genes and characters such as seedlessness or delayed ripening), and considering new target crops such as bio-energy plants. Generation and selection of mutations has proved to be an efficient system to increase the range of alleles available in crops.

The efficiency of mutation induction to enhance breeding depends on a number of parameters including: the species, the traits of interest, the necessary dissociation of chimaeras and the availability of efficient screening techniques. Cellular and molecular biotechnologies offer a panel of options to alleviate some of these bottlenecks and combined to mutation induction techniques, have led to the development of powerful technology packages triggering a renaissance in mutation breeding. But which mutation technology packages are appropriate?

A tremendous variety of production systems and environmental constraints are found in developing countries and differences may even be found within an individual country. There are four broad agro-ecological zones accounting for 90% of agricultural production in the developing world: 1. Humid and peri-humid lowlands, 2. Hill and mountain areas, 3. Irrigated and naturally flooded areas, 4. Dry-lands and areas of uncertain rainfall. Within each of these zones, a range of farming and cropping systems are found as well as a mixture of traditional and modern production systems. The Joint FAO/IAEA Division with its long experience in technology transfer has achieved several success stories in fostering the implementation of mutation induction and efficiency enhancing supportive biotechnologies to nurture agricultural development, not only as a local tool, but also as a paradigm.

The type of biotechnology related research activities to be performed is dependent on the reproduction system - vegetatively or seed propagated plants. The important and well known research biotechnologies include plant tissue culture, green houses and cold room facilities; for micro-propagation of fruit crops and ornamentals with further technological developments leading to bioreactors and cryopreservation, somatic embryogenesis; double haploid technology (microspore culture, anther culture), and cell suspension systems leading to *in-vitro* selection methods (e.g. salinity, drought, disease resistance/toxin tolerance). An example of the nature and impact of genomic technologies on breeding for tropical fruit crops comes from a project on banana where coordinated international efforts are making significant improvements [1, 2, 3].

In this chapter we will assess a range of techniques of molecular biology, developed since the late 1980s which can be considered for application. These techniques can assist with plant multiplication, improvement and selection with unprecedented accuracy and speed, even though they require specialized knowledge and equipment and may be expensive. The range of opportunities for plant breeding in the future may be considered as ‘superdomestication’ [4] allowing improvements to be made to plants based on consideration of the needs and design based on knowledge of crop behaviour.

The choice of molecular techniques depends upon:

- The “pro’ and “cons’ of the various technologies regarding their appropriateness to the specific project and its specific goals;
- The comparison of the molecular technologies with existing conventional methods in the specific crop given the realities of life in developing countries,
- The identification of certain regions in the developing world where these technologies can be applied in a better way and which may be considered “Centres of Excellence” for further development in selected geographical regions.

In addition, one should also consider the following:

- The relative costs (e.g. financial, social, and political) of molecular techniques *vs*. the relative benefits (e.g. productivity and food security);
- The appropriateness of the various molecular techniques regarding their environmental impact, their impact on human health, the status with respect to intellectual property rights, the status with respect to bio-safety regulations and controls, the existing level of access to these technologies, the level of capacity-building or resources required to use them, their impact on food production and food security and the desire to definitely bridge the scientific gap.

In this chapter we give a short review of the different technologies, which in association with mutation induction are readily available to all breeders and scientist, namely: DNA markers genomics, genetics and cytogenetics, reverse genetics, TILLING and high throughput sequencing. The authors present the potential of such approaches and discuss their applicability.

2. Genomes and DNA markers

Plant genomes consist of DNA encoding for genes that are expressed and ultimately produce the phenotype. While all diploid plants have approximately 25,000 protein encoding genes and their associated regulatory sequences, the sizes of plant genomes vary between less than 10^8 nucleotide base pairs [(bp), or 100 million bp, 100Mbp] and more than 10^{10} bp (10,000 Mbp, Table I), divided between 4 and more than 200 chromosomes [5]. The coding part of the DNA consists of only a few percent while most of the DNA is not coding and its role although assumed to be associated with gene regulation, still remains obscure.

TABLE I. GENOME SIZES AND CHROMOSOME NUMBER IN TROPICAL AND SUBTROPICAL FRUITS, *ARABIDOPSIS* AND RICE

Common Name	Genus	Genome size (Mbp)	Ploidy (n)	Chromosome number 2n
Arabidopsis	*Arabidopsis*	140	2	10
Rice	*Oryza*	400	2	12
Mango	*Mangifera indica*	439	2	20
Avocado	*Persea americana*	875	2	24
Papaya	*Carica papaya*	372	2	18
Citrus	*Citrus sinensis*	160	2	18
Banana	*Musa* spp.	600	2	22

Genetic variation and polymorphisms are common to all species and most of it results from variation in the DNA sequence [6]. Genetic variation is the “Currency of Genetics” and lately became a major target of modern biological research. The goals of these studies are to assess the variation, to understand the mechanism that maintains it and to apply various approaches to identify genes of interest. Polymorphism is the existence together in the same population of two or more variants of the same trait when the frequency of the rare variant is higher than the frequency of mutation. Eye colours and blood groups are examples of polymorphisms. Polymorphism exists in all sexually propagated organisms although the level of polymorphism varies among organisms and traits.

The various types of sequence variation are:

A. Chromosome Aberrations (deletions, duplications, inversions and translocations);
B. Variable Number of Tandem Repeats (VNTR);
C. Copy Number Variants (CNV);
D. Point Mutations-Single Nucleotide Polymorphisms (SNPs).

DNA sequencing of each individual is the ultimate tool for identification of sequence variation. This explains the tremendous effort put into determining the sequences of living organisms including bacteria, fungi, insects, animals, plant models and humans. The current goal is to be able to sequence not only a representative sample from each species but many

individual samples as well. Thus, the NIH aims for reducing the cost of a full sequence of an individual sample to US $ 1,000.

Sequencing of the various genomes reveals high homology among conserved parts of genes in various species. This homology is a very important tool for identification and isolation of genes from crops of interest (see degenerate primers below). Figure 1 shows an example of the sequence of a fragment of the cellulose synthase DNA from banana, rice, Arabidopsis and poplar. This economically important gene which is involved in cellulose synthesis and necessary for plant cell walls development is similar but not identical between the various species.

Figure 1. Sequence of a fragment of cellulose-synthase genes from two species.

3. Assessment of genetic variation

3.1. Assessment of chromosomal variation and genomes by cytogenetics

Crop plants vary in their ploidy levels and chromosomal numbers and several commercial crops varieties belonging to the same species may have different ploidy levels. While most such cells do not regenerate or give rise to obviously abnormal phenotypes, a few might be advantageous. For example, the large cells associated with tetraploidy might give rise to larger fruits, while triploidy might give seedless characters. In micropropagation-based research programmes it is worthwhile to analyse karyotypes or to check for homogeneity prior to regenerating plantlets bearing in mind the possibility of treatments giving rise to aneuploidy, polyploidy or other chromosomal aberrations. Where abnormalities or large changes in morphology are observed, chromosomal (karyotypes) and/or flow cytometric analyses should be considered as an early approach. Flow-cytometry allows relatively easy assessment of ploidy levels and detection of mixoploidy by comparison of different accessions. Microscopy and handling for chromosome counting and checking karyotypes requires skills which are relatively widespread.

In wide hybrids, whether made through protoplast or sexual methods, cytogenetic analysis is an excellent approach for examining transmission of genomes/chromosomes and recombination and segregation in subsequent generations.

Molecular cytogenetics (particularly *in-situ* hybridization), is proving to be a very valuable tool to characterize genomes and chromosomes in different crop species. For example, *Citrus* and *Poncirus* rootstocks as well as their hybrids may be identified on the basis of chromosomal constitution [7] (Figure 2).

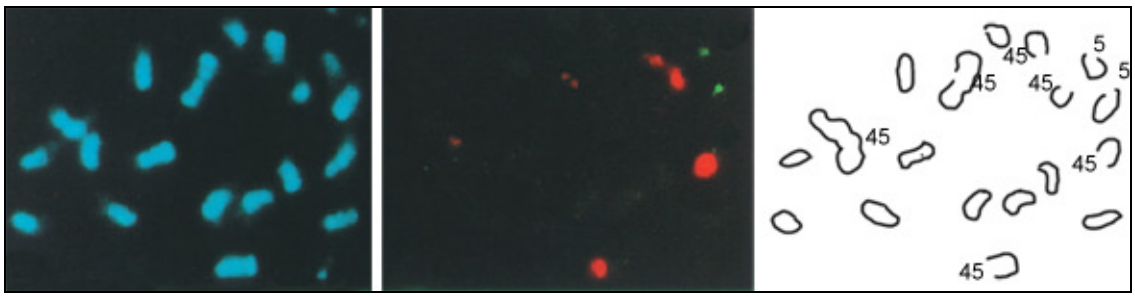


Figure 2. Characterization of Citrus in sweet orange by In-situ hybridisation.
***Left:** Chromosomes stained with the fluorochrome DAPI; **Center**: In-situ hybridisation showing 5S and 45S rDNA sites; **Right:** diagram showing 18 chromosomes, with 5S (one terminal pair of sites) and 45S (two major terminal pairs and one centromeric minor) sites marked. These chromosomes can be identified in hybrids used for breeding programmes (after [8]).*

3.2. Assessment of sequence variation – DNA markers

The currently available tool for the assessment and applications of sequence variation is DNA markers. All DNA marker applications are indirect ways to study DNA sequence – either at the base level or by homology with known probes – and each one has advantages and disadvantages. DNA markers are divided into "single locus markers" (with acronyms such as RFLP, SSRs and SNPs; see Mutant Germplasm Characterization using Molecular Markers Training Course Series No.19. IAEA, Vienna (2002), free download from http://www.iaea.org/nafa/d2/public/tcs19-manual.pdf) and "multi-locus markers" (like ISSRs and AFLP). The most important characteristics of "good" DNA markers are their abundance and distribution in the genome, their level of polymorphism (the number of alleles in a certain locus in the population must be appropriate for the study and germplasm being used) and the ability to apply them for genotyping in a high throughput setting.

A useful manual describing various technologies regarding the applications of these markers can be downloaded from the IAEA website as a PDF file: http://www-naweb.iaea.org/nafa/pbg/public/d2_pbl_1_6.html.

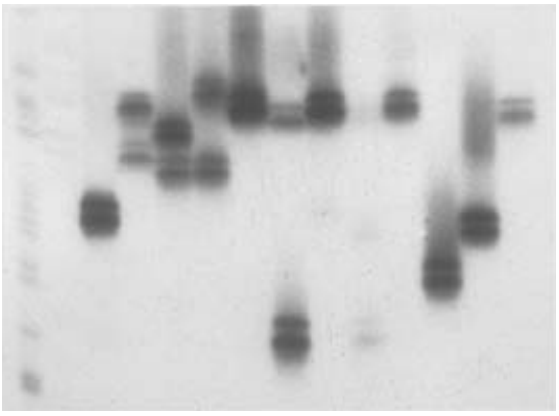
The choice of the suitable marker depends on the goal of the research project, the availability of the technology and the costs involved. The methodology can be chosen on the basis of laboratory resources (training and equipment), with variation for example from SSRs, requiring only PCR and electrophoresis equipment, compared to RFLPs, which need restriction digests, radioisotopes or fluorescent labelling techniques, and other molecular biology methods. While early results were promising with RAPDs (Randomly Amplified DNA Polymorphisms) using PCR with short arbitrary oligonucleotides primers, the reliability and reproducibility of this approach has been found to be much too low to be of use.

In general, the "multi-locus markers" such as AFLP, RAPD and ISSR are, someadvantageous for analysis of DNA variation between and within species including cultivars, wild populations and wild relatives. In assessment of sequence variation, DNA samples from each accession are used with the appropriate procedure to give a band pattern along a gel and these bands typically are analyzed in a binary (0-1) matrix. Depending on the marker system and nature of the accessions being investigate, their level of polymorphism may be rather low (existence or absence of a certain band) but one assesses several to tens of loci in each genotyping reaction. Most of these methods need no previous sequence or molecular knowledge

Using freely accessible software such as PHYLIP (www.evolution.genetics.washington.edu/phylip.html) or PowerMarker (www.statgen.ncsu.edu/powermarker), one can quantify and group (cluster) the genetic variation (Figure 3). These markers need no previous sequence/molecular knowledge and can be applied to all species thus are universal and simple to use.

The "single locus markers" are advantageous for linkage analysis and for the study of syntheny (RFLP). These markers are mainly species specific and easy to apply although more difficult to generate. Their level of polymorphism varies: RFLP and SNPs have usually two alleles per locus in the population, while the number of alleles in SSRs can reach tens and more. The frequency of single nucleotide polymorphisms (SNPs) for example, within a species may be extremely low (there are only a few alleles of each gene with very small difference), or as high as 1 in every 10 bps. The sequence information available for species like Arabidopsis and rice

The DNA sequence of the expressed sequence tag (EST) from Musa is aligned with the DNA homologous sequence from the database of other species, and shows more than 90% similarity. There is one indel (insertion/deletion) of three bases.



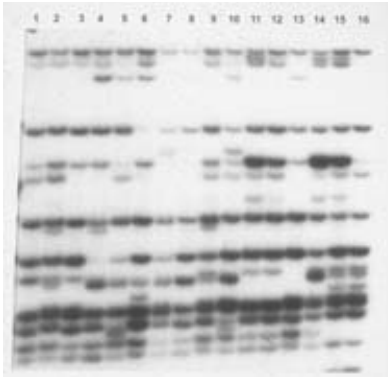


Figure 3. Avocado cultivars genotyped with SSRs *Mango cultivars genotyped with AFLP*

3.3. Applications of DNA markers

DNA markers are applied in various species for various purposes in humans they are applied mainly for medical and forensic needs. Here we discuss the applications of DNA markers for plants, which are basically aimed at identification and improvement of breeding projects.

3.3.1. Identification of individuals and populations

Identification is based on unique patterns of markers associated with species and/or individuals. Moreover, comparisons of markers' patterns allow the assessment of genetic relationships. The higher the homology between the patterns, the closer they are genetically (see also above: 3.2 Assessment of Sequence Variation). DNA markers each analyse directly small parts of the genomic DNA sequence. For example, RFLPs interrogate the twelve or sixteen bases which are homologous to the restriction enzyme used, and that the probe used is at least 85% similar to part of the target sequence between the enzyme sites. SSR markers measure the number of repeats, typically, of a two or three base pair motif that lies between

the primers. Sequencing of PCR products can look at 400 to 700 bp of sequence. However, these approaches using DNA markers only examine a tiny proportion of the hundreds of millions of base pairs in crop genomes (Table I).

Identification of individual cultivars and varieties may also serve for the protection of breeders' rights, for solving paternity dilemmas (mainly in subtropical trees having huge numbers of flowers and characterized by large fruitlet drop making controlled pollination unfeasible) and for assessment of genetic relationships and phylogeny studies. In addition to their biological interest, such assessments may aid in an efficient selection of accessions to be maintained as seeds, in tissue culture or as growing plants in genetic resource collections or Gene Banks with minimum redundancy while representing most of the genetic variation. For example, all the cultivars in one country may be unrelated to those in another country or alternatively, the cultivars may not be different from wild accessions. In addition, these markers may be used to assess the level of heterozygosity (and inbreeding) and other populations' characteristics.

In this respect, it is imperative to emphasize the difference between "exclusion" and "inclusion". When comparing patterns of DNA markers it is quite obvious that when two patterns are different one from the other and if one makes sure that no mistake has occurred in the genotyping and that the number of differences can not be explained by mutation - the two individuals are indeed different (exclusion). However, obtaining the same band patterns from two individuals does not mean that the two individuals are the same. It is possible that the level of polymorphism detected by the markers is not sufficient to distinguish between the tested individuals (inclusion). Until full sequence of individual samples is feasible, one should increase the number of tested marker loci, in order to achieve unique individual patterns. This point demonstrates the limitation of DNA markers compared with full DNA sequence of each individual.

3.3.2. Improvement of breeding projects

Three examples of applications of DNA markers to improve classical breeding projects are listed below:

3.3.2.1. Gene introgression

Breeding projects of gene introgression are based usually on a cross between elite cultivar lacking a certain valuable allele (like resistance to a specific disease) and a wild species lacking the desired agronomic trait but carrying this specific allele. In order to dilute out the undesired alleles in the wild-species, the breeder carries out 6-10 generations of backcrosses (BC). The length of time needed for this procedure makes it expensive and unfeasible for some species having long juvenile period (mainly fruit trees). Based on the pattern of DNA markers, one can select the individuals sharing the highest homology with the genome of the recurrent parent, in each generation (this selection is carried out in addition to the selection of the resistant individuals). Thus, and due to the fact that the individuals in each BC carries a different percentage of the genome of the recurrent parent, one can accurately select the most suitable individuals in each BC generation rather than using a random sample of progenies. In this way the breeder can "buy time with money". Namely, by genotyping large number of individuals one can obtain the goal of gene introgression in as little as 2 BC generations [8].

3.3.2.2. Hybrid seed production

In order to produce hybrid seeds one crosses two pure cultivars. Classically the process is based on “trial and error”. When the number of pure cultivars is in the hundreds or more, the number of possible crosses becomes unfeasible. It was found that the higher the genetic distance between two pure cultivars in a certain species, the bigger the hybrid vigour of their progeny. DNA markers allow the assessment of genetic distances between potential pure cultivars and thus allow a tool for the choice of cultivars to serve as parents for the production of hybrid seeds [9].

3.3.2.3. Marker assisted selection (MAS)

The generation of RFLPs resulted in the expectation that DNA markers would replace classical breeding by identification of DNA markers associated with the genes of interest thus selecting the markers rather than selecting the traits. However, this goal turned out to be very difficult to attain. Only a few examples of MAS are available and currently most of the plant breeding projects are based on classical breeding. Nevertheless, MAS appears effective in selecting for specific traits in targeted breeding projects. For example the DNA markers associated with resistance to viruses such as TMV, or fungi including *Verticillium* and *Fusarium* are widely used in tomato breeding projects [10,11].

The bottlenecks for wide applications of MAS in breeding projects result from the lack of markers closely linked with most of the genes of interest and from the high cost of genotyping large numbers of progeny with large numbers of markers needed for both the identification of the linkage and the application of MAS in the breeding project.

In evaluating the applicability of MAS one should consider: impact, utility and efficiency. The relative cost-effectiveness of various conventional and MAS schemes depend on the detailed circumstances of each particular application. An important consideration is the cost of the phenotypic assessment of the trait compared with the cost of MAS. In addition, it is crucial to understand that identification of markers linked to gene (s) controlling the trait of interest, depends on clear identification of the various phenotypes. Decisions about whether to incorporate MAS into a breeding scheme are likely to require a case-by-case analysis: the rationale of MAS differs between rice and oil palm breeding, the comparative advantage of MAS over field performance tests drops with shorter life cycles of crops and with the ease of phenotypic assessment.

On the other hand, the idea of Marker Assisted Selection (MAS) or Marker Assisted Breeding (MAB) is still of high interest for breeders, and scientists may apply DNA markers for the identification and isolation of specific genes of interest and of unique and new alleles (generated for example by induced mutations). A new generation of markers (SNPs), has the potential to be applied for MAS due to their high abundance in all genomes and the available technologies to genotype SNPs in very high throughput [12]. This results in the potential to identify linkage of these markers to the genes of interest. **T**he developments in SNP’s genotyping opened a new era in identification of genes of interest. It is noteworthy, that many of these technologies are developed for the identification of the genes controlling the generation of complex diseases and hopefully will be eventually applied to plant breeding as well.

In order to identify markers for all genes, there is a need to have a saturated map of markers similarly to the importance of a dense number of “mile-stones” on a highway. Thus, it is

important to emphasize that the high frequency of SNPs and the available technologies to genotype them in a high throughput, offer new options for applications of DNA markers. Examples of these applications are the 500,000-1,000,000 SNP chips of Affymetix: http//:www.affymetrix.com and Illumina: http//:www.Illumina.com.

In summary, we believe that in spite of the tremendous potential of MAS to turn classical breeding into more effective and less expensive projects, it is still a complicated procedure especially for developing countries. On the other hand MAS has a significant potential in terms of shortening the time of the breeding project especially in crops having a long juvenile periods (such as nuts, oil-palms and date palms). Thus, initiation of research projects aimed at the identification of markers linked to traits of interest should be considered very thoroughly on a case by case basis and will probably be justified in rare cases only, where the crops of interest (consider oil palm vs. rice) and the traits in question are appropriate. For farmers, the availability of either new genotypes or the ability to better select which genotypes are appropriate to grow offer significant advantages. In fact, for some traits, field evaluation is a better choice than genotyping of a linked marker. In order to identify a linked marker one needs a clear reliable screening test of the trait, the appropriate populations (F2, RILs, ILs, BCs), the right technology and the necessary funds.

4. Assessment of patterns of gene expression

4.1. Assessment of DNA methylation patterns

While DNA sequence variation is the ultimate source of genetic variation, it is increasingly recognized that methylation of cytosine residues in DNA (5-methyl cytosine) is critical to the control of gene expression (genetic imprinting, epi-genetics). Where a gene is methylated, there will be a tendency for it not to be expressed, and the methylation pattern of the gene can be transmitted from one cell or plant generation to another through the activities of methyl transferase enzymes. These methylation changes may be affected by environmental conditions, and in some particular tissue culture may change the methylation patterns across the whole genome (Figure 4). Changes in methylation patterns may be responsible for soma-clonal variations.

There are several ways to assess DNA methylation patterns:

- Methylation sensitive restriction enzymes
  - Application of methylation-sensitive AFLP (*Msp*I vs *Hpa*II)
  - Use of McrBC enzyme restriction patterns (Figure 4)

- DNA sequencing following bisulphite treatment of DNA [13] \

- *In situ* hybridisation using anti-methylcytosine antibodies

An example of methylation pattern changes is seen following tissue culture in oil palm. Figure 4 shows the differences in McrBC digestion patterns in trees fragments and tissue culture material in a group of trees where a flowering abnormality, ‘mantling’, was observed. Therefore, where unstable changes – symptoms of changes are detected over time, including several generations – are detected in breeding, methylation should be investigated.

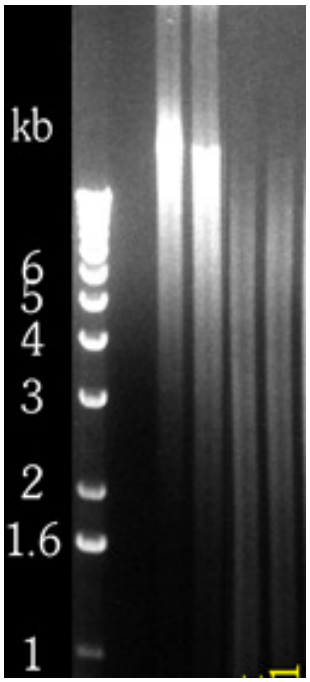


Figure 4. McrBC digests of oil palm DNA. ***Left:*** *Marker lane sizes of cut DNA fragments.* ***Central two lanes:*** *digests of DNA from callus culture; much DNA remains uncut at the top of the gel, showing it has limited amounts of methylated DNA, while the two lanes from trees.* ***Right:*** *most DNA cut to fragments between 1 and 10kb long, indicating that many cytosine residues are methylated [14].*

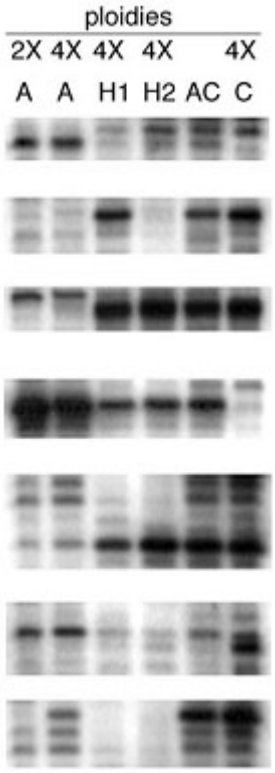


Figure 5. A cDNA-AFLP analysis. Pattern of Arabidopsis thaliana Gene Expression.
(A), Cardaminopsis arenosa (C) and synthetic allotetraploids of Arabidopsis suecica (H1 and H2) were compared. AC is an artificial reconstruction of the expected allotetraploid profile done by mixing equal amounts of cDNAs. [15].

4.2. Molecular technologies applied for the understanding of biological systems

In order to close the Phenotype Gap (PhG) - "the gulf between the available mutant resource and the full range of phenotypes that is essential to exploit fully investigated species [16]" -, mutation induction is an essential tool. Mutation induction can increase both the breadth (finding and mining new loci not previously known to relate to a character) and depth (mining new alleles at loci known to affect a trait) of available gene-based resources, in order to obtain the full range of phenotypes needed for the investigation of basic developmental, biochemical and physiological mechanisms.

In addition to the application of molecular technologies to make classical breeding more effective and more efficient, there is a need to look for the next stage of new technologies. Similarly to the need to identify the genes controlling human diseases in order to diagnose and treat them, there is a need to identify the genes controlling the important agricultural traits. Identification of these genes and understanding their control and orchestral activities is a prerequisite for the generation of better cultivars by either classical or molecular ways.

Bridging the "Phenotype- Genotype Gap" means the molecular understanding of the genes controlling a specific phenotype. A list of some important technologies to identify genes of interest is presented below.

4.2.1. Radiation techniques in crop genome mapping

Radiation hybrid (RH) mapping is a technique based on exposing somatic cells to lethal doses of γ radiation or X-ray, in order to fragment the chromosomes, which are then rescued by introduction into host cells, which subsequently are fused with suitable recipient cells. It was developed to facilitate the analysis of the human genome and recently its improved methodology has been transferred to plant systems. Using multiple RH lines, each with different chromosome fragments from the original target species, DNA markers can tested and grouped because they are found in the same RH line, allowing whole genome linkage mapping. Radiation hybrid maps in a number of crops such as barley, maize, wheat and cotton have been developed for gene discovery and detailed linkage analysis [17]. This would facilitate the identification and transfer of genes affecting useful agronomic, quality and stress tolerance traits in crop improvement.

4.2.2. Analysis of mutations for functional genomics

The identification of gene sequences will enable more efficient breeding (both cross breeding through MAS and transgenics). In fact, knowledge of gene sequences will assist programmes involving identification of parents for crossing and genes for transformation. Gene sequencing will identify the nature of induced mutations in the genes and these mutated genes can then be introduced through conventional and biotechnology breeding programmes in diverse germplasm.

Mutation grids of some of the major staple crops such as rice, maize, barley and wheat are now available, and allow researchers to identify plants and acquire seeds carrying particular mutant traits for crop improvement, reverse genetics and functional genomics. For example, in potato the focus lies on understanding the interaction between potato and the major pathogens affecting its production (Streptomyces), in *Medicago truncatula* on the isolation of mutants defective in calcium oxalate crystal formation, and in rice on mutational analysis of the phytic acid pathway as supported by a Regional Technical Cooperation Project (RAS/5/014). The Agency, through partnerships with advanced international laboratories under with the Consultative groups on International Agriculture Research centres (CGIARs) and some national institute in Member States (NARs) is developing mutation grids in banana and cassava and contributing to reducing the "phenotype gap" in functional genomics of rice.

4.2.2.1. Degenerate Primers

Known genes from one species are likely to have sequence homology with counterparts in the tropical and subtropical crops where there is little knowledge of the DNA sequence. Thus, the genes in the tropical crops can be isolated by PCR using degenerate or low-stringency hybridisation of primers made to the consensus sequences known in other species. Several programmes can be used to identify consensus primers which provide universal tools for isolation of important genes from a wide variety of species [18] and (www.generationcp.org). An example for the homology between different species for the cellulose synthase gene is shown in Figure 1. Primer could be designed to amplify a fragment of the gene from the species of interest.

4.2.2.2 Gene Knockouts

The generation of knockout mutants by T-DNA, transposons and knockout by RNAi, serve as a tool for gene identification and understanding gene function by isolating the "tagged sequence" from an individual carrying the knockout mutation (T-DNA/ transposons) or assessing the phenotype of knockout by RNAi.

4.2.2.3. Targeting Induced Local Lesions IN Genomes (TILLING)

The large amounts of DNA sequence information and induced mutants are rapidly becoming key elements in genetic studies as they provide the resources for the systematic discovery and functional analysis of genes. TILLING (Targeting Induced Local Lesions IN Genomes) is one example of an emerging reverse genetics technique where mutants for targeted genes can be rapidly identified in a high-throughput approach (Figure 6). This technology based on mutation induction and high throughput SNP (Single Nucleotide polymorphism) detection, has been developed into a method for inducing and identifying novel genetic variation, and has been demonstrated in the model plant, *Arabidopsis thaliana* [19, 20,21]. At present, TILLING moves beyond functional genomics into crop improvement by demonstrating a proof of concept in improving a quality trait like waxy starches, in diploid wheat which is now being applied to rice [22], hexaploid wheat and barley.

Improving mutation induction techniques and enhancing efficiency is an ongoing and constant activity of the Joint FAO/IAEA Program (http://www-naweb.iaea.org/nafa/pbg/index.html), through Technical Cooperation Projects (http://www-tc.iaea.org), Coordinated Research Projects (http://www-crp.iaea.org) and R&D at the Plant Breeding Unit (PBU) of the FAO/IAEA Agriculture & Biotechnology Laboratory (ABL). Technology packages based on mutation induction and supportive biotechnologies, in order to enhance efficiency of mutation induction for breeding purposes, are developed and optimized in order to fit the various laboratory infrastructures of the FAO and IAEA Member States. The R&D, service and training activities at PBU rest on three pillars: (i) rice for abiotic stress (salinity tolerance), (ii) banana for biotic stress (resistance to Black Sigatoka, aka. Black Leaf Streak Disease, BLSD), a mutation grid and a collection of deletion mutants (aneuploids) are being developed; (iii) cassava for quality traits (starch), a mutation grid is being developed. A TILLING platform has been successfully started up based on these three crops.

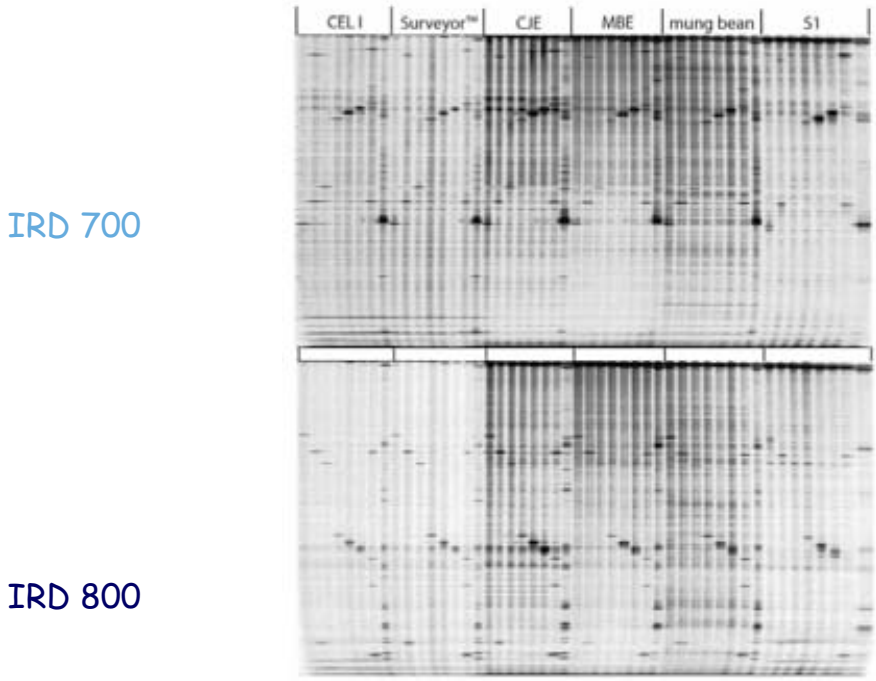


Figure 6. Mismatch cleavage by S1-related enzymes and crude extracts [23].

4.3. Assessment of patterns of gene expression

Identification of gene function and regulation is a major goal of research in many biological laboratories. An increasing understanding of genes of agronomic and economic importance is stemming from these studies, particularly for single-gene characters. Complex traits, such as response to abiotic stresses or quality characters involve a multitude of interacting genes, which demand longer times of study, but they may still be very useful in gene expression studies.

Several technologies are available to assess pattern of gene expression. These patterns serve as a tool for evaluation of genes' function because genes with similar patterns of gene expression are involved in similar or related biological activities. Function of genes can be identified on the basis of comparison of their expression patterns between different plant tissues (e.g. roots *vs.* ripening fruit), and different treatments (e.g. infected or drought treated *vs*. controls).

Such technologies include:

- **Differential display and cDNA-AFLP:** These technologies are used to identify genes which are altered in their expression pattern by treatments such as drought or exposure to a pathogen. They are rather simple to exploit and involve comparison of band patterns of cDNAs (made from messenger RNA, mRNA) generated from the tested samples. Bands which are not shared by the samples are isolated and characterized by cloning and sequencing, giving candidate genes where the expression is regulated by the treatment.

- **DNA chips and microarrays**: These are more sophisticated technologies that require expensive equipment [24, 25,26, 27]. Alternatively, several companies and research centers offer the assessment of RNA samples as a service. One limitation of this technology is that the commercial "chips" are available for only a limited number of plant species. On the other hand, this list includes both the model species and some of the major crops. See the Websites of 'Affymetrix', 'Agillent' and 'Illumina'.

- **High throughput sequencing:** Major efforts are invested in the development of high-throughput and reasonably priced technologies for sequencing. Such technologies can be useful to a vast list of applications. Their major goal is genome sequencing and it is anticipated that that not only new species will be sequenced but that eventually it would be possible to fully sequence the tested individual samples.

  Among the current available applications of these technologies is sequencing transcriptomes and thus assessment of patterns of gene expression. By sequencing large number of RNA (cDNA) molecules, one can compare the number of "calls" of a specific sequence between various phenotypes, tissues or treatments and thus obtain patterns of gene expression. The advantage of these technologies is that no prior sequence information is required. Two such technologies are currently available and one can either buy the expensive equipment and kits or approach companies which offer this service. The two major technologies are:

  - **'Illumina - Solexa':** Double stranded cDNA is synthesized on a template of mRNA and digested with specific restriction enzymes. 3' single stranded cDNA is attached to beads on a "chip". Two specific adapters are ligated to each strand. The resulting cDNAs are amplified by PCR to generate "colonies". Sequencing is carried out by primer extension using sequencing primer for 36 cycles. Each nucleotide is labelled with a different flurophore and blocked on the 3'end. During each cycle only one single base is added to each "colony". The 3' ends are de-blocked and the fluorophores removed. A new nucleotide is added by the polymerase at each cycle to each "colony". Four images are taken to identify the added nucleotide at each colony at each cycle. The output is 1-2 million reads of 17-26 bp each, organized by the number of calls for each sequence.

  - **'Roche – 454':** Double stranded cDNA is synthesized on a template of mRNA and fragmented mechanically. Fragments are clonally amplified in emulsion on beads. Sequencing is based on Pyro-sequencing where at each cycle a certain nucleotide is added. When a nucleotide is incorporated, PPi is released and ATP is generated. Based on the luciferin reaction, a light signal can be detected (see www.454.com). The output is the sequence of several hundred thousands of fragments each about 250 bp.

5. Discussion and conclusions

Molecular technologies are valuable and applicable for generating information about the genetic variation in germplasm and cultivars for breeders and farmers. It is advisable that scientists have background information about the gene pool that they are working with and the genetic variation of the plant material in their programmes. It is important to assess the genetic variation of this material in the context of the variation available within the species. Thus, construction of 'phylogenetic' trees is valuable when initiating a complex breeding programme.

The basic laboratory infrastructure for the implementation of mutation technology packages includes a cellular biology laboratory set up for *in-vitro* culture, somatic embryogenesis, micropropagation and when needed - Double Haploid Technology.

Supportive molecular technologies include genotyping with DNA markers as described above. Note that the choice of marker depends on several factors including mainly the goal of the research, the available technical knowledge and equipment as well as the cost. These technologies have become common in many labs and to prevent developing countries from lagging behind, we would highly recommend that interested laboratories look for collaborative research projects and training periods in experienced laboratories. The Plant Breeding Unit of the FAO/IAEA offers international and regional courses on the subject and will be happy to assist those scientists looking for collaborative projects and training.

Countries interested in more sophisticated biotechnologies should be at a higher stage of development. In order to implement molecular techniques, a country should be able to ensure the sustainability of this type of paradigm. This stage requires trained manpower at the research, the technical, and the educational level, as well as a need for facilities and infrastructure. Capacities have to be built and sustained in the appropriate political, economic, scientific and technical domains. At the local level, it is advantageous that farmers be organized in associations, which are supported by the national governmental agencies. National academic institutions are necessary for building the research capacities and as focal points for empowerment. These technologies usually accompany crop improvement projects and should be part of them.

One of the most difficult challenges faced by policy-making and financing organizations is the choice of strategy and molecular techniques. In order to address this challenge, it is advisable that governments develop a policy and agenda for molecular research, including human resources management infrastructure development, biosafety regulations and regulations regarding intellectual property rights (IPR). Partnerships between developing-country research systems, international research institutions, as well as private and public-sector research organizations in industrialized countries, are highly recommended. Finally, it is advisable that governments provide incentives for the private sector to undertake research and development (R&D) using molecular technologies that focuses on farmers' problems. Ideally, a body of scientists, research managers, and policymakers in public agricultural organizations should be constituted to reach the above listed goal. The beneficiaries should be rural communities and urban populations in these developing countries as well as the country as a whole which will have an advanced level of research with an impact on medicine, education and more.

Summary

The *conditio sine qua non* for a developing country to introduce molecular techniques into crop improvement programmes is the existence of crop breeding programs beyond germplasm evaluation. The breeding goals are to optimise genetic gain in the current varieties, to adapt new varieties to local environments and to produce improved varieties quicker and more efficiently.

The above mentioned level of implemented molecular techniques allows carrying out the following types of studies and more:

- ***Studies of genetic diversity*** - Assessment of phylogenetic relationships between plant species and plant populations.
- ***Monitoring*** - Confirmation of identity and homogeneity of varieties (seed propagated crops) and cultivars (vegetative propagated crops).
- ***Marker-assisted introgression*** - Decreasing the number of backcross generations needed for introgression of a gene from a wild species to an elite cultivar.
- ***Choosing the parents for the generation of hybrid seeds*** - Choice of parents is based upon assessment of the genetic distances between the potential accessions.
- ***Marker-assisted selection (MAS)*** - Application of markers to increase the response to selection by selection based on DNA markers rather than on phenotypical traits.
- ***Genetic mapping*** - Localisation of genes/QTL of interest.
- ***Studies of genetic mechanisms*** – The study of mechanisms controlling horticultural traits such as: flowering, pollination, fruit maturation, yield and more.

An advanced molecular laboratory is proficient in modern molecular technologies including: cloning, sequencing, expression and BAC libraries, cytogenetic techniques including fluorescent in situ hybridization (FISH) and molecular cytogenetic approaches, and TILLING, and is able to study functional genomics. Once an advanced stage is reached, the laboratory is capable to implement new technologies as they are available. As an example, CIAT in Columbia has recently implemented a diversity array platform on Cassava.

The major goal of research projects to be carried out in these laboratories is identification of genes controlling important traits. This become a highly important goal and challenge in various biological systems (humans, animals, plants and more). While in humans the interest is in genes controlling diseases, in plants the interest is in genes controlling agricultural important traits. Various technologies to reach this goal are available including linkage analysis and association studies based on DNA markers, generation of mutants by T-DNA, transposons and RNAi and study of gene expression by micro-arrays, Quantitative PCR and high throughput sequencing.

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