CONCURRENT SESSION 8

Mutation Induction and Breeding of Ornamental and Vegetatively Propagated Plants

A Report on 36 Years of Practical Work on Crop Improvement Through Induced Mutagenesis

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Abstract

Induced mutagenesis work was conducted from 1971 to July 2007, using both physical and chemical mutagens for improvement of a wide range of crops viz. vegetables, medicinal, pulse, oil-bearing, and ornamental crops. All classical and advanced methods were extensively used for the success of induced mutagenesis for the development of new and novel cultivars of economic importance. Being deeply engaged for the last 30 years on improvement of ornamentals through Gamma-ray induced mutagenesis, I have produced a large number of new and promising varieties in different ornamentals. A good number of ornamental mutant varieties have already been commercialized. A novel technique has been developed for management of floral chimeric sector in chrysanthemum through direct regeneration of mutated individual florets. A series of *in vitro* experiments were conducted and solid mutants developed through direct regeneration. *In vitro* mutagenesis has been successfully used for development of a salt-resistant strain in chrysanthemum, supported by biochemical analysis and field trials.

Introduction

Physical and/or chemical mutagens cause random changes in the nuclear DNA or cytoplasmic organelles, resulting in gene, chromosomal or genomic mutations. Induced mutagenesis is an established method for plant improvement, whereby plant genes are altered by treating seeds or other plant parts with chemical or physical mutagens. Voluminous work has been done worldwide for the improvement of both seed and vegetatively propagated crops through induced mutation. In the present paper, I will highlight important results of induced mutagenesis work carried out for the last 36 years, on both seed and vegetatively propagated crops. Appreciable knowledge and literature have been generated during 36 years of practical experiments on crop improvement using classical and modern induced mutagenesis techniques on aspects like radio-sensitivity, selection of material, methods of exposure to mutagens, determination of suitable dose of mutagen, combined treatment, recurrent irradiation, split dose, colchi-mutation, detection of mutation, mutation frequency and spectrum of mutations, nature of chimerism, classical and modern methods for management of chimera, *in vitro* mutagenesis, isolation of mutants, cytological, biochemical and molecular characterization of mutants, commercial exploitation of mutant varieties, etc.

Materials and Methods

Experimental materials

Crops selected as experimental materials included vegetables (*Trichosanthes anguina* L, *T. cucumarina*, *Cucurbita maxima* L, *Cephalandra indica, Luffa acutangula* Roxb., *Lagenaria ciceraria*), medicinal plants (*Trigonella foenum-graecum* L, *Mentha citrata* Ehrh), pulses (Winged Bean - (*Psophocarpus tetragonolobus* L. D.C.), oil-bearing crops (*Jatropha curcas* L, *Rosa damascena*, *Cymbopogon flexuosus* (Nees) Wats) and ornamentals (Amaryllis, Asiatic Hybrid Lily, Bougainvillea, Canna, Chrysanthemum, Dahlia, Gerbera, Gladiolus, Hibiscus, *Lantana depressa* Naud, *Tagetes erecta*, Rose, Tuberose, *Narcissus tazetta* etc.).

Mutagen and treatment methodology

Both physical (X-rays and Gamma-rays) and chemical (EMS, MMS, Colchicine) mutagens were used for improvement programmes (**Table 1**).

Table 1. Crops where mutation breeding was performed, mutagens used and treatments applied

Crop	Propagule	Mutagens	Dose	Treatment
T. anguina	Dry seeds	X-rays	6–30 kR	
		Colchicine	0.25–1.00%	[for 18 hrs]
T. cucumarina	Dry seeds	X-rays	6–30 kR	
		Colchicine	0.25–1.00%	[for 18 hrs]
C. indica	Dry seeds	X-rays	6-48 kR	
		Colchicine	0.25–1.00%	[for 18 hrs]
C. maxima	Dry seeds	X-rays	6-30 kR	
		Colchicine	0.25–1.00%	[for 18 hrs]
L. siceraria	Dry seeds	Colchicine	0.25–1.00%	[for 6 hrs]
L. acutangula	Dry seeds	Colchicine	0.25–1.00%	[for 6 hrs]
T. foenum-graecum	Dry seeds	Gamma-rays	30–50 Krad	
	2hrs H_2O soaked seeds	MMS	0.02–0.06%	[for 6 hrs]
		EMS	0.3–0.6%	[for 6 hrs]
M. citrata	Rooted cuttings	Gamma-rays	2–8 Krad	
P. tetragonolobus	Dry seeds	Gamma-rays	10–30 Krad	
J. curcas	Dry seeds	Gamma-rays	6–24 Krad	
		Colchicine	0.25–1.00%	
R. damascena	Stem cuttings	Gamma-rays	1-2Krad	[for 18 hrs]
		Ornamentals		
Amaryllis	Bulb	Gamma-rays	250rads-5 Krad	
Bougainvillea	Stem cuttings		250–1250 rads	
Canna	Rhizome		2 and 4 Krad	
Chrysanthemum	Rooted cuttings/ suckers		1.0 -3.5 Krad	
Gerbera	Rooted plantlet		1 and 2 Krad	
Gladiolus	Bulb		250 rads–5 Krad	
Hibiscus	Stem cuttings		1.0–4 krad	
Narcissus tazetta	Bulb		250,500,750 rad	
Perennial portulaca	Stem cuttings		250–1250 rads	
Polianthus tuberosa	Bulb		250 rad–8 Krad	
Rose	Stem with budding eyes		2–6 Krad	
Tagetes erecta	Rooted cuttings		500 rad–2 Krad	
Lantana depressa	Stem cutting		1–4 Krad	

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Combined treatment

Dry seeds of *T. anguina* were first treated with 24 and 30 kR X-rays and then kept immersed in 0.25, 0.50 and 1.00% aqueous solution of colchicines for 18 hours.

Treated seeds were sown in the field in randomized block design beds for biological assessment.

In vitro culture

Chimeric florets (developed through sport or gamma irradiation) were cultured for direct regeneration on MS medium [1, 2].

Results

Oil bearing crops

Both tall and dwarf, high-branching, high fruit and oil yielding and high biomass yielding variants were induced in *J .curcas*, the seed oil ("Curcas Oil"), an efficient substitute fuel for diesel engines [3, 4]. Gamma radiation was also found to be effective to induce suitable strains of *J. curcas* for growing on alkali soil [5]. Genetic variability in different morphological and agronomical characters could be induced in the essential oil-bearing plant *Cymbopogon flexuous* [6]. *Rosa damascena* or Damask rose, which contains essential oil considered to be the best, was found to be very sensitive to gamma radiation and variability could be induced in different morphological characters. The original flower color of *R. damascena* was light pink, while one plant in 1Krad treated population showed white flowers. The mutant has been isolated and established in pure form [7].

Pulse crops

A dwarf mutant with determinate growth habit has been developed in Winged Bean, this trait being highly economic to solve stalking problems in cultivation [8]. An early fruiting mutant has also been isolated from the Gamma-ray-treated population [9].

Medicinal crops

Response of *M. citrata* to gamma radiation was very promising in developing hairy mutants and mutants with higher herbage yield [10]. Gamma-rays, EMS and MMS were most successful in developing a series of mutants of economic importance in *T. foenum-graecum*, an important condiment, medicinal and fodder cum green vegetable crop. Mutants with small seeds, bold seeds, green and chocolate seed coat color against normal green, dwarf and high-branching, etc., were induced. Interestingly, a number of mutants with phylogenetic significance could be isolated. Mutants with uni to octa-foliate leaflets were developed against normal tri-foliate leaflets, playing a relevant role in understanding phylogenetic affinities [11-17].

Vegetable crops

Fruits of *T. anguina* are used as a summer vegetable. Its seeds also yield a kind of drying oil. One of the essential constituents of this oil is punicic acid, which is an isomer of alpha-eleostearic acid of tung oil. Mutants have been induced and isolated with promising and economic early flowering, short thick fruits, increased fruit weight, yellow fruits, high oil and punicic acid yield, crinkled leaves etc. [18-26].

Promising genetic variability in different economic characters could also be induced by X-rays in *C.maxima*, *L. siceraria*, *C. indica* and *T.cucumarina* [27-28].

Ornamental crops

Classical mutagenesis

Extensive work has been carried out for the last 30 years for improvement of different ornamental crops. The main objective was to develop new and novel varieties for floriculture trade. Large numbers of studies on mutagenesis in ornamental plants by using physical and chemical mutagens has been carried out in both applied and basic aspects. Gamma-rays have been most successfully used and 76 new mutant varieties with changed flower color/shape, and chlorophyll variegation in leaves have been developed and released in different ornamentals. Voluminous information has been generated on various basic aspects for successful application of classical mutagenesis. Work by the author on exploitation of mutagenesis for improvement of different ornamentals has been reported separately (IAEA-CN-167-283P).

Management of chimera and in vitro mutagenesis

The main bottleneck of induced mutation with vegetatively propagated crops is the formation of chimera. Chimeric tissues cannot be isolated using the available conventional propagation techniques. Both *in vivo* and *in vitro* techniques have been standardized for management of chimera. A novel technique has been standardized for the management of such chimeric tissues through direct shoot regeneration from flower petals. The technique has been standardized and a series of new solid flower color/shape mutants have been developed in chrysanthemum using *in vitro* mutagenesis through direct regeneration of mutated cells in florets without diplontic selection. These aspects are reported separately (IAEA-CN-167-284P).

Recently, systematic efforts have been made to develop trait-oriented mutation. Efforts were made to develop NaCl-tolerant chrysanthemum plants through *in vitro* mutagenesis. One such NaCl-tolerant chrysanthemum variant has been developed in a stable form through whole plant selection in *in vitro* mutagenesis using ethylmethane sulfonate (EMS) as the chemical mutagen [29-30]. Data reflects that a proper balance between enzymatic and non-enzymatic defence system is required for combating salinity stress in chrysanthemum. A better performance of the progeny of selected lines under the same salinity stress condition, even in the second year, confirmed the genetic stability of the salt-tolerance character. In a separate experiment, an attempt was made to develop stable NaCl-tolerant chrysanthemum plants by selection of a NaCl-tolerant callus line and subsequent differentiation under NaCl stress [31]. Enhanced tolerance of the variants was attributed to an increased activity of superoxide dismutase, ascorbate peroxidase, and dehydroascorbate reductage, and, to a lesser extent of membrane damage than NaCl-treated control plants. Salt tolerance was evaluated by the plant capacity to maintain both flower quality and yield under stress conditions. It has been concluded that a stepwise increase in NaCl concentration from a relatively low level to a cytotoxic level was a better way to isolate NaCl-tolerant callus lines, since direct transfer of callus to high saline medium was detrimental to its survival and growth [32].

More recently, attempts have been made to regenerate chrysanthemum plants from single cells, i.e. through somatic embryogenesis, for a management of single cell mutation events. An efficient somatic embryogenesis protocol has been standardized in chrysanthemum, which will open up a new way for isolating new flower color/shape ornamental cultivars through retrieval of single mutated cells [33].

Combined effects

Radiation induced genetic and physiological damages, and mutation frequency can be modified and influenced by pre and post-irradiation treatment of seeds with chemicals. Combined effects of mutagens i.e. post x-irradiation colchicine treatment were studied in *T. anguina*. Protective effects were estimated in combined treated population to reduce the damages caused by individual mutagens [34-35].

Colchi-Mutation

Colchicine was found to be very effective to induce genetic variability in *L. acutangula*, *L. siceraria* [36], *C. maxima* [18], *T .anguina* and *T. cucumarina* [18, 25]. High fruit, seed, oil and punicic yielding mutants were isolated from a colchicine-treated population of *T. anguina* [25].

Successful application of colchicine to induce somatic flower color mutations in vegetatively propagated ornamentals (Chrysanthemum and Rose), are reported separately in this volume (IAEA CN-167-283P).

Analysis of colchi-mutants (developed both in seed and vegetatively propagated crops), clearly indicated that instead of inducing complete polyploidy in the plant, colchicine produced gene mutations in both the presently experimental seed and vegetatively propagated plants. It may be pointed out that, normally, after colchicine treatment, attention is paid to chromosome duplication and its effect on phenotype. When there is no polyploid formation and when there are no gigantism in desired characters in induced polypoid in particular taxa, colchicine breeding is thought to be unsuccessful. But careful observations have led to the understanding that although colchicine is known more familiarly as a polyploidizing agent, it may also be used as a very good mutagen (37).

Domestication

It is commonly accepted that domestication of wild species has been conditioned by mutation following selection. *L. depressa* (*Verbenaceae*) is a semi-wild herb with creeping habit and yellow flowers with little genetic variability. It is grown on roadsides and boarders of gardens for decoration. Gamma-rays have successfully induced one chlorophyll-variegated mutant i.e. *L. depressa* 'variegata,' and two flower color mutants (one canary yellow – 'Niharika,' and another yellow and white bicolored – *L. depressa* bicolored). These mutants are very attractive and can be grown as potted ornamentals, supporting the concept that induced mutagenesis can also be used as an efficient technique for domestication [38].

Radiosensitivity

The study of radiosensitivity is a prerequisite for large-scale mutation breeding work. A wide range of parameters known to influence radiosensitivity were studied to determine the radiosensitivity and their correlation, including nuclear factors (chromosome number and size, centromere number and position, Interphase Chromosome Volume, Interphase Nuclear Volume, degree of polyploidy, nuclear DNA content etc.), seed moisture content, seed size, seed coat, flower type, flower color, flower shape, etc. Direct, fractionated dose and storage effects of Gamma-rays on radiosensitivity were studied. Radiobiological responses of a large number of plant species clearly indicated that radiosensitivity varies according to the propagules. Different cultivars were differentially sensitive to gamma radiation, and it is very clear that radiosensitivity is a genotype-dependent mechanism [39-42].

Discussion

These results and those available elsewhere in the literature, clearly show that mutation by using both physical and chemical mutagens has successfully produced quite a large number of new and promising varieties in different seeds and ornamental plants, and is considered to be a most successful tool for breeding ornamental plants [43-46]. The main advantage of mutation induction in vegetatively propagated crops is the ability to change one or a few characters of an otherwise outstanding cultivars without altering the remaining and often unique part of the genotype [47]. Mutation breeding has been more successful in ornamental plants because changes in any phenotypic characteristics like color, shape or size of flower and chlorophyll variegation in leaves can be easily detected. In addition, the heterozygous nature of many ornamentals offers high mutation frequency. The capability of Gamma-rays in inducing desirable mutations in ornamental plants is well understood from a significant number of new varieties developed via direct mutation breeding. Recent genetic engineering techniques appear to be most promising and exciting for development of desirable transgenic ornamentals, but this technology is at the early stage of development. Every technique has its own advantages and disadvantages. After more than three decades of applied mutagenesis work, it is established beyond doubt that mutation breeding will constitute an excellent supplement to the conventional methods in practice. Studies have clearly proved that mutation breeding techniques using nuclear radiation can be exploited for the creation of new and novel ornamental cultivars of commercial importance, by inducing genetic variation in already adapted, modern genotypes and can also enrich the germplasm of ornamental horticulture. Although mutation breeding is a random (chance) process, reports are available for directive mutation in flower color with some starting colors [48]. Mutation breeding at its present status appears to be well standardized, efficient and cost-effective. Classical mutagenesis combined with management of chimera and *in vitro* mutagenesis are most promising and standardized techniques for developing new and novel varieties. Voluminous practical knowledge generated by the author on basic and applied aspects for successful application of induced mutagenesis for improvement of seed and vegetatively propagated crops has been published in the form of research papers (258), review papers (13), book chapters (29), books (2), edited books (4), bulletins (8), popular articles (78), Symposium abstracts (123), etc.

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Citrus Improvement Using Mutation Techniques

E S Louzada

Citrus cultivar improvement is hampered by several biological factors inherent to most citrus species. Facultative apomixis, self and cross-incompatibility, long juvenility period, and high heterozygosis are some of the vast arrays of impediments faced by citrus breeders in conventional hybridization.

Since oranges and grapefruits are highly polyembryonic, the production of enough numbers of zygotic offspring for selection of superior genotypes of these species is basically impossible; hence, most commercially important cultivars of these species have originated through natural or induced mutation. Star Ruby, a deep-red-fleshed grapefruit, was developed by irradiation of Hudson grapefruit seeds with thermal neutrons [1]. Unlike Hudson, which contains over 50 seeds per fruit, Star Ruby is nearly seedless. Hensz [2] irradiated buds of Ruby Red grapefruit with thermal neutrons and a tree that originated from one of the buds produced fruits three times redder than Ruby Red. It was named A&I-1-48. Ten trees were propagated from A&I -1-48, and out of one of the trees, a budsport mutation was found producing fruits five times redder than Ruby Red. Called Rio Red, it is currently the variety of choice for Texas and is known worldwide for its sweetness, red flesh and beautiful blush. Currently, 37 years after A&I -1-48 was first propagated, the trees are still producing several budsport mutations. So far, in the 2007/2008 season, more than 100 new mutations were obtained from a 100-tree block.

In the mandarin group, the existence of several monoembryonic cultivars facilitates conventional breeding, but still, induced mutation is part of most mandarin breeding programmes, and proprietary, new seedless cultivars have been produced in the US, Italy, Israel and elsewhere [3, 4, 5, 6]. Seedless mandarins produced by the University of California Riverside include Dayse, Fairchild, Encore, and Nova. The USDA-ARS, U.S. Horticultural Research Laboratory in Florida released a seedless Pineapple orange, and the University of Florida a seedless Murcot tangor.

Mutation has been also important in lemon breeding, and a seedless lemon, with tolerance to a devastating lemon disease was recently reported [7], in addition to earlier reports of a thornless lemon mutant produced by gamma irradiation [8].

Gamma irradiation is currently an important component of our breeding programme and several potentially improved cultivars of grapefruit, pummelos, and lemons are in the pipeline. Additional details of citrus irradiation programmes in the US will be provided.

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Mutation Breeding of Chrysanthemum by Gamma Field Irradiation and *In Vitro* Culture

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Abstract

The purpose of this work is to clarify the effect of chronic (gamma field) and acute (gamma room) radiation and *in vitro* culture on mutation induction of flower color in chrysanthemum. The combination of both methods yielded a mutation rate 10 times higher than the conventional chronic cutting method, and also produced non-chimeric mutants. Somaclonal variation often occurred in plants regenerated from callus, but no significant variation appeared in callus regenerants from non-irradiated plants. Therefore, proper mutagenic treatment on cultured materials is indispensable for effective mutation induction. The chronic culture method clearly yielded the widest color spectrum in chrysanthemum, while the acute culture method resulted in a relatively low mutation rate and a limited flower color spectrum. Flower color mutation could be more readily induced in plants regenerated from petals and buds, than from leaves. In this respect, it is supposed that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture, but could perhaps induce mutation in a desired direction. A possible reason why the chronic culture methods showed higher frequencies than the acute, is discussed. Nine out of 10 registered mutant varieties were derived from chronic irradiation, and only one from acute. The combined method of chronic irradiation with floral organ cultures proved to be of particularly great practical use in mutation breeding, not only of flower species but of other species as well.

Introduction

Mutation breeding has been successfully applied for variety improvement of many crop species. About 70 % of the world's mutant varieties have been induced through Gamma-rays. There are two streams of Gamma-ray irradiations, chronic and acute. Since the 1960s, 14 chronic irradiation facilities have been constructed worldwide, but all facilities were shut down, except for a gamma field in Japan, which has been operating for almost half a century.

A Japanese pear variety resistant to black spot disease called "Gold Nijisseiki" or "Osa Gold" had been induced and selected in the gamma field, supported by a single and quick selection technique using leaf disks taken from irradiated trees and AK toxin of the disease [1, 2]. An ornamental mutant with pink and yellow striped leaves was induced in a gamma greenhouse and registered as a new variety of pineapple. New mutant varieties of evergreen and dwarfness in Manila grass were selected during the winter in the gamma field [3,4]. This variety can provide green turf throughout the year and reduce lawn-mowing frequencies. A wide spectrum of different shapes and color mutants in roses was induced in the gamma field, and three mutants were registered as new varieties.

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The notable results derived from chronic irradiation using gamma field and gamma greenhouse in IRB were reevaluated in cooperation with nine countries in East Asia under the collaborative framework of "Forum for Nuclear Cooperation in Asia" [5]. Recently, new chronic irradiation facilities have been constructed one after another in member countries. A gamma bio-room in Thailand, a gamma greenhouse in Malaysia, and a gamma phytotron in the Republic of Korea are now operating. A gamma field is currently being planned for construction in Vietnam. A new venue for chronic mutation breeding has also opened in East Asia.

The purpose of this study was to clarify the effect of chronic (gamma field) and acute (gamma room) radiations and *in vitro* culture [6] on mutation induction of flower color in chrysanthemum [7, 8], and to establish an effective mutation breeding method for vegetatively propagated plant species.

Materials and Methods

Using a cut flower variety called "Taihei," a number of plants were regenerated from explants of petals, buds, and leaves excised from chronic, acute, and non-irradiated plants. Flower color mutation was then induced through the combined effects and analyzed in comparison with the conventional chronic cutting method.

For chronic irradiation, the gamma field in IRB was used to grow plants on field at optimal dose rates. Gamma-rays were irradiated from a cobalt source (^{60}Co 88.8 TBq, 2400 Ci) on the tower in the center of round field (100 m in radius). The growing plants were irradiated at dose rates ranging from 0.25 to 1.5Gy/day for 20 hours every day except Sundays and national holidays. Total treatment doses of plants were from 25 to 150Gy for 100 days until the flowering season of the chrysanthemum.

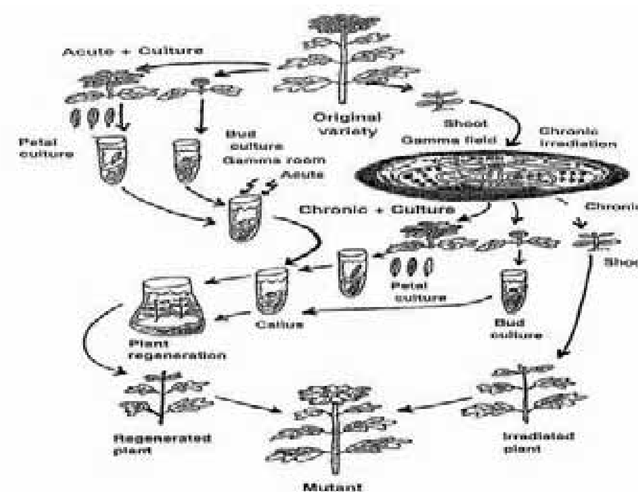

Figure 1 Experimental flowchart

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Floral petals, buds, and leaves were excised from the irradiated plants and used as explants for callus induction culture (**Fig.1**). In the petal culture, the explant sources were divided into their sectorial patterns.

The callus was induced on a callus medium comprised of Murashige and Skoogs's basal formula [9], supplemented with 0.2 mg/l NAA (naphtaleneacetic acid), 1 mg/l BA (benzylamino purine) and 10% vw coconut milk. After the calluses were subcultured on the medium, regenerated plants were obtained on a medium composed of MS basal medium supplemented with 0.1 mg/l NAA and 1 mg/l BA. All the cultures were kept under 3000 lux light for 16 hours at a temperature of 27°C. The regenerated plants were acclimatized in a greenhouse, then transplanted to a field nursery to investigate mutation induction. The plants used as the control were established by cutting back twice the lateral shoots of the chronically irradiated plants.

For acute irradiation, the explant sources of leaves, buds and floral petals dissected from unirradiated plants were incubated on callus induction medium after sterilization. After three days of incubation, the explants were irradiated at a dose rate of 10Gy/hr, total doses ranging from 20 to 100Gy in a gamma room (44.4 TBq., ^{60}Co source). Soon after irradiation, the segments were transferred to fresh callus induction medium. The induced calluses were subcultured on callus medium and then on the regeneration medium. Regenerated plants derived from each explant source of unirradiated plants were treated for comparison. All of the regenerated plants obtained were transplanted to a field nursery at IRB. The mutant flower colors of the regenerated plants were observed and recorded using a TC-1800 MK-II spectrophotometer.

Results and Discussion

Radiosensitivity of chronic and acute irradiated materials

The radiosensitivity of the growing point of the main stem was estimated to be 100Gy at a 50% survival dose (LD 50) and 150Gy at a lethal dose (LD 100). The number of flowers decreased sharply as irradiation dose rate rose, and few flowers appeared at 1.25Gy.

Figure 2 Flower color mutant lines derived from chronic irradiation using floral petal cultures of chrysanthemum (Upper right: Original "Taihei" variety).

The radiosensitivity of cultured explants to acute irradiation was estimated, and although the callus was induced on explants irradiated at a dose as high as 80Gy, the subsequent regenerability of the callus stayed normal up to 20Gy, but diminished sharply at 40Gy. The critical dose for plant regeneration was assumed to be 40 to 50Gy, and none of the callus retained its generability at 80Gy. Accordingly, the optimum dose of acute irradiation for cultured explants was estimated to be 20Gy. However, the optimum dose of acute irradiation for cuttings was estimated to be 15-20Gy, and the lethal dose 50Gy. Therefore, the optimum dose of chronic irradiation can be extended to almost 2.5 times that of acute irradiation. The chronic irradiation method employed in the study caused a relatively high accumulation of radiation in regenerants, but reduced adverse radiation damage to the proliferation and differentiation of the callus.

Mutation frequency in flower color

A total of 549 mutants (15%) were obtained from 3,688 plants in the field nursery; 79% of them fell into the category of light to dark pinks similar to the original variety and 21% were a different color from the pinks. A wide spectrum of flower color appeared in individual regenerants derived from floral organ culture. Wide, continuous variations also appeared in the shape and size of the flowers and leaves in regenerants from petal and bud cultures (**Fig. 2**).

At chronic irradiation, average mutation rates of flower color were 38.7%, 37.5%, 13.8% and 4.7%, respectively, in regenerants from petal, bud, and leaf cultures and shoots (conventional) (**Fig. 3**). The former two were approximately eight times higher than the latter. At acute irradiation, average mutation rates in regenerants from petal, bud, and leaf explants were 29.7%, 12.0%, and 12.9%, respectively.

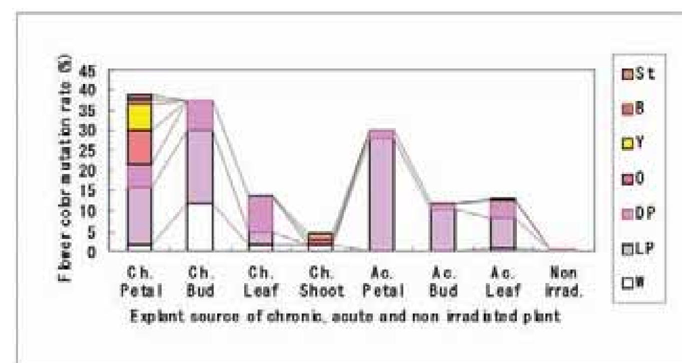

Figure 3 Comparison of flower color mutation rate and color spectra in regenerants derived from cultured explant sources and ordinary shoots from chrysanthemum, induced by chronic, acute and non-irradiation methods. Flower color of regenerants: W=white, LP=light pink, DP=dark pink, O=orange, Y=yellow, B=bronze, and St=striped.

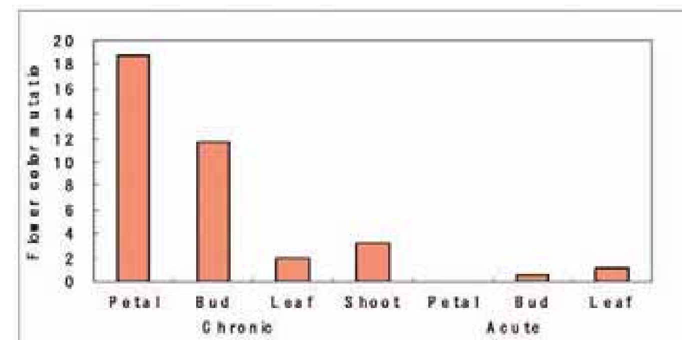

Figure 4 Comparison of flower color mutation rate of all colors, excluding the light and dark pinks that are similar to the original color, in regenerants derived from cultured explant sources and ordinary shoots, induced by chronic and acute irradiation methods.

At non-irradiation, the average mutation rate was a mere 0.5% in regenerants from all types of explants indicating somaclonal variation. Somaclonal variation often occurred in plants regenerated from the callus, but no significant variation appeared in callus regenerants from non-irradiated plants. Therefore, proper mutagenic treatment on cultured materials is indispensable to effective mutation induction.

The method using chronic petal culture gave the highest mutation rate, followed by chronic bud and acute petal cultures. The methods using a leaf with either chronic or acute irradiation provided a lower mutation rate than those using petals and buds. It was noted that most regenerated mutants directly induced from chronic shoots displayed chimera formation.

The mutation rate of all flower colors of the regenerants, excluding the light and dark pinks shown in Fig. 4, indicates which methods extended the mutated color spectrum. Thus, the highest mutation rate was obtained from the chronic petal culture, followed by the chronic bud culture. The other methods that used chronic shoot and chronic leaf culture and all acute irradiation methods proved to have lower mutation rates, with a narrow spectrum.

The chronic culture method clearly yielded the widest color spectrum in chrysanthemum, while the acute culture method resulted in a relatively low mutation rate and a limited flower color spectrum.

Flower color mutation could be more readily induced in plants regenerated from petals and buds, than from leaves. In this respect, it is assumed that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture, and therefore results in higher and wider mutations. The choice of desired organ as *in vitro* explants could perhaps induce mutation in a desired direction.

Comparison of mutation induction in chronic and acute irradiations

In short, chronic irradiation using petal and/or bud culture could be an effective method for inducing flower color mutant varieties. A possible reason why the chronic culture methods showed higher frequencies than the acute, is that most of the cells composing the tissue, organs and plant continually irradiated into a cell division and became more sensitive and mutable to irradiation (**Fig. 5**). It is well known that cells at the G_2 and M stages are more sensitive to radiation than those at the G_1 and S stages.

Under chronic irradiation, if continual low dose Gamma-rays are irradiated onto each cell division every day, mutated cells emerge (**Fig. 6**). These mutated cells then bear a couple of daughter cells with accumulated mutation in growing organs. Contrarily, under acute irradiation, when the explants composed of various cell stages are irradiated at high doses and dose rates at one time, the dormant cells (G_1, S stages), less sensitive to radiation, were able to survive, but the more sensitive dividing cells (G_2, M stages) were inactivated. In this state, there would be no more accumulation of mutation under acute irradiation. This is the reason why mutants from chronic irradiation yielded a wider color spectrum than those from acute irradiation.

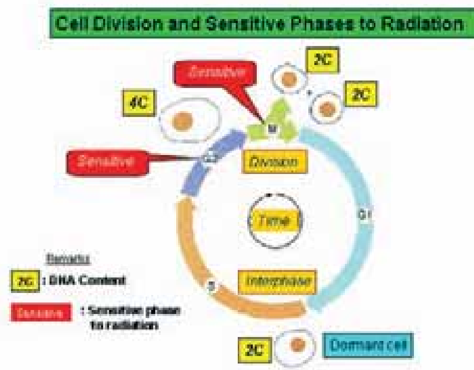

Figure 5 Cell division and phases sensitive to radiation.

In this study, nine out of 10 registered flower color mutant varieties were derived from chronic irradiation. As for culture techniques, five varieties were derived from chronic petal culture, three from chronic bud culture, one from chronic shoot, and one from acute leaf culture (**Fig. 7**).

The combined method of chronic irradiation with floral organ cultures proved to be of practical use in mutation breeding, not only of flower species like *Cytisus and Eustoma* [10, 11], but also of other species such as sugarcane, pineapple, banana and so on [12, 13, 14].

Figure 6 Relationship between cell cycle and mutation induction under chronic and acute irradiations.

Figure 7 The original variety, "Taihei," its registered mutant varieties and their induced methods using *in vitro* cultures of explants taken from different organs in chrysanthemum under chronic, acute and non irradiations. Chr.: chronic irradiation, Acu.: acute irradiation.

Conclusions

1. The optimum dose of chronic irradiation can be extended to almost 2.5 times that of acute irradiation. The chronic irradiation method employed in this study caused a relatively high accumulation of radiation in regenerants, but reduced adverse radiation damage to the proliferation and differentiation of the callus.
2. The combined methods of irradiation and *in vitro* culture yielded a mutation rate eight times higher than the conventional chronic cutting method, also producing non-chimeric mutants (**Fig. 3**).
3. Somaclonal variation often occurred in plants regenerated from callus, but no significant variation appeared in callus regenerants from non-irradiated plants. Therefore, proper mutagenic treatment on cultured materials is indispensable for effective mutation induction (**Fig. 3**).
4. The chronic culture method clearly yielded the widest color spectrum in chrysanthemum, while the acute culture method resulted in a relatively low mutation rate and a limited flower color spectrum (**Fig. 4**).

5. Flower color mutation could be more readily induced in plants regenerated from petals and buds, than from leaves. In this respect, it is supposed that the gene loci fully expressed on floral organs may be unstable for mutation by mutagenesis or culture, but could perhaps induce mutation in a desired direction (**Fig. 4**).
6. A possible reason for why the chronic culture methods showed higher frequencies than the acute, is that most of the cells composing the tissue and organs continually irradiated into a cell division and became more sensitive and mutable to irradiation (**Fig. 5**). Under these conditions, many small mutated sectors accumulated in the cells of growing organs. In contrast, when explants composed of various cell stages were irradiated at high doses and dose rates, the dormant cells, less sensitive to radiation, were able to survive, but the more sensitive dividing cells were inactivated (**Fig. 6**).
7. In this study, nine out of 10 flower color mutant varieties registered were derived from chronic irradiation (**Fig. 7**). The combined method of chronic irradiation with floral organ cultures proved to be of particularly great practical use in mutation breeding, not only of flower species but of other species as well.
8. By using this technique, a number of useful mutant varieties could be developed in many kinds of crops, such as sugarcane, pineapple, *Eustoma and Cytisus*.

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Enhancing Genetic Diversity Through Induced Mutagenesis in Vegetatively Propagated Plants

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Abstract

Conventionally, crop improvement strategies rely not only on the availability of heritable genetic variations within utilizable genetic backgrounds, but also on the transferability of the traits they control through hybridizations between the parental stocks. Procedures for producing hybrids of sexually reproducing plants are routine, while for vegetatively propagated plants, hybridizations are usually impractical. Therefore, the improvement of crops that lack botanical seeds necessitates alternative strategies for generating and utilizing genetic variations. Induced mutagenesis generates allelic variants of genes that modulate the expression of traits. Some of the major drawbacks to the widespread use of induced mutations for vegetatively propagated plants include the difficulties of heterozygosity of the genetic backgrounds, the incidence of chimeras and the confounding effects of linkage drags in putative mutants. In general, the inherent inefficiencies of time and space economies associated with induced mutagenesis are further exacerbated in vegetatively propagated crops mostly on account of the need for continual propagation. We highlight the mitigating roles on these drawbacks of judicious integration of validated biotechnologies and other high throughput forward genetics assays in induced mutagenesis pipelines. Using cassava and banana as models, we demonstrate the use of cell and tissue biology to achieve homozygosity, minimize or eliminate chimeras, and significantly shorten the duration of the generation of mutants. Additionally, use of these biotechnologies to attain significantly reduced propagation footprints while evaluating putative mutants without compromising population size is also presented. We also posit that molecular biology approaches, especially reverse genetics and transcriptome assays, contribute significantly to enhancing the efficiency levels of the induced mutagenesis processes. The implications for crop improvement and functional genomics via the concerted application of biotechnologies in the generation, identification, and tagging of mutation events in the genomes of vegetatively propagated crops are also discussed.

Introduction

Mutation, the heritable change to the genetic make-up of an individual, is a naturally occurring phenomenon. The outcome of the interplay of the accumulation of mutation events and the modulating influences of natural and artificial selection pressures is manifested in the evolutionary process. A vivid contribution of mutation to agriculture is crop speciation and domestication. Characterized mutation events that discriminate cultivated plant species from their wild relatives include the abolishment of shattering in cultivated peas and cereals such as barley and wheat. Other examples include non-bitter edible almonds and parthenocarpy that confers seedlessness in grapes and other fruits, e.g. bananas. A recent exploitation of a mutation event that is amply chronicled in literature is the isolation of the semi-dwarf characteristics in wheat by scientists at the International Center for Maize and Wheat (CIMMYT, its Spanish acronym) in Mexico. The massive generation and deployment of wheat varieties with the mutant *sd1* gene, characterized to be a mutation leading to a deficiency in the growth hormone, gibberellic acid, led to the Green Revolution [1].

Early in the 20th century, X-rays and other forms of radiation were discovered; the demonstration of the ability of these physical agents to cause mutations followed quickly afterwards [2, 3, 4]. Naturally occurring mutations are referred to as spontaneous mutations, as opposed to induced mutations that are brought about through artificial means. For the past 80 years, mutation induction has been a routine tool for the generation of genetic variation in crop germplasm, and has been overwhelmingly used in crop improvement, a strategy that is known as Mutation Breeding. The use of these mutant stocks has since been expanded to include the more upstream activities of functional genomics which encompasses a body of procedures relating to the discovery of genes and elucidation of their functions. This has been achieved through the production of insertional and gene knockout lines. More recently, plants harboring induced subtle genome alterations, usually single base pair changes, are becoming commonly used in gene discovery and functional studies through the association of changes in their DNA sequences to modified phenotypic expressions.

Vegetatively propagated crops

A wide range of crops, grown for their edible roots, tubers, fruits, aerial stems and leaves, are not planted using botanical seeds. When plant parts other than true sexual seeds are used for propagating a plant, the mode of propagation is referred to as vegetative. Vegetatively propagated crops play critical food security roles in the tropical and neo-tropical regions of Africa, Asia and Latin America and the Caribbean. Increased and stable yields in these crops are therefore imperative for meeting the dietary needs of the rapidly growing populations in these parts of the world. Conventional crop improvement, predicated upon the availability of utilizable desirable genetic variation, is dependent upon the breeders' ability to generate progeny from hybridizing parental stocks. This is not an option for plants which, for a variety of reasons, are vegetatively propagated crops.

Due to this handicap, the development of improved varieties with stable high yields that enhance the nutritional status of the populace, are tolerant of the myriad of biotic and abiotic stresses, and suffer minimal post-harvest losses, remain veritable challenges to breeders for many of the staple food security crops in the tropics and neo-tropics. This daunting task is exacerbated by the other biological constraints of high heterozygosity of vegetatively propagated crops, the high levels of systemic disease and pest inoculums.

Survey of induced crop mutants

Considering the aforementioned drawbacks to the use of conventional hybridization-based strategies for the genetic improvement of veg-

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etatively propagated crops, it would be reasonable to assume that plant breeders and other scientists engaged with the generation of new varieties of these crops have been making use of induced mutations and other novel genetic improvement interventions. The facts however belie this assumption.

The elegant surveys of officially released induced crop mutants in [5] and [6] indicate that seed propagated crops constitute the overwhelming majority of crop mutant varieties. Of the 1,700 officially released crop mutant varieties surveyed in [5], only 97 were vegetatively propagated crops (**Fig. 1**). A similar skewed trend in favor of a disproportionately large ratio of seed propagated mutant crop varieties is evident when all mutant plant varieties (and not only crops) are surveyed. A search of the Joint FAO/IAEA Mutant Varieties Database [7] in November of 2008 showed that out of the total entry of 2,797 mutant plant varieties, only 310 vegetatively propagated mutant plant varieties (including food crops, ornamental plants and animal feeds crops) were listed (**Fig. 1**).

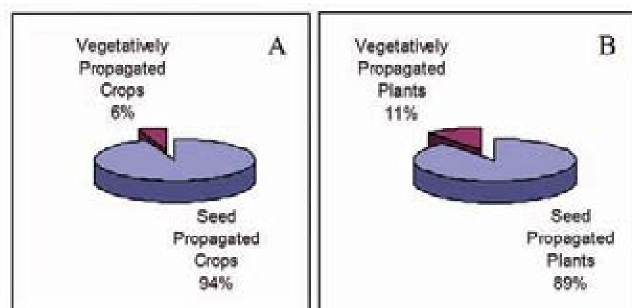

Figure 1 Officially released mutant crop varieties by mode of propagation: A, in 2000 [5]; B, in November 2008 [7].

Ahloowalia, *et al.* [6] highlighted this relative paucity of vegetatively propagated mutant crop varieties. Interestingly, according to these authors, the few vegetatively propagated mutant crop varieties were enthusiastically adopted by farmers and have been contributing to enhanced income for the growers. The most striking examples of these mutants, excluding the ornamental plants, are shown in **Table 1**.

Table 1. Summary of some widely cultivated vegetatively propagated mutant crop varieties with significant economic values for growers. Adapted from [5] and [6]

Crop type	Plant	No. of mutants
Oil	Peppermint	2
Fruit	Orange (Mandarins)	5
	Apple	10
	Banana	2
	Apricot	1
	Sweet Cherry	8
	Sour Cherry	4
	Peach	2
	Pomegranate	2
	Pear	5
	Japanese Pear	2
	Black Currant	1
	Ribes	1
	Raspberry	1
	Grape	1
Root	Cassava	1

Of note are the two varieties of peppermint, 'Todd's Mitcham' and 'Murray Mitcham,' whose tolerance to *Verticillium* have made them varieties of choice in the US. Others are the 48 mutant varieties of fruits including the 'Gold Nijisseiki' Japanese pear (*Pyrus pyriforia*), which is resistant to black spot disease (caused by *Alternaria alternata*), and the 'Rio Star' grapefruit which accounts for 75% of the grapefruit production area in Texas, USA. The inference from the widespread adoption and cultivation of these vegetatively propagated mutant crop varieties is that the relatively depressed number of mutant varieties in this class of crops is not for want of end-users. The reasons derive from the peculiarities inherent in the reproductive biology of these crops; overviews of these bottlenecks will be reviewed in subsequent sections of this paper.

Drawbacks to the induction of mutation in vegetatively propagated crops

In general, a major drawback to the routine application of induced mutagenesis to both crop improvement and genomics studies (through forward and reverse genetics strategies, respectively) remains the drudgery of producing, handling and assaying the requisite large populations of mutant stocks. This is because since the success of induced mutations is a function of statistical probability, protocols are not yet available (nor readily feasible) for targeted gene modifications, and large population sizes must be created in order to have a fair chance of detecting desirable mutations. This is expensive, laborious and time-consuming. For obligate vegetatively propagated plants, the starting materials for inducing mutations cannot be botanical seeds, usually more convenient to handle than the bulkier vegetative propagules, as is the case with the seed propagated crops. This immediately poses the first problem of footprint as at the very least, more space is required for handling the propagules and more time and resources will have to be invested in the actual exposure of the propagules to the mutagenic agent. This drudgery continues into the evaluation of the putative mutants, as this typically also requires more time and resources. Other peculiarities of vegetatively propagated plants go beyond this drudgery and require deliberative strategizing in order to efficiently generate mutants. The most common issues include the absence of meiotic sieves; the concurrent fixation of deleterious alleles; the transmission of pathogens to subsequent generations, and most importantly, the preponderance of chimeras [8].

Absence of meiotic sieves

Point mutations, physiological damage (primary injury), and chromosomal aberrations have often been identified as the three main effects of mutagenesis. In seed propagated crops, gross chromosomal aberrations – which are lethal – are 'sieved' out at the first mutagenic generation (M_1), as well as the physiological damages that are extra-nuclear. In practice, only point mutations and other non-lethal mutations are carried over to the next generations in seed propagated crops. With meiosis not intervening in the advancement of putative mutants to subsequent generations, this 'sieve' mechanism is obviated leading to the accumulation of inordinate levels of unintended mutation events.

Concurrent fixation of deleterious alleles

Pleiotropic effects, occasioned by creation of new alleles through mutagenesis, or linkage drags (the co-segregation of desirable with deleterious alleles), are easily mitigated through hybridization, usually backcrossing to the desirable genetic background. In vegetatively propagated crops, this facility cannot be exploited, leading to the accumulation of undesirable genic effects in the induced mutant. An otherwise excellent induced mutant could be dispensed with on this account, a situation that may have some contributory roles in the relatively lower numbers of induced mutants in this class of crops.

Transmission of pathogens to next generations

Plant pathogens - fungal, bacterial and viral – through systemic infection are propagated along with the host from one generation to the next. The presence of disease agents in a putative mutant may confound detection of the phenotypic expression of the mutation event. While systemic infection could also result in transmission of pathogens through seeds, the sheer bigger size of vegetative propagules ensures greater loads of inoculums of disease-causing agents which accumulate over generations.

Chimeras

A result of using a multi-cellular tissue (as most vegetative plant propagules do) as the starting material for mutation induction is the incidence of chimeras, i.e. sectorial differences whereby different mutation events exist side-by-side in the same individual. Bearing in mind the characteristic totipotency of plant cells, the ontogeny of the progeny from the same putative mutant would be distinctly dissimilar, a situation that is clearly undesirable in generating a mutant population; such a population should be uniform for the mutation event. The handling of the mutagenic population therefore requires significant effort being invested in the dissociation of chimeras, an added drudgery.

Strategies for mitigating the drawbacks to inducing mutations in vegetatively propagated crops

The identified bottlenecks to routine induction, isolation and deployment of mutations in vegetatively propagated crops can be ameliorated through a strategic use of biotechnologies; cell and tissue biology techniques are useful for the efficient production of mutants while molecular biology techniques enhance efficiency in the rapid genotyping of the mutation events, as reviewed below.

Cellular and tissue biology strategies

A critical bottleneck in the routine application of induced mutations in plants is the low level of efficiency of the processes; quality and quantity of induced mutant populations are sub-optimal when conventional strategies are used. Also, mechanisms for discovery and deployment of the mutated segments of genomes could be significantly improved. The requirement for generating and evaluating large population sizes remains imperative being the only way to ensure a fair chance of recovering the desired mutation events. Another drawback, the inherent problem of chimeras, is exacerbated in vegetatively propagated plants. A further significant hurdle is the need to have the mutated segment in a homozygous state so that the mutation, usually recessive, could manifest as a phenotype. A number of *in vitro* techniques have been shown to circumvent or significantly mitigate these bottlenecks to induced mutations. These include cell suspension cultures including somatic embryogenesis, and rapid *in vitro* multiplication. Strategies for mitigating the drawbacks of inducing mutations in a heterozygous state include the recovery of dominant alleles and exploiting existent haplo-insufficiency and limited sexual reproduction.

Somatic embryogenesis

The presence of chimeras, especially in vegetatively propagated crops, confounds strategies employed in the development of homohistonts. The validated methodology for mitigating this problem is to pass the putative mutant through several cycles of vegetative regeneration (*in vitro* and *in vivo*). The added expense, in terms of time and resources, could be circumvented through the use of single cells as starting materials for inducing mutations and subsequently regenerating whole plantlets from them, a path that mimics classical embryos (involving zygotes) and is referred to as somatic embryogenesis. This process typically involves mass proliferation of undifferentiated cells – callus – and subsequently through the modification of culture media, the induction of embryogenic properties in the cells. Typically, the most common paths taken include the production of cell suspension cultures and friable embryogenic calli (FEC). The individual cell is then exposed to the mutagen, and taking advantage of the potential of each plant cell to regenerate into a whole plant, a phenomenon known as totipotency, plantlets are regenerated on appropriate culture media. Cell suspension culture processes include the production of cell lines from callus followed by the regeneration of plantlets through somatic embryogenesis. The FEC path circumvents the culturing of cells on liquid media, rather through the manipulation of growth media, embryogenic structures are induced which subsequently regenerate whole plantlets. Since Nickell [9] demonstrated the cell suspension technique with *Phaseolus vulgaris*, reproducible protocols have been validated for other plant species. This ability to grow individual plant cells under aseptic conditions, and from them, regenerate whole plantlets, permits the exposure of individual cells to mutagens. Arising from an individual cell, each plantlet is devoid of chimeras. Obviating therefore the requirement for several cycles of regeneration, significant gains in time and resources are achieved through this path. Following the demonstration of somatic embryogenesis through cell suspension cultures in *Musa* [10], the use of cell suspension cultures as starting materials in induced mutations has been successfully used in the generation of banana mutants [11, 12]. Lee, *et al.* [13] had also generated homohistonts in cassava through gamma irradiation of FEC in cassava.

Rapid *in vitro* multiplication

Protocols for somatic embryogenesis, being genotype-dependent, are not available for all crop species, therefore limiting the utility of this technology in the efficient induction of mutations in plants. In instances where somatic embryogenesis is an impractical option, or where protocols have not been validated, *in vitro* techniques, especially rapid multiplication systems using meristematic regions of plants, could be used to achieve some level of efficiency in induced mutations. Plantlets are regenerated, but not at the same levels of homozygosity and incidence of chimeras as in somatic embryos. Such plantlets are then passed through several cycles of regeneration to dissociate chimeras. Through this method (irradiation and *in vitro* culture of shoot tips), an induced mutant banana variety, Novaria, currently under cultivation in Malaysia, was developed [14]. Roux, *et al.* [11] reported that incidence of cytochimeras in *Musa* was reduced to 8% after three subcultures of plantlets arising from multi-apices. This could be used as a guide as it has not been possible to empirically demonstrate the complete elimination of chimerism through sub-culturing of the plantlets. Owoseni, *et al.* [15] reported the use of *in vitro* nodal segments as starting materials for the induction of mutations in cassava.

Molecular biology techniques for enhancing efficiency in induced mutations

While cell biology techniques could be used to address the bottlenecks imposed by the need to rapidly generate large mutant populations of suitable genetic backgrounds (homozygous for the mutation events, and devoid of chimeras), there is still a compelling need to query the mutant populations for the mutation events. This is of course not peculiar to vegetatively propagated crops, but nevertheless deserves a mention. Direct field trials or laboratory assays for the traits of interest for the large mutant population is usually laborious, expensive and hardly cost-efficient. Molecular biology strategies offer mechanisms for direct querying of target genes for changes. Molecular biology might not provide conclusive evidence of the desired mutation in itself, but such techniques will significantly obviate the need for field trials of large populations, enhancing the efficiency of the processes.

Reverse genetics strategies, especially Targeting Induced Local Lesions IN Genomes (TILLING), through its inherent high throughput platform, promise to be indispensable tools for the efficient and rapid identification of mutation events. Making use of a combination of polymerase chain reaction amplification of target regions of the genome and a subse-

quent identification of mutations in the target region through enzymatic mismatch cleavages [16, 17, 18], TILLING has been demonstrated in the identification of induced mutations in wheat [19].

Conclusions and perspectives

Continually, the scientific community is recognizing the critical roles of induced mutations in crop improvement and functional genomics. Thus, 'designer crop varieties,' such as high-yielding crops with targeted modifications to their genomes, are needed to address the uncertainties of global climate change and variations, and the expected increase in global food insecurity. There is a compelling need to invest significant efforts in the development of strategies for an efficient use of induced crop mutants in producing crops that are adapted to harsh environments and the development of genomics tools for use in marker-aided selection. On account of the almost total absence of sexual reproduction, genetic improvement of vegetatively propagated crops presents particular problems for which induced mutagenesis might be the most viable cost-effective solution. However, sub-optimal breeding strategies for these crops also affect the efficiency levels in the use of induced mutagenesis. One of the most serious identified bottlenecks is the incidence of chimeras, a problem that could be mitigated by a strategic deployment of appropriate cell and tissue biology techniques.

The need for recessive mutations to be in a homozygous state in order to manifest phenotypically, also poses a significant hurdle. However, the fact that vegetatively propagated plants that were induced to mutate using vegetative propagules constitute 11% of all reported officially released mutant plant varieties [7], demonstrates the feasibility of mutation breeding. The causative mutations underlying the mutant varieties are unknown. Phenotypic manifestations in the varieties could be ascribed to the abolishment of function in loci with only one active gene copy. The frequency of heteroalleles will be increased in vegetatively propagated species because of spontaneous accumulation of deleterious mutations and the lack of meiosis, independent assortment, and selection to cull such mutations from the population. Heterozygous mutations will also reveal haploinsufficient loci. It is also probable that dominant mutations could be in play for some of the traits in these vegetatively propagated crops.

As with seed propagated crops, the rapid and cost-effective identification of mutation events is desirable and could be achieved by using molecular biology tools to query targeted regions of the genome for mutations. Reverse genetics methodologies, especially those amenable to high throughput – in order to efficiently evaluate the requisite large mutant population sizes – such as TILLING, hold great promise for the use of molecular biology techniques to rapidly whittle down the size of putative mutants before field trials or other assays with cost implications that are required to confirm the phenotypic expression of the desired mutation. The reasoning is that if TILLING for instance, does not uncover a mutation in the target gene (already implicated in the expression of a trait), it is unlikely that the putative mutant would express a phenotypic variation from the wild-type, negating therefore the need for any further evaluation of that candidate. This could be a major contribution to easing the drudgery associated with induced mutations in crops, especially for crop breeding.

Validated protocols exist for these different biotechnologies, but a need remains for the assemblage, adaptation and interlacing of these novel cell and molecular biology techniques into components of the induced mutations process, as well as the adaptation to specific crops. The generation of protocols along these themes, in manners that achieve a seamless dovetailing of validated processes into a modular pipeline, will significantly ameliorate above drawbacks and consequently aid the routine application of induced crop mutations in crop improvement and in gene discovery and elucidation.

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Induction and Identification of Useful Mutations for Root Quality Traits in Cassava

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Abstract

A population of about 1,500 M_2 genotypes (from five different cassava families), were screened for relevant traits. Roots could be harvested from 800 of these genotypes and thoroughly screened for starch quality traits. Four distinctive phenotypes were identified: a small granule, high-amylose starch mutation with almost no paste viscosity, a group of genotypes with tolerance to post-harvest physiological deterioration, a "hollow" starch granule mutation with intermediate viscosity peaks, and a group of genotypes with a starchless (potentially "sugary") root mutation. The last two mutations need to be further characterized and confirmed. The small granule mutation has been fully characterized and a lesion in the isoamylase one or two loci has been postulated to be the most likely site. The frequency of this mutation (present in two lineages out of the 800 M_2 genotypes analyzed) suggests that mutation at the putative locus is easy to attain, or else that the repair mechanisms are less efficient at that site. Several of these unusual phenotypes identified offer important commercial advantages.

Introduction

Cassava (*Manihot esculenta* Crantz), is a perennial crop originated and domesticated in the neo-tropics [1, 2]. Its most common product is the starchy root, but the foliage has an excellent nutritional quality for animal and human consumption [3]. Cassava is the fourth most important basic food and is a fundamental component in the diet of millions of people [4, 5]. The crop produces well under marginal conditions, particularly due to its tolerance to drought and infertile soils and its ability to recover from disease and pest attacks. It can also produce well in non-marginal areas. Cassava offers the advantage of a flexible harvesting date, allowing farmers to hold the roots in the ground until needed [6, 7].

In addition to its role in subsistence farming and food security, cassava is increasingly used as raw material for many industrial applications: as a source of energy in animal diets, for the starch industry and, more recently, in bio-ethanol production [7]. The presence of cyanogenic glucosides in the root limits their use [8]. Another constraint is the short shelf life of roots due to post-harvest physiological deterioration (PPD). PPD begins within 24 hours after harvest [9, 10], and rapidly renders the roots unpalatable and unmarketable. Consequently, cassava roots need to be consumed or processed soon after harvesting [11]. Finally, cassava has the disadvantage of low genetic variability for root starch traits [12, 13, 14]. The fact that roots are not reproductive or multiplicative organs may offer cassava (and other root crops) an advantage over seed-propagated crops.

In spite of their low frequency, generally negative effects and unpredictability of results, the induction of mutations has been a successful approach to generate new variability in other crops where natural genetic variation is limited and insufficient. As a result, several varieties have been developed after induction of mutations [15, 16]. CIAT and National University of Colombia have implemented a joint mutation-breeding project in search of genetic modifications for useful traits. As a result of this project, cassava genotypes that showed distinctive root quality characteristics were identified in March 2006, and further confirmed in harvests in March of 2007 and March of 2008.

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Materials and Methods

As part of the project to identify cassava germplasm with novel root or starch quality characteristics, CIAT and Universidad Nacional de Colombia (Palmira Campus) initiated a project to induce mutations in botanical seed from five different families. The project benefited from the financial and technical support of the International Atomic Energy Agency and the Rockefeller Foundation.

Seed Irradiation to obtain the M_1 generation, field growth and production of the M_2 generation

About 1400 botanical seeds from five different full or half-sib families (**Table 1**) were irradiated with 200Gy Gamma-rays (from Cobalt^{60}). The irradiated seed (M_1 generation) was germinated and grown in the greenhouse for two months. Seedlings were then transplanted to the field and normal cultural practices were used to maintain the crop in good growing conditions. By August 2004 several M_1 plants started to flower. Flowering in cassava does not follow a pre-defined pattern. Some genotypes flower few months after planting (four to five months), some do later in the season (six to nine-month-old plants) and some just fail to flower. Whenever possible, the M_1 plants were self-pollinated to generate M_2 seed. This action was fundamental to eliminate chimeras which are very common in M_1 generation. It is also important to increase the level of inbreeding, thus facilitating the phenotypic identification of recessive traits (a typical feature of mutations). As is frequently the case, irradiation affected vigor negatively.

Screening for useful traits in the M_2 generation

M_2 seeds were germinated and transplanted to the field in May 2005. About 1,500 M_2 genotypes were derived from the self-pollinations made during the previous season. In most cases, several M_2 genotypes were obtained from the same M_1 plant. M_2 plants were weak because, in addition to the mutations they were carrying that affected their general performance, they also suffered from the natural inbreeding depression. Nonetheless, up to 800 of these M_2 plants were vigorous enough to produce roots from which starch could be extracted. Harvest took place in March 2006. Only one plant per genotype was available because evaluations were made on individual plants obtained from botanical seed. At least one commercial-size root was harvested per genotype. Whenever possible, up to five roots per plant and genotype were harvested. Two tests were made immediately after harvest: iodine staining and analysis of starch granule morphology with an optic microscope. Roots were

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then washed and peeled before samples were prepared for the different analyses performed.

Iodine-stained field evaluation of roots

As soon as roots were harvested they were cut and one of the transversal sections was sprayed with iodine solution 2% (2g KI and 0.2g I2 in 100cm^3 of distilled water). Reddish-brown staining is typical of amylose-free starch, whereas cassava starch with normal amylose-amylopectin mixture stains dark-blue.

Optic and scanning electron microscopy (SEM)

A microscope slide was rubbed against the freshly cut section of the roots and stained with iodine solution 0.2%. The slide was observed through a light microscope (Olympus CX41) using a 40X magnification lens. If the initial optic analysis suggested changes in starch granule morphology, more detailed analysis was conducted with a scanning electron microscope. Dehydrated starch granules were sprinkled on double sided sticky tape, mounted on circular aluminium stubs, coated with 35 nm of gold-aluminium, and then photographed in a Scanning Electron Microscope (JSM 820 Jeol, Tokyo, Japan) at an accelerating voltage of 20 kV.

Granule size and distribution

Starch granule size and distribution was determined with a laser diffraction particle size analyzer (SALD-3001-Shimadzu Japan). This kind of study was only done on samples whose preliminary results suggested changes in starch granule morphology and/or size. Starch samples were mixed with distilled water and one drop of sodium hexametaphosphate (0.2%) was added. Suspension was mixed using a mechanical stirrer and sonicated to obtain a laser light obscuration level of ~ 30%. Refractive index of 1.600±0.101 was set for the starch. Measurements were run in triplicate on two different starch samples per genotype at room temperature.

Other tests

Other tests, following the standard procedures of the root quality laboratory at CIAT [17] were conducted in roots, flour or starch from the "seedling plants" (plants derived from botanical seed, not vegetative cuttings): cyanogenic potential, root and starch moisture content, paste clarity, colorimetric amylose determination, pasting properties, swelling power, solubility and dispersed volume fraction measurements. Any genotype whose analyses suggested a change in root quality trait(s) or modifications in plant architecture or physiology, was immediately selected and stem-cuttings were obtained to vegetatively multiply it. Cloned genotypes were then harvested in March 2007 and March 2008 to confirm the stability of the changes and their genetic nature and to provide further phenotypic information.

Results

A total of 11 M_2 genotypes derived from botanical seed irradiated with Gamma-rays were selected because of their unusual characteristics. As explained above the M_2 genotypes, initially selected in "seedling plants," were cloned as soon as their unusual characteristics were first identified.

Small-granule, high-amylose starch mutation

Results from the discovery of a small-granule mutation (SGM) have already been published [18]. A total of four genotypes with a similar starch phenotype were identified. Three of these genotypes (5G160-13; 5G160-16; 5G160-18) originated from self-pollinations the same M_1 genotype (5G160), which traces back to family C4 from the TME3 x TMS30555 cross (**Table 1**). They were therefore, siblings from the same mother plant and their special starch phenotype originated in a common mutation event induced in the M_0 seed. The fact that these three related genotypes expressed the same unusual starch phenotype was a strong indication that their special starch characteristic was, indeed, genetically controlled. The fourth genotype (3G43-1) was the only M_2 plant derived from the M_1 genotype (3G43) expressing the same starch phenotype observed in the 5G160 M_2 family. 3G43 originated in SM 3015 (**Table 1**), which is an open-pollinated family whose female progenitor was MCOL 1505 and different known potential male progenitors acted as source of pollen.

Table 1. Cassava germplasm irradiated with Gamma-rays (from Cobalt^{60})

Family	Mother	Father	No. Seeds	
1	CM 9331	SM 1210-10	MNGA 1	150
2	SM 3015	MCOL 1505	Unknown	150
3	SM 3045	HMC 1	Unknown	150
4	GM 155	MTAI 1	SM 2102-34	153
5	C-4	TME3	TMS30555	787

The initial evidence of unusual starch granule morphology was first observed through light microscope analysis of the starch from the four genotypes, which were distinctively smaller than wild-type (WT) (**Fig. 1**). Further evaluation through scanning with an electron microscope not only confirmed the smaller size of starch granules, but a surface that was not as smooth as in WT starch granules (**Fig. 1**). Results from the laser diffraction particle size analyzer indicated that granule size was about one third the typical average of WT cassava starch granules (**Table 2**).

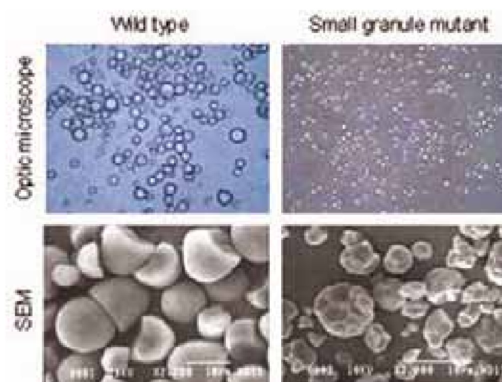

Figure 1 Microscopic (transmission and scanning electron) comparison of starch granules between the WT and small granule mutant of cassava.

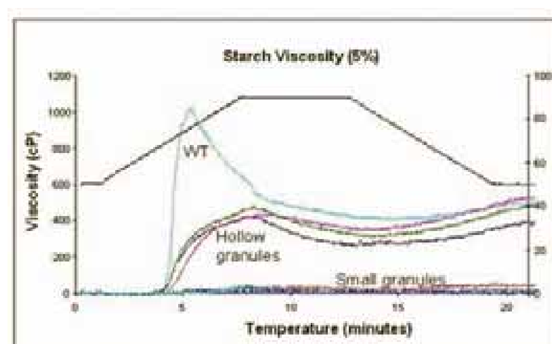

Figure 2 Amylograms of WT and various mutants of cassava.

The most outstanding difference in the amylograms relates to the very low viscosity of SGM (peaks ranging only from 19 to 76 cP compared to 976 to 1080 cP in WT cassava). This striking difference is clearly illustrated in **Fig. 2**. Hot and cool paste viscosities, breakdown, setback

and consistency were very low and difficult to quantify in the almost flat amylograms of SGM (**Fig. 2**). Another interesting observation was that the average amylose content in SGM using the colorimetric method ranged from 28-36%, a considerably higher value compared with the 20-22% values observed in the WT genotypes presented in **Table 2** or the average of 20.7% obtained after the analysis of more than 4,000 cassava genotypes [19]. Paste clarity was also much lower in SGM (13-26%) compared with WT starch (59-64%).

Table 2. Analysis of the starches from SGM and three WT genotypes. Data from one SGM came from a seedling plant (March 2006 harvest) and five cloned plants in later harvests. Analyses for these five plants were made individually so SD (in brackets) values could be provided. For the second SGM (5G160-16), only data from the seedling plant is available. Peak viscosity and pasting temperatures are based on 5% starch suspensions.

Parameter	Seedling		Cloned			
	5G 160-16	5G 160-13	5G 160-13	MTAI 8	MCOL 1505	MNGA 11
Average granule size (µm) (St. Deviation)	n.a.	n.a.	5.80 (0.327)	16.17 (0.086)	18.73 (0.099)	13.97 (0.118)
Paste clarity (%) (St. Deviation)	17	26	13 (1.18)	63 (1.76)	63.5 (0.14)	58.7 (1.06)
Amylose (%) (St. Deviation)	36.23	28.49	30.07 (0.87)	20.67 (1.62)	22.18 (0.76)	22.6 (0.60)
Pasting temperature in °C (St. Deviation)	67.4	67.15	60.17 (1.06)	64.23 (0.53)	61.60 (0.49)	63.55 (0.07)
Peak viscosity in cP (St. Deviation)	44	76	22 (4.5)	976 (2.82)	1052 (6.36)	1080 (4.94)
Solubility (% db) (St. Deviation)	33.83	37.17	36.84 (0.67)	10.15 (0.03)	12.20 (0.73)	8.34 (0.55)
Swelling Index (g g^{-1}) (St. Deviation)	16.45	14.19	9.25 (0.26)	21.77 (0.44)	27.92 (0.57)	26.55 (0.66)
Volume fraction dispersed phase (Φ) (St. Deviation)	0.32	0.28	(0.66)	0.70 (0.002)	0.72 (0.002)	0.68 (0.009)

A preliminary screening for the most likely cause of the small granule SGM was conducted for two candidate enzymes: starch branching enzyme (SBE) and isoamylase (ISA). The activities of SBE and ISA were assayed using native gels. The native gel analysis also gave information about the activities of other enzymes [18]. These preliminary results suggested that the SGM does not lack SBE, one of the common causes of a high-amylose phenotype in mutants of other species. However, a consistent difference between WT and SGM in the banding pattern of isoamylase on native gels was observed. Whether the changes in ISA bands shown here for the cassava SGM are due to a lesion in an isoamylase gene or due to secondary effects remains to be determined. Other enzymes such as starch synthase are potential sites for the mutation in cassava and will also be investigated after the next harvest. In addition to isoamylase, other enzymes (pullulanase, disproportionating enzyme, and α-glucan water dikinase) have also been related to amylopectin biosynthesis in different crop species and are also potential site(s) of the observed mutation. Although on native gels of the mutant no effect on SBE activity could be observed, this enzyme cannot be entirely ruled out at this stage. TILLING [20] using primers from as many as 16 different enzymes known to be related to starch biosynthesis is also underway. Until further information is produced, the tentative site of the lesion causing this mutation is in one of the isoamylase genes.

A remarkable feature of this mutation is that two totally independent events with the same phenotype have been identified (5G160 and 3G43), in a relatively small population of about 800 M_2 genotypes from which roots could be harvested and starch extracted. After an extensive evaluation of the cassava germplasm collection at CIAT (more than 4,000 genotypes) in no case has such SGM phenotype been reported, including in the female progenitor of 3G43 (MCOL 1505). A large number of cassava genotypes (including MCOL 1505) have been self-pollinated in search of useful recessive traits [13], and SGM has not been observed in any of these S_1 families either. Also very important is the fact that only one M_1 genotype from the many representatives of families C4 and SM3015 produced M_2 plants with the SGM phenotype. If the mutation was already carried by one of the progenitors of these families, it should have surfaced in more than a single lineage of each of these two families. Therefore, a reasonable working hypothesis is that these two SGM phenotypes were created *de novo*, as a result of the irradiation of the botanical seed. The effect of irradiation overlaps and confounds with the normal genetic segregation that made each botanical seed unique and different from each other. To rule out the possibility that progenitor(s) of each of the two families whose seed was irradiated (C4 and SM 3015) were already carrying the mutation, they were retrieved and self-pollinated. Several of the potential male progenitors in the open-pollinated SM 3015 family will also be recovered.

The higher-than-normal level of amylose in the small-granule mutation has important commercial implications. Increased amylose levels lead to slowly digestible and resistant starches, which have distinctive advantage in health, particularly in diabetes management and other industrial uses [21, 22]. Crosses between the two different SGM have been made. Since they are genetically unrelated, the resulting F_1 cross will not have the negative effects of inbreeding observed in the M_2 cloned genoytpes currently available, and may produce reasonable yields. Moreover, the F_1 crosses will be segregating for many loci, including those causing the small-granule phenotype (in case the mutations in 5G160 and 3G43 affected different loci). This segregation could allow selecting for amylose levels higher than the maximum level of 36% already observed in one of the quantifications made in this study.

Alternatively, the starch granule characteristics of SGM may have other commercial applications. The reduced granule size and the obvious irregularities in their surface (**Figure 2**) would lead to a facilitated hydrolysis [23], thereby offering advantages for the bio-ethanol industry. It is acknowledged, however, that while the starch granule appearance would facilitate bio-ethanol production, the higher proportion of amylose would tend to make it less efficient. Only when proper fermentation studies are conducted, the relative importance of these contrasting and opposed trends will be clarified. A similar problem has already been analyzed for starches from other crops [23].

Tolerance to post-harvest physiological deterioration (PPD)

The processes involved in PPD resemble typical changes associated with the plant's response to wounding that triggers a cascade of biochemical reactions, which are frequently oxidative in nature. Specific genes involved in PPD have been identified and characterized, and their expressions evaluated [9, 24, 25]. As a consequence, there are several potential loci where the induction of mutations could result in the alteration of this cascade of biochemical reactions. It is therefore, not surprising that, in preliminary evaluations, five different genotypes have shown tolerance to PPD: two M_2 genotypes from family C4 (5G108-3 and 5G108-4), two from family SM 3045 (4G77-4 and 4G77-5), and a single genotype from family GM155 (2G15-1).

Reaction to PPD is difficult to assess. PPD is influenced by environmental conditions [26] and cultural practices [11]. Root to root variation in PPD, even when they have been harvested from the same plant can be high. The preliminary evaluations made on few roots harvested from "seedling plants" and the first cloned generation, needs further confirmation. A total of 67 plants from the five cloned genotypes were grown in the field and were supposed to be harvested in March 2008. Unfortunately, weather conditions during the 3-4 months prior to the harvest were unusually wet, resulting in generalized root rots and atypical root quality conditions that made the assessment of the reaction to PPD unreliable. Further confirmation for these phenotypes will have to wait until March

2009. PPD implies high marketing costs for cassava roots and causes frequent losses. The logistic complications derived from having to consume or process cassava roots within one or two days after harvest are severe. Therefore, any action directed towards an alleviation of PPD would have very significant and positive impact on the livelihood of millions of people who depend on this crop, either producing or processing it [27].

Hollow-granule phenotype

Genotype 2G28-9 (from family GM155) showed atypical starch granule morphology. Granules looked empty or hollow inside. Unfortunately, this genotype was very weak and produced little amount of starch for extensive evaluation. Amylograms showed a pasting property with an intermediate viscosity peak (**Figure 2**), but this kind of behavior is not totally unusual and similar viscosity curves have been observed occasionally before although they were not necessarily linked to a "hollow granule" phenotype. The heavy rains before the harvest of March 2008 did not help to harvest enough roots for adequate starch extraction to perform extensive analyses on the properties of this suspected mutation.

Starchless / sugary phenotype

A fourth unusual phenotype was identified during the early screening of the M_2 generation. Genotype 5G190-11 from family C4 failed to produce roots with the normal levels of starch. In fact, it was very difficult to extract any starch even from the cloned plants harvested in March 2007. Carvalho and co-workers [14] described a landrace in cassava, which produces very little starch (<3%). However, the roots of this interesting group of "*sugary*" cassava landraces have high levels of free sugars (mostly glucose) and a glycogen-like molecule. The roots from these genotypes have reduced levels of amylose as well. As for the previous mutations, a large number of roots are required for the proper characterization of this putative starch mutation. Because of the limited amount of starch they contain and the environmental conditions during the harvest in March 2008 which did not allow an adequate production of roots, further studies on this mutation will have to wait until the harvest in March 2009.

Conclusions

At least four different root or starch phenotypes have been identified. One of them has already been confirmed and fully characterized [18]. Relevant to the understanding mutation induction, genetic lesions and DNA repair mechanisms, is the fact that the SGM may be a high-frequency event either because locus or loci involved are particularly susceptible to radiation damage, or else because repair mechanisms are more relaxed for this kind of locus/loci given the relatively neutral impact for the fitness of the genotypes carrying the mutations (little selective disadvantage). Given the frequency of mutations affecting amylose and amylopectin biosynthesis and their relative contents in cereals, potato and sweet potato, this hypothesis may be true not only for cassava but for these other crops as well.

The other mutations need further confirmation and characterization. PPD tolerance, if confirmed, would imply a breakthrough discovery given the huge and negative economic implications of PPD. For decades cassava researchers worldwide have been searching for a solution to this problem [27]. Induction of "*sugary*" cassava, if confirmed, is more relevant from the scientific (suggesting also the possibility of a high frequency mutation rate for the involved loci) than from the economic perspective, considering that sugary phenotypes already exist [14]. The relevance of the "hollow granule mutation" can only be assessed after it has been confirmed and starch quality characteristics properly evaluated.

As in the case of other crops the induction of mutations in cassava through irradiation with Gamma-rays has proven to be an effective tool for the development not only of germplasm suitable for basic research but also capable of offering important benefits in the livelihood of farmers and processors of cassava.

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Application of Induced Mutation Techniques in Ghana: Impact, Challenges and the Future

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Abstract

Over two decades of application of induced mutation techniques toward crop improvement in Ghana have led to the production of improved mutant varieties in two crops. In cassava (*Manihot esculenta* Crantz), irradiation of stem cuttings using gamma irradiation resulted in the production of "Tek bankye," a mutant variety with high dry matter content (40%) and good poundability from the parental line which was a segregant of a hybrid between the Nigerian landrace Isunikaniyan (ISU) and the breeder's line TMS 4(2)1425, both from IITA, Nigeria. Similarly, irradiation of vegetative buds of 'Amelonado' (P30), 'Trinitario' (K5) and 'Upper Amazon' (T85/799) cocoa varieties resulted in the production of Cocoa Swollen Shoot Virus (CSSV)-resistant mutant variety. Multi-locational on-farm trials of the mutant line indicate significant increases in yield by farmers with no symptoms of the disease. Despite these achievements, application of induced mutation in Ghana has been challenged by low funding, inadequate statistics on small-holder farms, high attrition rate of researchers, low rate of useful mutant regeneration and lack of indicators for early mutant selection. Recent advances in plant breeding, which combine *in vitro* techniques with mutation induction hold better prospects for generating useful mutants.

Introduction

Mutation induction is a useful technique in plant breeding used to improve traits without disrupting the original genetic constitution of the crop [1]. It has been used extensively in the improvement of several crops, especially vegetative propagated species without extensive hybridization and backcrossing. Mutation breeding has led to release of more than 2,250 plant varieties in the past 70 years [2, 3].

The application of nuclear techniques in Ghana dates back to the mid-1980s. Research efforts in various plant breeding programmes have used these techniques to develop improved varieties of several food crops including cassava, yam, plantain, banana, oil palm, winged bean and cocoa.

Whereas most of these programmes yielded no useful mutants, a few have been successful, having a positive impact on farmer output with a highly significant improvement in the general economy of the nation. This paper reports the achievements made in induced mutation breeding over the past 20 years in the face of frustrating challenges, and also examines the continued application of the technique in future breeding programmes.

Historical perspective

The establishment of the Department of Biology, Food and Agriculture (now Biotechnology and Nuclear Agriculture Research Institute) in the Ghana Atomic Energy Commission in the early 1980s marked the beginning of the application of nuclear techniques in crop improvement in Ghana. Early scientists were trained by the International Atomic Energy Agency (IAEA) in Vienna and in Italy to acquire basic skills in the use of physical and chemical mutagens through Technical Cooperation projects. The training was later extended to other scientists in universities and research institutions in Ghana. The acquisition of these skills provided the impetus for the commencement of efforts at the induction of mutations for improvement of several food crops including yam, plantain, cocoyam, oil palm, coconut, winged bean, African Yam bean, bambara groundnut, groundnuts and maize. Beside the establishment of the LD50 for various plant parts and plant regeneration protocols in the case of pineapple [4] and cocoyam [5], the initial enthusiasm did not result in the release of mutant varieties. Furthermore, there is no peer-reviewed documentation on the outcomes of most of these programmes, suggesting that they were truncated for one reason or another. In the case of cassava and cocoa, results have been well documented by [6,7] and [8], respectively.

Induction of mutation in cassava

Cassava is a staple food crop in Ghana, used for human consumption in various forms. Systematic breeding of cassava was initiated in 1930 when the Cassava Mosaic Virus Disease (CMD) was first observed in the country [9]. Use of local landraces in a hybridization programme with exotic varieties imported from other West African countries and the Caribbean led to the production and release of four varieties, namely, Queen, Gari, Williams, and Ankrah which were high yielding, of good taste and highly resistant to the CMD (ACMV). However, resistance to CMD broke down in all except Ankrah, which later succumbed to the disease due to increased vector population and probably the resurgence of other strains of CMD virus by 1950. Later, another set of four varieties, namely, *K357*, *K162, K680* and *K491* were released as a result of hybridization between local varieties and four closely related *M. esculenta* species [9].

Incorporation of mutagenesis in the cassava improvement programme began in the 1980s. In 1984, seeds of two high-yielding cassava varieties Isunikaniyan or TME117 (ISU) a landrace from Western Nigeria and the improved breeders line, TMS 4(2)1425 introduced from IITA, Nigeria, served as parents for mutation induction using gamma radiation to improve the poundability (mealiness, elasticity and smoothness of the pounded paste). In 1988, cuttings from the ISU X TMS 4(2)1425 F_1 plants were irradiated at 25 and 30Gy Gamma-rays. Selection at the M1V4 stage produced a mutant line with good poundability, high dry matter content (40%) and low incidence of CMD (**Tables 1 and 2**). This was adopted as a new variety after a series of multi-locational trials and officially released in 1997 by the Ministry of Food and Agriculture known as "Tek bankye."

Cocoa development in Ghana

Since its introduction in 1878, cocoa has been a major foreign exchange earner playing a key role in the national economy with about 17% of the

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country's labor force depending on it for the family budget. The rapid expansion of the cocoa industry in the early 1900s declined in the 1980s due to climatic changes, bushfires and other socio-economic factors. In addition to these factors, the cocoa industry was seriously constrained by the black pod disease caused by *Phytophthora palmivora* and *P. megakarya* and the cocoa swollen shoot virus disease (CSSV) transmitted by the mealy bug *Planococcoides njalensis* (Laing) [10]. The CSSV disease could not be controlled either by chemicals [11] or biological methods [12]. Earlier attempts to eradicate the disease by removal of infected trees from their symptomless neighbors [10], which initially proved successful, also failed after over 190 million infected trees had been removed [13]. Thus, genetic improvement of the crop to produce resistant CSSV varieties remained the most suitable option for the control of the disease.

Table 1. Tuber yield, cooking quality and cassava mosaic disease (CMD) incidence of cassava varieties

Variety	No. of tubers/ plant	Tuber yield/ plant	CMVD* incidence	Cooking quality		
				Fufu Mealiness	Ampesi Elasticity	Gari
Abasa Fitaa	5.3	25.1	0.8	2.3	2.3	2.9
Afisiafi	7.4	28.8	0.8	1.0	1.0	3.0
Gblomoduade	7.4	42.6	1.6	1.0	1.0	3.0
ISU-W	5.7	27.0	0.5	2.5	2.0	2.9
Local Variety	4.8	15.5	2.8	2.5	2.5	2.9
C.V.	16.8	27.2	2.4	30.9	36.1	8.2
L.S.D.	1.0	7.6	0.6	0.6	0.6	0.67

Source: Asare and Safo-Katanga [6]. Note: Score of CMD: 0-3, None- very heavy incidence.

Table 2. Root tuber yield of cassava varieties in different locations

Variety	1	2	3	4	5	6	7	8
Abasa fitaa	22.2	23.6	15.4	18.0	16.0	49.4	30.4	26.0
Gblemoduade	25.8	26.8	20.6	56.0	44.8	90.0	48.5	28.5
Afisiafi	22.7	28.1	13.2	32.0	34.0	60.4	25.0	15.5
ISU-W (Mutant)	27.8	18.0	13.0	35.0	25.2	58.7	24.3	14.1
Local variety	14.8	9.8	11.7	12.0	13.5	36.3	15.3	11.0

Source: Asare and Safo-Katanga [6]. Locations: 1- Duase; 2-Akomadan; 3-Offinso; 4-Datoyili; 5-Nyeshe; 6-Subinso; 7-Techiman; 8-Nkoraza.

Development of hybrid cocoa variety

Genetic improvement of cocoa in Ghana has included introductions of exotic varieties, conventional hybridization and induced mutations. According to [14] there have been seven cocoa varietal releases dating from as far back as 1887 (**Table 3**).

Mutation induction for improvement of cocoa started in the 1980s when vegetative buds of three cocoa varieties, 'Amelonado' (P30), 'Trinitario' (K5) and 'Upper Amazon' (T85/799) were irradiated with 15, 20 and 25Gy of Gamma-rays, respectively and budded on to root-stocks to generate MV1 shoots. At MV3, shoots were screened for Cocoa Swollen Shoot Virus (CSSV)-resistance by infecting developing shoots with strain 1A of CSSV, and screened for resistance to the virus using patch-graft inoculation and enzyme-linked immunosorbent assay (ELISA). Mutant lines were selected and budded to produce MV4 and again indexed for resistance and multiplied to produce MV5. MV5 mutant lines with high yield and producing good quality beans have been planted in multi-locational on-farm trials in farmers fields for over 10 years with no symptoms of the CSSV virus disease.

Table 3. Stage in cocoa varietal development programme in Ghana.

Variety no.	Variety name	Parent	Source	Extension period	Years to bearing
"Traditional varieties"					
1	Amelonado	Amelonado	Equatorial Guinea	Before 1887	6-8
2	Trinitario	Trinitario	Trinidad, Jamaica, Venezuela	1900-1909	6-8
3	Mixed Amazon	Mixed Amazon	Peru via Trinidad	1950s	5-6
4	Original Series II Hybrids	Upper Amazon x Amelonado and local Trinitario	Peru and WACRI	1966-1970	4-6
5	Modified Series II	Upper Amazon x Amelonado hybrids	WACRI	1971-1985	2-3
"New Varieties"					
6	BRT collection	Inter-Amazon	British Research Team	Mid-1980s	2-3
7	Mutant Hybrids	Irradiation techniques	Current CRIG collections	1990s	4

Source: Edwin and Masters [14].

The challenges

Although the impact of induced mutations has been highly significant, it has not been without challenges. One major constraint has been the low level of efficiency of mutant production resulting in screening of large populations of both cocoa and cassava for mutant selection. For instance, between 1,500 to 3,050 individual cassava and cocoa seedlings respectively were screened at various stages for screening and selection of high-yielding mutants. Although mutation breeding is considered to be cheap and easy to use, with the minimum recommended planting space at 1x1m for cassava and 3x3m for cocoa, the cost involved in handling such large populations is high and may hinder progress.

This problem is further compounded by the absence of efficient screening methods *in vitro*, or earlier in the seedling nursery stages due to the absence of simple phenotypic markers for the desired traits, and either lack of or inadequate biotechnology and/or molecular biology facilities at the early stages of the breeding programme. Until recently, molecular biology equipment was lacking and thus their application for mutant selection was limited. Both crop plant species used for mutation induction are heterozygous vegetatively propagated crops and may carry undesirable traits, which may need three or four generations of selfing before homozygous lines are produced.

There is also no data available to assess the direct economic impact of the mutants produced both at the small-holder farmer and at a national level. Thus, the direct benefit from mutation breeding cannot be assessed. In the case of cassava, it is extremely difficult to determine the contribution of the "Tek bankye" mutant to food security in the country. Similarly, the Mutant Vegetative bud 5 (MV5) is currently being extended to farmers in the Eastern region; it is very difficult to estimate its economic benefit to farmers involved in the planting of this mutant.

Another major bottleneck to mutation breeding in Ghana is the low level of funding allocated for research. This problem is also compounded by the wrong perception that people had of the use of nuclear energy. The implication of this wrong perception is enormous as it often hinders policy decisions on the use of nuclear techniques, as well as release of funds to replace expired or weak gamma irradiation sources.

Future prospects

In spite of these bottlenecks, the use of induced mutation has a bright future as the technique continues to be a cheap and the only option for

breeding vegetatively propagated crops. Additionally, the combination of induced mutation with *in vitro* cell culture may resolve the problem of early selection, thereby making the technique attractive.

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Molecular Characterization of Somatic Mutation in *Musa acuminata* 'Red'

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Abstract

Musa acuminata 'Red' (AAA) is a South Indian dessert banana cultivar (2n = 3x = 33) with a characteristic red color in the pseudostem, petiole and fruit peel. Red banana undergoes the process of somaclonal variation, producing the 'off types' *Musa acuminata* 'Green' cultivars. The frequency of the production of this 'green variant' is high during *in vitro* multiplication. In plants, anthocyanin pigments are assembled from phenyl alanine and acetyl CoA by the enzyme chalcone synthase (CHS). To understand the molecular mechanism for the somaclonal variation in Red banana, the chalcone synthase gene sequences were amplified using PCR products and sequences were compared with those of 'Green variants (AAA)', 'Dwarf Cavendish (AAA)' and diploid 'Pisang lilin' (AA). Sequence variations were observed only in amplified product from Red cultivar. Predicted amino acid sequences of the longest ORF indicated changes in seven amino acids such as arginine, glutamine, alanine, aspartic acid, isoleucine, phenylalanine and asparagine to serine, leucine, proline, alanine, valine, tyrosine and serine respectively. *Musa acuminata* cv. Red with anthocyanin content might have originated as a natural mutant, selected and maintained by vegetative propagation through generations.

Introduction

Musa acuminata 'Red' has originated as a backyard clone of dessert banana in South India. It is cultivated in the states of Maharastra, Tamil Nadu and Kerala [1]. Cultivation of red banana has also been reported from Indonesia, Papua New Guinea, Eastern Australia, Fiji, Samoa, Hawaii, Philippines, Thailand, Myanmar, Sri Lanka, East Africa, the West Indies and continental America. The plants are tall and vigorous, slightly susceptible to Panama disease, and resistant to leaf spot disease. The fruit bunch in red banana is compact and bears 50 to 100 red rind fruits that are good in size, slightly curved and possess a blunt apex. The fruits do not easily fall off from the pedicel. The pulp is cream-colored and has a characteristically strong flavor. Because of the attractive size and coloration, the fruits always fetch high prices in the local markets.

The cultivar is named 'Red' because of its very distinctive color in the pseudostem, petiole, midrib and in the fruit rind. The epidermal peels from petiole showed characteristic accumulation of anthocyanin pigments in the vacuoles of parenchymatous cells in the sub epidermal layer. In nature, the red cultivar rarely produces green suckers; while the reverse change from green to red has never been recorded [2]. *In vitro* propagation of the red cultivar on MS medium with 8mg/l BA increased the shoot multiplication rate as well as the frequency of the green variants [3]. The green variants produced green fruits that on ripening turned yellow, with low market demand. In this study, our primary goal was to understand whether red banana is a chimera consisting of red skin on a green core and variegation results from developmental accidents rather than mutation. Chalcone synthase (CHS) is involved in the beginning of anthocyanin biosynthesis. Molecular analysis CHS sequences of Red and Green cultivars were carried out to investigate if any changes in the gene are linked to red banana.

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Materials and Methods

Musa acuminata Red (AAA), Green variants (AAA), Dwarf Cavendish (AAA) and Diploid (AA) cv Pisang lilin were selected in the present study. While 'Red' has red coloration in the pseudostem, petiole and fruit rind, the other three have green colored pseudostems, petioles and fruits. In Red cultivars the fruits remained red even after ripening, whereas in others the green fruits turned yellow on ripening.

Genomic DNA was isolated from young leaves of field grown plants (Banana Agriculture station, Peringamala, Kerala, India), packed in plastic bags and kept in ice-boxes for two days before DNA extraction. DNA was extracted using a modified CTAB (hexadecyltrimethylammonium bromide) method [4]. The percentage of polyvinylpyrrolidone with molecular weight 40,000 (PVP-40 SIGMA) was increased to 6% due to the high phenolic compounds in banana leaves.

The two primers used for PCR amplification reactions were 5'- TCTCCGACGCCTTCAGCACG-3' (forward primer) and 5'-AACATGGAGCGGAGCCTGCG 3' (reverse primer). All PCR reactions were carried out in a final volume of 20µl reaction mixture consisting of 100ng DNA, 1X PCR buffer (cat # M 1861, Promega), 1.5 mM $MgCl_2$, 200µM dNTP mix, 10 pmol of each primer and 1U *Taq polymerase* (cat # M 1861, Promega). Amplification was performed using a Biometra T-Gradient (Gottingen) thermocycler. The programme was run for five minutes at 94˚C followed by 30 cycles of 60 S at 94°C, 60 S annealing at 56°C and two-minute extension at 72°C. Amplification products were resolved in 1.5% agarose gels. The products obtained from the gel were purified using QIAquick gel extraction kit (QIAGEN, cat # 28706). Purified products were cloned in the pGEM Teasy vector system 1 (Promega, cat # A1360). Cloned products were sequenced commercially using an ABI3700 capillary sequencer and compared with GeneBank sequences using NCBI BLASTN services. The similarities between the sequences were studied using ClustalW multiple sequence alignment programme (www.ebi.ac.uk).

Results and Discussion

The PCR produced single bands of ~500 bp in size (**Fig. 1**). Following the cloning of the PCR products from all samples, the identity of the putative target segments was determined by sequencing followed by a blast search and by alignment with heterologous GeneBank sequences (**Fig. 2**). The products showed more than 99% homology with products in a *Musa* EST database (C_600145587T1). ClustalW analysis produced multiple sequence alignments of the CHS sequences and calculated the similarities between samples. The neighbor joining tree showed that sequences from Green, Dwarf Cavendish, and Pisang lilin come under a single cluster, whereas the sequence from Red falls into a separate group. When the DNA sequences were translated using the biology workbench

3.2 (http://workbench.sdsc.edu), the red cultivar differed in the seven amino acids arginine (R), glutamine (Q), alanine (A), aspartic acid (D), isoleucine (I), phenyl alanine (F), and aspargine (N) to serine (S), leucine (L) proline (P), alanine (A), valine (V), tyrosine (Y), and serine (S), respectively (**Fig. 3**). The amino acid changes in the ORF obtained from the red cultivar also showed more transversion mutations (**Table 1**).

Color differences in fruits are easily scorable features of genetic diversity and are important for taxonomic classification. In India, red fruits fetch high prices and color is widely used to assess the market value and quality. Extensive studies in anthocyanin biosynthesis were reported in petunia, snapdragon, and maize [5, 6]. The enzymes in the anthocyanin pathway are encoded by structural genes and regulatory genes control the expression of structural genes in response to environmental stimuli such as light and temperature [6, 7]. Mutations in either structural or regulatory genes can produce mutants with reduced or without pigmentation [8]. The first committed step in the biosynthesis of flavanoides and anthocyanins is catalyzed by chalcone synthase. Four genes encoding CHS have been isolated from Petunia, two from Maize and one from Snapdragon [9]. In banana, the distribution of anthocyanins in the bracts of wild and cultivated varieties separated them into different chemotypes [10], but the molecular analysis of the genes involved in pigment synthesis was not reported. The primers used in this study produced ~500 bp fragment in all the samples and amino acid variation was observed only in the red cultivar, indicating the red cultivar as a genetic variant. Despite the large number of CHS sequences in the Gene Bank database, only the identity of a comparatively small number was verified by heterologous expression [9]. Therefore, several sequences in the data base which are annotated as CHS by similarity may have different metabolic roles.

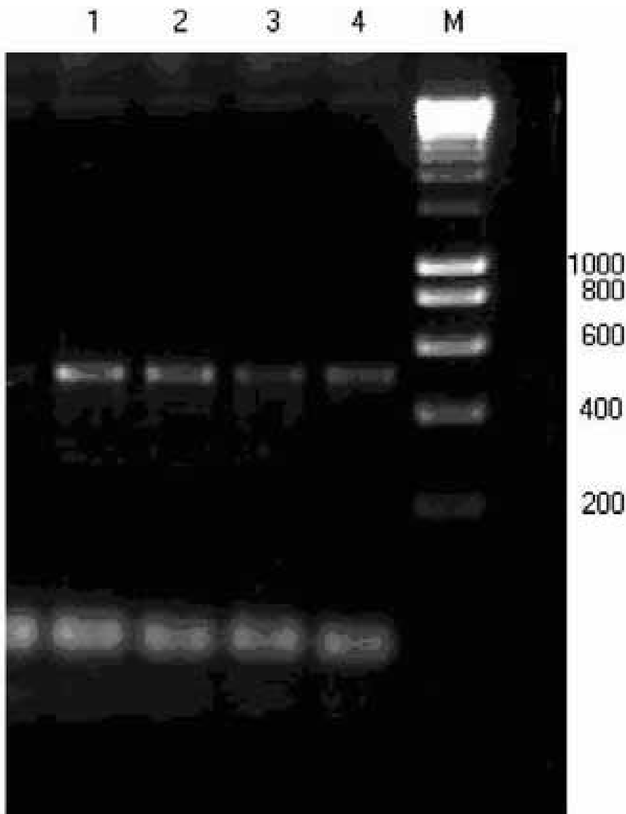

Figure 1 PCR analysis of DNA isolated from young leaves of banana cultivars: lane 1-Red, lane 2- Green variants, lane 3- Dwarf Cavendish, lane 4 Pisang lilin. The numbers to the right indicate the DNA size markers (kb, lane M).

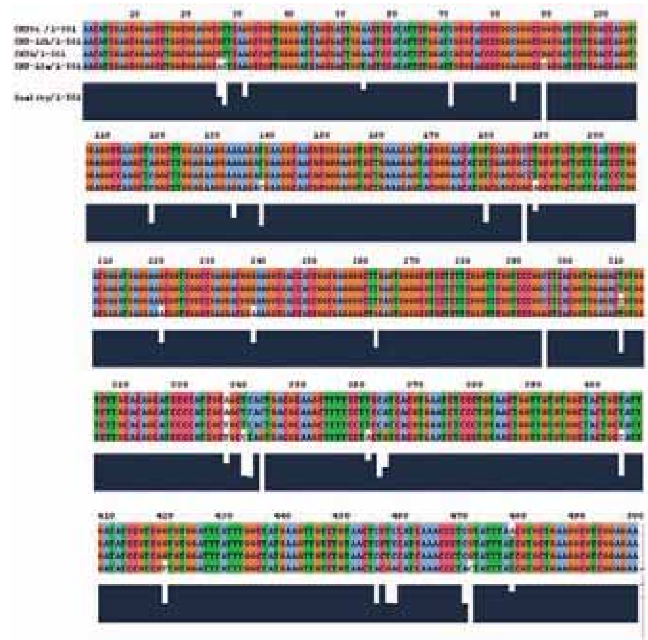

Figure 2 Alignment of 500 bp CHS gene sequences. Chse (Pisang lilin) Chs12b (Green) chsA (Dwarf cavendish) and CHS 13 (Red)

Table 1. Mutational changes in the ORF region in CHS sequences from *Musa* cultivars

Nucleotide position	Genetic code		Base change	Type of mutation	Aminoacid change	
	*Green	Red			Green	Red
39	agg	agc	g→c	Transversion	Arginine	Serine
146	cag	ctg	a→t	Transversion	Glutamine	Leucine
148	gcg	ccg	g→c	Transversion	Alanine	Proline
155	gac	gcc	a→c	Transversion	Aspartic acid	Alanine
198	atc	gtc	a→g	Transition	Isoleucine	Valine
281	ttc	tac	t→a	Transversion	Phenylalanine	Tyrosine
307	aac	agc	a→g	Transition	Asparagine	Serine

* Other three DNA has exactly like the sequences in Green

Anthocyanins are secondary plant products that play a major role as pigments of flowers and fruits. They also protect leaves from ultraviolet radiation [10]. In vegetatively propagated, sterile triploid banana cultivars genome modifications occur through spontaneous mutations. In nucleotide sequence models of evolution, the transition transversion (TI/TV) is an important aspect because it expresses the relative probabilities of different types of nucleotide changes [11]. When a base mutates it can undergo a transition in only one way, but a transversion occurs in two ways. Amongst spontaneous mutations, there are about twice as many transversion mutations as there are transitions. Also, replacement of aminoacid proline probably changes the enzyme activity. In praline, the nitrogen atom of the amino group is incorporated into a ring and can cause a sharp transition in the organization of polypeptide. This speculation needs more experimental evidence, however, this study indicates the potential of CHS gene as a molecular marker to study the evolution of *Musa* germplasm.

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Gamma Irradiation-Induced Mutation for the Improvement of Josapine Pineapple Against Bacterial Heart Rot Disease and Improved Fruit Quality

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Abstract

Bacterial heart rot disease, caused by *Erwinia chrysanthemi*, is one of the most serious diseases of the susceptible cultivars of pineapple in Malaysia, namely, Josapine, Sarawak, Gandul and N36. Using acute irradiation of Gamma-rays and *in vitro* cultured meristems, selection of resistant mutants to bacterial heart rot disease with improved fruit quality has been carried out in the most popular variety, Josapine. Suckers were collected from the experimental field plot at MARDI Research Station in Pontian Johor. Explants from meristem tissues were transferred to MS (Murashige & Skoog) solid media with 2.5mg/l benzyl aminopurine (BA) and incubated at 23°C at a 16-hour photoperiod. After 10 days in culture, meristem explants were irradiated with a series of Gamma-ray doses of 0, 20, 40, 60, 80, 100, 120, 140 and 160Gy and radiosensitivity was investigated based on shoot formation and survival rate. Radiosensitivity of meristem tissues producing shoots was inversely proportional to the irradiation dose. As the dose was increased mutations appeared more frequently, indicated by the formation of albino and striped leaves. The dose required for 50% lethality (LD_{50}) and 100% (LD_{100}) of meristem tissues for shoot formation were 40Gy and 83Gy, respectively. On the other hand, the LD_{50} and LD_{100} for survival rate were 77Gy and 147Gy, respectively. Suitable doses for mutation induction are suggested in the lower region alongside with LD_{50} curve, where multiple shoots are regenerated from the irradiated meristem tissues. Therefore, for field screening, four lower doses of gamma irradiation viz. 10, 20, 30 and 40Gy were applied. The limit for shoot induction from irradiated explants generally agreed with the LD_{100} curve. Shoots derived from irradiated meristem explants at 10, 20, 30 and 40Gy were sub-cultured up to M1V5 to minimize chimerism. Multiplication of irradiated shoots was carried out in Temporary Immersion Bioreactor System using MS liquid media with 5 mg/l BA and incubated at 23°C with a 16-hour photoperiod. Rooting of shoots was promoted on MS media with 2 mg/l of Indole-3-butyric acid (IBA). Rooted plantlets measuring 10 to 12.5cm were transplanted to soil-mix in plastic trays for hardening. Mutation of regenerated plants was investigated two months after acclimatization. Preliminary screening of 20,000 irradiated plants in the nursery was first undertaken to select for potential mutants with desired characters, and 60% of the irradiated plants were observed to have smooth leaves and vigorous growth. Hardened irradiated plants with smooth leaves measuring 17.5 to 20cm were field planted in hot spot until fruiting. Potential mutants were selected based on desired characters such as a) resistance to bacterial heart rot disease, b) single crown, c) cylindrical fruit shape with big fruit size and weight, and d) high sugar content. At 10 and 20Gy, there were no potential resistant mutants with a significantly improved total sugar content and fruit weight. However, at higher doses of 30 and 40Gy, 11 and five resistant plants with significant increase in total sugar content and fruit weight were recorded, respectively.

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Introduction

The Third National Agriculture Policy stressed that the fruit industry will be expanded to cater the increasing demand for fresh and processed tropical fruits, both for local and export markets. Fifteen fruits have been identified as priority crops for development: banana, papaya, pineapple, watermelon, starfruit, mango, durian, jackfruit, rambutan, citrus, duku langsat/ dokong, cempedak, guava, ciku and mangosteen. The world trade in pineapple is dominated by canned fruit. Some fresh fruit for table consumption is also traded but the volume is small compared with to market for canned fruit. Pineapple in Malaysia is grown for both table consumption and canning, though the majority of production is for canning by far. Pineapple is the most important fruit in terms of revenue earner in this country. There are about 10,000 ha cultivated and half of this is owned by estates and planted for the canning industry. The export of canned pineapple is about two million standard cases annually valued at RM 60 million, while the export of fresh pineapple is about 40,000 ton and worth about RM 10 million. However, the industry for canning an ailing one, with production on the decline since the 1970's.

The current varieties Josapine (fresh fruit) and Gandul (canning) have several weaknesses. For Josapine, improving the resistance to bacteria heart rot and occurrence of multiple-crown will increase production and fruit marketability by 25% in some instances. The estates have complained that Gandul had declined in vigor and yield (from 25 t to 10-13 t/ha) over many years of replanting and a vigorous variety with early 'annual' bearing habit and improved cannery recovery is required to boost the flagging industry. Cannery recovery from 20% currently to 25-28% (close to Cayenne) would significantly improve profits. There is also a shift to a demand for canning in natural juices (30% of current production). But Gandul has low TSS% content and sugar has to be added during canning. A new variety having TSS% of 18% or higher can be processed into a health product in its natural juice with 'no-added-sugars.' Canneries would stand to save at least RM three million annually in sugar input.

Somaclonal variations and induced mutation using irradiation in breeding are least invasive in changes to genetic make-up of an established variety and will be useful for improving the Josapine cultivar. This has proven to be an excellent fresh fruit variety with the exception of two weaknesses: susceptibility to black heart rot and multiple crowns. The use of tissue culture to generate somaclones with minute genetic changes that do not damage the overall varietal identity would be the most suitable tool for improving the variety. This breeding tool has been successfully used for development of several local banana varieties. Recent work by Chan & Lam (2002) has developed protocols to produce Josapine plants by tissue culture. Nuclear techniques using gamma-irradiation that causes small 'point mutations' are employed for the improvement of this variety, with variants that arise from this technique screened for tolerance to heart rot and occurrence of multiple-crown.

The specific research objectives of the programme are: a) to develop a generation of economically important mutations for crop improvement, clonal propagation and field evaluation of mutants that exhibit desir-

able traits such as disease resistance, long shelf-life, resistance to abiotic stresses; b) 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 improved economic viability of growers and the nutritional component; and c) to access the impact of induced mutants on early fruit bearing, fruit yield, fruit vigor, and quality components (high sugar content for canning, and high cannery recovery rate) depending on the fruit tree life cycle, under field conditions.

Materials and Methods

Establishment of tissue culture protocols for micropropagation

Suckers of cultivar Josapine were obtained from our collaborator, Malaysian Agricultural Research & Development Institute (MARDI) at Pontian Experimental Station in Johor. Outer leaves were removed, roots were cut off and they were excised into small pieces of meristem tissues about 2.0-2.5cm in size. These meristem explants were first rinsed under running tap water for 20 minutes in a beaker added with a few drops of Tween 20 solution. Bacterial contamination is very high using suckers collected from the field, and explants were soaked in 0.3% Mercury chloride for five minutes and then rinsed three times with sterile distilled water. They were then surface sterilized using 20% sodium hypochlorite for 30 min and then rinsed three times with sterile distilled water. These meristem tissues were explanted onto MS media with 0.3% Phytagel and 2.5mg/l benzyl aminopurine (BA) and incubated at 23°C at a 16-hour photoperiod. Cultures were regularly observed for the formation of adventitious buds and *in vitro* shoots for two to three months.

Radio-sensitivity test and *in vitro* mutagenesis

Radiosensitivity test (dose response) for cultivar Josapine was carried out by irradiating meristem explants which had been cultured for 10 days on MS media plus 2.5mg/l BAP with a series of 0, 20, 40, 60, 80, 100, 120, 140 and 160Gy Gamma-ray doses. For each dose, 100 meristem explants were irradiated and divided into five replicates for statistical analysis. Radiosensitivity was determined based on:

1. Percentage survival of irradiated explants
2. Average number of *in vitro* shoots

Irradiated meristem exlants were transferred to fresh MS media with 2.5mg/l BAP and incubated at 23°C at a 16-hour photoperiod. Cultures were regularly observed for the formation of adventitious buds and *in vitro* shoots for a period of two to three months. Data on percentage survival of irradiated explants and formation of adventitious buds and *in vitro* shoots was recorded after three months of culture and compared with the control. Effective doses for the main field experiment were selected based on the observed LD_{50} for both percentage survival of irradiated explants and average number of *in vitro* shoots formed after three months of culture.

Mass propagation of *in vitro* plantlets using bioreactor system

Shoots derived from irradiated meristem explants at selected effective doses were sub-cultured onto fresh MS liquid media supplemented with 2.5mg/l BA in Temporary Immersion Bioreactor System at 23°C with a 16-hour photoperiod up to M_1V_5 generation in order to minimize chimerism.

In vitro rooting of regenerated shoots from M_1V_5 generation was on MS solid medium containing 1% activated charcoal and supplemented with 2.0mg/l Indole-3-butyric acid (IBA), with cultures incubated at 23°C with a 16-hour photoperiod for 30 days. Rooted plantlets measuring from 10.0 to 12.5cm in height were transplanted to soil-mix in plastic trays for hardening in the nursery for a period of two months before being transferred to the field. Mutated plants were screened and selected for desired characters such as smooth leaves and vigorous growth.

Field screening in hot spot

A total of 20,000 irradiated plantlets regenerated from four selected effective doses of 10, 20, 30 and 40Gy, measuring 10.0 to 12.5 cm in height were acclimatized and hardened in the nursery. During this stage, plantlets with smooth leaves and vigorous growth resulting from mutation were carefully recorded. Hardened irradiated plants with those two traits and measuring 17.5 to 20cm were first isolated and field planted in hot spot. Slow growth irradiated plants with smooth leaves were also transferred to the field when they reached 17.5 to 20cm. Plants with spiny leaves were discarded. Selected irradiated plants together with the controls were grown in hot spot for black heat rot disease until fruiting for a period of 10-12 months after field planting. Potential mutants were selected based on desired characters compared to the control such as a) resistance to bacterial heart rot disease, b) single crown, c) cylindrical fruit shape with bigger fruit size and weight (>1.0kg/fruit), and d) higher sugar content.

Results and Discussion

Establishment of tissue culture protocols for micropropagation

From previous tissue culture work on pineapple, it was known that the use of sodium hypochlorite alone or in combination with ethyl alcohol for surface sterilization was not very suitable since high rate of both bacterial and fungal contaminations occurred (>70%). Pretreatment of meristem explants with 0.3% mercury chloride for five minutes before surface sterilization using sodium hypochlorite was able to reduce contamination rate drastically (>90%).

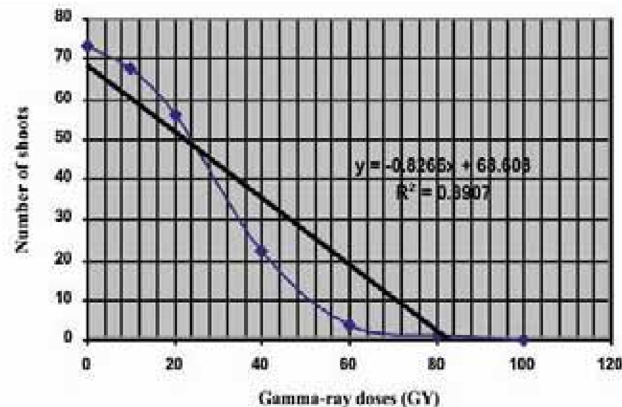

Figure 1 Radiosensitivity test - Percentage shoot formation.

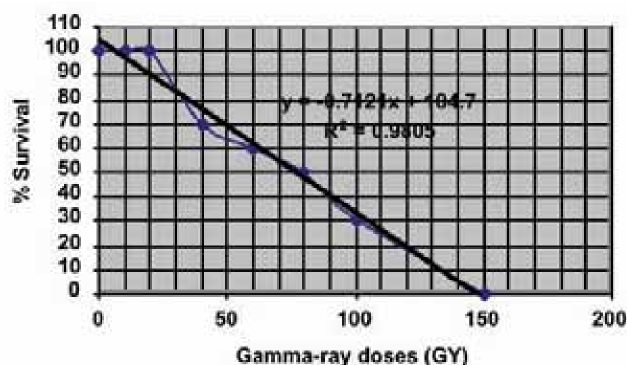

Figure 2 Radiosensitivity test - Percentage of survival.

Adventitious buds began to appear after two months in culture and shoots started to proliferate when meristem explants from suckers were explanted on MS medium with 2.5mg/l BA. Media with higher BA concentration (>3.0mg/l) have provoked high rates of somaclonal variation in pineapple, including plants with multiple crowns when planted in the field. Newly formed *in vitro* shoots were transferred onto liquid MS medium with 2.5mg/l BA and multiplication was routinely carried out every three to four weeks using Temporary Immersion Bioreactor System.

Radio-sensitivity test and *in vitro* mutagenesis

In a mutation breeding experiment, LD_{50} gives an indication of the response of different types of explants of a species to radiation, so that the right dose(s) for the main field experiment can be selected. When selecting the most effective dose(s) for the main experiment, it is advisable to select a few (more than one) which are 20-30% lower than the LD_{50} value. Induction of desired mutations by radiation being by chance, it is safer to choose the doses that can cause less damage and give higher multiplication and survival rate.

Radiosensitivity of meristem tissues producing shoots of pineapple was inversely proportional to the irradiation dose. As the dose was increased, mutations appeared more frequently, indicated by the formation of albino and striped leaves. The doses required LD_{50} and LD_{100} of meristem tissues for shoot formation were 40Gy and 83Gy, respectively (**Fig. 1**). On the other hand, the doses required for LD_{50} and LD_{100} for survival rate were 77Gy and 147Gy, respectively (**Fig. 2**). Suitable effective doses for the main field experiment are suggested in the lower region alongside with LD_{50} curve where multiple shoots are regenerated from the irradiated meristem tissues. Therefore, for the main field experiment 10, 20, 30 and 40Gy were selected based on shoot multiplication rate. The limit for shoot induction from irradiated explants generally agreed with the LD_{100} curve.

Mass propagation of *in vitro* plantlets using bioreactor system

Mass propagation of irradiated shoots was carried out using Temporary Immersion Bioreactor System in MS liquid media with 2.5mg/l BA, at 23°C with a 16-hour photoperiod. Previous multiplication work done on pineapple using MS solid media with 2.5mg/l BA gave an average of 3.5 new shoots after 30 days in culture. In comparison, MS liquid media with 2.5mg/l BA using Temporary Immersion Bioreactor System produced an average of 15.5 new shoots after 30 days in culture. Shoots were routinely sub-cultured for multiplication using fresh MS liquid medium every 30 days. By the M_1V_5 generation, a total of 20,000 *in vitro* shoots were regenerated from selected doses of 10, 20, 30 and 40Gy.

In vitro roots started to form after two weeks of culture on MS solid media containing 1% activated charcoal and with 2.0mg/l IBA. Plantlets with fully formed *in vitro* roots measuring 10.0 to 12.5 cm in height were transferred to soil-mix in the nursery for acclimatization and hardening for a period of two months.

From a total of 20,000 irradiated plantlets, which were pre-screened in the nursery for desired characters, 60% of the mutant plants had smooth leaves with vigorous growth compared to spiny leaves of control plants. Percentages of mutant plantlets with smooth leaves recorded were 15% for 10Gy, 20% for 32Gy, 30% for 30Gy and 35% for 40Gy. In addition, 10 and 20Gy produced mutant plantlets, which were vigorous in growth compared to higher doses of 30 and 40Gy. This experiment clearly indicates that *in vitro* mutagenesis of meristem tissues of pineapple using low doses of gamma irradiation (10, 20, 30 and 40Gy) was able to produce useful mutant plants with smooth leaves and vigorous growth. Pineapple plants with smooth leaves are very much preferred for large-scale field planting since harvesting of fruits is done manually and this will result in fewer injuries to the workers. In addition, vigorous growth mutants were also specially selected so as to screen for early fruiting mutants (less than 12 months) in the field.

Field screening in hot spot

After pre-screening in the nursery for a period of two months, a total of 12,000 mutant plantlets with smooth leaves and vigorous growth were selected for field planting. Only rooted plantlets reaching a height of 17.5 to 20 cm were first transferred to the field for screening against bacterial heart rot disease. Previous work done by MARDI has indicated that small rooted plantlets (10.0 to 12.5cm in height) were unable to survive and almost 90% died after one month field planting, while tissue culture derived plantlets of pineapple with specific height of 17.5-20cm have a >95% survival when transferred to the field.

After field planting and screening for bacterial heart rot disease in hot spot for a period of 10-12 months, performance of mutant plants were recorded and compared with the control. Out of 12,000 mutants plants screened in hot spot during the first planting season, a total of 21 potential mutant plants were selected based on desired characters, such as: a) resistance to bacterial heart rot disease, b) single crown, c) cylindrical fruit shape with bigger fruit size and weight (>800g/fruit), and d) higher sugar content (>18 TSS). A total of 2,100 mutant plants were regenerated using suckers from selected 21 mutant lines and screened again in hot spot for a second planting season. After harvesting, 16 potential mutant lines were selected. At doses of 10 and 20Gy, there were no potential resistant mutant plants with a significant improvement in total sugar content and fruit weight. However, at 30 and 40Gy, 11 and five resistant plants with significant increase in both total sugar content (20-25 sugar index compared to control, only 18) and fruit weight (800-900g/fruit compared to control, 500-600g/fruit). Further investigations on molecular analysis using AFLP technique will be conducted to identify markers for the selected characters.

The present method of gamma irradiation in combination with tissue culture, proved to be excellent in increasing efficiencies of useful induced mutation and it is an innovative technology which represents high improvement to the somaclonal variation and conventional breeding of pineapple (Chan, 1993). Potential mutants will be micropropagated and released to the farmers for large-scale planting.

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Natural Genetic Variation in Cassava (*Manihot esculenta* Crantz) Landraces: A Tool for Gene Discovery

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Abstract

Cassava landraces are the earliest form of the modern cultivars and represent the first step in cassava domestication. Our forward genetic analysis uses this resource to discover spontaneous mutations in the sucrose/ starch and carotenoid synthesis/accumulation and to develop both an evolutionary and breeding perspective of gene function related to those traits. Biochemical phenotype variants for the synthesis and accumulation of carotenoid, free sugar and starch were identified. Six subtractive cDNA libraries were prepared to construct a high quality (phred > 20) EST database with 1,645 entries. Macroarray and microarray analysis was performed to identify differentially expressed genes aiming to identify candidate genes related to *sugary* phenotype and carotenoid diversity. cDNA sequence for gene coding for specific enzymes in the two pathways was obtained. Gene expression analysis for coding specific enzymes was performed by RNA blot and Real Time PCR analysis. Chromoplast-associated proteins of yellow storage root were fractionated and a peptide sequence database with 906 entries sequences (MASCOT validated) was constructed. For the sucrose/starch, metabolism a *sugary* class of cassava was identified, carrying a mutation in the BEI and GBSS genes. For the pigmented cassava, a pink color phenotype showed absence of expression of the gene CasLYB, while an intense yellow phenotype showed a down regulation of the gene CasHYb. Heat shock proteins were identified as the major proteins associated with carotenoid. Genetic diversity for the GBSS gene in the natural population identified 22 haplotypes and a large nucleotide diversity in four subsets of population. Single segregating population derived from F_2, half-sibling and S_1 population showed segregation for *sugary* phenotype (93% of individuals), waxy phenotype (38% of individuals) and glycogen like starch (2% of individuals). Here we summarize our current results for the genetic analysis of these variants and recent progress in mapping of loci and with large-effect genes.

Introduction

Biochemical phenotypes are essential for the definition of gene functions and to understanding gene regulation. Currently, such functional analysis uses either forward or backward genetic approaches with laboratory-induced mutants. Studies on genetics of cassava are rare, incomplete and most of the time difficult, because of long life cycle of the plant. Mutants have not been found in cassava. Some reports have attempted to identify phenotype variants in landraces for starch type without genetic analysis [1], laboratory-induced mutants in starch [2], and linamarin cleavage [3] in a restricted number of genetic backgrounds. In cassava, an alternative to laboratory-induced mutants is identifying biochemical phenotypes in landraces in their center of origin and domestication. Since cassava is not a selfing species, most of the collected plants are highly heterozygous, making it practically impossible to find in nature an inbred line that could carry a recessive character like a mutation in phenotype using conventional genetic analysis. Therefore, exploitation of such genetic variation for either quantitative or qualitative traits needs molecular species-specific tools for genetic analysis. In considering the constrains of producing offspring and the complexity of the cassava genome, our two complementary strategies are based on association genetics analysis using natural populations and single segregating population, the latter involving more time and field experiments.

For association studies, we accessed the center of origin and domestication of cassava [4] to explore the possibility of isolating useful natural mutants. Our search was focused on starch and carotene accumulation because relatively few major genes are involved. Because resources were often limited, our study was performed in a two-stage approach by using a subset of samples to identify biochemical phenotypes and SNPs. Instead of genotyping hundreds of controls for the characterization of haplotype tag SNPs (htSNPs), we genotyped sample cases and carried out preliminary tests of association to aid the selection of htSNPs. Once the subset has been genotyped, the whole set of loci will be tested for equilibrium to proceed. In addition, cross populations are being prepared, based on a modified backcross breeding design, to obtain single segregating populations with alternate new local adapted parental divergent from the antecessor parental identified by marker assisted recurrent selection (MARS). Here, we summarize our advances on this systematic exploitation of the naturally occurring variation as a complementary resource for the functional analysis of the cassava genome.

Biochemical phenotype characterization

The use of the candidate gene approach requires considerable knowledge of the physiology and biochemistry of the phenotype. This knowledge is available for starch and carotenoid accumulation in model plants as well as grain crops and has been applied successfully in carotenoid candidate gene analysis in *Solanaceae* [5]. However, the biology of biochemical phenotypes is usually species-specific and varies with the organ and storage tissue studied. Consequently, different mechanisms of regulating starch and carotenoid accumulation are involved, including genetic background of the cultivar, as well as the general environment.

***Sugary* cassava:** Normal cassava storage root accumulates a large amount of starch with distinct features such as clarity of the gel, excellent thickening (swelling capacity), neutral flavor and good-texture quality. All these properties are largely determined by the starch type and composition (amylose/amylopectin proportion). Some variation in amylose proportion has been observed, but the lack of variation in amylopectin structure is remarkable. A new class of cassava (named *sugary*) was reported [1], with high free sugar content, several variants in the starch type and composition, including amylose-free starch, glycogen-like starch, and a unique amylopectin structure when compared with normal cassava (**Fig. 1A**). Landrace CAS36.1 showed the highest glucose content (248.2 mg g^{-1} DWt) and a glycogen-like starch [1]. The

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sugar profile separated in a GC-MS analysis revealed free sugars such as arabinose, glucuronic acid, galacturonic acid, manose and xylose that were not observed in the profile for normal cassava (**Fig. 1B**). Enzyme activities and protein blot analyses revealed that the branching enzyme I (BEI), and its corresponding protein, is either low or not detected in landrace Cas36.1 [1]. Therefore, the *sugary* cassava phenotype revealed biochemical variants related to free sugar content and composition that could involve starch synthesis/degradation, as well as cell wall degradation and modification in amylopectin structure associated to the missing activity of BEI [1].

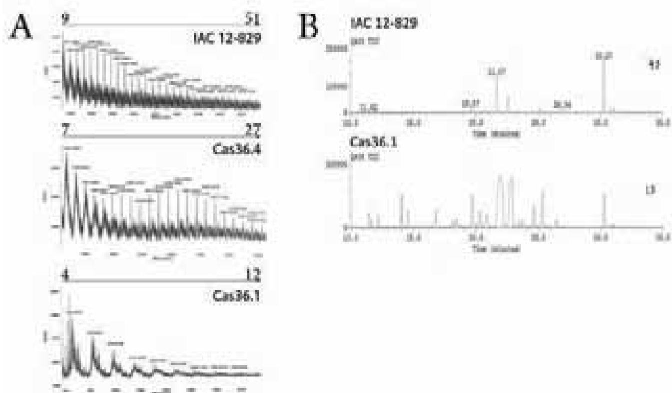

Figure 1 Sugar composition and amylopectin structure variation in cassava. **(A)** Amylopectin structure as revealed by digestion of amylopectin with β-glucosidase and polyglucan fragment identified by mass spectrometer analysis (MALDI-TOF apparatus). Polyglucan fragments varied from 9-51, 7-27 and 4-12 units of glucose for cv. IAC 12-829 and landraces Cas36.4 and Cas36.1, respectively. **(B)** Free sugar profile revealed by GC-MS analysis.

Pigmented cassava variants: Naturally occurring color variation associated with carotenoid accumulation was observed in cassava storage root. Carotenoid separation, identification and quantification by HPLC analyses indicate that total β-carotene is the major carotenoid form present and accounts for 54% to 77% of the total carotenoid in cassava storage roots. The carotenoid biosynthetic pathway is fully activated in cassava storage root, including the white phenotype. No detection of α-carotene in 24 landraces studied was observed, but variable amounts of lutein (an β-ring xanthophyll) were present. Yellow color intensity variation was associated with the accumulation of different carotenoids. Landraces with white storage root showed a profile with eight types, whereas intense yellow showed 17 types. Variation in total β-carotene content ranged from none in landrace Mirasol (pink) that accumulates only lycopene (99.81 μg g^{-1} DWt), to 49.91 (μg g^{-1} DWt) in landrace MC008 (deep yellow) that accumulates β-carotene (66%) its colorless precursor phytoene (31%) and its derived intermediate β-criptoxanthin (3%). Variations, in α and β-ring xanthophyll content, preferentially accumulate high amounts of lutein or violaxanthin, together with β-carotene, such as in landraces MC002 and MC016, respectively. The possible mechanisms responsible for this genetic variation include genetic regulation by genes coding for enzymes in the syntheses pathway as observed with the pink landraces, variants in the yellow that differentially synthesize β-carotene and β-ring xanthophylls (violaxanthin, neoxanthin, luteoxanthin and auroxanthin) but not α-ring xanthophylls (lutein) and carotenoid cleavage enzymes. In addition, the differential accumulation of β-carotene could possibly be associated with the sink capacity of chromoplasts related to carotenoid sequestering proteins as shown below.

Chromoplast-associated proteins analysis: Carotenoid accumulation is likely to be regulated by the carotenoid sequestering proteins in chromoplasts and may have a three-fold importance to cassava: firstly, to explain the range of β-carotene content in the yellow color gradient, secondly, to better understand the carotenoid protein sequestering mechanism in non-photosynthetic tissue and, thirdly, to account for protein content enhancement in cassava storage root due to the need for more functional proteins in the root tissue. In exploring this alternative, we first observed that protein content was 40-60% higher in intense yellow than in white cassava [6]. Correlation studies between total carotenoid and protein content indicated that the level of significant correlation is dependent on the method and procedure used to estimate protein and that protein is linearly correlated with β-carotene (**Fig. 2**). To dissect this correlation, a procedure combining chromoplast-enriched suspension, fractionation of carotenoid-protein complex in size exclusion chromatography (SEC) and protein blot analysis was developed. **Figure 3A and 3B** show two protein peaks separation in the SEC profile for white and yellow root. The carotenoid-protein complexes in peak 1 revealed β-carotene (**Fig. 3C**) as the major pigment present in yellow and traces of it in white landraces. 2DE gel [6] and SDS-PAGE analysis of fractions of peak 1 and peak 2 showed a large protein number difference between yellow and white color phenotypes (data not shown). To identify which proteins are specifically associated with β-carotene present in peak 1, the protein complex in each peak was sequenced.

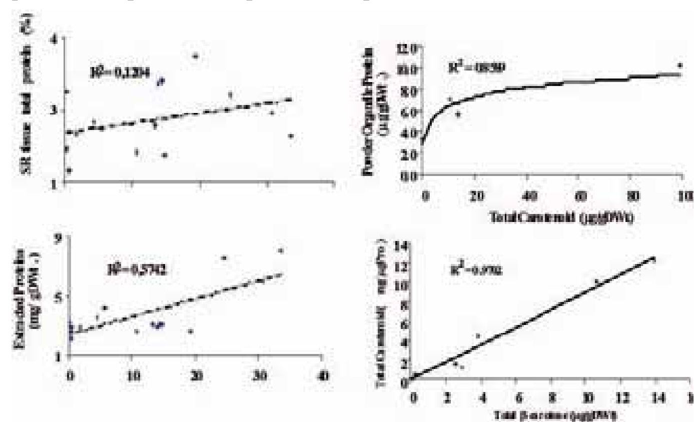

Figure 2 Correlation between total carotenoid and protein content. **(A)** crude protein (%, factor 3.24). **(B)** saline buffer soluble protein (mg/ gDWt, Bradford). **(C)** Enriched chromoplast suspension proteins quantified (Bradford). **(D)** Total carotenoid expressed on protein base. Total carotenoid was extracted and quantified by spectrometry. β-carotene was separated by HPLC and amount estimated by peak integration.

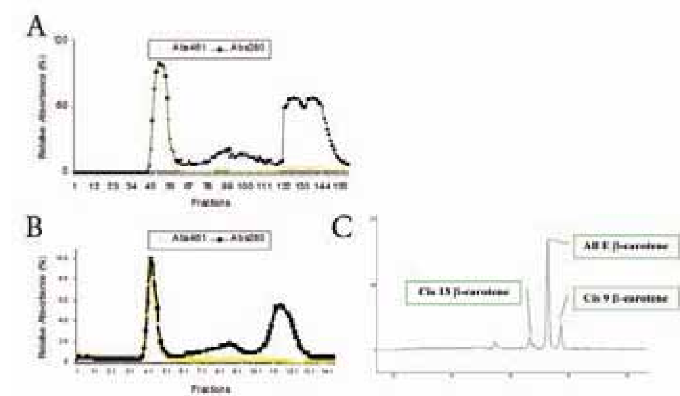

Figure 3 Protein-carotenoid complex characterization. Size exclusion chromatography separation of carotenoid-protein complex (Peak 1) and non-associated protein (Peak 2) in yellow **(A)** and white **(B)**. **(C)** HPLC carotenoid profile present in Peak 1 identifying β-carotene.

Molecular isolation and characterization of candidate genes responsible for naturally occurring variation

To find candidate genes of interest, global (macroarray and microarray) and specific gene expression analysis was performed. Six subtractive

cDNA were constructed and an EST database was assembled for *sugary* and pigmented cassava. Clones of cDNA fragments for genes coding for enzymes related to starch and carotenoid synthesis were sequenced. Chromoplast-associated proteins sequences and their corresponding genes were identified using three EST cassava databases (http://genoma.embrapa.br/genoma/, http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=cassava, http://www.brc.riken.go.jp/lab/epd/Eng/catalog/pDNA.shtml) to identify differentially expressed proteins and genes.

Differentially expressed gene in *sugary* cassava: Global differentially expressed genes between normal and *sugary* cassava were accessed with a membrane base cDNA chip built up with 264 genes and hybridized with cDNA obtained by reverse transcription of total RNA population. Results indicated that the major genes differentially expressed are largely related to stress response such as up-regulated gene for ABA synthesis (two genes), transcription factor homolog related to hypoxia (2 genes), transport proteins for glucose/ABA (one gene)], nitrogen (one gene), and three unknown genes. Due to the low resolution of this technique, seven cDNA clones for gene coding for enzymes related to starch synthesis were cloned, sequenced and used for mRNA blot analysis. Results showed that the expression of the gene coding for BEI is missing in the *sugary* phenotype with glycogen-like starch [1]. For the waxy starch type, the analysis of cDNA and genomic DNA sequence of the GBSSI gene for normal and sugary cassava carrying waxy starch type showed a nonsense mutation on the N-terminal in the coordinates 337-372 of the mRNA (data not shown).

Differentially expressed genes in pigmented cassava: Global differentially expressed genes (microarray) between normal and pigmented cassava was accessed with a cDNA chip built up with 24,000 Euphorbiaceae genes and hybridized with cDNA obtained by reverse transcription of total RNA population. Preliminary results indicated that three carotenoid cleavage enzyme coding genes were upregulated in relation to pigmented cassava (not shown). Sequences of cloned cDNA fragments for the genes coding phytoene synthase (CasPSY), phytoene desaturase (CasPDS), carotenoid isomerase (CasCRTISO), lycopene β-cyclase (CasLCYb), β-ring hydroxilase (CasHYb), and neoxanthin synthase (CasNXS) were obtained. Transcript profiles for those genes across landraces contrasting carotenoid HPLC profiles consistently correlated with end products of carotenoid synthesis. Transcript levels for CasPSY were equivalent in all landraces evaluated, while trace values of CasLCYb and CasHYb were observed in landraces that accumulate only lycopene (Mirasol) or β-carotene (MC008), respectively. Taken together, these results indicated two kinds of color mutations related to carotenoid synthesis: the pink color genotype, which accumulates only lycopene with traces expression value detected for the gene CasLCYb, and the intense yellow genotype (MC008), which accumulates mainly β-carotene, indicating a down regulation of the gene coding for CasHYb.

Identification of carotenoid-associated proteins: From the SEC results above, proteins recovered from Peak 1 were sequenced by a classic PROTEOMIC procedure. The majority of the pigment-associated proteins in Peak 1 belong to the small heat shock proteins (sHSP) family class I and II. This provides evidence to support roles of sHSPs on the specificity of β-carotene accumulation in cassava, as observed for chaperonin21 for lycopene in tomato [7, 8]. The mechanism by which sHSPs promotes carotenoid accumulation is yet unknown. To gain knowledge on the sHSPs effects on β-carotene differential accumulation in yellow root phenotype, cDNA sequence for the gene coding for all the sHSPs identified in the carotenoid-protein complex were obtained and tested with Real time PCR. The results confirmed the protein blot experiment for fibrilin protein (not shown) and indicated traces of HSP18.1 protein in white cassava and a 7.2-fold higher expression value for the intense yellow phenotype. Together, these results indicate a possible gene mutation related to the carotenoid protein-sequestering mechanism in white cassava. Genes coding for sHSPs are not single copy but are distributed in six different classes, hence making it difficult to be used as a candidate gene in the genetic analysis.

Forward genetic studies of naturally occurring variation

Our forward genetic analysis is focused on two alternative experimental designs. In the first one, a population genetic analysis is carried out with candidate genes derived from the biochemical phenotypes as described above and tested under an evolutionary perspective in a subset sample of a population including cassava ancestors (33 individuals), and landraces (121 individuals). The analytical procedure follows the rational and computer soft as (9,10). In the second, crossing populations based on a modified backcross breeding design to obtain single segregating populations for mapping, field evaluation and new cultivars are being prepared.

Genetic analysis of natural populations: Two candidate genes coding for starch synthesis (CasBEI and CasGBSSI) and three for carotenoid synthesis (CasPSY, CasLCYb and CasHYb) were selected and are sequenced across a population of 154 individuals. Here we show our preliminary analyses in a subset sample for the N-terminal region of the CasGBSSI. Table 1 summarizes the statistics describing the sequence diversity in the combined and each subset of the four classes of population. Larger nucleotide diversity is observed in the cassava ancestor followed by the pigmented cassava. Tagima's D value was highly significant for the combined subset sample indicating genetic neutrality. Haplotype number and diversity was also high for combined and ancestor subset.

Table 1. Genetic diversity estimates and neutrality test for GBSS (N-Terminal).

Penotype	Sample	Nucleotide Diversity (π)	Theta (θ)	Tagima's D	Haplotype number	Haplotype diversity
Ancestor	4	0,02044 (0,00689)	0,02187 (0,00157)	-0,66711 (NS)	22	0,966 (0,00031)
Normal	14	0,00978 (0,00021)	0,01659 (0,00688)	-1,73316 (NS)	12	0,978 (0,00119)
Sugary	8	0,00749 (0,00165)	0,00889 (0,00458)	-0,77880 (NS)	6	0,929 (0,084)
Pigmented	6	0,01992 (0,00619)	0,02416 (0,01212)	-1,10378 (NS)	5	0,933 (0,01481)
Combined	32	0,01232 (0,00245)	0,03068 (0,01006)	-2,29800 **	22	0,966 (0,018)

Values in parenthesis are standard deviation.
(NS) is not statistically significant.
** is statistically significant at P<0.0001.

The likely genealogical history of the genetic diversity showed in **Table 1** was inferred from RM networks constructed for haplotypes in the combined (**Fig. 4A**) and separate population samples (**Fig. 4B-E**).

Genetic analysis in single segregating populations: Field-tests for agronomic performance and morphological descriptors have been performed in the Cerrado region of Brazil since 2002 in a provenance test. Results showed identical *sugary* and pigmented phenotypes as observed early in the center of origin and domestication. In 2006, a breeding programme was initiated to transfer these new identified traits to local varieties. In addition, RAPD and SSR markers were also applied and showed a large genetic diversity among the *sugary* accessions. Agronomic performance indicated that the landrace Cas36.17 (waxy starch type) yielded about 9 ton/ha (12 month growth season base) while the best-adapted local variety (cv. Japonesinha) yielded 28 ton/ha. All the other *sugary* accessions showed mainly lower yield due to a severe attack of Xanthomonas axonopodis pv. manihotis. This information was used to better orientate the selection of landraces within individuals obtained in the crossed populations (F_1 segregating and self pollination population). Preliminary results for a half sibling population (55 individuals) tested in the 2006/2007 growth season revealed segregation of *sugary* phenotype

(93% of the individuals), waxy phenotype (38% of the individuals) and glycogen-like starch (2% of the individuals). Individuals (185) of the S_1 population for the best-adapted *sugary* landrace were planted in the 2007/2008 growth season in the Cerrado region and are being evaluated as above.

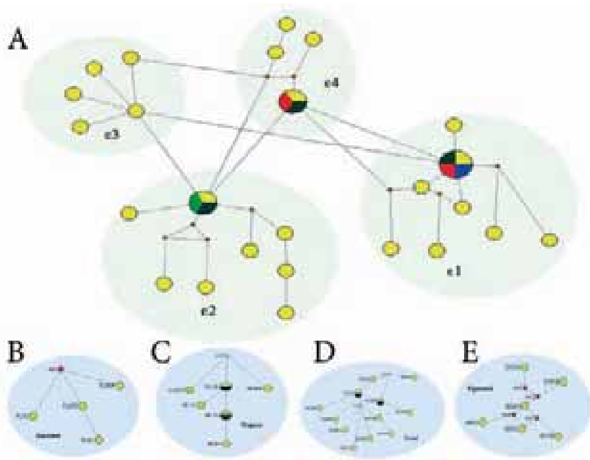

Figure 4 RM network of GBSS sequence haplotype. **(A)** Combined samples and sample subset for Cassava Ancestor **(B)**, *sugary* cassava **(C)**, normal cassava **(D)** and pigmented cassava **(E)**.

Concluding remarks and prospects

Cumulatively, our results confirm the importance of landraces, the efficiency of developing species-specific molecular tools directly from the naturally occurring variations and point out new research for cassava, related to protein enhancement in the storage root.

The exploitation of these variants will increase and become more systematic and efficient with the development of more permanent mapping populations, high-throughput genotyping technologies, improved QTL mapping statistical methods and more precise assays for phenotype analysis.

ACKNOWLEDGEMENTS

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Induced Mutations in Coleus (*Solenostemon rotundifolius*) (Poir. J.K. Mortan) – An Under-Utilized Medicinal Tuber

M Abraham* & V V Radhakrishnan

Abstract
Sixty Coleus accessions collected from different eco-geographical regions of Kerala and neighboring states exhibited genetic diversity. High heritability was observed for tuber yield, harvest index, biological yield per plant, tuber volume and tuber weight. The 60 accessions were grouped into 10 clusters and there was no parallelism between genetic diversity and geographical distribution. Representative genotypes from the 10 clusters were subjected to mutagenic treatment using Gamma-rays and EMS. The response of Coleus to mutagen varied with concentration as well as with genotype. Mutation has changed the plant height, size of tubers and tubers per plant. In addition, selected mutants showed photoinsensitivity to tuberization and acceptable qualitative changes. Based on their field performance, mutants M 131 and M 61 were identified for year round cultivation.

Introduction
Solenostemon rotundifolius (syn. *Coleus parviflorus*), commonly known as Chinese potato, is a native of Africa and possesses elite flavor, taste and unique medicinal properties due to the presence of flavanoids which help to lower cholesterol level in blood [1]. The crop has high consumer preference and is extensively grown in southern peninsular India, but year-round cultivation is not possible as it is photosensitive. Furthermore, the yield is also low. As it is vegetatively propagated, seed set is absent and there is no appreciable variability in the germplasm for genetic improvement. Hence, an attempt was made to explore and assess the genetic variability present in the major growing areas and to supplement it through mutation for further exploitation in breeding of the crop.

Materials and Methods
An eco-geographical survey of the cultivation of Coleus was conducted in three southern states of India viz., Kerala, Karnataka and Tamil Nadu. Samples of Coleus cultivars along with its wild progenitors were collected from farmers' fields and main markets of the area. Sixty accessions thus collected, formed the basis of the study. The seed tubers collected from various sources were multiplied in primary nurseries and 45-day-old cuttings were planted in the main field at the College of Horticulture, Vellanikkare from June to November 1999. The first experiment was laid out in randomized block design with two replications. Each plot consisted of 10 plants in two rows 60 cm apart, with 30 cm between each plant. The data of 13 important quantitative characters were recorded from five randomly selected plants and subjected to statistical analysis, namely tuber yield, tuber girth, point of tuber formation (base of stem / leaf node / base of stem and leaf node), volume of tuber, weight per tuber, tuber density, tubers per plant, days to tuberization, nematode susceptibility, days to flowering, plant height, biological yield and harvest index.

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The variability in present population was partitioned into heritable and non-heritable components with the aid of genetic parameters like genotypic coefficient of variation (gcv), heritability (h^2), and genetic advance (ga), (**Table 1**).

Cluster analysis was carried out following the methods compiled by Singh and Chaudhary [2]. The 60 accessions were grouped into 10 clusters based on the economic traits. Based on the ranking, the overall best tuber yielder from each cluster was selected and subjected to physical and chemical mutagenetic treatment with EMS as the chemical mutagen, and Gamma-rays as the physical mutagen.

Table 1. Estimates of genetic parameters for thirteen characters in Coleus

Character	P.C.V	G.C.V	h2 (%)	G.A	G.G
Tuber yield (g)	49.97	41.89	70.70	162.56	72.34
Tuber girth (mm)	18.18	10.35	33.16	5.96	12.14
Point of tuberization	22.72	9.34	17.74	0.18	7.89
Volume per tuber (cc)	42.71	37.90	79.08	4.13	69.30
Weight per tuber (g)	42.63	38.27	80.89	4.56	70.81
Tuber density (g/cc)	7.53	5.70	57.92	0.09	8.49
Tubers per plant	39.69	22.09	31.72	13.75	25.32
Days to tuberization	5.24	1.46	8.64	1.07	0.85
Nematode susceptibility	48.54	22.68	22.63	0.52	21.94
Days to flowering	52.94	1.33	0.01	0.0004	0.16
Plant height (cm)	23.13	19.89	74.28	34.70	35.21
Biological yield	48.80	42.98	77.92	520.84	77.99
Harvest index	51.81	45.15	76.31	0.22	81.09

The seed tubers from selected genotypes were grown in the primary nursery and 45-day-old single node cuttings were taken for mutagenic treatment using physical and chemical mutagen. Estimation of LD50 was done for both Gamma-rays and EMS. Twenty different doses of Gamma-rays ranging from 1Gy to 100Gy were used under laboratory conditions, and survival was recorded based on the percentage of variation of sprouting of cuttings over the control. Based on this, LD_{50} for Gamma-rays was found to be 40Gy. Hence, selected doses for gamma irradiation were 10, 20, 30, 40, & 50Gy.

For the estimation of LD_{50} for EMS aqueous solutions of 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.50, 1.00, 1.50, 2.00 and 2.50 % were prepared, and 10 cuttings were immersed in different solutions for 30 minutes. The treatment duration was selected based on results from a preliminary study involving four durations viz., 15, 30, 60 and 90 minutes. The LD_{50} value was fixed at 0.4 %. Based on this, selected doses of EMS were 0.2, 0.4, 0.6, 0.8 and 1.0 %. The treated cuttings were raised in 10 x 3 factorial RBD with two replications. Spacing and after care were given as per Package of Practice recommendations [3]. Data of 13 economic traits was taken from individual plants and subjected to statistical analysis in

which genotypes were taken as main effects and doses as sub effects. The effect of the mutagen was evaluated on the basis of the percentage of sprouting of single node cuttings and establishment of the mutant under field conditions. Superior genotypes were identified based on yield and yield-contributing characters and advanced to further generations to study their yield and photo insensitivity. Fourteen superior mutants thus isolated from mutagenic treatments were tested for photo insensitivity in three seasons viz., December 1999, February 2000 and April 2000. Observations were subjected to statistical analysis. Analysis of variance for different factors and its interactions were worked out as per [2] (**Table 2**).

Results and Discussion

Genetic variability

Sixty Coleus genotypes from different ecogeographical regions of Kerala and neighboring states were collected. The highest number of genotypes was collected from Thrissur district followed by Malappuram, since these two districts form the major Coleus growing tracts in Kerala, India. During the course of collection, wild progenitors were not seen in the areas of cultivation, which indicated that this is an introduced crop. Analysis of variance showed significant differences among genotypes for the 13 characters, suggesting the existence of genetic diversity among the Coleus genotypes. Variability for different characters was previously reported already [4, 5].

A high magnitude of gcv and pcv followed by h^2 and genetic advance were observed for tuber yield, harvest index, biological yield/plant, volume/tuber yield, harvest index, biological yield/plant, volume/tuber and weight/tuber. This indicates the heritable nature of these characters with negligible influence of environment. Tuber density showed low values for ga (0.09), pcv (7.53), gcv (5.70) and high values for h^2 (57.92), indicating an additive gene action. The same held true for point of tuber formation. Mean girth of tuber showed low values for h^2 (33.16), pcv (18.18,), gcv (10.35) and genetic advance (5.96), stressing the higher influence of environment on this trait. Similar results were also observed in sweet potato [6]. Correlation and path analysis studies conducted in this investigation revealed that for tuber yield improvement in coleus, the breeder should give emphasis on high harvest index with optimum plant height and a larger number of tubers with medium size and high density. Such plant types should also be shorter in duration and coupled with high nematode resistance.

Genetic diversity

D^2 analysis employing a combined classificatory approach in respect of 13 selected characters recorded that the 60 accessions could be grouped into 10 clusters. This grouping indicated that some genotypes belonging to the same location grouped into different clusters, while certain genotypes habitating in different locations grouped in the same cluster. The results indicated that factors other than geographical diversity may be responsible for such clustering and that there was no parallelism between geographical distribution and genetic diversity, as also observed in rice [7] and in Colocasia [8]. Genotypes in a cluster with a high order of divergence among themselves would be the best breeding material for achieving maximum genetic advance with regard to yield.

Mutagenesis

Coleus mutants exhibited variation in yield attributes and photoinsensitivity with varying doses of Gamma-rays and EMS. The effect of the mutagen on Coleus was assessed in terms of mutant survival.

The sprouting percentage at 30 days after planting was found to decrease with increase in the concentration of mutagens, as previously reported in Canna [9] and in Ginger [10]. Delay in sprouting was another observed effect with increased concentrations of mutagen. The response of Coleus to mutagens varied with concentrations as well as with genotypes, which may be due to differences in the genetic architecture of the plant, which responded differentially to the mutagen. Similar mutagenic concentration and genotype relationships were reported in Kacholam [11]. Gamma-rays and EMS-induced positive variation in the number of tubers per plant. EMS created significant influence altering the density of tubers of various genotypes. Plant height, which had a significant negative correlation with yield in the parental population reversed to a positive correlation in mutants due to the mutation.

The field performance of selected mutants and parents for growth and yield traits during April 2001 to October 2001 (**Table 3**) indicated that the effect of mutation was related to concentration of mutagen and genotype. EMS at 1 % was effective in inducing variability in tuber yield. Chemical mutagens changed the size and number of tubers and plant height, both positively and negatively in Coleus.

Another important positive feature due to mutation was that some of the mutants showed photoinsensitivity for tuberization.

All the mutants differed significantly in many of the characteristics like tuber yield, tuber girth and harvest index for season as well as for its interactions. M 131 ranked first for all seasons for tuber yield taken

Table 2. Characteristics of coleus mutants in different seasons

Mutant	Season				Season				Season			
	I Feb 99-May 00	II Feb-May 00	III Apr 99-Oct00		I Feb 99-May 00	II Feb-May 00	III Apr 99-Oct00		I Feb 99-May 00	II Feb-May 00	III Apr 99-Oct00	
No	No. of tubers/plant			Mean	Tuber yield (g/plant)			Mean	Girth of tubers			Mean
131	**12.0**	**29.7**	**27.3**	**23.0**	**103.3**	**13.3**	**275.0**	**130.6**	**5.3**	**5.8**	**5.8**	**5.6**
61	**25.7**	**6.3**	**22.7**	**18.2**	**175.0**	**38.3**	**153.3**	**122.2**	**5.0**	**5.6**	**7.4**	**6.0**
112	5.67	40.7	7.7	18.0	14.0	30.0	30.0	24.7	4.5	4.4	4.2	4.3
641	4.67	11.7	11.7	9.3	26.7	258.3	13.3	99.4	6.4	4.7	5.7	5.6
422	4.3	21.3	17.7	14.3	15.7	228.3	140.0	128.0	4.4	6.7	6.7	6.0
111	7.0	10.3	10.3	9.2	43.3	103.3	121.7	89.4	6.2	5.5	5.7	5.6
121	5.3	5.7	32.0	14.3	34.3	173.3	134.0	113.9	4.1	4.7	5.3	4.7
62	8.3	10.3	31.0	16.6	22.0	170.0	106.7	99.6	3.6	5.7	7.7	5.6
Check	5.3	5.8	4.0	5.0	41.7	52.3	38.5	44.2	6.2	4.8	5.3	5.4
Mean	9.1	17.0	20.0		54.3	126.9	121.8		5.0	5.7	6.0	
CD (Mutants)		2.6				27.0				0.4		
CD (seasons)		4.2				44.1				0.6		

together (130.556 g/plant). In the off-season it was M 61 which recorded maximum tuber yield (175 g/plant). When comparing first and third season, M 61 ranked first in season one (off season 175 g/plant) second in third season (normal season 153.333 g/plant), with a mean of (122.222 g/plant), when all three seasons were taken together. The same trend was also shown for the harvest index and tuber girth. This result indicated that Mutant 131 and Mutant 61 are suitable for year-round cultivation for higher numbers of tubers, tuber yield per plant and size of tubers (**Table 2**).

Table 3. Growth and yield characteristics of 14 mutants and parents

Mutants and Parents	Plant height (cm)	Biological yield (g)	Tuber number	Tuber yield (g)	Tuber girth (cm)	Harvest index	Tuber density (g/cc)
111	78.33	843.33	10.33	121.67	5.17	0.13	0.58
112	94.67	850.00	7.67	30.00	4.20	0.04	1.24
121	86.67	640.00	32.00	134.00	5.33	0.18	1.19
131	85.33	836.67	27.33	275.00	5.80	0.32	0.72
352	82.00	773.33	10.67	310.00	4.40	0.32	1.57
412	66.33	796.67	7.67	116.67	4.30	0.13	0.37
421	64.67	321.67	15.33	90.00	5.67	0.22	1.20
422	70.33	1016.67	17.33	140.00	6.87	0.12	1.53
632	62.67	923.33	24.00	220.00	4.47	0.21	0.73
641	79.33	843.33	11.67	13.33	5.70	0.02	1.00
1012	75.67	426.67	19.67	128.33	5.67	0.30	1.79
1042	80.33	570.00	12.67	59.00	5.87	0.11	1.04
61a	77.00	730.00	22.67	155.33	7.40	0.16	2.87
61b	78.00	420.00	31.00	106.67	5.57	0.21	0.39
Parlikad local	89.00	1076.67	6.67	36.67	4.00	0.04	0.45
IC – 65724	63.00	1023.33	5.00	46.67	4.53	0.05	0.70
Kolazhy local	68.00	650.00	6.00	20.00	5.60	0.03	0.64
Paipra local	55.00	790.00	5.33	41.67	6.20	0.05	0.40
Mullurkkara III	64.33	450.00	7.00	34.33	6.53	0.07	1.00
SE	± 2.14	± 44.08	± 1.37	± 12.95	± 0.20	± 0.02	± 0.10
CD	4.45	91.69	2.85	26.94	0.42	0.04	0.21

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Resistance of Mutants of Sweet Orange Induced by Gamma-rays to Citrus Canker *(Xanthomonas citri* subsp. *citri*) Under Artificial Inoculation

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Abstract
The sweet orange holds great economic and social importance for Brazil, but it is susceptible to citrus canker as is the majority of citrus species. In cases of high incidence, this disease caused by the bacterium *Xanthomonas citri* subsp. *citri* can result in great economic damage. More resistant cultivars are the best long-term solution for management of citrus canker and one of the approaches can be the production of mutant plants. In a previous work, several induced mutant clones of sweet orange cv. Pera were selected. They showed a lower intensity of symptoms of citrus canker in leaves and fruits in evaluations under natural incidence of the disease, in the field. The objective of this study is to assess the resistance to citrus canker of six mutant clones of cultivar Pera and control plants (three different varieties), in experiments of artificial inoculation. The parameters evaluated were: incubation period, diameter of the lesions and area under the disease progress curve (AUDPC), in evaluations every 15 days, until the 147th day. Only the clones 9-1, 9-2 and 9-3 showed lower incidence of disease, represented by the longest period of incubation of the disease, smaller diameter of lesion and lower AUDPC, using average data of the three experiments. This study is one of the first reports of success in citrus-induced mutations aimed to obtaining greater resistance to diseases.

Introduction
The citrus industry can be considered as one of the most globalized segments of Brazilian agribusiness. Most of its production is exported thus bringing more than 2.5 billion USD to the country every year [1]. One of the main products of the Brazilian Citrus Industry is frozen concentrated orange juice (FCOJ), which represents over 80% of the world's export [1]. In Brazil, oranges represent 89.3% of the production of citrus species, followed by mandarins, with 5.8%, and limes and lemons with 4.9%.

Asiatic citrus canker (ACC) is one of the most serious diseases of citrus species [2, 3]. Caused by *Xanthomonas citri* subsp. *citri*, the pathogen is distributed in many countries and can infect plants of the *Rutaceae* family [4]. It is a quarantine disease with regulated trade of fruits between countries by measures of exclusion, aimed at preventing the pathogen entrance in areas free of disease [2, 5, 6].

The first occurrence in Brazil was in 1957, in Presidente Prudente, São Paulo State [7], and was subsequently spread to almost the entire country [8, 9, 10]. Measures of exclusion and eradication were adopted in the same year in the form of a campaign for eradication of infected trees that remains active until the present days [11, 12]. The only exception occurs in Parana State, where the eradication is not mandatory, but the adoption of disease management practices is accepted, such as application of cupric agrochemicals and protection by windbreak curtains [13].

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Citrus canker disease is characterized by the formation of circular and water soaked lesions that become raised and blister-like, growing into yellow spongy pustules, then to darken and thicken into a light tan to brown canker, often surrounded by a chlorotic halo [7]. On heavily infected trees, citrus canker causes defoliation and premature fruit drop in the most susceptible varieties [14, 15].

All citrus varieties are susceptible to citrus canker. Grapefruit, acid limes and lemons are highly susceptible. Sour oranges and oranges are moderately susceptible, while mandarins are termed moderately resistant [16]. In experiments with artificial inoculation it was shown that susceptibility varies according to the tissue infected and to the different modes of infection [17, 18, 19]. Young leaves and fruits are more susceptible than mature tissues [20], and inoculation methods that cause direct contact of the bacteria with mesophyll cells (pinprick inoculation, for example) are more efficient, often resulting in disease [21].

More resistant cultivars are the best long-term solution for citrus canker management. One of the approaches can be the induction of mutants. Mutant plants of citrus have been experimentally induced in several countries. The main characteristics selected were seedlessness or reduction in the number of seeds [22, 23, 24, 25], absence of thorns [22], better color of fruits [23], salinity tolerance [26] and increased resistance to diseases [27].

In a previous work, [28] reported the selection of 12 sweet orange mutant clones with less intensity of citrus canker symptoms in leaves and fruits. These mutants were obtained from mutagenic treatment with Gamma-rays and with selection in field conditions, on natural incidence of the disease. Two of those clones (9 and 41) were confirmed as more resistant to citrus canker, after five years of evaluation in the field [27].

The objective of this study was to assess the resistance to citrus canker of three mutant clones of orange Pera selected by [28], in experiments with artificial inoculation by wounding.

Materials and Methods
In the present study, we evaluated the resistance of six mutant clones of 'Pera' (numbers 9-1, 9-2, 9-3, 42-2; 61-1 and 61-2), four non-mutant clones of 'Pera' ('Pera Fischer', 3-1, 3-2 and 3-3), and three control genotypes, in experiments with artificial inoculation. The control plants used were 'Ponkan' mandarin (highly resistant), 'Pera IAC' sweet orange (control non-irradiated - medium resistant) and 'Hamlin' sweet orange (less resistant).

The experiment was carried out using a randomized design with four replications, each consisting of four one-year-old plants, maintained in vessels (4L), in a greenhouse. Plants were pruned 90 days before inoculation.

Ten leaves per plant were inoculated by pinprick inoculation using a needle (0.55 x 0.2 mm) previously immersed in a 10^6 CFU m.L^{-1} of bacterial suspension (strain IBSBF 1421). Each leaf was pricked six times. Plants were kept under a humid condition in growth chambers for 18 hours after inoculation and then, transferred to a greenhouse. The experiment was repeated three times.

The parameters evaluated were: a) Incubation period (number of days after inoculation to visualize at least one lesion in more than 50% of the inoculated leaves), b) Average diameter of the lesions, measured in four lesions taken at random at 15-day intervals for a period of 147 days, and c) Area under the disease progress curve (AUDPC) that was calculated from the average diameter of the lesions, in the same period of time (147 days). This parameter allows a comparison between treatments considering the whole assessment period.

Treatments were compared in pairs. Linear regression equations were fit to data for lesion diameter obtained in each treatment for each experiment. The curves were compared by analysis of variance (F test for coincidence between curves) and the angular coefficient (b) of each curve was also compared using *t* test (for parallelism between curves), separately for each experiment. Incubation period and AUDPC data were analyzed by ANOVA and means were compared using Tukey test ($P \leq 0.05$).

Results and Discussion

All plants showed disease symptoms but with large variability among treatments. The incubation period of all treatments ranged from eight to 12.8 days after inoculation (DAI), on average in the three experiments. The first genotype that showed lesions was Hamlin, after 9.2 DAI, while in Ponkan the incubation period was 10.7 DAI (**Fig. 1**). Four mutant clones, 'Pera Fischer' (non-mutant clone) and the control genotype (Pera IAC) did not show symptoms before 10 DAI. Mutant clone 9-3 was the only one that showed an incubation period longer than 11 days (11.2 DAI).

The inoculum concentration (10^6 CFU mL^{-1}) was appropriate in carrying out the screening between resistant and susceptible genotypes. Optimum inoculum density needed for infection varies depending on the mode of infection, [21] having recommended 10^4 and 10^5 CFU mL^{-1} for infection via stomata (without wounding) and 10^2 CFU mL^{-1} for infection by wounding. However, other authors used larger concentrations for screenings of resistance in citrus varieties using pinprick inoculation. Viloria, *et al.* [29] only saw consistent differences between genotypes after increasing the inoculum concentration from 10^3 to 10^4 CFU mL^{-1}, while [30] used 10^6 CFU mL^{-1} to assess citrus canker resistance of somatic hybrids between Hamlin sweet orange x Montenegro mandarin, and [31] evaluated ACC resistance of 54 citrus species using pinprick inoculation method with inoculum concentration of 10^8 CFU mL^{-1}.

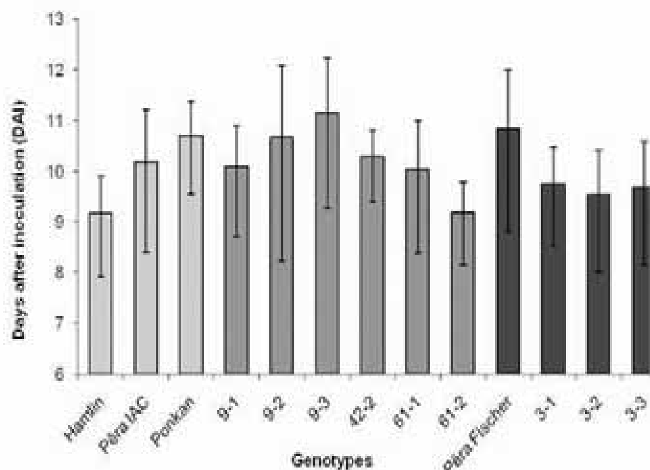

Figure 1 Incubation period up to lesion occurrence. Values represent average of three experiments.

The diameter of lesions increased in all treatments throughout the evaluation period of the experiment, which extended for 147 DAI (**Fig. 2**).

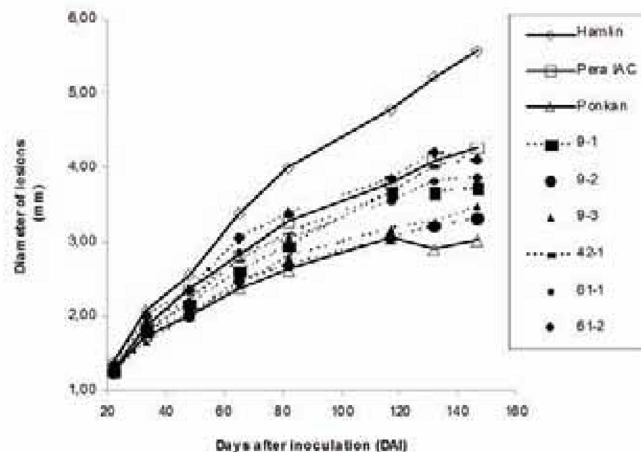

Figure 2 Diameter of lesions, measured for four lesions taken at random, at 15-day intervals until 147 days. Values represent the average of three experiments.

The ranking of genotypes, based on the assessment of lesion diameters, was the same in the three experiments. 'Hamlin' sweet orange showed lesions with greatest diameter (average of 5.6 mm, in 147 days), while 'Ponkan' mandarin was the genotype with the smallest lesions (3.0 mm, in 147 days). Among the mutants, only clones 9-1, 9-2 and 9-3 showed lesions with diameters significantly lower (3.3 to 3.7 mm) compared to 'Pera IAC' sweet orange (non-irradiated control - 4.3 mm) and similar to 'Ponkan' (3.0 mm). The other mutants and non-mutated clones showed no differences in relation to control plants (Pera IAC).

The results observed for the diameter of lesions confirm observations made by [16] that Ponkan mandarin is more resistant to infections by citrus canker and sweet oranges are more susceptible, but with differences among varieties. Hamlin, that is considered one of the most susceptible sweet orange varieties to citrus canker, showed lesions with larger diameter in all experiments, while Pera IAC and Pera Fischer had intermediate resistance.

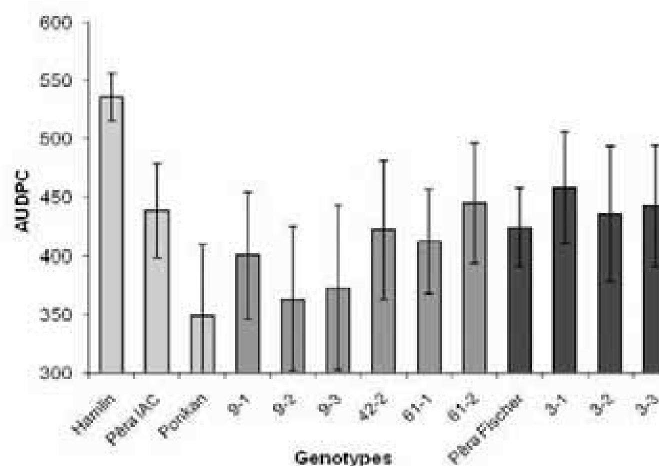

Figure 3 Area under disease progress curve (AUDPC), calculated from average diameter of the lesions of citrus canker. Values represent the average of three experiments.

The average AUDPC value obtained for Hamlin was higher than all other genotypes (536 ± 35) in all experiments, while smaller values were observed for 'Ponkan' (349 ± 104) and for three mutant clones (9-2, 9-3 and 9-1 – 363 ± 106 to 401 ± 93) (**Fig. 3**). Mutant 9-3 was the only one that did not differ from 'Ponkan' in all experiments, while mutants 9-2 and 9-1 differed in one or two experiments. Other mutant and non-

mutant clones did not differ from 'Pera IAC' in some experiments, nor when we analyzed the data as an average of the three experiments.

AUDPC is the most complete and informative variable for epidemiological studies and for studies of resistance of citrus canker, because it can retain maximum information such as disease onset, rate of increase and final disease incidence [21].

The causes of increased resistance of plants to direct inoculation of citrus canker in mesophyll are not fully clarified [31]. According to [21], the greater resistance of leaves by wounding inoculation may be associated with the process of healing, tissue age or other mechanisms of resistance because, in this type of inoculation, bacteria can be put directly in contact with the leaf mesophyll tissue. In leaves with maximum size or with two thirds of their maximum size, it has been proven that the increased resistance is correlated to water congestion [21], while [32] considered that in mature leaves, the mesophyll resistance is expressed by limitation of the multiplication of bacteria, which varies between species and cultivars.

The results obtained in all experiments suggest that mutant clones 9-1, 9-2 and 9-3 have higher resistance to citrus canker than control plants, comparable to that observed in 'Ponkan' mandarin (the most resistant cultivar) under conditions of artificial inoculation. Other experiments will be conducted to assess the fruit yield of these mutants and the resistance to citrus canker in field experiments, under natural infection. If they show yield and fruit quality suitable for commercial use, these mutant clones should be recommended for regions where citrus canker is endemic.

This study is one of the first reports of success in the induction of mutations in citrus aimed to obtaining greater resistance to diseases.

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Use of Irradiation for the Induction of Mutations in Oyster Mushrooms for Improvement of Strains

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Abstract
In order to induce mutants with improved characteristics in terms of good yield and adaptability to a wider range of temperatures, five parent strains of *Pleurotus*, a species of edible mushroom, were subjected to gamma and UV irradiation. Mycelial plugs of the actively growing parent strains were subjected to doses of gamma radiation ranging from five to 400Gy using a ^{134}Cs radioisotope. Irradiated mycelia were assessed in laboratory experiments and were used to prepare mother flasks, spawn and fruiting bags for production experiments. Certain stimulatory effects of gamma-irradiation were noted on mycelial growth rate and yield of fruiting bags at different doses. However, these effects were not consistent. Similarly, for UV irradiation, multispore suspensions of the five parent strains were subjected to exposure times varying from three to 20 hours to UV-rays. After plating, vigorously growing mycelia were evaluated in laboratory assessments and production trials. Decreased viability was noted in several strains after repeated subcultures and storage. Viable UV-irradiated strains exhibited similar stimulatory effects as in gamma irradiation at certain exposure times. Again, an erratic effect at varying exposure times was noted. Based on these results, irradiated strains showing stimulatory effects on mycelial growth and yield have been selected for further evaluation at different agroclimates in Mauritius as well as for other breeding work.

Introduction
Oyster mushrooms (*Pleurotus* sp) are edible fungi, commercially cultivated for consumption worldwide. Mauritius imports various mushrooms, both fresh and processed, to a value amounting to around one million euro annually. This demand is supplemented by the local production, on a major part, of oyster mushrooms. Oyster mushroom cultivation in Mauritius is chiefly carried out in low cost growing houses at ambient conditions with no temperature and humidity control. As such, mushroom productivity is heavily dependent on agroclimatic conditions occurring in different areas of the island and on the seasonal variations. Mushroom growth and adaptability as well as quality and quantity produced are intrinsically linked to temperature, which is an important factor in mushroom development [1]. It would be beneficial to local producers to have oyster mushrooms strains adapted to a wider range of temperatures for their development and fruiting phases as well as strains of a higher productivity at a particular agroclimate. For seasonal mushroom growers, improved strains with better adaptability to a range of temperatures will enable an extension of their growing season or even year round production. Identification of higher yielding strains at low, medium or high temperatures will increase the productivity and eventually the revenue of growers. Thus, the cultivation of better-adapted strains for each agroclimate will help to ensure optimal mushroom development and yield. This can lead to an increase in local production and a decrease in importation. Mutation breeding of oyster mushroom strains available in Mauritius was selected in order to increase the potential of obtaining strains with desirable characteristics.

Five Pleurotus mushroom strains were selected from the 52 strains maintained at the Culture Collection (CC) of Agricultural Research and Extension Unit detailed in [2]. The selection procedure was based on the fitted model of mycelium growth of these strains at eight different temperatures ranging from 13° to 34°C. Two *Pleurotus sajor-caju* strains, CC 46 and CC 116, were selected for good performance at low temperatures (16°-19°C). Similarly, *P. columbinus* (CC 66) and *P. hybrid* (CC 71) showing good mycelial growth rate at higher temperatures (28°-31°C) and the commercially cultivated *P. sajor-caju* CC 114 were selected for breeding and improvement purposes.

The principal aim of this study was to improve the five selected strains of oyster mushroom by irradiation to induce mutations. Two types of irradiation, namely irradiation using Gamma-ray and UV radiation were used in order to attempt to improve the five promising parent strains.

Materials and Methods
The work was divided into two distinct steps. The first consisted of the laboratory procedures including the induction of mutagenesis using irradiation, subculture of irradiated strains and mycelial growth assessment as per the procedure described in [2]. For all laboratory culture preparations and for plating for vegetative growth assessment of strains, Potato Dextrose Agar (PDA) medium (Hi Media laboratories, India) was used. Media and glassware were autoclaved. All mycelium transfers and culturing were carried out in aseptic conditions under a laminar flow hood. The second step consisted of the evaluation of irradiated strains in production trials. This involved multiplication of irradiated strains by the production of mother cultures followed by spawn and fruiting bag production for evaluation of mushroom yield.

Laboratory experiment
Induction of mutation in oyster mushrooms using gamma irradiation
The five parent strains were aseptically inoculated on PDA petridishes and incubated at 25°±1°C for five days. From the perimeter of the actively growing culture, mycelium covered agar plugs of 6 mm diameter were aseptically excised using a cork borer. Each plug was centrally inoculated onto a PDA petridish (diameter 50mm). The Gammator Cesium-137 was used to subject four replicated petri-dishes per strain to each dose of gamma irradiation (ranging from fiveto 400Gy). Exposure time was determined for each dosage of irradiation. After irradiation, petri-dishes were placed in an incubator set at 25°±1°C. The rate of mycelial colonization was noted.

Induction of mutation in oyster mushrooms using UV irradiation
Multispore suspensions were used for UV irradiation. Only mature carpophores harvested from the first flush of the five parent strains were used to produce spore prints. Suspensions were obtained by streaking an inoculating needle on spores from spore prints into sterile distilled

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water. Before exposure to mutagenic treatment, the optimal multispore dilution level was determined, by evaluating four dilution levels (undiluted, 10^{-1}, 10^{-2}, 10^{-3}). The 10^{-1} multispore dilution level was selected for future work based on the incidence of fungal colony formation upon streaking of suspension onto a PDA petri-dish.

Spore suspensions of the five parent strains at 10^{-1} dilution level were subjected to varying periods of exposure to UV irradiation. Test tubes of spore suspensions were subjected to irradiation from the UV lamp within a laminar flow hood for seven exposure periods (0, 3, 6, 9, 12, 18, 20 hours). This procedure was carried out in darkness to inhibit photoreactivation. After irradiation, spore suspensions were plated and incubated at 25° ±1°C. The growth of surviving spores was noted. Actively growing mycelia from these developing spores were then subcultured followed by rate of growth evaluation.

Production experiment

Both the UV and gamma (0, 100, 200, 300, 400Gy) irradiated strains were assessed in production trials for their ability to fructify and their yield. All strains were subcultured onto PDA petri-dishes. Agar plugs were cut out and used to inoculate mother spawn flasks. The latter consist of a sterilized millet, talc, calcium carbonate and sugar cane bagasse medium. After inoculation, flasks were incubated at 25° ±1°C. The time taken for complete colonization was noted. Mother spawn flasks were then used for the inoculation of sterilized spawn bags comprising of a maize seeds, bagasse and lime media. Spawn bags were incubated in a darkened room at ambient temperature. The rates of colonization of spawn bags were noted. Standard bagasse based substrate for local oyster mushroom cultivation comprising of bagasse, crushed maize and lime was prepared and pasteurized at 60° to 70°C. After cooling, the fruiting bags were inoculated with the colonized spawn. Each replicate spawn was used to inoculate nine fruiting bags of 0.75 kg capacity and seven bags of 3 kg capacity. These bags were allowed to colonize in an incubation room. After spawn run, bags were transferred to a growing house and fructification was initiated. Regular daily spraying of water was carried out. Yield of fruiting bodies from bags of each strain were noted. Production experiments were replicated over time for confirmation of observation and outcome.

Results

The highest gamma irradiation dose used in the laboratory experiments to induce mutation in oyster mushroom strains was 400Gy. At this dose, no mortality was induced in the five *Pleurotus* parent strains and all irradiated mycelial plugs successfully showed growth onset and mycelium development. Analysis of rates of mycelial growth during petridish incubation showed that increases in gamma irradiation dose beyond 200Gy resulted in a decrease in vegetative growth. There was not much difference in growth rate in doses below 200Gy. Doses of 300Gy and 400Gy resulted in a slight decrease in growth rate reflected in a longer time taken for media colonization, i.e. to reach a radial mycelial diameter of 50 mm (from seven to eight days for unirradiated and doses of less than 200Gy up to 14 to 15 days for 400Gy).

For UV-ray irradiation, the multispore dilution level of 10^{-1} was determined to be optimal for plating of spore cultures. After irradiation, a good spore survival rate was observed in irradiated strains with the exception of CC 71 irradiation duration 18 hours and CC 66 irradiation duration 12 hours, which showed no spore survival. Actively growing mycelia from surviving spores were transferred to fresh PDA medium for further evaluation. All UV and gamma-irradiated strains were also assembled in a culture collection. It was observed that some irradiated strains showed loss of vitality over a two-year period and were not included in further trials.

The mother spawn flasks prepared from all viable gamma and UV-irradiated strains for production experiments, took on average four days for onset of mycelial growth, and colonization was completed on average in 11 days. The time taken for colonization of strain CC 116 UV-irradiated for three hours was 18 days, with onset of growth noted after five days. CC 46 irradiated at 100Gy showed faster colonization rate (nine days) compared with the unirradiated and the three higher irradiation doses of 200, 300 and 400Gy. The colonization rates for the respective (conventional 0.75 kg) spawn bags, however, varied greatly, and ranged from 21 to 35 days. No definitive trend alterations in spawn colonization rates were observed with increasing gamma-radiation doses or with increasing exposure time to UV radiation. For instance, colonization rate of CC 71 subjected to 0, 100, 400Gy gamma-radiation was 21 days with a marginal increase in replicate colonization rate (25 days) at a dose of 200Gy. Moreover, time taken for colonization of CC 46 irradiation doses 0, 200, 300Gy was 35 days, with a marginal decrease of three days noted at an irradiation dose of 100Gy. Increasing UV radiation exposure duration of CC 116 resulted in a decrease of colonization rate (from 27 days for unirradiated strain to 22 days for 18 hours UV radiation exposure). The strains subjected to gamma or UV radiation likewise exhibited alterations in colonization rates of fruiting bags. Similarly to spawn bags, no smooth trend was noted in the colonization rates of both the 0.75 and 3 kg fruiting bags. For example, time taken for colonization of fruiting bag of 0.75 kg of CC 46 (0Gy) decreased from 22 days for the unirradiated to 20 days after irradiation at 100Gy, followed by an increase in time taken at higher gamma-radiation doses. However, no change in time taken for colonization (33 days) was noted in gamma-irradiated CC 66 fruiting bags of 3 kg capacity unlike those of 0.75 kg. Furthermore, it was also noted that strains such as UV-irradiated CC 71 (six hours exposure) and gamma-irradiated CC 66 (100Gy) failed to colonize adequately.

The color of pilei surface and the gross morphology of the fruiting bodies of the irradiated strains were not markedly different from the original parent types. It was noted that CC 46 and 66 generally fructified later than the other parent strains. For ease of comparison, yield from the two sizes of fruiting bags were worked out per kilogram of substrate.

For gamma-irradiated strains, analysis of variance (two factors without replication at $p \leq 0.05$) of the compiled results of fruiting bodies yields was carried out. No significant difference in average number of fruiting bodies produced was obtained with respect to the gamma-radiation treatment. Average number of fruiting bodies varied significantly with respect to the fruiting bag type and strain. Fruiting bags of 0.75 kg produced numerous (average 17) but smaller fruiting bodies as compared 3 kg bags producing less in number (6) but larger fruit size. Strain CC 71 produced a higher number of fruits (23.5) than strains CC 46 and 66 as well as CC 114 and 116.

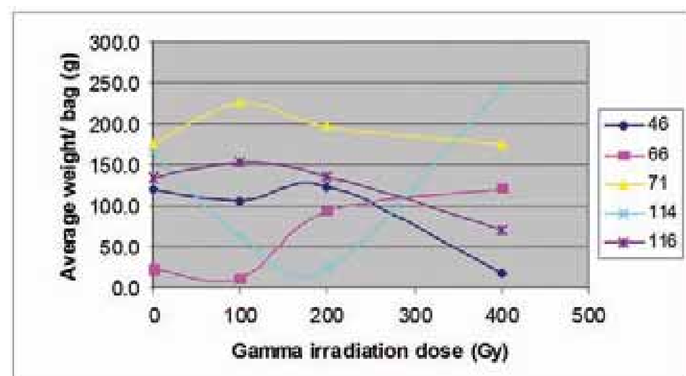

Figure 1 Average weight of oyster mushrooms obtained per kilogram of substrate from fruiting bags of 0.75 kilogram capacity for the five parent strains irradiated with Gamma-rays at doses 0-400Gy.

As shown in **Fig. 1**, the average total weight of fruiting bodies is influenced by different irradiation dosages for the parent strains. The analysis of variance of average fruiting bodies yield per kilogram of substrate indicated a significant difference, which was influenced by irradiation dosage, parent strain and size of the fruiting bag. The average weights of fruiting bodies obtained per kilogram of fruiting bag substrate irrespective of size of bag are summarized in **Table 1**.

In the case of UV irradiation, several strains showed loss of viability subsequent to subculture and storage as a result of which insufficient strains with different exposure times remained for possible comparison. CC 116 with exposure times of zero to 18 hours was the exception. The analysis of variance of the production results indicated that no significant difference in yield per kilogram of substrate occurred following exposure at varying periods to UV radiation. However, yield varied significantly with respect to fruiting bag size such that per kilogram of substrate, bags of 0.75 kg produced greater number of fruiting bodies (13 compared to 4 from 3 kg bags) and a higher average weight per bag (107g compared to 52 g from 3 kg bags).

Table 1. Yield of fruiting bodies of *Pleurotus* mushrooms harvested per kg of fruiting bag substrate after induced mutation using gamma radiation

	Average Yield (g)			
Strain	**0** Gy	**100** Gy	**200** Gy	**400** Gy
CC 46	109.8	97.1	120.2	24.6
CC 66	8.5	12.6	94.1	126.0
CC 71	180.9	203.6	176.3	168.2
CC 114	142.2	36.7	161.7	218.2
CC 116	94.5	110.1	120.0	44.4

Discussion and Conclusion

Gamma radiation induced mutants have been reported in several works on *Pleurotus* sp [3-5] and the use of UV-ray irradiation has been reported in others [6-7]. In the present study, it was demonstrated that improvement of parent strains of oyster mushroom could be induced by artificial mutagenesis using both gamma radiation and UV radiation. Mutation breeding of *Pleurotus* species by subjecting non-sexual spores and hyphal segments (mycelial plugs) to irradiation, a physical mutagen, so as to create variants as defined in [8], was carried out.

In this study, mycelial plugs were subjected to gamma radiation doses to a maximum of 400Gy after which all plugs showed mycelial growth during incubation. This indicates that the doses used were below the lethal doses for the parent strains. This is in line with the results of [4] where lignocellulolytic mutants were irradiated to doses of 1 to 2 kGy with no strain mortality. As discussed in [3], faster mycelial growth, decreased spawn run time and increased productivity in terms of yield were noted after gamma irradiation of *P. sajor-caju* spawn at 0.1 to 0.25 kGy. In this study, no changes in mycelial growth rate in petridishes were noted after irradiation up to 200Gy . At higher doses though, a marginal inhibitory effect on mycelial growth rate was noted. A stimulatory effect of gamma-irradiation on mycelial growth rate at the mother spawn and spawn bag level and decreased colonization time during spawn run as seen in [3] were noted in certain cases. For instance, gamma irradiation of CC 46 at 100Gy increased mycelial growth rate so that colonization was completed in nine days instead of the mode of 11 days. An increase in average yield of fruiting bodies was also noted following gamma irradiation at specific doses (CC 114 irradiated at 400Gy, CC 116 at 200Gy and CC 46 at 200Gy, etc). However, these stimulatory effects were neither consistent for the different parent strains throughout all the gamma radiation doses nor through the different stages of laboratory and production experiments. Erratic trends and slight inhibitory effects of both irradiation types were noted. This may be due to the limitations of mutation breeding [9] in its basic form. These limitations arise due to the random and undirected nature of mutations as well as the possible occurrence of 'unsolicited' mutations having no bearing on the breeding objectives. Irradiation-induced desirable mutation in a cell not contributing to the next generation, decreased cellular fitness, poor regeneration after irradiation, chromosomal damage, chimerism especially in the case of use of mycelial plugs, can all be possible reasons accounting for the erratic trends in growth rate and yield of oyster mushroom.

Pleurotus species exhibit bifactorial heterothallism [10] and as such, their single spore cultures are sterile. Multispore cultures where thus chosen for UV-ray irradiation. The presence of clamp connections indicating fusing of compatible hyphae [8] were sought in order to select actively growing mycelium from irradiated spores. All UV irradiated strains showed mycelial growth after subculturing on PDA. After repeated subcultures and storage in the Culture Collection over the course of two years, it was observed that several UV irradiated strains lost their viability and failed to show mycelial growth when plated and incubated. This differs from the stable mutant character of UV irradiated strain maintained over more than 10 generations of subculturing as reported in [7]. This may be due to the lower exposure period of 75 minutes to UV-rays in [7], or to a faster degeneration of multispore cultures propagated by mycelial cultures than the single spore cultures explained by Fritsche as reported in [11]. A stimulatory effect similar to that of the reported Gamma-ray irradiation was also noted in the surviving UV irradiated strains. Increasing exposure times to UV radiation stimulated yield in certain cases. However, due to insufficient strain survival, a detailed analysis was not possible.

In production experiments, fruiting bags of smaller size, of 0.75 kg capacity, generally showed better productivity. This may be attributed to faster substrate colonization and less susceptibility to contamination of smaller bags, or to improved substrate utilization.

To conclude, it has been demonstrated that irradiation can improve oyster mushroom strains. Yield has shown to enhance for certain strains after different types of irradiation, different doses or different exposure times. Also the inherent productivity of strain CC 71 was found to be higher than that of CC 114, the actual commercial oyster mushroom strain, and can be considered as a potential substitute. All promising irradiated strains identified in these experiments will be evaluated during successive production experiments and will be assessed for growth and yield in different agroclimates for identification of better suited strains for each agrozone of Mauritius.

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The Breeding of *Arthrospira platensis* Mutants with Good Quality and High Yield Induced by Space Flight

X Hou*, Y Lu, Z Zhang & W Wang

Abstract

Arthrospira platensis mutant PNK-2 has been induced by space flight. PNK-2, with good quality and high yield, is suitable for outdoor large scale production. Compared with the initial ST-6, the helix number of PNK-2 was 12~18; the average length of algae body, thread pitch, helix width, diameter of trichome and the rate of large-scale production were 764.31μm, 52.98μm, 18.75μm, 6.02μm and $10g\bullet(m^2\bullet d)^{-1}$, respectively, representing increase rates of 166.52%, 5.88%, 8.19%, 12.31% and 22.89%, respectively. The content of protein, chlorophyll, β-Carotene and phycocyanin in PNK-2 were 69.57%, 1.01%, 0.16% and 14.70%, respectively, with respectively raising rates of 8.31%, 8.60%, 6.67% and 6.68%. The γ-linolenic acid content of PNK-2, at 0.63%, was reduced by 3.08%. The results showed that PNK-2 was a new *A.platensis* strain with good quality and high yield.

Introduction

Spirulina is a photosynthetic, filamentous, spiral-shaped, multicellular cyanobacterium. It is particularly rich in protein and also contains carotenoids, vitamins, minerals, and essential fatty acids [1]. The nutritional composition of *Spirulina* is comprehensive and balanced for humans as a high-quality health product. Test tube and animal studies have demonstrated several properties of large amounts of spirulina or spirulina extracts, including antioxidant [2], antiviral [3], anticancer [4,5], antiallergy [6,7], immune-enhancing [8], liver-protecting [9], blood vessel-relaxing [10], and blood lipid-lowering [11] effects. It is recommended as a fine food resource for humans by WHO and FAO.

The two most common species used for human consumption are *Spirulina maxima* and *S. platensis*. *Arthrospira platensis* is one of the two species of *Spirulina* that is applied for production. Its mutation is easily induced by environment factors such as temperature, radiation, etc. [12]. In order to improve the output and quality of *Spirulina* and decrease the cost of production, some new species had been bred by domestication, natural selection, and physical or chemical mutation [13,14].

Space mutation breeding is a new fast breeding technology. The biological material is taken to 200-400 km up into space by an airship or a return satellite. Variation occurs in space for factors such as microvacuums, cosmic rays, energetic particles, cosmic magnetic fields, microgravity, etc. The new idioplasm, material and species will be selected from the returning biological material. Space mutation breeding is characterized by inducing a high frequency and large amplitude variation, with good heredity and beneficial modifications [15].

Materials and Methods

Original *Spirulina*

ST-6, *Arthrospira platensis* (Shenzhen Nongke Group Corporation) was carried to space and returned in the 20th return S&T satellite of China.

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Culture

Zarrouk culture indoors, NO.4 culture of Noneke (per liter culture components: sea salt 0.5g, urea 0.05g, $NaHCO_3$ 5g, $NaNO_3$ 0.7g, $MgSO_4$ 0.2g, $FeSO_4$ 0.01g, $CaCl_2$ 0.02g, H_3PO_4 0.15g, KCl 0.5g, Na_2SO_4 0.25g) outside.

Breeding

The returned *Spirulina* from the satellite was cultured at 25°C, illumination intensity 3200-4000 lx. After 30 d, robust trichomes with a helix number above 10 are separated by capillarity with the help of a microscope, and then cultured separately in a tube. The separating operation was repeated several times until the appearance of uniform, fast-growing trichomes with good quality of the species.

Comparison of growing speed

The selected species and original ST-6 were all inoculated in conical beakers at the same concentration (absorbance value at OD_{560}) and cultured stationarily, with the beaker shaken four times every day and the OD_{560} value determined. The *Spirulina* was collected with the 50 eye silk cover after 336h. Test samples were prepared through rinsing, drying and weighing.

Determination of protein

The protein contents of samples were determined by Kjeldahl method for nitrogen [16].

Large-scale production

Large-scale production was carried in the *spirulina* farm of the Nongke base. Each pond area was about 1143 m^2. In order to obtain the best quality and most stable species, the shape, productivity and nutriment contents of *spirulina* must be determined and compared.

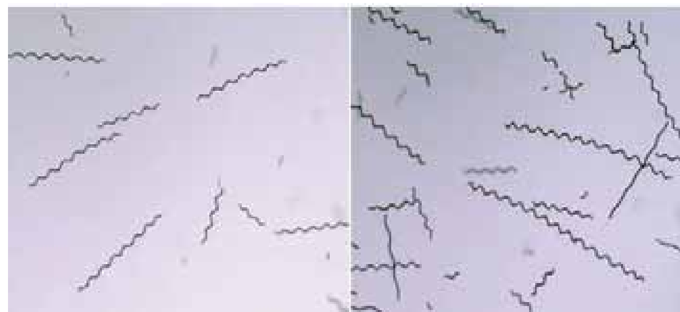

Figure 1 Differences of *A. platensis* mutants (left: before induction; right: after induction).

Results

Breeding of stable species

The space-mutated *spirulina* showed much variation in shape and length through the microscope observation (**Fig. 1**). Some were regular and robust, some linear and weak, others two to three times longer than the original.

Spirulina variation is mainly caused by the external factors. Cultured in an open environment, *spirulina* shows variations such as straightening, thinning and irregularity. However, space mutation could induce more significant changes in its shape and other aspects. Through single *spirulina* separation and breeding, seven stable shapes of *spirulina* species were obtained and numbered PNK-1 to PNK-7.

Selection of high yield species

Each species of PNK-1 to PNK-7 was inoculated in four bottles (500ml/ bottle). OD_{560} value is the average of the four bottle sample. The results of one-factor analysis of variance are showed in **Table 1**. PNK-2, PNK-4 and PNK-5 were significantly different from ST-6. PNK-2 and PNK-5 were selected as the high yielding species due to OD_{560} value at 280h and final biomass. The biomass of PNK-2 and PNK-5 increased 22.89% and separately in comparison with ST-6.

Table 1. Comparison of OD560 of different A.platensis

Species	Inoculation time/h						
	0	48	96	144	192	240	288
PNK-1	0.097±0.005	0.178±0.018	0.316±0.011	0.597±0.057	0.815±0.052	1.108±0.143	1.348±0.201[b]
PNK-2	0.097±0.004	0.188±0.021	0.310±0.037	0.633±0.052	0.893±0.063	1.201±0.084	1.457±0.066[a]
PNK-3	0.097±0.011	0.172±0.016	0.288±0.022	0.577±0.028	0.815±0.052	1.056±0.083	1.276±0.100[c]
PNK-4	0.097±0.010	0.177±0.019	0.319±0.022	0.580±0.038	0.801±0.046	1.137±0.110	1.398±0.091[a]
PNK-5	0.097±0.004	0.194±0.026	0.323±0.016	0.611±0.035	0.845±0.038	1.201±0.084	1.421±0.062[a]
PNK-6	0.097±0.013	0.177±0.025	0.288±0.026	0.593±0.057	0.839±0.050	1.108±0.053	1.369±0.048[a]
PNK-7	0.097±0.005	0.174±0.028	0.297±0.025	0.588±0.016	0.815±0.052	1.137±0.146	1.319±0.205[b]
ST-6	0.097±0.007	0.163±0.026	0.271±0.029	0.547±0.037	0.714±0.047	0.921±0.096	1.164±0.137

[a] P<0.01 (greatly significant difference),
[b] 0.01<P<0.05 (significant difference),
[c] P>0.05 (no significant difference).

Breeding of good quality species

The nutrients of *spirulina* were the significant standard of good quality. However contents of nutrients were affected by factors such as culture condition, collecting time and method [17]. PNK-2 and PNK-5 were cultured at a large scale and collected twice at logarithmic phase. The protein content of PNK-2 was 70.3% and 68.3%, and better than PNK-5 which had 68.7% and 64.7%.

Large-scale production

PNK-2 and ST-6 were cultured in the farm as a comparative trial for four batches. Comparative trial results are shown in **Table 2**. In the four batches comparative trial productivity of PNK-2 increased by 21.38%, 20.00%, 27.25% and 33.75%, separately than ST-6.

Spirulina is an exclusive photosynthetic autotroph. It can reduce the CO_2 to carbohydrate energized by light. Nutrition, temperature, light and pH can affect *spirulina* growth rate, output, chemical components and its contents. In fact, light is the major growth-limiting factor. Deficient sunshine on rainy days can weaken the photosynthesis of *spirulina* and lead to a lower output. In the outdoor trial, productivity of PNK-2 amounted to 9.55 and 9.62 $g\bullet(m^2\bullet d)^{-1}$, respectively, at 50mm rainfall and 30mm rainfall. From **Table 2**, it can be concluded that PNK-2 has a high adaptability to the environment.

Morphological characters of PNK-2

PNK-2 and ST-6 had obvious differences in shape at logarithmic phase (**Fig. 3**). Compared with the initial ST-6: the helix number of PNK-2 was 12~18; the average length of algae body, thread pitch, helix width, diameter of trichome and rate of large-scale production were 764.31μm, 52.98μm, 18.75μm, 6.02μm and $10g\bullet(m^2\bullet d)^{-1}$, respectively with respective rates of increase of 166.52%, 5.88%, 8.19%, 12.31% and 22.89% against ST-6 (**Table 3**).

Table 2. Records of outdoor production data of *A.platensis*

Batch	Species	OD_{560}			Quantity of dry powder (g)	Productivity $g\bullet(m^2\bullet d)^{-1}$	Weather
		Inoculation at 0 h	Collection	Filtrate			
1	ST-6	0.155	1.097	0.092	103000	8.19	1 d; rainfall 20 mm
	PNK-2	0.155	1.187	0.046	125000	9.94	
2	ST-6	0.167	1.125	0.108	105000	8.35	1 d; rainfall 15 mm
	PNK-2	0.167	1.222	0.056	126000	10.02	
3	ST-6	0.187	1.119	0.125	95000	7.56	2 d; rainfall 35 mm
	PNK-2	0.187	1.208	0.071	121000	9.62	
4	ST-6	0.180	1.131	0.143	98000	7.14	2 d; rainfall 50 mm
	PNK-2	0.180	1.268	0.086	131000	9.55	

Table 3. Comparison of morphology between PNK-2 and ST-6 (in μm)

Character	ST-6		PNK-2	
	Length range	Average length	Length range	Average length
Length of algae body	233.34~400.78	286.77±52.78	629.82~852.58	764.31±83.33
Thread pitch	48.28~52.11	50.04±1.38	50.93~59.00	52.98±2.71
Helix width	15.70~18.92	17.33±1.07	17.46~20.44	18.75±0.90
Diameter of trichome	5.07~5.66	5.36±0.20	5.83~6.42	6.02±0.18
Helix number	5~8	12~18		

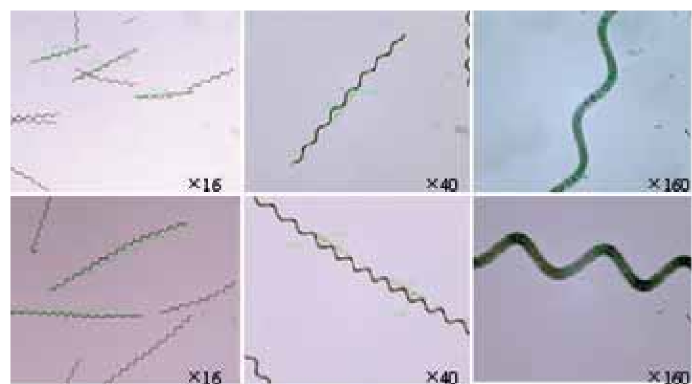

Figure 3 Microstructure of ST-6 (upper row) and PNK-2(lower row)

Biochemical character of PNK-2

The nutrients of the same batch of spirulina powder were analyzed by the authoritative institution (**Table 4**). The protein content in PNK-2 was 69.57% and increased by 8.31% than ST-6. The contents of chlorophyll, β-Carotene and phycocyanin in PNK-2 were 1.01%, 0.16% and 14.70%, respectively, having increased respectively by 8.60%, 6.67% and 6.68% compared to ST-6. Conversely, the γ-linolenic acid content of PNK-2 was 0.63%, i.e. reduced by 3.08% compared to the original strain.

Table 4. Comparison of nutrition contents between PNK-2 and ST-6

Species	Protein	Chlorophyll	β-carotene	Phycocyanin	γ-linolenic acid
ST-6	64.23%	0.93%	0.15%	13.78%	0.65%
PNK-2	69.57%	1.01%	0.16%	14.70%	0.63%

Discussion

Arthrospira platensis underwent great changes under the integrative effect of the space environment. The productivity of PNK-2, which was selected from the mutants, was increased by 22.89%. Yield of large-scale production increased by over 20%. The contents of protein, chlorophyll, β-Carotene and phycocyanin in PNK-2 were greatly increased. The protein content in particular reached 69.57% in PNK-2. All suggested that PNK-2 was a good quality and high-yielding *A. plantensis* species, and could be retained for large-scale production.

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Improvement of Taro (*Colocasia esculenta* var *esculenta*) Through *In Vitro* Mutagenesis

S Seetohul, V Maunkee* & M Gungadurdoss

Abstract

An *in vitro* mutation programme was implemented to improve taro (*Colocasia esculenta* (L). schott) for resistance to the fungus *Phytophthora colocasiae*. Apical shoot tips used as explants were cultured on Murashige and Skoog medium (1962) with varying concentrations of Indole-3- acetic acid, Thidiazuron (1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) and N6-benzylaminopurine (BA). Optimal culture initiation and multiplication was obtained on MS supplemented with 10 mgL^{-1} of IAA and 0.9 mgL^{-1} TDZ/BA at 20 mgL^{-1}, respectively. Explants were exposed to various doses of gamma radiation and the effective mutation dose that causes 30% reduction in growth (LD_{30}) was found to be 7.65 grays. Nine accessions of *colocasia species* (dasheen and eddoes type) and two from *Xanthosoma* species were used for morphological and molecular characterization. 44 morphological characters were assessed and analysed with an unweighed pair group method using an arithmetic average (UPGMA). For RAPD analysis, eight 10-mer random primers were selected as they amplified more than five polymorphic bands. UPGMA cluster analysis using Nei and Li's distance coefficient were then performed. Both morphological and molecular analysis revealed low genetic diversity among germplasm accessions. RAPD primers screened will be useful for characterization of mutant lines showing resistance to leaf blight while the micropropagation methodology developed will be useful for rapid multiplication of mutants.

Introduction

Taro (*Colocasia esculenta* (L) *Schott*), which is considered to have originated in South East Asia [1] [2], is the most important edible member of the aroid family (*Araceae*). In Mauritius it is a highly appreciated commodity. However, its production has dropped drastically from 480 tons in 1995 to about 40-50 tons in 2003 due to severe attacks caused by the fungus *Phytophthora colocasiae*. Phytophtora leaf blight disease, which is known as the most destructive disease of taro and was identified for the first time in Mauritius in 1995, affected all local taro germplasm. Taro is a vegetatively propagated crop, which limits genetic variation within the species, and local quarantine regulations prohibit introduction of new resistant germplasm. Hence, an *in vitro* mutation breeding programme was implemented to develop mutant lines showing resistance to *Phytophtora colocasiae*.

This study focuses on the development of modern *in vitro* culture and molecular techniques to accelerate the improvement of taro for increased disease resistance.

Materials and Methods

In vitro mutagenesis of taro

In vitro initiation and multiplication media test

Explants were taken from axillary buds of developing suckers of taro. These were cleaned and rinsed with running tap water for one hour and washed in a solution of benomyl (Benlate 50WP, 0.06%) for 15 minutes. They were then dipped in sodium hypochlorite (2%) for 15 minutes. Explants were established on Murashige and Skoog [3] medium with different concentrations of Indole–3-acetic acid (IAA) (0, 10, 15, 20 and 25 mgL^{-1}) for culture initiation. For multiplication, media based on MS medium with Thidiazuron (1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) at 0, 0.3, 0.6, 0.9 1.0 and 1.2 mgL^{-1} and N6-benzylaminopurine (BA) at 0, 1, 2, 3 and 4 mgL^{-1} were tested. All media were solidified with Phytagel (0.18%) and had a pH adjusted to 5.7±0.1 before autoclaving for 15 min at 121°C. Explants were cultured at 23±2°C under 12-hour photoperiod.

Determination of effective mutation dose (LD30)

Thirty apical shoot-tips of 3-4mm, excised from tissue culture plantlets, were irradiated with 2, 4, 6, 8, 10, 12, 14, 16, 20, 40 and 60GY. The control consisted of non-irradiated shoot tips. Explants were cultured on MS media with IAA (10mgL^{-1}). Data on percentage survival, number of leaves and roots, length of leaves and roots and number of buds was recorded weekly for eight weeks.

Determination of optimal hardening substrate for taro plantlets

Ten treatments of various combinations of composted scum, flyash, bagasse, vermiculite and peat were tested against control, imported substrates, which consisted of a mixture of lecca, peat and vermiculite in the ratio of 2:5:1. Each treatment consisted of three trays with 50 tissue-cultured plantlets per tray. Data on percentage mortality, number and length of leaves and root length was taken after 30 days.

Morphological and molecular studies of taro germplasm

Survey and Germplasm collection

Germplasm collection activities were undertaken from various parts of the country. Detailed data on place of collection, sample type, type of vegetation, habitat/ecology for collected germplasm accessions as shown in **Table 1** was recorded. From each location, random samples of plants were collected to constitute the population sample.

Table 1. Germplasm used for characterization

Colocasia esculenta spp			Xanthosoma spp
Dasheen type		Eddoes	
Arouille violette – giant (Avg,)	Songe Blanc (Sb)	Arouille carri – Marron (Acm)	Arouille Popette-Blanc (Apb)
Arouille violette – dwarf (Avd)	Songe Noire (Sn)	Arouille carri – vert (Acv)	Arouille Popette-marron (SI)
	Songe Réunion (Sr)	Arouille carri – blanc (Acb)	
	Songe wild type (Wt)		

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Morphological characterization

Morphological traits and taxonomical values in taro

The collected germplasm accessions, including *Colocasia esculenta* (*Dasheen and Eddoes* type) and two accessions from the *Xanthosoma* species were included in this study. The original collections were first multiplied and then grown at Reduit Crop Research station. All the accessions were grown in replicated trial (three replicates in randomized block design) following optimum agronomic practices. Forty-four morphological traits were described and quantified according to the IPGRI 1999, descriptors for taro (*Colocasia esculenta* var. *esculenta*). Data was recorded on five randomly selected plants per accession.

Molecular Characterization of collected germplasm

DNA extraction and quantification

Total genomic DNA was extracted from young rolled leaves of taro following the cetyl trimethylammonium bromide (CTAB) method [4] with modifications. The DNA concentration (after RNase treatment and phenol chloroform extraction) was quantified using a UV spectrophotometer.

RAPD analysis of taro germplasm

A series of optimization experiments was conducted where concentrations of template DNA, primers and $MgCl_2$ were varied to determine which conditions gave the strongest and most reproducible patterns. The optimized PCR reaction mixture (30ml) consisted of 40ng DNA, 1 x PCR buffer, 4mM $MgCl_2$, 200 µM dNTP mix of dATP, dTTP, dCTP,and dGTP, 0.2µM primer and 1U *Taq Polymerase* (Bioline, USA). PCR amplification was performed in a PTC-100 programmemable thermal cycler, programmed to pre-denature DNA for 5 min at 94°C, denature DNA at 94°C for 1 min, anneal DNA to primers at 35°C for 1 min and polymerize DNA for 2 mins at 72°C. After 45 cycles, the programme allowed a final extension of 5 min at 72°C before maintaining at 4°C. Following amplification, RAPD products (10µl) were loaded in 1.5% agarose gels in TBE buffer (Tris /Borate /EDTA), separated by electrophoresis at 90-100 V for about 2½ hours, and photographed on a UV transilluminator. The size of amplification products was determined by comparison with Hyperladder II.

Initially, 62 random decamer primers from commercially available primer series (Inquaba Technology) were screened using the DNA from four accessions.

Data Analysis

In this study, pair-wise comparisons of taro genotypes, based on the presence or absence of unique and shared fragments produced by RAPD amplifications, were used to generate similarity matrices based on Nei and Li's [5] definition of similarity which is as follows:

$$Sij = 2a/(2a + b + c)$$

where Sij is the similarity between two individuals *i* and *j*, a is the number of bands present in both *i* and *j*, b is the number of bands present in *i* and absent in *j*, and c is the number of bands present in *j* and absent in *i*; and this also known as the Dice coefficient.

The similarity matrix was then analyzed using UPGMA clustering methods. Analyses were performed using the software " Population." (CNRS;http://www.cnrs-gif.fr/pge).

Results and Discussion

In vitro mutagenesis studies of taro

In vitro initiation and multiplication media test for taro

In initiation media, healthier and more vigorous growth in terms of highest average number of leaves and roots was obtained with IAA at 10 mg L^{-1} (**Fig. 1**). There were significant differences at a 5% level in number of leaves between the different IAA concentrations. MS media with beyond 15mg L^{-1} IAA did not support growth of taro explants compared to hormone-free MS medium, where growth may be attributed to the effect of endogenous growth regulators. Similar observations regarding the role of endogenous growth regulators in determining the shoot forming capacity of leaf disks have been reported in tomato [6, 7]. Another study [8] has also demonstrated that a critical endogenous level of growth regulators has to be attained before cell division and organogenesis can occur.

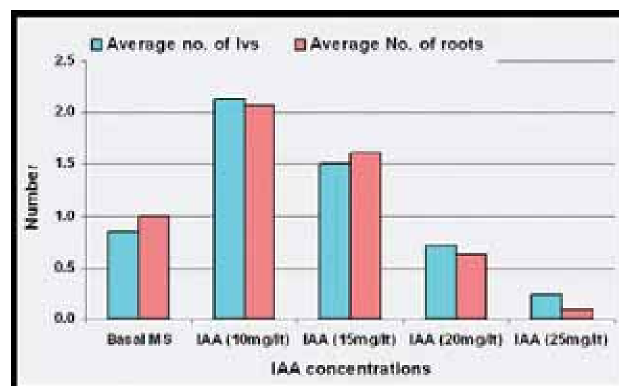


Figure 1 Effect of different concentrations of IAA on *in vitro* initiation of taro at eight weeks.

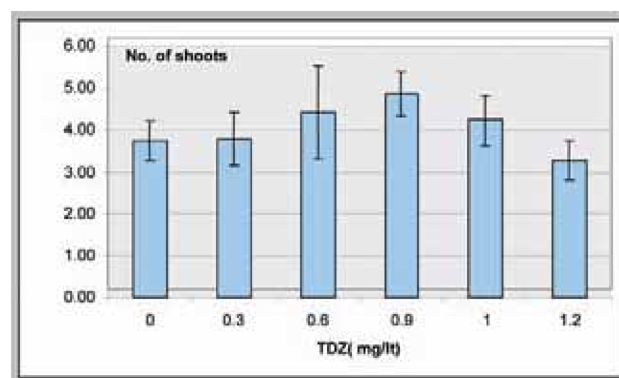


Figure 2 Effect of different concentrations of TDZ on *in vitro* multiplication of taro at eight weeks.

Determination of effective mutation dose (LD_{30})

Among all the parameters studied, number of leaves was considered in determining LD_{30} because it resulted in the least experimental error. The average number of leaves after 18 days, of shoot-tips treated with 2Gy was greatest, even more than that of the control, indicating the boosting effect of this dose (**Fig. 3**). A similar response was obtained when Anthurium Andreanum *in vitro* leaf explants were irradiated with 5Gy. The calli and seeds also expressed better responses at the 5Gy, but lethality at 15Gy [9]. In this study, irradiation doses above 20Gy were lethal to taro explants. Effective mutation dose is controlled by a number of parameters including the genotype, the type of explant, the orientation of explant on the culture medium, and the origin of the explant from the mother plant [10]. Data recorded on the number of leaves showed that the effective mutation dose, which caused a 30% reduction in growth, was 7.65Gy as shown in **Fig. 4**.

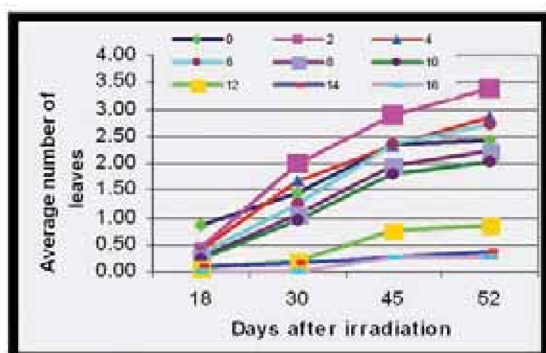
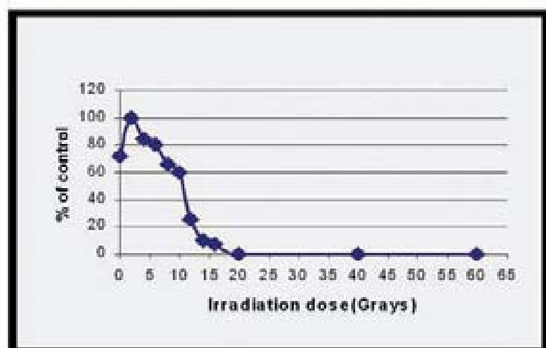


Figure 3 Response of *in vitro* culture of shoot tips of taro after irradiation.



Days after irradiation	LD30(Gy)
30	6.50
45	8.45
52	8.00
Average	**7.65**

Figure 4 Effective mutation dose which caused 30% reduction in growth.

Determination of optimal hardening substrate for taro plantlets

Almost no mortality was recorded in three treatments, viz. 50% scum + 50% bagasse, 70% Scum + 30% bagasse, and 100% peat. The number of leaves per plant ranged from three to five for all treatments. However, the highest growth of leaves in terms of length four weeks after hardening was recorded with 60% scum + 40% fly ash (13.60cm), and with 50% vermiculite + 50% peat (13.63cm). Vermiculite and peat being very expensive, the locally available substrate 60% scums + 40% fly ash from sugar cane industry was selected for hardening of *in vitro* taro plantlets.

Morphological characterization of taro germplasm

The UPGMA tree based on morphological traits is shown in Fig 6. Morphological observation showed that the Dasheen type could be distinguished from Eddoes by the presence of a main corm, stolons and absence of suckers, while Eddoes are characterized by a presence of cormels and suckers. However, it was difficult to distinguish between the two dasheen types "*Songe*" (*Sb* and *Wt*) and "*Arouille*" (*Avg* and *Avd*). Pair-wise genetic distances based on morphological traits for the 11 taro genotypes, ranged from 0.22-0.83. The dendogram indicates that the majority of taro genotypes were clustered in three groups. As expected, both AP and SI, belonging to the *Xanthosoma species*, are clustered together, distantly apart from others. The Eddoes type (*Colocasia esculenta* var. *antiquorum*) namely *Acv, Acb* and *Acm* were separated in another group.

RAPD Analysis of taro germplasm

Table 2 lists the eight primers selected as useful for RAPD markers of taro based on their ability to generate complex and scorable amplification patterns. Figure 8.0 shows the RAPD banding profile obtained with primer ONP 07. A total of 91 RAPD bands ranging from 200bp to 3.5Kbp were obtained, of which 95.7% were polymorphic with an average of 11 bands per primer. Pair-wise genetic distance based on RAPD data for the 11 taro genotypes ranged from 0.12 to 0.56 indicating low diversity. The analysis method UPGMA was used to provide detail insights into the genetic relationships (**Fig. 7**).

Table 2. Most informative RAPD primers, total number of bands, number of polymorphic bands and proportion of polymorphic bands

Primer	Sequence	Total No. of amplicons	No. of polymorphic bands	% Polymorphism	Fragment size (bp)
OPO 03	CTGTTGCTAC	10	10	100	500-1750
ONP 07	CAGCCCAGAG	16	16	100	750-2750
OPC 16	CACACTCCAG	8	8	100	500-1400
OPA 17	GACCGCTTGT	15	14	93	350-2000
OPC 08	TGGACCGGTG	8	8	100	400-1000
OPP 16	CCAAGCTGCC	14	13	92.8	1250-3500
OPO 19	GGTGCACGTT	11	10	91	800-1400
OPO 07	CAGCACTGAC	9	8	88.8	200-1000

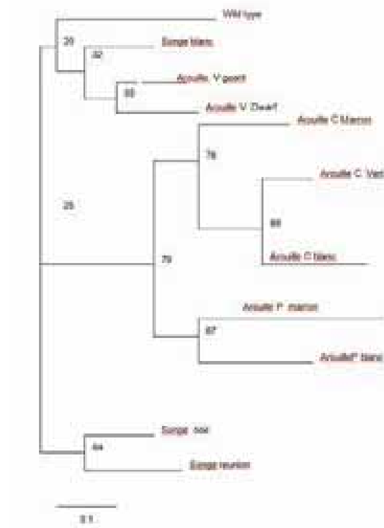


Figure 6 UPGMA dendogram based on 44 morphological data.

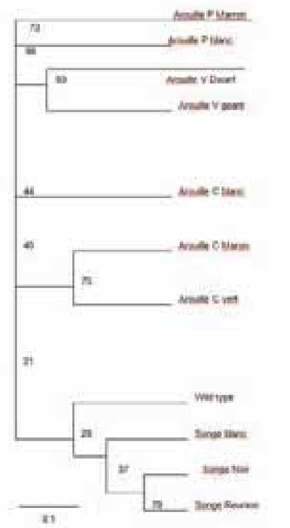


Figure 7 UPGMA dendogram on 11 taro genotypes based on RAPD data. Numbers represent percent bootstrap support for each node.

Based on RAPD data, the taro germplasm has been classified in three groups, with *SI* as an out-group. *SI*, which is also known as "*Arouille Popette marron*" together with *AP* (*Arouille popette blanc*), belong to the *Xanthosomas spp*. The three types of "*Arouille carri*" (*Acm, Acv, Acb*) were grouped together. This is expected as they form part of the eddoes type of *Colocasia esculenta*, var *antiquorum* (*triploid*), as compared to *Avd* and *Avg*, which belong to the variety *esculenta (diploid)*. *SR*, believed to be introduced in Mauritius from Reunion Island was found closely related to *SN* (songe noire), hence grouped together, while songe "wild type" *Wt* is more related to *SB* (songe blanc) within the group. Resistance to *Phytophthora* leaf blight disease caused by *Phytophthora* colocasiae is observed in *Wt, SR, SI* and *AP*.

Analysis of morphological and RAPD markers to assess diversity

The dendograms obtained from both morphological markers and genetic variations indicate that the majority of the 11 taro genotypes

were clustered in three groups, with low genetic diversity. Studies carried out by [11] revealed high genetic diversity among Indonesian germplasm and were able to distinguish between Hawaiian accessions by using RAPD markers. The low genetic diversity of our local germplasm can be explained by the fact that taro in Mauritius is propagated mainly by vegetative means, and although it bears flowers, these are sterile.

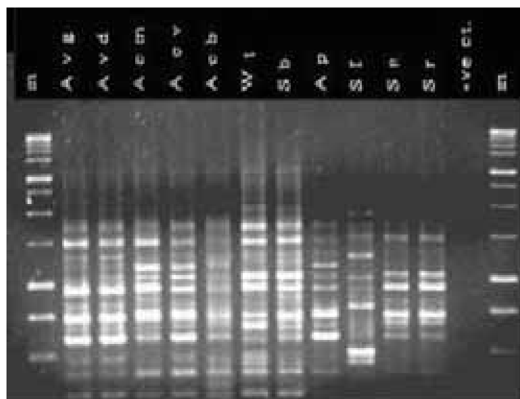


Figure 8 RAPD profile obtained with primer ONP 07. Lanes 1-11 accessions of taro (nine *Colocasia esculenta* spp. & 2 *Xanthosoma* spp) spp. Lane m: DNA size marker. A total of 16 amplified products ranging from 750 -2750 bp were produced. A unique fragment of 1000 bp was obtained with *SI*, differentiating this genotype from the rest of the group. Similar fingerprints were obtained with Avd and Avg and a common band of 2,500 bp is shared among Acm, Acv and AP. **1: A.Violette (giant); 2: A.Violette (dwarf); 3: A.Carri marron; 4: A.carri verte; 5: A.Carri blanc; 6: Wild type; 7: Songe Blanc; 8: Arouille Popette blanc; 9: Arouille Popette Marron; 10: Songe Noire; 11: Songe Reunion; 12: Negative control.**

In the dendogram based on RAPD data, *AP* and *SI* belonging to *Xanthosoma* spp, although branched out from the *colocasia spp*, were found to be related to the Dasheen Type (*Avd* and *Avg*) of this species. However, based on morphological traits, *AP* and *SI* although separated from others were closely related with the Eddoes type (*Acv*, *Acb*, *Acm*). This inconsistency in both RAPD and morphological analysis can be explained by several factors. The genetic origin of each RAPD marker is different, while morphological expression (phenotype) is conditioned by the state of the plant, by agricultural management and by the environmental conditions in which it develops. On the other hand, it is possible that the dominant nature of RAPD markers and RAPD technique can be limited to detecting polymorphism in cases of heterozygosity [12]. In such cases, only variables with strong genetic control should be quantified in morphological trait analysis, and other more effective molecular markers such as AFLP, SSR or ISSR should be used to obtain more precise taxonomic clusters within taro genotypes of low genetic diversity.

Conclusion

In this study the optimal initiation, multiplication and hardening media, along with the effective mutation dose were determined for taro. Such micropropagation methodology will be very useful for the rapid multiplication of promising mutant lines developed. The RAPD primers already identified for taro will be very useful for characterizing taro mutant germplasm showing resistance to Taro Leaf Blight disease, caused by *Phytophthora colocasiae*. Furthermore, these primers will also be very useful for future genetic analysis and provide taro breeders with a genetic basis for selection of parents with disease resistance in other crop improvement programmes.

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Role of Classical Mutagenesis for Development of New Ornamental Varieties

S K Datta

Abstract

Mutation techniques by using ionizing radiations and other mutagens have successfully produced and commercialized a large number of new promising varieties in different crops including ornamental plants worldwide. Considering the importance of induced mutagenesis, extensive work on classical mutagenesis has been carried out by the author, who has successfully developed 76 new mutant varieties using gamma radiation in different ornamentals. Research carried out generated voluminous literature on radio-sensitivity, selection of materials, methods of exposure to gamma-rays, suitable dose, detection of mutations, isolation of mutants and commercial exploitation of mutants. Different treatment methodologies such as recurrent irradiation, colchicine treatment, and mutation frequency and spectrum, have been precisely determined for successful development of new varieties. Changed flower type, development of appendage like structure on florets, striped flowers and induction of tubular florets are a few of the interesting observations in chrysanthemum. Development of late blooming varieties in chrysanthemum has tremendous commercial impact. Studies have clearly proved that classical mutagenesis can be exploited for the creation of new and novel ornamental varieties of commercial importance.

Introduction

As a result of science-based techniques and steady supply of improved plant materials floriculture has become a very important industry in many countries. The possibilities of creating different forms and improving ornamentals are infinite and a breeder will always have goals to work towards. Mutation is recognized as one of the most important technologies for the development of new varieties through genetic manipulation. Mutation techniques by using ionizing radiations and other mutagens have successfully produced a large number of new promising varieties in different ornamental plants. It will not be possible to consider all the scientific papers published in the field of application of mutagenesis for improvement of ornamental plants due to limitations of space. The details of utilization of induced mutations and its prospects and released mutant varieties have been reported [1-6]. The present paper will cover most of the basic information generated by the author during his practical work at National Botanical Research Institute (NBRI), Lucknow, India, for the successful application of classical induced mutagenesis for improvement of different ornamental crops. While describing the work carried out by the author on bougainvillea, chrysanthemum, hibiscus, gladiolus, perennial *portulaca*, *Polianthes tuberosa*, rose, *Lantana depressa* etc., attempts will also be made to incorporate important results obtained by other workers for improvement of ornamental plants.

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Materials and Methods

Plant materials

For practical experiments different ornamentals like bougainvillea (80 cvs.), chrysanthemum (150 cvs.), gladiolus (8 cvs.), *Hibiscus rosa-sinensis* cv. Alipur Beauty, perennial *portulaca*, *Polianthus tuberosa* (3 cvs.), rose (50 cvs.), *Lantana depressa* etc. were selected as materials.

Treatment methodology

Suitable propagules of above mentioned ornamentals were treated with different doses of Gamma-rays (cobalt 60 radiation source) as follows :

Ornamental plant	Propagule	Dose
Bougainvillea	Stem cuttings	250– 250 rads
Chrysanthemum	Rooted cuttings/suckers	1.0-3.5 krad
Gladiolus	Bulb	250 rads–5 krad
Hibiscus	Stem cuttings	1.0–4 krad
Perennial portulaca	Stem cuttings	250–1250 rads
Polianthus tuberosa	Bulb	250 rad–8 krad
Rose	Stem with budding eyes	2–6 krad
Lantana depressa	Stem cutting	1–4 krad

Recurrent irradiation

Recurrent irradiation experiments were carried out on chrysanthemum cv. 'Sharad Bahar' and rose cv. 'Contempo' as per experimental design mentioned earlier [7-8].

Colchicine treatment

Budwoods of rose cv. 'Contempo' and rooted cuttings of chrysanthemum cv. 'Sharad Bahar' were dipped in 0.0625 and 0.125% aqueous solution of colchicine for 4 and 5 hrs., respectively. Treated materials were washed thoroughly and planted in bed following all cultural practices [9-10].

Treatment of mutant genotypes

Gamma-ray induced mutant genotypes of chrysanthemum were treated with different doses of Gamma-rays for further improvement.

Results

Radiosensitivity and suitable mutagen dose (LD_{50} dose), a prerequisite for large-scale irradiation for induction of mutations of all experimental cultivars have been determined precisely [11-13]. Radiosensitivity has been estimated with respect to influence of various factors including flower type, shape and color, chromosome number, Interphase Chromosome Volume, Interphase Nuclear Volume and 2c DNA content after treatment with different doses of Gamma-rays. On the basis of these observations and available literature it has been very clear that radiosensitivity in different ornamentals is a genotype-dependent mechanism [14-15].

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The frequency of mutation varied with the cultivar and dose of Gamma-rays. Some cultivars were moderately sensitive, some were more sensitive and some were resistant to mutagens. Like mutation frequency, the spectrum of mutation also varied with the cultivar and dose of Gamma-rays. NBRI has developed 76 new mutant varieties using gamma radiation in different ornamentals (5 bougainvillea, 6 perennial *portulaca*, 43 chrysanthemum, 1 *Hibiscus*, 16 rose, 2 tuberose, 3 *Lantana depressa*) [16]. The mutations concerned mainly flower color/shape and chlorophyll variegation in leaves. Thus, almost all the experimental colors could be mutated, including yellow color from white. More than one flower color mutation could also be induced in different cultivars. Phenotypic variation, including several interesting novel changes in flower form, have been recorded. Induction of tubular florets was one of the interesting observations in chrysanthemum [5].

Chlorophyll variegation in leaves provides additional beauty to plants not only at the time of blooming but also even when there is no flower. A wide range of variegated plants have been developed through mutagenic treatment in different ornamentals. A number of chlorophyll variegated mutants have been developed and released in Bougainvillea, Tuberose and *L. depressa* [17]. Some of the most promising and beautiful variegated mutants induced by gamma irradiation in bougainvillea are 'Arjun,' 'Pallavi,' 'Mahara Variegata,' 'Los Banos Variegata' and 'Los Banos Variegata Jayanthi' [18]. 'Rajat Rekha' and 'Swarna Rekha' are the most promising chlorophyll variegated mutants of single and double flower Tuberose respectively.

In most cases, chlorophyll variegation developed as a mericlinal chimera. For establishment of mericlinal chimeric branches into a new variety (as periclinal form), all green leaves of the mericlinal branch were removed to avoid diplontic selection and to provide better growth for the auxiliary buds of the variegated leaves. Ultimately, new branches developed from the auxiliary buds of the variegated leaves, which were either periclinal or mericlinal. For a mericlinal branch, the same process was repeated, while periclinal variegated branches were multiplied directly by cuttings for establishment of a new variety. This technique was standardized with *Lantana depressa* and bougainvillea to convert mericlinal chimera into periclinal chimera [19-20].

For the first time, colchicine has been used to develop new flower colors in chrysanthemum (21-23) and rose (24-26). Chrysanthemum cv 'Sharad Bahar' was purple, whereas the mutant ('Colchi Bahar') color was terracotta red. The original color of rose cv 'Contempo' was orange with yellow eye and the mutant colors were tangerine orange and empire yellow.

Recurrent irradiation experiments with chrysanthemum and rose resulted in more genetic variability. Percentage of mutations and spectrum of mutations was more in recurrent irradiated populations in both of these species. In chrysanthemum, two new flower colors were detected in the recurrent irradiated population. It is concluded that recurrent mutagen treatment may provide an even greater range of genetic variability than a single mutagen treatment in vegetatively propagated ornamentals. This method can be successfully used in routine mutagenesis programmes for inducing novelties in flower color and shape [7-8].

Somatic mutations in all the above mentioned plants were mostly detected in M_1V_1. But mutations have also been detected in M_1V_2, M_1V_3 and later vegetative generations from normal looking irradiated plants in M_1V_1. It has been observed that the chances of getting solid mutants were higher in M_1V_2 and later generations. The mutated cells of the lower auxiliary buds remain in the dormant stage (M_1V_1) and express their mutant character when included during vegetative propagation in M_1V_2 [5].

Sensitivity of mutant genotypes of chrysanthemum was found to be the same as the original cultivars. A wide range of flower color mutants were obtained, not only largely including the outstanding characteristics of the original cultivars, but sometimes even with an appreciable improvement in quality and yield [27-30].

A major drawback of classical mutagenesis is the formation of chimeras. The concept of management of chimera and *in vitro* mutagenesis has opened new possibilities for inducing increased number of mutants and solid mutants in a relatively short period of time [31-33]. Extensive practical work has been carried out by the author and his team on management of chimera and *in vitro* mutagenesis which has been discussed in more detail in another paper elsewhere in this volume (number IAEA-CN-167-284P).

Discussion

The main advantage of mutation induction in vegetatively propagated crops is the ability to change one or a few characters of an otherwise outstanding cultivar without altering the remaining and often unique part of the genotype [1]. Mutation breeding has been most successful in ornamental plants due to some additional advantages. Changes in any phenotypic characteristics like color, shape or size of flower and chlorophyll variegation in leaves can be easily detected. The heterozygous nature of many of the cultivars offers high mutation frequency. Mutation techniques by using ionizing radiations and other mutagens have successfully produced a large number of new promising varieties in different ornamental plants. Voluminous literature has been published in induced mutagenesis. Different basic and applied aspects of induced mutagenesis on ornamental plants have been reported earlier [1, 4, 5, 15, 17, 34-36]. Radiation-induced phenotypic variation including several novel changes in flower form have been reported earlier – double flower type begonia [1], chrysanthemum [34], gladiolus [37], hibiscus [38], *Hyacinthus* [1], semi-double or single carnation [3, 40], increase and decrease in petal number in rose [1], variegated and dwarf mutant in *Coleus* [41], dwarf mutants in *Impatiens platypetala* [42], changed flower color in *Lilium* [1, 43], begonia [44], *Streptocarpus* [45], etc. Effects of fractionated doses and different basic aspects of radiation-induced changes have been studied in African violet [1].

Out of all presently available crop improvement techniques, it is very clear that induced mutation breeding is well standardized for the development of new ornamentals, whereas a molecular technique is in progress. Although mutation breeding is considered to be a random (chance) process, the possibility of inducing a desired flower color mutation has been proved in chrysanthemum. If white flower color varieties are irradiated, and if there is any mutation, the mutation will be yellow. Red varieties, on the other hand, will produce either a completely yellow mutation, or a mixture of red and yellow. If yellow varieties are irradiated, and if there is any mutation, the mutation will be either different shades of yellow or white or a mixture of yellow and white (46). The product of molecular breeding is expected to be as per desire, but it will take time to develop a reproducible regeneration and transformation system. Induced mutants are mostly stable. Flower color mutants induced 25 years ago are still maintaining purity at NBRI, Lucknow, India. There are numerous reports for the variation and instability of genes in transgenic plants. It has been reported that a gene can lose its transcriptional activity after crosses into the next generation or after the introduction of another foreign construct into such a transgenic line. Induced mutagenesis and mutant induction through the application of classical mutagenesis is a relatively easy-to-apply technology for production of novel varieties in ornamental breeding. At this stage, induced mutation breeding combined with *in vitro* chimera management has tremendous potential for developing new flower colors and shapes.

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Management of Chimera and *In Vitro* Mutagenesis for Development of New Flower Color/Shape and Chlorophyll Variegated Mutants in Chrysanthemum

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Abstract
Mutation breeding is an established method for crop improvement and has played a major role in the development of many new flower color/shape mutant varieties in ornamentals. The main bottleneck with vegetatively propagated plants is that the mutation appears as a chimera after treatment with physical and/or chemical mutagens. A small sector of a mutated branch or flower cannot be isolated using the available conventional propagation techniques. A novel technique has been standardized for the management of such chimeric tissues through direct shoot regeneration from chrysanthemum florets. This direct novel regeneration protocol has been successfully used not only for the isolation of chimeric mutant tissues developed through sports, but also to develop a series of new flower color/shape mutants through induced mutagenesis. Gamma radiation and tissue culture techniques have been optimized to regenerate plants from stem internodes, stem nodes, shoot tips and ray florets for *in vitro* management of chimera and for *in vitro* mutagenesis. Chimera isolation has practical importance not only for chrysanthemum but for breeding of other ornamentals also. The present technique will open up a new way for isolating new flower color/shape ornamental cultivars through retrieval of mutated cells.

Introduction
Induced mutagenesis is an established method for plant improvement, and the technique has been successfully exploited for development of new and novel ornamental varieties in general and chrysanthemum in particular [1-3]. A large number of new flower color varieties have developed in chrysanthemum through sports. The main bottleneck of induced mutagenesis with vegetatively propagated plants is that the mutation appears as a chimera after treatment with physical and/or chemical mutagens. Sports are also chimeric in nature. Mutated cells are present along with normal cells in chimeric tissues. The size of the mutant sector varies from a narrow streak on a petal to the entire flower head and from a portion of a branch to the entire branch. The mutated tissue can be isolated in pure form when a portion of a branch or an entire branch is mutated. However, a small sector of a mutated branch or flower cannot be isolated using the available conventional propagation techniques. Therefore, many mutagen-induced new flower color/shape mutants or spontaneously developed mutants are lost due to lack of microtechnique for management of such chimeric tissues either *in vivo* or *in vitro*. *In vitro* regeneration methods for chrysanthemum are well established [4-8]. Reports of adventitious shoot regeneration from floret explants of chrysanthemum are also available, but in all cases shoots were produced from floret-derived callus [9-11], and there is always a loss of somatic genetic homogeneity with a lengthy callus phase [12-13]. Here, we report a novel technique, standardized for isolation of new chimeric flower color/shape mutants through *in vitro* direct shoot regeneration from ray florets and development of solid mutants through *in vitro* mutagenesis in chrysanthemum.

Materials and Methods

Plant materials
Chrysanthemum morifolium Ramat was selected as material for management of chimera and *in vitro* mutagenesis work.

Plant sport
In the germplasm collection of National Botanical research Institute, Lucknow, India, one plant of large white flowered chrysanthemum cv. 'Kasturba Gandhi' developed a few yellow florets due to a spontaneous mutation. Yellow florets were collected and subjected to direct regeneration of shoot buds using suitable tissue culture medium [14-15].

Gamma irradiation
Rooted cuttings (13 cm height) of different December-January flowering chrysanthemum ('Puja,' 'Lilith,' 'Maghi,' 'Purnima' and 'Colchi Bahar') cultivars were treated with 1.5, 2.0 and 2.5 Krad of Gamma-rays (^{60}Co radiation source) and grown in the field u pto flowering. Plants showing chimeric mutated florets were selected, and mutated ray florets were cultured on agar-solidified basal medium [14] supplemented with sucrose and different combinations of BAP/kinetin and NAA [16-20].

Surface sterilized ray florets of different cultivars ('Lalima,' 'Flirt,' Puja,' 'Maghi' and 'Sunil') were treated with 0.5 and 1.0 krad Gamma-rays before transfer to culture. All the regenerated shoots were rooted in suitable medium and rooted plantlets were subsequently planted in field and observations were taken until flowering [21-22].

Results and Discussion
All the direct and callus regenerated plants from chimeric florets of 'Kasturba Gandhi' produced flowers true to the chimeric floret color i.e. yellow [16]. The other morphological characters of 'Kasturba Gandhi' and its new yellow flower color mutant were the same (**Fig. 1V-Y**).

'Maghi' is a pompon type, mauve color, small-flowered, late-blooming variety of chrysanthemum. One plant each from 1.5 and 2.0 krad treatment showed few chimeric florets with new colors of one flower-head. The new colors were white and yellow. Plants were regenerated in pure form from both the mutated florets on MS medium containing 0.2 mg l^{-1} NAA and 1 mg l^{-1} BAP [15]. 'Maghi' is a late-blooming variety, flowering in the end of January under the sub-tropical Lucknow climate, India, when no other chrysanthemum flowers are available. The isolated white and yellow varieties are very attractive and will create a good impact in the floriculture industry (**Fig. 1A-H**).

'Lilith' is a double Korean, white small-flowered chrysanthemum. Two and three plants showed flower color mutations in 1.5 and 2.0 krad respectively. In all cases the flower color mutation was yellow. A total of 59 yellow ray florets from sectorial flower head chimera were inoculated in media and 14 explants showed shoot bud formation. The floret color

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of all the tissue culture raised plants was yellow, i.e. true to the explant color [19] (**Fig. 1R-U**).

Chlorophyll variegation in leaves was detected after treatment with gamma radiation in cvs. 'Maghi' and 'Lilith.' Chlorophyll variegations were regenerated subsequently through vegetative propagation, i.e. by cuttings. *In vitro* direct regeneration from variegated leaf explants could not be standardized.

Figure 1 Management of chimera for development of new flower color/shape and chlorophyll variegated mutants in chrysanthemum. **(A)** Original chrysanthemum cv. Maghi, **(B)** Sectorial chimera (yellow) of cv. Maghi, **(C)** Sectorial chimera (White and Yellow) of cv. Maghi, **(D)** Shoot bud regeneration from mutated segments of ray florets of Maghi, **(E)** Plant established in pure form as new variety from sectorial white mutated tissue of Maghi, **(F)** Plant established in pure form as new variety from sectorial yellow mutated tissue of Maghi, **(G-H)** Chlorophyll variegated mutants isolated from Maghi, **(I)** Sectorial chimera (yellow) of cv. Puja, **(J)** Mutated florets of Puja, **(K)** Shoot bud regeneration from mutated segments of ray florets of Puja, **(L-M)** Plant established in pure form as new variety from sectorial mutated tissue of Puja, **(N)** Original chrysanthemum cv. Purnima, **(O)** Sectorial chimera (yellow) of cv. Purnima, **(P)** Shoot bud regeneration from mutated segments of ray florets of Purnima, **(Q)** Plant established in pure form as new variety from sectorial yellow mutated tissue of Purnima, **(R)** Original chrysanthemum cv. Lilith, **(S)** Sectorial chimera (yellow) of cv. Lilith, **(T)** Shoot bud regeneration from mutated segments of ray florets of Lilith, **(U)** Plant established in pure form as a new variety from sectorial yellow mutated tissue of Lilith, **(V)** Original chrysanthemum cv. Kasturba Gandhi, **(W)** Spontaneous sectorial chimera (yellow) of cv. Kasturba Gandhi, **(X)** Shoot bud regeneration from mutated segments of ray florets of Kasturba Gandhi, **(Y)** Plant established in pure form as new variety from sectorial yellow mutated tissue of Kasturba Gandhi.

'Purnima' is a ball type, white, large-flowered chrysanthemum mutant, developed through gamma irradiation from 'Otome Zakura' with mauve flower color (**Fig. 1N-Q**). 'Colchi Bahar' is a decorative type terracotta red flower color mutant chrysanthemum, developed through colchicine treatment from 'Sharad Bahar' with purple flower color. Sectorial yellow floret mutations were detected after irradiation with 1.5 and 2.0 krad of Gamma-rays in both varieties respectively. Mutated ray florets were cultured on agar-solidified Murashige & Skoog [14] basal medium supplemented with sucrose and different combinations of BAP/kinetin and NAA. Plantlets developed through direct shoot organogenesis flowered true-to-explant floret color in both the cultivars [17, 18].

'Puja' is a decorative type chrysanthemum. Few florets of two flowers from treated population showed chimeric mutations. The original floret color of 'Puja' was red-purple and florets were flat spoon shaped. One of the mutant floret colors was yellow-orange with original flat florets and another mutant floret color was yellow-orange with tubular florets. Regenerated plants flowered true-to-explant floret color and shape [20] (**Fig. 1I-M**).

Ray florets of five decorative type chrysanthemum cvs. 'Lalima,' 'Flirt,' 'Puja,' 'Maghi,' and 'Sunil' after inoculation were irradiated with gamma radiation and all the regenerated shoots were isolated, rooted and transplanted in the field after hardening. Somatic mutations in flower color and floret shape were detected in Gamma-ray-treated population but no chimeric nature of mutation was detected in any plant. All the mutations were solid in nature. 'Lalima' was grey-red and florets were flat spoon shaped. Two mutants were obtained in the 0.5Gy irradiated plants. Both mutants were yellow colored but one had flat spoon shaped ray florets similar to the original cultivar, while the other had tubular florets. The original ray floret color of 'Flirt' was grey-purple. Two types of flower color mutations were observed with 0.5Gy treatments. In one mutant ray floret color was red with yellow tip, while in another mutant the ray floret color was yellow with very fine red stripes. Slight changes in ray floret morphology were observed in both mutants. The ray floret color of 'Puja' was purple, while its mutant developed in 1Gy treatment was darker in color. The mutant detected in 'Maghi' with 1Gy treatment showed change in floret shape. The original shape of ray florets was flat type, while it was tubular in the mutant. The original ray floret color of 'Sunil' was red-purple, but the mutant developed at 1Gy treatment was darker in color. The shape of the mutant ray florets changed from flat type to tubular type. The mutants were propagated vegetatively and have produced true-to-type florets [21, 22].

The difficulty in regenerating whole plants from sectorial mutated tissues is the main bottleneck in induced mutagenesis technique and a huge number of such spontaneous and/or induced mutant tissues were lost. Standardization of such regeneration protocol was most essential for isolation of chimeric tissues for commercial exploitation. The present *in vitro* technique has opened a new way for isolating new ornamental varieties through retrieval of chimeric tissues. This technique has practical importance not only for chrysanthemum but for other ornamentals also. The present experiments also indicated that solid flower color/shape mutants can be developed through direct *in vitro* mutagenesis by avoiding a chimeric phase.

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Development of Promising Seedless *Citrus* Mutants Through Gamma Irradiation

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Abstract

Citrus are highly heterozygous, polygenic plants with a long juvenile period, whereby conventional breeding is laborious, time-consuming and expensive. Therefore, mutation breeding was carried out. Bud woods of two Indonesian local commercial mandarin (*Citrus reticulata* L. Blanco) cv. SoE and Garut, and pummelo (*Citrus grandis* L. Osbeck) cv Nambangan were exposed to Gamma-ray doses of 20, 40 and 60Gy, and irradiated bud woods were then budded onto rootstocks cv. Japanche citroen. Three-year-old untreated and irradiated plants grown in pots were checked for fruit characters such as seeds number per fruit, and color of flesh and skin. Selected promising mutant lines were found in terms of seedlessness in cvs SoE mandarin and Nambangan pummelo, and nearly seedless cultivars were found in cvs Soe, Garut and Nambangan when bud woods were irradiated at 20 and 40Gy. The performance of promising mutant lines obtained is now being observed and are propagated in the field to confirm their stability.

Introduction

Citrus are among the most important fruit crops in Indonesia, and their consumption has increased steadily with an increase in population. In modern and industrialized citriculture, highly adaptable varieties with high quality should be cultivated to secure high profitability. In many countries, the target of *Citrus* improvement has focused on high fruit qualities (e.g., seedless, easy peeling and mandarin types) and disease resistance. These breeding programmes have been carried out through conventional breeding (hybridization), mutation breeding, and biotechnological techniques. However, *Citrus* breeding has been confronted with difficulties such as high heterozygosity, polygenic traits and a long juvenile period, regardless of breeding techniques used.

Several important commercial *Citrus* varieties, such as Washington navel orange, Marsh grapefruit, Shamouti Orange, and Salustiana orange, have arisen as bud "sprout" mutation. Mutation induction techniques such as radiation or chemical mutagens are good tools for increasing variability in crop species because spontaneous mutations occur with an extremely low frequency. Mutation techniques have significantly contributed to plant improvement worldwide, and have made an outstanding impact on the productivity and economic value of some crops [1].

This study describes recent progress of induced mutation by Gamma-rays for breeding promising mutant lines of seedless *Citrus*, namely mandarin (cvs. *Citrus reticulata* L. Blanco cvs. SoE and Garut) and pummelo (*Citrus grandis* L. Osbeck cv Nambangan).

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Materials and Methods

Gamma irradiation and plant maintenance

Fresh, non-dormant bud woods of mandarin (*Citrus reticulata* L. Blanco) cvs. SoE and Garut, and pummelo (*Citrus grandis* L. Osbeck) cv Nambangan were exposed to Gamma-rays at the doses of 0, 20, 40, 60 and 80Gy on April 2003, and were chip-budded onto rootstock 'Japanse citroen' (JC), two days after irradiation. Plants originated from irradiated bud woods along with plants from untreated bud woods were grown in the same plot for further observation. The number of surviving bud woods was measured four months after irradiation and the survival percentage of bud wood was calculated. 'Japanche citroen' (JC) was used as rootstock. Mother plants were maintained in the screen house of the Indonesian Citrus and Subtropical Fruits Research Institute (950 m above sea level) in Batu, East Java, Indonesia. Shoot length of growing bud woods was observed six times during four months.

Morphological Fruits Characters

Morphological characters of fruits were determined based on the International Plant Genetic Resources Institute (IPGRI) protocols [2]. Fruits were collected from the fifth flushes originating from irradiated bud woods (MV5) and more. Fruits were grouped based on the number of seeds per fruit as either seedless (0-5 seeds per fruit) or nearly seedless (6-10 seeds per fruits), according to Varoquaux, *et al.* [3]. Mutated branches for seedless were taken as bud woods, and grafted on to JC rootstocks. These plants were planted on individual pots and harvested fruits were evaluated for seedless.

Table 1. Number of bud wood per treatment and survival percentage of bud wood

No	Cutlivar/Treatments	Survival	Total	Survival %
1	Keprok SoE (20Gy)	33	40	82.5
2	Keprok SoE (40Gy)	16	35	45.7
3	Keprok SoE (60Gy)	0	27	0
4	Keprok Garut (20Gy)	47	52	90.4
5	Keprok Garut (40Gy)	12	23	52.2
6	Keprok Garut (60Gy)	9	38	23.7
7	Pamelo Nambangan (20Gy)	20	21	95.2
8	Pamelo Nambangan (40Gy)	19	24	79.2
9	Pamelo Nambangan (60Gy)	16	25	64.0
Total		**172**	**285**	**59.2**

Results

Survival of bud wood

About 285 bud woods of Keprok SoE and Garut (mandarin) and Nambangan (pummelo) were gamma irradiated to induce variability.

Survival percentage of bud woods to gamma irradiation was different among doses and varieties. Keprok SoE survived up to 40Gy only, while Keprok Garut's bud woods were able to produce new shoots after irradiation at a dose of 60Gy . These two mandarins showed higher survival rate at low doses of Gamma-rays. As indicated in **Table 1**, survival percentage of bud woods of mandarin decreased as gamma irradiation doses increased. Survival percentage on pummelo has shown that this variety was able to survive at a higher percentage at doses ranging from 20-60Gy . In total, only 172 out of 285 bud woods were flushed in the first three months after grafting.

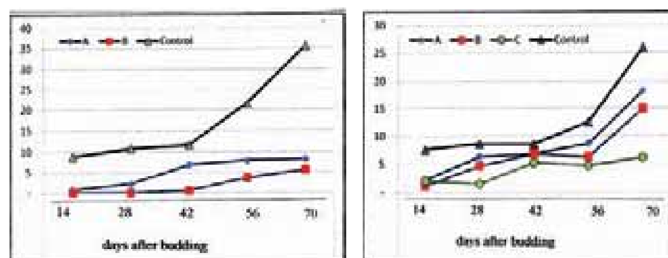

Figure 1 Development of shoot-length (cm) of mandarin from K.Soe and K. Garut (left) and pummelo of Nambangan (right) derived from irradiated shoots observed in the nursery.

Table 2. Variation on seed number per fruit collected and observed from seedless plants

Varieties	Number of seed per fruit	Number of seedless plants			Number of seeds/fruit in untreated plants
		20 Gy	40 Gy	60 Gy	
C. reticulata cv. Keprok SoE	0-5	11	9	0	12-14
	6-10	47	9	0	
C. reticulata cv Keprok Garut	0-5	14	1	0	10-33
	6-10	9	4	0	
C. grandis cv. Nambangan	0-5	3	1	0	65-140

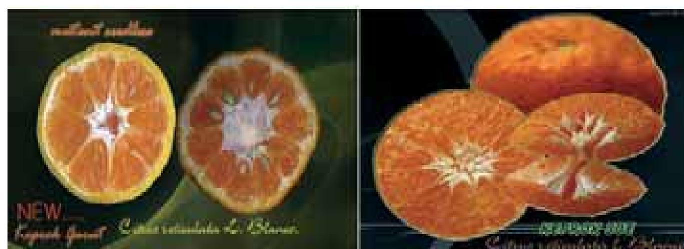

Figure 2 Citrus seedless mutants on *Citrus reticulata* L. Blanco cvs Keprok Garut and Keprok SoE.

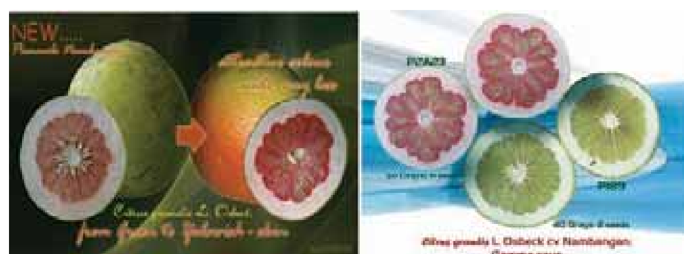

Figure 3 *Citrus* seedless and changed color mutants on *Citrus grandis* L. Osbeck cv. Nambangan.

Time to produce new shoots was also different among doses of gamma and varieties. In Keprok SoE, irradiation delayed the first shoot growth (**Fig. 1**). After four months, there was no difference in shoot growth between gamma irradiation at 20Gy and that at 40Gy .

Long-term observation showed that pumello was resistant to low doses of Gamma-rays (20-40Gy). Seedlings derived from 20-40Gy irradiation treatment repressed shoot growth slightly. On the other hand, plants derived from 60Gy of gamma radiation grew slower than other treatments.

Fruit characters

Fruits were collected from three-year-old trees. In mandarin, 11 and nine fruits checked were seedless (0-5 seeds per fruit) in 20 and 40Gy gamma irradiation treatment, respectively (**Table 2**). Some fruits were completely seedless in Keprok Soe **(Fig. 2**). Gamma irradiation on mandarin also produced nearly seedless fruits (47 and nine seeds at 20 and 40Gy , respectively). All branches were labelled and grafted for further observations.

Gamma irradiation induced variations in fruit characters other than seedlessness, i.e., skin color (yellowish skin), flesh color (green flesh) and endocarp thickness, in pummelo. Four typical new characters were recorded from those branches, namely yellowish skin, green flesh, seedlessness and endocarp thickness (**Fig. 3**). Two branches derived from 20 gray irradiated bud woods bore completely seedless fruits. On the other hand, fruits with two seeds and green flesh were produced in a branch derived from 40 gray irradiated bud woods.

Discussion

Spontaneous and induced mutations have already played an important role in breeding of many fruit crops. Mutagenesis has already been used to improve many useful traits affecting plant size, flowering time and fruit ripening, fruit color, self-compatibility, self-thinning, and resistance to pathogens. Nowadays, the number of cultivars derived from mutation induction increases constantly [4, 5, 6].

Inducing mutations by Gamma-rays has been effectively used with several species of *Citrus*. Irradiation of Gamma-rays on bud wood can produce higher frequencies of mutation, leading to the creation of new variants compared to the control. Selection and testing of *Citrus* trees derived from mutations takes several years before they can be used commercially. In the present study, induced mutagenesis on *Citrus* can improve characters derived form the original variety three years after obtaining mutation bud woods showing seedless characters as well as new color flesh and skin. Even though it is not a final result, this procedure is much faster than conventional hybridization.

In *Citrus,* several attempts at inducing variability were conducted with some reports on seedless, spineless, changed color of fruit and juice [7]. Radio-sensitivity (LD_{50}) of acute exposure of *Citrus* ranges from 40-100Gy [8, 9], depending on species and varieties. Scion (bud wood), seeds, floral stage embryos, and *in vitro* material of *Citrus* were exposed to Gamma-rays.

Citrus sunki was irradiated with 20 or 40Gy of Gamma-rays at three different floral stages [10] and nucellar seedling [11]. A spine free mutant was selected from irradiated nucellar. In *Citrus sinensis*, immature seeds were exposed to Gamma-rays at the doses 80-100Gy [9], while those polyembryonic seeds were exposed to Gamma-rays of 100Gy [12]. Two seedless mutants were selected, leading to release of a new cultivar "Hongju 418 and Hongju 420" [12]. *Citrus paradisi* cv Hudson were exposed with thermal neutron, leading to release of a seedless mutant 'Star Ruby' [13]. Another five nearly seedless mutants of *Citrus paradisi* cv Foster were also selected from irradiated bud wood at the dose 50 gray of Gamma-rays [14].

Citrus limon cv Eureka and Israeli Villafranca were irradiated by 60 and 50Gy of Gamma-rays respectively, and completely new seedless varieties were released [15]. A red color of flesh and juice mutant derived from 80Gy gamma irradiation of *Citrus paradisi* cv Ruby Red was released as

cultivar Rio Red in 1984 [16]. Bud woods of pummelo, mandarin and Navel Orange irradiated by Gamma-rays at doses of 30 – 75Gy showed high sensitivity at higher dose, while Valencia and grapefruit produced more seedless fruits from those at the higher doses [17]. Irradiation of Gamma-rays (10-60Gy) on calli *in vitro* proliferation stage resulted in high mortality at the dose of 60Gy [4] When nucellus and embryonic mases of *Citrus sinensis* cv. Pera were exposed to gamma irradiation at the dose 0 – 160Gy and 0 – 189Gy respectively, normal growth of plantlets was obtained from irradiated nucellus exposed to 20 – 80Gy [18].

Here, complete seedless and seedless were found from doses of 20 and 40Gy on Keprok SoE and pumello Nambangan. One branch of pumello derived from gamma irradiation with a dose of 40Gy produced different juice color and skin. However, survival percentage of mandarins decreased largely at the dose of 60 gray. In pummelo, survival percentage decreased little at 60 gray, but no promising mutation lines were obtained. Therefore, it is likely that appropriate dosage in Indonesian cultivars of mandarin and pummelo is lower than that reported by other researchers.

Selected branches of trees have been grafted to rootstocks in the field. The new seedlings mutants obtained are now under investigation in the field to confirm their stability, agronomic advantages and for genetic tests with molecular markers.

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The Development of New Genotypes of the White Yam by Mutation Induction Using Yam Mini-tubers

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Abstract

A method for the genetic improvement of the white yam, *Dioscorea rotundata* by mutation induction was investigated by irradiating mini-tubers of the yam with Gamma-rays. Batches of 150 mini-tubers of the well adopted local cultivar "Obiaoturugo" were gamma irradiated at doses of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 Gray (Gy) using the ^{60}Co gamma source at the Center for Energy Research and Development, Obafemi Awolowo Universitym, Ife-Ife in 2004. Each irradiated tuber including the control was divided into setts weighing 10-15g and planted in the field to establish the first mutated vegetative generation (MV_1), separating setts from the head (H) and the tail (T) regions. In 2005, first generation tubers harvested from the MV_1 generation were used to establish the MV_2 populations. At the MV_1 generation, increasing doses of Gamma-ray irradiation progressively inhibited sprouting of sets isolated from treated mini-tubers. These effects were more severe on sets from the tail (T) region than those from the head (H) region. Also, plant height, number of leaves, number of nodes and mean tuber yields per stand decreased with increased Gamma-ray doses. LD_{50} and GR_{50} were observed at 40Gy and 30Gy, respectively. At the MV_2 generation, the observed differences among the treatments means disappeared (or were not significant). MV_2 yam lines with modified vegetative characteristics were isolated. Distinct lines with bunchy and bushy vegetation and bushy with spreading vines were isolated. This is relevant, as one of the genetic improvement objectives apart from high tuber yields in yam, is for the development of lines that may be cropped without staking.

Introduction

Yams are very important in the economic and social life of Nigerians. Nigeria is the largest world producer of the crop accounting for about 75% of the crop produced in West Africa [1]. Among yams, *Dioscorea rotundata* (White yam) is the most widely cultivated. Although, *D. rotundata* is believed to be indigenous to West Africa, the species has one of the lowest yields [2]. The low yield is partly due to limited attempt at breeding the crop for high yields. Coursey and Martin [3] have the belief that it is possible to increase the yield of yams though hybridization. The successful germination of true yam seeds by various authors [4, 5, 6, 7] heightened this belief. However, more than 25 years after the successful germination of true yam seeds, it is only recently that new white yam hybrid varieties have been developed and released by breeders from IITA, Ibadan, in collaboration with National Root Crops Research Institute, Umudike. Many workers believe that the improvement of yams over the years has been through the selection of tubers with appealing characters and the vegetative propagation of such tubers by farmers. As a result of this asexual method of propagation, many important species and cultivars have lost the ability for efficient sexual reproduction [8]. In *D. rotundata*, many good and important cultivars have never been known to flower while others produce only male or female flowers. Only few cultivars are truly dioecious [6, 9]. These and the problem of high ovule abortion after crosses [10], low fruit and seed production, and low genetic variability for many characters in yam cultivars [11], make yam a difficult crop to improve by hybridization. Therefore, mutation induction offers a veritable tool for the genetic improvement of yams. In fact, some important agronomic traits in yams are lacking in the Nigerian yam germplasm, such as dwarf erect vegetation that may not require staking for high yield. Such traits need to be induced.

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Materials and Methods

Batches of 100 mini-tubers of *Dioscorea rotundata*, cv. "Obiaoturugo" were irradiated with Gamma-rays at doses, 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 Gray (Gy) using the ^{60}CO gamma source at the Center for Energy Research and Development, Obafemi Awolowo Universitym, Ife-Ife in 2004. Each irradiated tuber including the control was divided into setts weighing 10-15g and planted in the field to establish the first mutated vegetative generation (MV_1). The sets from the head region (H) were planted separately from those from the tail region (T), using a split-plot experiment laid out in the field in a randomized complete block design (RCBD) with four replications.

Data collected was plant height (canopy height), number of leaves per stand and number of vines per stand. Others included the number of days from planting to the register of the first sprout and 50% sprout, the total number of sets planted and the number that sprouted. These were used to calculate percentage of sprout, relative survival and lethality. The relative survival (RS) was calculated as

RS = (% survival of dose treatment X 100) / % survival of control
Lethality was estimated as Lethality = 100 – RS [12]

Tubers were harvested when senescence set in and the data collected from the harvested tubers was the number of tubers per stand and the tuber yield. Other traits assessed were the diameter (D) and length (L) of individual tubers, which were used to calculate the tuber shape index (TSI) as TSI = D/L [13].

All data was subjected to statistical analysis based on RCBD method.

A total of 5,000 first-generation tubers (lines) harvested from MV_1 generation were used to establish MV_2 population. The field lay out in the present trial was similar to that of the earlier trial. Each plot contained equal number of lines and a minimum of five stands per line was planted. During data collection, at least three stands per line were sampled. During replication, care was taken to plant out each line (tuber) for easy identification and follow-up. However, during statistical analysis, data were pooled and expressed on per stand basis.

The MV_2 generation population was also screened and mutant lines with modified vegetative characters were tagged and isolated.

Results, Discussion and Conclusion

Increasing doses of Gamma-ray progressively inhibited sprouting of sets of treated mini-tubers (**Table 1**) as indicated by the number of days to first sprout emergence in the MV_1 generation.

The number of days to 50% sprout and the mean percentage sprout (**Table 2**) in the MV_1 generation also showed the same trend. When the number of days to 50% sprout was compared among the treatment means, setts from the H region treated to 0 – 60Gy gamma irradiation recorded a range of 25.5 days to 81.1 days and those from the T regions recorded a range of 38.3 days to 95.0 days. Setts, isolated from mini – tubers treated to 70Gy and above Gamma-ray failed to sprout or did not record up to 50% sprout. In all cases, the effects were more severe on setts isolated from the tail (T) region than those from the head H region indicating that the tail region is more radiation sensitive than the head region.

Table 1. The effect of Gamma-ray dose rate on the mean number of days to first sprout of yam tuber setts isolated from the head (H) and tail (T) regions of irradiated yam tubers at the MV_1 and MV_2 generations

Gamma-ray Dose (Gy)	MV_1			MV_2		
	H	T	Mean	H	T	Mean
0	18.0	22.2	20.1	19.8	19.7	19.8
10	19.8	27.3	23.5	18.1	20.1	19.1
20	19.8	27.3	23.5	20.1	21.1	20.6
30	20.5	32.0	26.3	19.0	18.2	18.6
40	24.0	32.8	28.4	18.6	19.1	18.9
50	27.3	39.3	32.3	18.7	18.6	18.7
60	30.5	63.0	46.8	20.1	20.2	20.2
70	32.8	73.5	51.9	20.0	19.9	20.0
80	62.5	83.3	72.9	19.7	18.9	19.3
Mean	28.3	44.5	-	19.3	19.5	-

F – LSD (P=0.05) for comparing MV_1 means between two tuber sett source means = 7.8; between two Gamma-ray dose means = 5.1; between two Gamma-ray dose means at the same and different sett source level = 10.2. MV2 means are not significantly different.

Table 3 illustrates how the mean canopy heights (plant heights), the mean number of leaves and the mean tuber yield per stand of stands from treated setts also decreased with increased Gamma-ray dosages. These effects were also more severe on setts isolated from the T regions than those from the H regions.

Gamma-ray irradiation did not seem to affect the mean number of vines per stand, the mean number of tubers and the mean tuber shape index (TSI) of yam tubers raised from tuber setts isolated from the H and T regions of treated mini-tubers. LD50 (50% lethality) and GR50, (50% growth reduction) were obtained at 40Gy and 30Gy respectively. There was complete inhibition of sprouting (100% lethality) at doses over 80Gy, as shown in **Table 4**. In the MV_2 generation, however, there were no significant differences among the treatment means. The significant differences among the treatment means observed in the MV_1 generation disappeared in the MV_2 generation.

Gamma irradiation of crop plant parts results in three types of effects, physiological damage, (primary injury), factor mutations (gene mutations) and chromosome mutations, (chromosomal aberrations) [14]. All these effects are referred to as radiation injury and may manifest in several forms. They may include reduced sprouting ability when vegetative organs are used, survival ability, reduced plant heights, and reduced number of plant organs such as number of leaves. From the work reported here, there was a decreased sprouting ability with increasing doses of Gamma-ray. The same trend was repeated when the plant heights, and the number of certain plant organs, such as the number of leaves were considered.

The induced variability observed in the MV_1 generation (as a result of gamma irradiation) was the result of the cumulative effect of physiological damage, gene mutations and chromosome mutation. However, the contribution of physiological damage, (primary injury) is much higher than the contributions by gene mutation and chromosome mutations in the MV_1 generation. This is because gene and chromosome mutations occur at very low frequencies [15]. Gene and chromosome mutations can be transferred from MV_1 generation to the subsequent ones but physiological damage may not. For practical purposes, selection for mutants started from MV_2 generations [16], once physiological effects may have disappeared.

The most effective dose range for mutation induction in vegetatively propagated crop plants like the yams must be between dosages that may cause lethality of not more than 50% and growth reduction of not more than 50%. Brunner [17] recommended a dose treatment range that must allow the survival of 40 –50% and or a retardation in growth of not more than 50%. Within this range, enough populations of the treated plant parts will survive the lethal effects of the mutagen as well as inducing

Table 2. The effect of Gamma-ray dose rate on the mean number of days to 50% sprout[a] and mean percentage sprout[b] of yam tuber setts isolated from the head (H) and tail (T) regions of yam tubers treated to Gamma-ray irradiation

	Mean number of days to 50% sprout						Mean percentage sprout					
Gamma-ray Dose (Gy)	MV_1			MV_2			MV_1			MV_2		
	H	T	Mean	H	T	Mean	H	T	Mean	H	T	Mean
0	25.5	38.3	31.9	26.1	25.0	25.6	95.0	80.0	81.5	90.0	91.1	90.5
10	29.5	50.3	39.9	25.5	25.2	25.4	85.3	69.3	77.3	89.2	90.0	89.6
20	33.0	57.5	45.3	25.9	27.0	26.5	74.8	59.3	67.0	85.5	91.5	88.5
30	36.3	58.5	47.4	26.1	25.5	25.8	48.5	40.5	44.5	92.0	88.5	90.3
40	45.8	59.5	52.6	24.9	26.0	25.5	46.0	34.3	40.1	80.7	89.4	85.0
50	60.8	75.0	67.9	25.6	25.5	25.6	39.0	28.8	33.9	92.1	90.3	91.2
60	81.8	95.0	88.4	28.1	27.0	27.7	29.0	15.0	22.0	87.7	95.0	91.4
70	0.0	0.0	0.0	25.5	26.1	25.8	21.3	7.5	14.4	90.9	92.0	91.5
80	0.0	0.0	0.0	26.1	27.3	26.7	7.55	1.3	4.4	89.8	88.7	89.3
Mean	34.7	48.2	-	26.0	26.1	-	49.6	37.3	-	88.7	90.7	-

[a] 0.0 = Setts treated to 70Gy and above Gamma-ray irradiation failed to sprout or did not record up to 50% sprout. F – LSD (P=0.05) for comparing MV_1 means. Between two tuber sett source means = 8.5 between two Gamma-ray dose means = 7.7 between two Gamma-ray dose means at the same and different sett source level = 10.9. MV_2 means are not significantly different.
[b] F – LSD (P=0.05) for comparing MV_1 means between two tuber sett source means = 2.7, between two Gamma-ray dose means = 4.7, between two Gamma-ray dose means at the same and different sett source level = 6.6 MV_2 means are not significantly different.

Table 3. The effect of yam ray dose on the mean canopy height[a] (cm) (plant heights), mean number of leaves[b] and on the mean tuber yield per stand[c] of yam plants raised from tuber setts isolated from the head (H) and tail (T) regions of yam tubers treated to Gamma-ray irradiation at the MV_1 and MV_2 generations.

Gamma-ray Dose (Gy)	Mean plant height (cm)						Mean number of leaves						Mean tuber yield per stand					
	MV_1			MV_2			MV_1			MV_2			MV_1			MV_2		
	H	T	Mean	H	T	Mean	H	T	Mean	H	T	Mean	H	T	Mean	H	T	Mean
0	125.4	104.9	115.1	159.0	158.9	159.0	18.0	22.2	20.1	19.8	19.7	19.8	126.2	81.5	103.9	240.00	260.00	250.0
10	109.3	97.0	103.1	149.2	159.9	154.6	19.8	27.3	23.5	18.1	20.1	19.1	110.6	67.5	89.0	220.0	230.0	225.0
20	88.9	73.0	80.9	159.7	154.6	157.2	19.8	27.3	23.5	20.1	21.1	20.6	98.1	60.7	79.4	260.0	260.0	260.0
30	62.2	53.1	57.7	159.2	153.3	156.3	20.5	32.0	26.3	19.0	18.2	18.6	85.0	49.7	67.3	230.0	230.0	230.0
40	59.2	43.9	51.6	148.9	149.1	149.0	24.0	32.8	28.4	18.6	19.1	18.9	75.1	45.7	60.4	260.0	270.0	265.0
50	52.0	36.7	44.4	145.4	149.5	147.5	27.3	39.3	32.3	18.7	18.6	18.7	63.5	37.3	50.4	270.0	250.0	262.5
60	47.4	28.7	38.0	155.8	150.1	153.0	30.5	63.0	46.8	20.1	20.2	20.2	38.6	19.6	29.1	250.0	230.0	240.0
70	39.3	8.3	23.8	153.5	158.1	155.8	32.8	73.5	51.9	20.0	19.9	20.0	29.1	12.8	20.9	260.0	260.0	260.0
80	24.5	0.0	12.3	158.9	157.2	158.1	62.5	83.3	72.9	19.7	18.9	19.3	16.5	6.0	11.2	240.0	250.0	245.0
Mean	67.6	49.5	-	154,4	154.5	-	28.3	44.5	-	19.3	19.5	-	71.4	41.6	-	247.8	248.9	-

[a] F-LSD (P=0.05) for tuber sett source means = ns; for Gamma-ray dose means = 10.9; for tuber sett source and Gamma-ray interaction = 15.5 ; F.LSD(0.05) for comparing MV_2 means = ns
[b] F – LSD (P=0.05) for comparing MV_1 means between two tuber sett source means = 7.8, between two Gamma-ray dose means = 5.1 between two Gamma-ray dose means at the same and different sett source level = 10.2 ns = not significantly different. MV_2 means are not significantly different.
[c] F- LSD (P = 0.05) for comparing MV1 Means Two tuber sett source means = 13.7 Two Gamma-ray dose means = 11.7 Two Gamma-ray dose means at The same or different setts source = 16. MV_2 treatment means are not significantly different.

Table 4. The effect of gamma irradiation on the percentage height of control, relative percentage sprout and lethality (%) of setts isolated from the head (H) and tail (T) regions of treated yam mini-tubers.

Gamma-ray dose (Gy)	Percentage height of control	Percentage sprout	Relative percentage sprout	Lethality
0	100	87.6	100	0
10	89.6	77.3	98.0	12.0
20	70.3	67.0	70.6	23.4
30	50.1	44.5	50.9	49.1
40	44.8	40.1	45.8	54.2
50	38.6	33.9	38.7	61.3
60	33.0	22.0	25.1	74.9
70	20.7	14.4	16.5	83.5
80	10.7	4.4	5.0	95.0
90	0.0	0.0	0.0	100

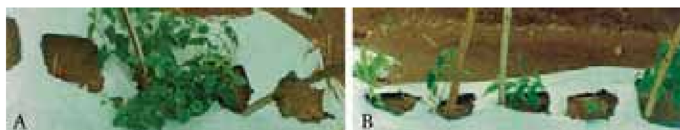

Figure 1 White yam mutants developed from Gamma-ray treated mini-tuber of Cvs. 'Obiaoturugo': **(A)** A dwarf non-climbing mutant; **(B)** Five stands of a dwarf mutant clone with erect non-climbing vegetation.

adequate genetic changes to allow for more efficient selection of desirable mutants. Thus, the Gamma-ray dose range from the results for mutation induction in the white yam, using mini-tubers was established to be 20 – 40Gy (GR_{50} was 30Gy and LD_{50} was 40Gy).

MV2 yam lines with modified vegetative characteristic (**Table 5, Fig. 1**) were isolated.

Distinct lines with bunchy and bushy vegetation, bunchy with conical canopies and bushy with spreading vine branches were isolated. In these lines, vines had reduced lengths, and possessed comparatively shorter canopies. One of the genetic improvement objectives apart from high tuber yields, include the development of yam lines that may cropped for high yield without staking [2]. The characters associated with these dwarf mutants have proved stable over three generations.

Table 5. Lines isolated from irradiated mini-tubers with modified vegetative structure at the MV_2 generation

S/No	Line	Yield Per Stand (Kg)	Description
1.	10GyHR$_2$009	0.10	Dwarf erect vegetation
2.	10GyHR$_4$013	0.09	"
3.	10GyHR4014	0.10	"
4.	20GyHR$_3$015	0.07	"
5.	$H_{30}R_2$019	0.10	"
6.	$H_{30}R_3$007	0.15	"
7.	$H_{40}R_4$003	0.18	"
8.	$H_{40}R_4$013	0.16	Bunchy, non-climbing
9.	$H_{60}R_3$013	0.10	Erect vegetation
10.	$H_{70}R_2$001	0.15	Non-climbing with spread vegetation
11.	$H_{50}R_3$002	0.15	"
12.	$T_{10}R_1$010	0.10	Erect with bunchy vegetation
13.	$T_{40}R_2$002	0.09	Bunchy, non-climbing
14.	$T_{20}R_1$013	0.17	Non-climbing, spreading.
15.	$T_{70}R_4$001	0.05	Non-climbing, erect
16.	$T_{30}R_1$002	0.14	Non-climbing, spreading
17.	$T_{30}R_4$004	0.15	Non-climbing, spreading
18.	$T_{50}R_1$003	0.27	Non-climbing, Spreading
19.	$T_{50}R_3$001	0.10	Erect-dwarf
20.	$H_{40}R_4$020	0.10	Erect-dwarf
21.	$H_{50}R_1$004	0.20	Non-climbing, bunchy
22.	$H_{30}R_4$011	0. 06	Erect-dwarf
23.	$H_{40}R_3$005	0.04	Erect-dwarf
24.	$H_{40}R_4$015	0.21	Climbing, short canopy
25.	$H_{40}R_4$014	0.28	Climbing, bunchy, short canopy
26.	Control	0.45	Normally climbing and staked

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