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In Vitro Mutation Breeding of Anthurium by Gamma Radiation

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ABSTRACT

In vitro plantlet regeneration in a number of Anthurium (*Anthurium andreanum*) varieties was achieved from callus cultures derived from young, tender leaf explants on Nitsch's (1969) medium. Apart from the time period for callus induction, no differences in terms of regeneration were noted amongst the three varieties tested. Callus induction was found to be more rapid and prolific when Nitsch medium containing BA (1 mg L^{-1}) and 2,4-D (0.1 mg L^{-1}) with a reduced concentration of ammonium nitrate (200 mg L^{-1}) was used. Shoot formation occurred when BA concentration was reduced to 0.5 mg L^{-1} and the ammonium nitrate level increased to 720 mg L^{-1}. Regenerated shoots rooted readily on Nitsch medium containing IBA (1.0 mg L^{-1}). Rooting was improved significantly by the addition of activated charcoal (0.04%) to the medium. When explants (leaves, seeds, in vitro plantlets) were irradiated, best response was observed with the 5 Grays (Gy) treatment in terms of callus formation and regeneration while the 15 Gy dose was lethal to the Anthurium tissues. The phenotypic results indicated a boosting effect of the 5 Gy dose on the leaf tissues. The variability in the responses observed seemed to indicate some mutation, both positive and negative, at the cellular level of the tissues. However, no difference in RAPD profiles were noted between the DNA fingerprints of the mother plant and that of the irradiated tissues using a limited number of primers.

Key Words: Mutation; Breeding; Anthurium; Gamma; Radiation

INTRODUCTION

Species within the genus Anthurium, family Araceae, are highly prized for their exotic flowers and foliage which make demand for propagating material and new cultivars high. In Mauritius commercial cultivation of anthurium started about 30 years ago and over the years, anthurium has undoubtedly become one of the most economically important crops in Mauritius. In the year 2000, 95% of the flowers exported consisted of anthurium, showing its significance in our horticultural industry. The number of blooms produced kept on increasing, with about 9.2 million exported in the year 1990 and increasing to over 15 million in 1995. The horticulture industry, in general, has flourished within a few decades. Statistics show that in the year 1996, income from flower export was only Rs 99,000 while by the year 2000; it increased to Rs 127 million. At present, however, Mauritius is facing severe competition from countries such as the Netherlands, Hawaii and China which is emerging as a potential producer of anthurium. Recently, there has been a continued decline in production and the price fetched on the world market. Our main anthurium export markets are Japan, Italy, France and Taiwan and a smaller amount is exported to Hong-Kong, South Africa, the United States and Germany. The main constraints faced by the anthurium sector presently are its high cost of production, the high air-freight charges, lack of proper marketing system, lack of adequate technical support, scarcity of growing medium, no insurance cover to protect against damage and no cold chain distribution facility. Also, the traditional locally grown commercial varieties are running out of fashion and have low market value. Earlier, Mauritius was over-dependent on the Dutch varieties. However, an imposition of ban on import of tissue-cultured plantlets in the early 1990's made access to new varieties difficult and growers were unable to respond to the changing market.

Anthurium is conventionally propagated by seeds and, therefore, cultivation is hindered by problems due to the inherent heterozygosity. Although traditional techniques of vegetative propagation such as the use of stem cuttings and suckers exist, they are tedious and not practical when carried out on a large scale. Tissue culture greatly increases the normal multiplication rate of plants and can provide a source of clean material which has become increasingly important due to outbreak of bacterial and other diseases such as anthracnose, blight, leaf spot, root knot and bacterial wilt caused by *Xanthomonas campestris* pv. diffenbachiae. The method for in vitro production of plantlets of *Anthurium andreanum* was first developed by Pierik *et al*. (1974). The production of in vitro plants directly from proliferating axillary buds (Kunisaki, 1980), adventitious buds (Cen *et al*., 1993), leaf or petiole organogenic callus culture (Pierik *et al.*, 1974; Pierik, 1975, 1976; Finnie & Van Staden, 1986; Kuehnle & Sugii, 1991) and from somatic embryos derived from in vitro grown leaf blade explants (Kuehnle *et al*., 1992) has been reported. Geier (1982) working on *Anthurium scherzeranium* was also able to develop plantlets from spadix explants and later from leaves. All workers found that there was great variation in the requirements of different genotypes. Methods for several other varieties may have been worked out by commercial establishments but these are not available for general use. Hence, we have undertaken to develop suitable methods or

modify existing ones for tissue culture of three varieties of Anthurium. This will be of great help in micropropagation and for future in vitro breeding work to develop resistant varieties.

Mutagenic agents have been used to induce useful phenotypic variations in plants for more than 70 years (Foster & Twell, 1996). A large number of mutant lines have been isolated from many plants and these have been used for plant research and crop breeding purposes (Evans, 1962). For anthurium, new techniques are needed for further improving crop cultivars apart from the traditional plant breeding. Mutation breeding is therefore being proposed as a means to create additional variation. The application of ionizing radiation, chemical mutagens as well as somaclonal variation from tissue culture is quite common in the creation of genetic variation. Novel plant mutants like maize, alfalfa, potato, banana, and barley among others and other cell lines of agricultural and industrial interests generated from tissue culture have also been quite popular (Collin & Dix, 1990).

Different sources of plant tissues such as seeds, pollen, and other cell systems have been employed in studies related with mutagenesis of a number of plant species (Redei, 1970; Lindgren, 1975; Feldmann *et al.,* 1994). For seeds, both chemical and irradiation methods for inducing mutations have proved to be highly efficient (Redei, 1970; Koornneef *et al.,* 1982; Haunghn & Somerville, 1987). Physical mutagens like ionizing radiations (X-rays, gamma rays and neutrons) and UV light, and also a series of chemical agents are common examples of mutagenesis techniques that have a high efficiency at generating mutations in plants, animals as well as bacteria. Furthermore, the outcomes of these treatments can at least be predicted to a certain extent.

As yet, relatively few sequences of irradiation-mutagenised genes in plants have been published. However, recent work with irradiation mutants of *Arabidopsis* supports the general assumption that irradiation causes single- and double-strand breaks which result in chromosomal rearrangements. These studies have also indicated that deletions may be accompanied by other local chromosomal alterations, such as insertions and inversions, not detectable at the cytological level (Wilkinson & Crawford, 1991). The commonly used mutagenic agents cannot produce new genes but in fact they only alter those present in the treated genotype. Ionizing radiation, for instance, generates chromosomal breaks which, following DNA repair, result in a variety of chromosomal aberrations. Gene mutations are less frequent than chromosomal mutations, which include translocations, inversions, deletions and deficiencies. Mutations in the narrow sense affect parts or sections of a gene, either single base pairs or groups of them. Exchange of base pairs or alterations of their sequence may change the primary gene product and by way of a more or less complicated chain reaction of events ultimately lead to a modified phenotypic expression of one or several traits. Sometimes the gene is affected in such a way that it cannot code for any product. The recovery of mutants induced by high levels of mutagens is limited by somatic effects, such as reduced viability, growth abnormalities and reduced fertility. Therefore, every mutagen has a most effective dose, which produces the maximum level of mutagenesis with minimal somatic effects. Mutated genes often appear to be somewhat weak in their phenotypic expression and under heterozygous conditions, can be classified as recessive.

The successful outcome of a mutation depends on the efficient induction of mutations as well as the efficient recognition and recovery of the desired mutant plants or mutant genes. From the variety of mutagenic agents that are available, each has their particular merits. For example, some may induce predominantly point mutations and others chromosomal rearrangements. Also, some can penetrate multi-cellular plant structures, while others cannot and they may be more easily available or safer than other mutagens. Apart from the choice of the proper mutagenic agent, the dosage and the treatment conditions are important. Consideration must also be given to the plant materials treated such as the stage in the life cycle of the plant or plant organs, the sensitivity of the plant species to the effects of the mutagenic agents and the possible genotypic differences in sensitivity to the mutagenic treatments.

MATERIALS AND METHODS

Tissue Culture Studies

Plant materials. *Anthurium andraeanum* plants grown in the University farm shadehouse were used for the experiments. All plants were fertilized monthly with 13:13:20:2 soluble fertilizer containing micronutrients. The plants were also treated with a fortnightly spray of a mixture of 4 g L^{-1} Welgro, 3 g L^{-1} Microthiol, 2 g L^{-1} Peropal, 4 g L^{-1} Lannate and 4 mL L^{-1} Fenitrothion.

Explant preparation and sterilization. Young unfolded leaves were collected and briefly washed under running tap water. Pre-sterilization was done on the whole leaf by soaking in a solution of 0.6% Benlate (benomyl) for 30 min. Sterilization consisted of washing the leaves with diluted liquid soap and thorough rinsing with tap water followed by a dip in 70% alcohol for 30 sec and soaking in 1.5% sodium hypochlorite, containing two drops of Tween 20. After 20 min of gentle agitation, the leaves were rinsed three times in sterile distilled water with 15 min in each rinse and a final rinse for 30 min. The leaves were then cut into explants of 1 to 2 cm^2 and inoculated abaxially onto callus induction medium.

Culture medium and culture conditions. The following culture media were used:

(1) Modified MS (Murashige & Skoog, 1962) medium with macronutrients at half strength, full-strength MS micronutrients, 100 mg L^{-1} myo-inositol and MS vitamins and (2) Modified Nitsch (1969) medium and vitamins. For callus culture, the ammonium nitrate concentration of this

medium was reduced to 200 mg L^{-1} but for regeneration and rooting, it was increased to 720 mg L^{-1}. All media contained 8.0 g L^{-1} agar (Oxoid, Technical Grade No.3), 30 g L^{-1} sucrose and different concentrations and molar ratios of 2,4 - D and BA as shown in Table I. Medium pH was adjusted to 5.8 with KOH before adding the agar. Media were autoclaved for 15 min at 121^0C and dispensed as 25 mL aliquots into 125 mL glass jars. In an attempt to speed up rooting, activated charcoal (0.04%) was added to the media. For callus induction, explants were grown in a culture environment at 25 ± 2^0C with continuous darkness. For regeneration experiments, the calli were grown at 25 ± 2^0C with a 16 h photoperiod and a light intensity of 5.0 Wm^{-2} provided by daylight-type fluorescent lamps. Plantlets were hardened by potting in vermiculite and growing them in a mist house with very low light intensity and keeping the humidity as high as possible.

Table I. Plant Growth Regulators additives (in mg L^{-1}) for each of the stages of the culture

Additive	Callus initiation	Shoot development	Rooting
BA	1.0	0.5	0.0
2,4 - D	0.1	0.0	0.0
IBA	0.0	0.0	0.1

Experimental design, data collection and analyses

A completely randomized design was used for all experiments. Each treatment consisted of six replications (jars) with two explants in each jar. All the experiments were run twice. The final data are reported as an average of 12 replications with 24 explants in each treatment. Data were recorded as the number of explants per jar producing callus after two months in culture, their fresh and dry weights and the percentage of shoots regenerated from the callus.

Irradiation Studies. The irradiation was carried out at the 'Entomology Department' of the Agricultural Research and Extension Unit (AREU). For the irradiation studies, seeds, callus and leaves of tissue-cultured plantlets of anthurium, variety Nitta, were used throughout the experiment. The apparatus used for irradiation is kept in a highly protected area with restricted access as strong radiation may be emitted. It consisted of a radiation cell and the dose rate had to be calculated. The cell was rotated to enable the dose rates to be uniform throughout. The gamma rays doses used were 5, 10 and 15 Grays and had to be regulated according to the time of exposure of the plantlets to the ionising radiation emitted from ^{137}Cs (Caesium) radioactive compound. The equivalent time lapse were obtained through a series of calculations as the irradiation fluctuates with time due to the changing half - life of the compound and with the capacity of the apparatus.

Following irradiation, the plantlets were kept in culture for at least 2-4 weeks before any molecular work was performed. DNA was extracted from each irradiated explants and their profiles compared to that of the mother plant.

Molecular Studies

DNA Extraction

Solutions. Extraction buffers consisting of 3% Sarkosyl (v/v), 0.2 M Tris-HCl (pH 8.8), 50 mM EDTA (pH 8.8), 0.5 M NaCl, 0.1% and 1% β-mercaptoethanol, and 2.5% polyvinylpyrrolidone (PVP - Mr 10,000), were prepared. In addition, chloroform : Isoamyl alcohol (24:1), 70% and 80% ethanol, sodium acetate and a TE buffer consisting of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0) were also needed.

DNA Isolation and Purification Procedures. Unbruised tender pieces of leaves were ground in liquid nitrogen into a fine powder. Two grams of leaf material was weighed and placed on a pre-cooled mortar. Liquid nitrogen was poured onto the sample and allowed to evaporate completely. The leaf sample was macerated with the pestle to produce small pieces. The latter were added to 15 mL of pre-heated (65^0C) extraction buffer. The mixture was incubated for 4 h at 65^0C with constant shaking at intervals followed by cooling to room temperature (R.T) with gentle shaking on a shaker at 45 rpm. An equal volume of chloroform : isoamyl alcohol was added to the mixture. The tubes were mixed gently for 5 min at R.T to produce a uniform emulsion. The latter was centrifuged at 5000 g for 10 min at R.T. The supernatant was transferred to a new Corning tube using a micropipette. Second chloroform: isoamyl alcohol extraction was performed. The supernatant was carefully decanted and transferred to a new tube followed by precipitation with 2/3 volume of isopropanol. The precipitated nucleic acids were collected and washed twice with the buffer (70% ethanol, 10 mM sodium acetate, TE (1X): 1 mM Tris, 0.1 mM EDTA, pH 8.0). The pellets were air dried and re-suspended in TE. The dissolved nucleic acids were brought to 1.4 M NaCl and re-precipitated using 2 volumes of 70% ethanol (If the pellet obtained was hard to re-suspend, this step was repeated one more time). The pellets were washed twice using 80% ethanol, dried and re-suspended in 100 µL of TE buffer. The tube was incubated at 65^0C for 5 min to dissolve genomic DNA followed by RNase treatment.

Measurement of Amount and Purity of DNA. The yield of DNA per gram of leaf tissue extracted was measured using a UV-VIS Spectronic Genesys 5 (Milton Roy) spectrophotometer a 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. Pure DNA has a ratio of 1.8 ± 0.1 (Clark, 1997). Polysaccharide contamination was assessed by calculating the ratio of absorbance at 260 nm to that of 230 nm.

Method for PCR (RAPD analysis). Reagents used - Target DNA (10-100ng), oligonucleotide primers (10-mers Primers OPA 18, OPB 17, OPB 18, OPB 20, OPC 05, OPD 01 and OPW 04), sterile de-ionised distilled water, *Taq* polymerase, dNTP mix (dATP, dCTP, dGTP, dTTP), light mineral oil, agarose (Sigma, Molecular biological grade), TBE buffer (X0.5), Molecular marker VI, Gel-loading buffer (ULB – 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30%

glycerol in water; Stored at 4 ^{0}C), Ethidium bromide (10 mg mL^{-1}).

The reaction mix was prepared on ice for the PCR analysis. Table II gives the reagents used per PCR tube.
The reaction mix was dispensed into the reaction tubes. The *Taq* Polymerase was added last. One drop of mineral oil was added to each tube to prevent evaporation during reaction. The tubes were placed in the thermal cycler and the PCR program for RAPD was then run - reaction initiation at 94^{o}C for 2 min followed by cycles at: 94^{o}C for 1 minute, 35^{o}C for 1 min and 72^{o}C for 1 min. Forty such cycles were done.

RESULTS

Tissue Culture Studies

Pre-sterilization and sterilization. Contamination was a major problem encountered during this study. Fungal contamination appeared during the first week of inoculation while contamination due to the presence of internal contaminants, appeared after three weeks in culture. The pre-sterilization and sterilization methods devised, reduced the contamination level considerably (<10%), independent of variety. However, the concentration of Benlate used during pre-sterilization and the number and duration of rinses in sterile distilled water following sterilization was crucial. Benlate at concentrations higher than 0.6% caused the leaves to become chlorotic while residual sodium hypochlorite caused the explants to become necrotic.

Effect of media and plant growth regulators concentrations. Various concentrations of cytokinin (BA) and auxin (2,4 - D) added to either modified Murashige and Skoog (1962) medium or modified Nitsch (1969) medium were tested in a preliminary experiment. Of the two media tested, Nitsch (1969) with reduced ammonium nitrate concentration (200 mg L^{-1}), proved to be the best for callus induction (Table III). For regeneration, best results were again obtained with Nitsch medium but with the ammonium nitrate concentration increased to 720 mg L^{-1} (Table IV) The same response was noted for all varieties under investigation.

BA (1 mg L^{-1}) and 2,4 - D (0.1 mg L^{-1}) induced callus in all three varieties. Callussing also occurred at lower and higher concentrations but at much lower frequencies. Callus induction began after two weeks in culture and was produced along cut edges of the leaf explants. Incubation in continuous darkness was found to enhance callussing. The calli were firm and pale yellow in colour. Callus formation was more prominent when veins, major or minor, were present on the explants. This can be explained by the presence of metabolically active phloem tissues which are capable of growth in culture as well as retaining some of their endogenous growth factors for additional stimulation of explant growth (Finnie & Van Staden, 1986). Division and subculture of the callus was done every 12 weeks. Fig. 1 shows the mean fresh and dry weights of callus of the three varieties over a period of 70 days.

Table II. Reagents for PCR reactions

Reagents	Stock molarity	Molarity required	Volume used (µL)
Water (Nanopure)	_	_	19.4
PCR Buffer	10 x		3.0
dNTP mix	100 mM	200 µM	2.4
Primer	50 µM	20 picomoles	4.0
Taq polymerase	250 U (5 U/µL)	1 µL	0.2
DNA Template	Variable	Variable (10-100 ng)	1.0
		Total Volume/tube	30.0

Table III. Influence of ammonium nitrate concentration on callus initiation from leaf segments of different varieties of *A. andraeanum* after 4 weeks in culture in complete darkness and supplemented with BA (1 mg L^{-1}) and 2,4 - D (0.1 mg L^{-1}).

Variety	NH_4NO_3 conc. (mgL^{-1})	% Explants forming callus
Osaki	200*	100
	720**	12.5
	825***	0
Nitta	200*	100
	720**	12.5
	825***	0
Anouchka	200*	100
	720**	8.3
	825***	0

* Modified Nitsch medium, ** original Nitsch medium, ***modified MS medium

Table IV. Influence of ammonium nitrate concentration on shoot development from callus, obtained from leaf explants grown on modified Nitsch medium, of different varieties of *A. andraeanum* cultured under low light intensity and supplemented with 0.5 mgL^{-1} BA

Variety	NH_4NO_3 conc. (mgL^{-1})	% callus forming shoots	No of shoots per callus
Nitta	200*	16.7	1-5
	720**	100	>10
	825***	8.3	1-5
Osaki	200*	20.8	1-5
	720**	91.7	>10
	825***	8.3	1-5
Anouchka	200*	12.5	1-5
	720**	87.5	>10
	825***	0	0

*modified Nitsch medium, **original Nitsch medium,*** modified MS medium.

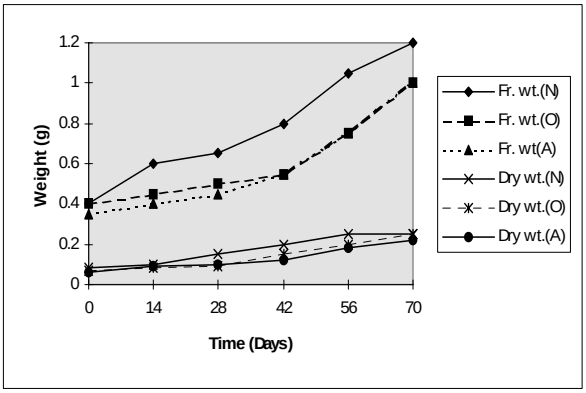
Transferring callus from the callus induction medium to Nitsch basal medium (720 mg L^{-1} NH_4NO_3) supplemented with BA (0.5 mg L^{-1}) and culturing for 16h per day to an illumination of 5.0 Wm^{-2}, caused shoot formation and a depression in callus growth. The shoots were both adventitious and axillary in nature. Table VI shows the effect of growth regulators on shoot formation. The number and size of shoots produced per culture varied considerably in all three varieties.

Allowing the regenerated shoots to stand for over two months on Nitsch (1969) basal medium supplemented with BA (0.5 mg L^{-1}) caused spontaneous rooting to occur. However, transferring the shoots to Nitsch (1969) basal medium supplemented with IBA (1.0 mg L^{-1}), improved

rooting and this was further enhanced when activated charcoal (0.04%) was added to this medium. The level of ammonium nitrate used (720 mg L^{-1}) was essential for rooting as a reduced level (200 mg L^{-1}) delayed rooting. (Table VII)

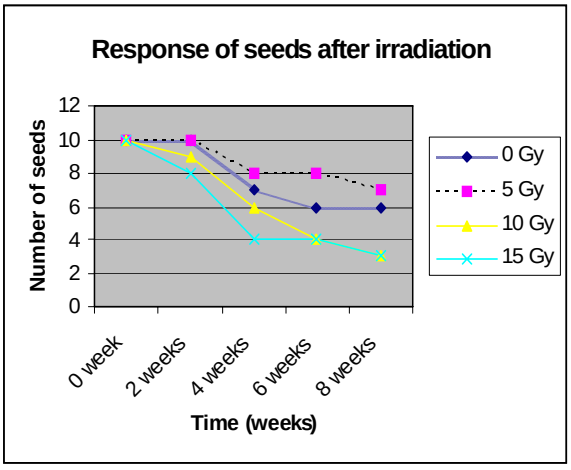
Illumination was also found to be an important factor in the rooting of shoots as callus of all three varieties, grown in the dark on the above medium, did not produce any shoots. As observed by Geier (1982) while working with leaf explants of *Anthurium scherzeranium*, the time of rooting was related to the extent of shoots and leaflet development; the larger shoots with more prominent leaflets forming roots quicker. Plantlets with well-developed roots were hardened by transplanting in vermiculite and growing in a mist house with very low light intensity and high humidity. No losses were observed and the plantlets were transferred to the shade house after two months. However, considerable losses were observed in plantlets without roots or poorly developed roots when transferred to vermiculite.

Fig. 1. Mean fresh weight and dry weight of callus of the three varieties



Fr wt.: Fresh weight. N: Nitta. O: Osaki. A: Anouchka.

Fig. 2. Number of seeds surviving after irradiation



Irradiation studies. Apart from the control where the explants were not irradiated, the other explants were subjected to three doses of irradiation (5, 10 & 10 Gy). The parameter to determine dose-dependent irradiation damage was the survival of the explants after irradiation. The effects of irradiation on seeds after eight weeks following the treatments are shown in Fig. 2.

The higher the dose of radiation, the higher was the mortality rate of seeds. Seeds irradiated at 5 Gy also showed better growth response in the modified Nitsch's (1969) medium. They grew faster and more vigorously, producing shoots within six weeks of culture.

The effects of increasing doses of gamma rays on callus during eight weeks in culture are shown in Fig 3. By the 4th week, the numbers of calli from the 5 Gy treatments were highest as compared to the 10 Gy, 15 Gy and even the control. By week 8, all the explants treated at 15 Gy died. *In*

Table V. Comparison of growth regulator supplements to modified Nitsch medium in callus induction from three *A. andraeanum* varieties incubated in complete darkness.

Medium	BA (mgL^{-1})	2,4 - D (mgL^{-1})	% Callus[a]
1	0.5	0.0	0
2	1.0	0.0	0
3	1.5	0.0	16.7
4	0.0	0.1	25
5	0.5	0.1	33.3
6	1.0	0.1	100
7	1.5	0.1	75
8	0.0	0.5	75
9	0.5	0.5	83.3
10	1.0	0.5	75
11	1.5	0.5	75

[a] Expressed as a mean for the three varieties.

Table VI. Effect of growth regulators on shoot formation from callus obtained from leaf explants grown on modified Nitsch medium supplemented with BA (1 mgL^{-1}) and 2,4 - D (0.1 mgL^{-1}) and transferred to original Nitsch medium.

BA (mgL^{-1})	2,4 - D (mgL^{-1})	% Shoots[a]
0.5	0.0	100
1.0	0.0	66.7
0.5	0.1	50
1.0	0.1	50
0.5	0.5	33.3
1.0	0.5	33.3

a Expressed as a mean for the 3 varieties. Evaluation after 2 months under low light.

Table VII. Effect of growth regulators on rooting (% response) after two months in culture on original Nitsch medium (720 mgL^{-1}) at low light intensity

		BA (mgL^{-1})		
		0	0.5	1.0
IBA (mgL^{-1})	0	0 (0)	66.6 (16.6)	20.8 (4.1)
	0.5	58.3 (20.8)	50 (12.5)	25 (8.3)
	1.0	83.3 (25)	50 (12.5)	41.6 (8.3)

Figures expessed as a mean for the three varieties. Figures in brackets represent% response using 200 mgL^{-1} NH_4NO_3. All media contained 0.04% activated charcoal.

vitro grown leaf explants also showed similar response.

Fig. 4 shows some of the response observed when the explants were treated at different gamma rays doses. Several morphological changes were observed in plantlets regenerated following irradiation. The variability in the responses indicates that the radiation doses may have caused mutation. Explants irradiated at 5 Gy gave very good response indicating the boosting effect of the 5 Grays doses. The 15 Gy dosages were lethal to most of the explants.

Molecular studies. The results of the different spectrophotometer readings and the amount of DNA obtained following extraction from leaves of non-irradiated and irradiated plantlets are shown in Table VIII

A series of PCR reactions were carried out to determine the optimum $MgCl_2$, DNA template, primers and dNTP concentrations to be used for the analysis. From the results obtained, it was found that using 2.0 mM $MgCl_2$, 200 µM dNTP and 20 picomoles of primer gave satisfactory banding patterns. Using either 10 or 20 ng of genomic DNA did not reveal any difference. Out of the seven primers of arbitrary nucleotide sequences chosen to amplify genomic DNA, all were able to amplify PCR products. Results of a preliminary study to compare the banding patterns obtained when using primer OPB 17 and OPC 05 are shown in fig. 5a and 5b respectively. Both monomorphic (Fig. 5b) and polymorphic bands were observed (Fig. 5a).

The sharp bands given for the marker VI correspond to base pairs of 2176, 1766, 1260, 1033, 653, 517, 453, 394 and 293, respectively).

The bands given in the DNA sample were compared with that of the given marker through visual estimations.

The results obtained using other primers following the optimization are shown in Fig 6a and 6b. Some of the results obtained from the DNA extracted from irradiated explants and using the limited number of available primers, are shown in Fig 7 and 8.

After DNA extraction from the irradiated calli and from leaves of anthurium, variety Nitta under the same conditions, the above results were obtained. All four calli DNA gave similar patterns, and these corresponded to that from the mother plant. Hence, at this level no difference in the banding patterns was observed.

Here again, the similar patterns that were obtained showed that the polymorphisms at the genome level of the Anthurium callus and leaf tissues were not demonstrable from the RAPD analysis.

DISCUSSION

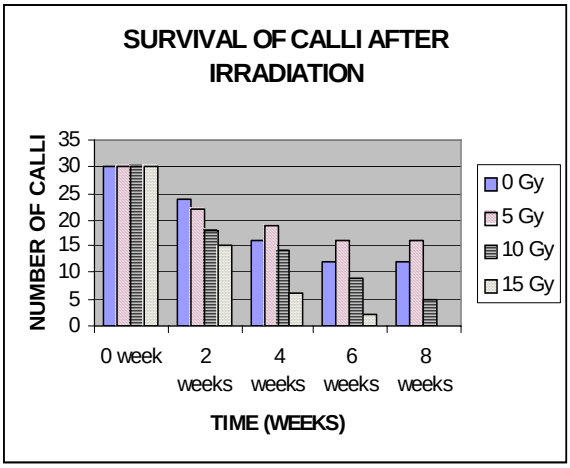
Several workers have reported the variation in the requirements of different genotypes of *Anthurium* in tissue culture (Fersing & Lutz, 1977; Kunisaki, 1980; Geier, 1982). Although methods for several commercial varieties have been worked out, they are not available for general use. The results reported in this paper demonstrate that a single medium can be used for the *in-vitro* culture of three different varieties of *Anthurium andraeanum*. Apart from the time taken for callus induction, there were no genotypic differences in the ability of the callus to regenerate shoots and eventually plantlets.

The influence of the ammonium level on shoot initiation from *Anthurium andraeanum* callus was first reported by Pierik and Steegmans (1976) and Pierik *et al*., (1979). In this study, the level of ammonium nitrate used had a significant effect on callus formation and regeneration. Callus initiation was quicker on modified Nitsch's medium (200 mg L^{-1} NH_4NO_3) supplemented with 2,4 - D (0.1 mg L^{-1}) and BA (1 mg L^{-1}) than original Nitsch's medium supplemented with the same growth regulators. The cultivar Nitta was the first to respond followed by Osaki while callus initiation took longer in the case of Anouchka. This beneficial effect of the low NH_4NO_3 level was also observed by Geier (1982) while working with *Anthurium scherzerianum*. The inability of the leaf explants to initiate callus on modified Murashige and Skoog's (1962) medium with half-strength macronutrients (825 mg L^{-1} NH_4NO_3) further confirms the efficiency of using a low level of ammonium nitrate during callus induction in the cultivars Nitta, Osaki and Anouchka. This is contrary to the findings of Finnie and Van Staden (1986). Geier (1982) obtained shoot regeneration from callus of *A.scherzeranium* on modified Nitsch's medium (200 mg L^{-1}) supplemented with BA (1 mg L^{-1}) and 2,4-D (0.1 mg L^{-1}). However, we observed no regeneration in all the three varieties, using this

Table VIII. Spectrophotometric readings and DNA concentration from irradiated explants

Gamma ray dose (Gy)	A 230	A 260	A 280	A 260/A 230	A 260/A 280	DNA conc (µg/mL)
0	0.063	0.128	0.068	2.032	1.882	0.640
5	0.057	0.108	0.057	1.895	1.895	0.540
10	0.045	0.095	0.054	2.102	1.759	0.475
15	0.019	0.034	0.021	1.789	1.619	0.170

Fig. 3. Survival rate of calli



level of ammonium nitrate. Instead, shoot regeneration from callus occurred when original Nitsch's medium (720 mg L^{-1} NH_4NO_3) supplemented with BA (0.5 mg L^{-1}) was used. This level of ammonium nitrate was also found to be necessary for rooting, which was more prominent when original Nitsch's medium supplemented with 1.0 mg L^{-1} IBA was used. This was true for all the three varieties tested.

In this experiment, rooting was enhanced by the addition of activated charcoal (0.04%) to the medium. Although the precise role of activated charcoal in tissue cultures is unknown, it seems to be involved in the removal of substances from media that promotes unorganised growth (Friedborg & Eriksson, 1975). Growing cells excrete large amounts of phenyl acetic acid and derivatives of benzoic acid (Friedborg *et al*., 1978), which accumulate in the medium and possibly have negative effects on differentiation. Phenolic compounds have also been demonstrated in plant tissue cultures (Butcher, 1977) and have been shown to affect differentiation in tobacco callus (Lee & Skoog, 1965). It is possible that phenyl acetic acid and benzoic acids are not in themselves responsible for the inhibition of root formation in tissue cultures, but their presence indicates a block of a biosynthetic pathway, which is necessary for normal organ development. Other compounds, alone or in combination, may also be active as inhibitors in plant tissue cultures and activated charcoal adsorbs these as well.

Regulation of auxin and cytokinin balance has long been recognized as a key factor in the control of cell division and organogenesis in tissue culture (Murashige, 1977). Our research demonstrated that exogenously applied BA (1.0 mg L^{-1}) and 2,4 - D (0.1 mg L^{-1}) was essential for callus induction from leaf explants of all the three varieties

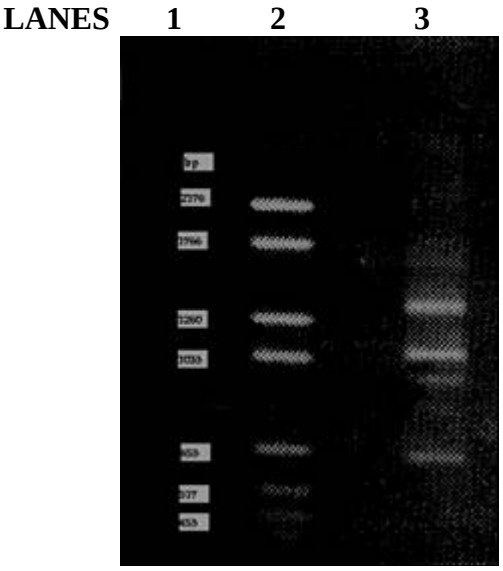
Fig 4 (a). Irradiated seeds (0 Gy, 5 Gy, 10 Gy & 15 Gy) after 8 weeks in culture

Fig. 4 (b). Plantlet regeneration on the 5 Gy treated explants

Fig. 4 (c). Plantlet showing sign of necrosis following 10 Gy treatment

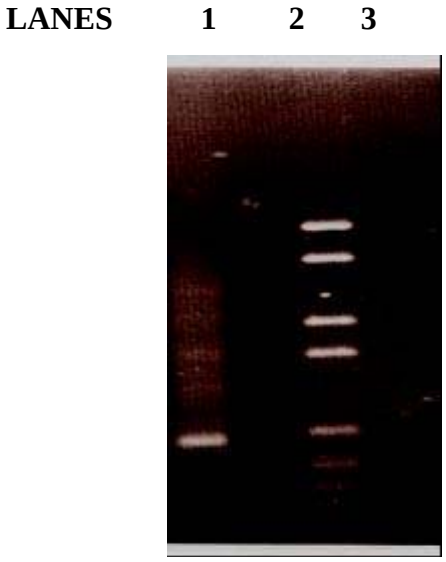
Fig. 4 (d). Plantlet following 15 Gy dose treatment

Fig. 5a. Results using Primer OPB 17



(Lane 1: DNA template (10 µg); Lane 2: Negative control; Lane 3: Marker VI)

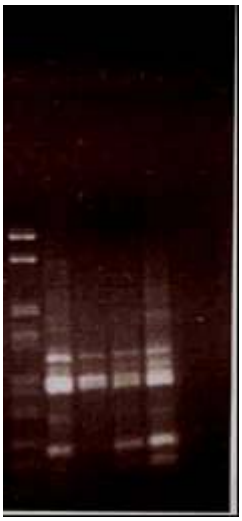
Fig. 5b. Results using Primer OPC 05



(Lane 1: DNA template (20 µg); Lane 2: Negative control; Lane 3: Marker VI).

Fig. 6(a) Amplification product using primer OPB 18

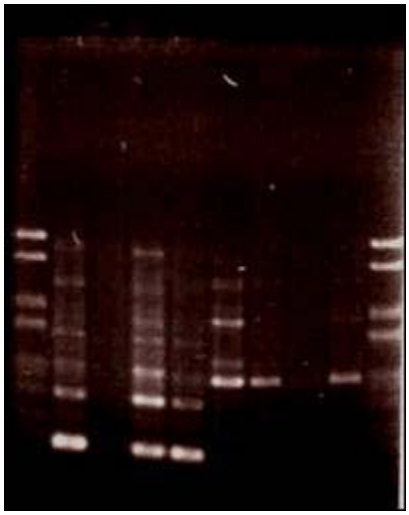
Lanes 1 2 3 4 5 6



Lane 1: Molecular marker VI; Lanes 2 – 5: DNA samples from the two replicates; Lane 6: Negative control.

Fig 6 (b) Amplification product using primer OPW 04 (Lanes 2 – 5) and OPD 01 (Lanes 6 – 9)

Lanes 1 2 3 4 5 6 7 8 9 10

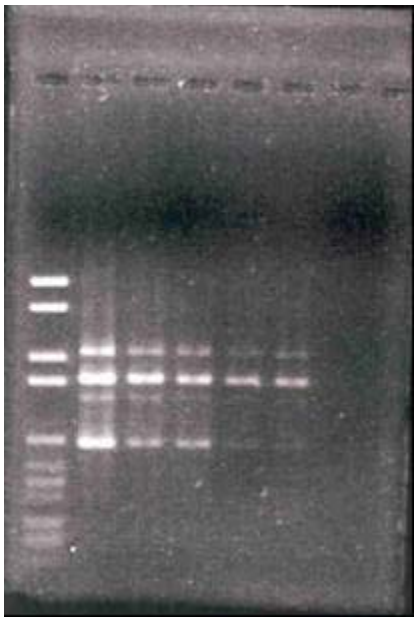


Lane 1 & 10: Molecular marker VI; Lanes 2 – 9: DNA samples from the different replicates.

of Anthurium. Shoot regeneration from the callus occurred when BA (0.5 mg L^{-1}) alone was used while rooting required removal of the BA from the medium and addition of IBA (1 mg L^{-1}). The difference in regeneration capacity and mode of regeneration at concentrations higher and lower than optimum may be explained on the basis of variation in the endogenous levels of these growth hormones in leaf tissues. Similar observations regarding the role of endogenous hormone levels in determining the shoot

Fig. 7. Using Primer OPB 20

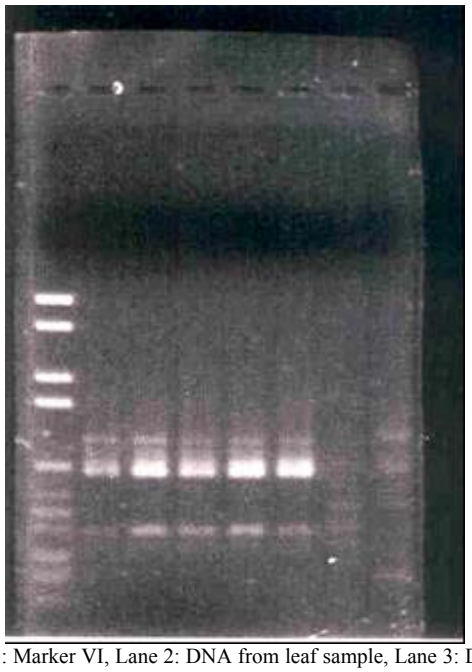
Lanes 1 2 3 4 5 6 7



Lane 1: Marker VI, Lane 2: DNA from leaf explants, Lane 3: DNA 5 Gy (irradiated), Lane 4: DNA 10 Gy (irradiated), Lane 5: DNA 15 Gy (irradiated), Lane 6: DNA 0 Gy (not irradiated)/ control, Lane 7: Negative control

Fig. 8.Using Primer OPA 18

Lanes 1 2 3 4 5 6



Lane 1: Marker VI, Lane 2: DNA from leaf sample, Lane 3: DNA 5 Gy, Lane 4: DNA 10 Gy, Lane 5: DNA 15 Gy, Lane 6: DNA 0 Gy (control)

forming-capacity of tomato leaf disks have been reported (Kartha *et al*., 1976; Frankenberger *et al*., 1981). Another

study (Elliot *et al*., 1987) has also demonstrated that a critical endogenous level of growth regulators has to be attained before cell division and organogenesis could occur.

Apparently, callus induction from leaf was not dependent on light, which is contrary to the findings of Finnie and Van Staden (1986), where light was found to be essential. However, we found that low light intensity levels considerably enhanced shoot regeneration. These results are consistent with the studies of Pierik *et al*., 1974, 1979 and Geier (1982) on plant growth regulator and light action in organ differentiation in a number of Anthurium varieties. Increased shoot formation with a slight increase in light intensity levels has also been reported in other plant species (Hughes, 1981).

In this study, it is also demonstrated that no genotypic variation exists in terms of regeneration from callus for the varieties Nitta, Osaki and Anouchka. Regeneration occurred in all genotypes *via* an intermediary callus phase; no direct shoot regeneration from leaf explants was observed. The phenomenon of genotypic differences in callus formation and regeneration capacity in other varieties of Anthurium has been reported earlier (Kunisaki, 1980; Geier, 1982; Kuehnle & Sugii, 1991).

A matter of interest for plant breeders is the use of mutagens, in combination with in-vitro cultures, to create genetic variation. The Anthurium variety "Nitta" was maintained for the irradiation studies since it had responded earlier than the other tested varieties under in vitro conditions. Here, explants like seeds, leaf pieces and plantlets of the same variety were subjected to the three doses of gamma-rays (5, 10 & 15 Gy) from the ^{137} Cs radioactive compound. Similar results obtained for the different explants showed the reproducibility of the radiation effects of the radiation on Anthurium tissues.

From the experiments on seed culture for example, the 5 Gy treatment showed a higher survival rate (70% at week 8) as compared to the other. The results were even better than those obtained from the "control" experiments, both in terms of survival rate (60%) and in the morphological variation of the seeds in culture. The calli and plantlets also expressed better responses at the 5 Gy but lethality at the 15 Gy doses, whereas the 10 Gy treated explants were moderately lethal to the radiation. These observations demonstrated the probable mutations that have taken place in the anthurium tissues due to the gamma radiation. Apart from the dose effects, the responses were controlled by a number of parameters, including the genotype, the type of explant, the orientation of the explant on the culture medium, and the origin of the explant from the mother plant (Douglas, 1985).

Tissue-cultured based mutagenesis has been employed for many years to generate novel plant mutants and cell lines of agricultural and industrial interest (Collin & Dix, 1990). Somaclonal variation, which can induce a range of gross chromosomal alternations, (as well as more limited gene mutations) has been studied as possible route for generation of novel genetic variation (Evans & Sharp, 1986). The factors responsible for the in vitro origin of chromosomal structural changes (or chromosome mutations) and gene mutations are not known. In several cases, hormones, especially 2,4-D or other hormone combinations are suspected of causing such changes. In fact, it is more likely that these hormones may act as mutagens and favour mutation by influencing metabolism (D'Amato, 1985). Moreover, the physical mapping and DNA sequencing of loci mutagenised by irradiation and chemicals in plants has provided more precise information about these different types of DNA alterations. For irradiation by gamma rays, the occurrence of insertion or inversion changes could explain the occurrence of mutation.

However, the mutation frequency may be influenced by a number of factors such as the mechanism of mutagen action (Sparrow, 1961; Griffiths *et al*., 1993), target gene size and nucleotide composition (Haughn & Somerville, 1987; Bichara *et al*., 1995), genomic location (Swoboda *et al.,* 1993; Brown & Sundaresan, 1991), chromatin structure (Jackson, 1991; Shaffer *et al.,* 1993), replication timing (Salganik, 1983), efficiency of DNA repair (Britt *et al.,* 1993; Veleminsky & Gichner, 1978) and transcriptional activation (Schlissel & Baltimore, 1989; Zehfus *et al.,* 1990; Lindahl, 1991). The frequency of a particular mutation can be underestimated if a degree of elimination occurs (Butler, 1977; Dellaert, 1980; Vizir *et al.,* 1994). The recovery of mutants induced by high levels of mutagens is limited by somatic effects, such as reduced viability, growth abnormalities and reduced fertility. Therefore, every mutagen has a most effective dose, which produces the maximum level of mutagenesis with minimal somatic effects.

This could be the case for the 5 Gy treatment that produced minimal damages to the Anthurium tissues. In fact, the better responses observed could suggest that the type of chromosomal alterations that took place eventually produced a change in the morphology. This was expressed under in vitro conditions. The higher gamma-ray doses may have produced other modifications that caused necrosis of the tissues and calli. However, from the RAPD-profiles, these genomic changes could not be detected. The base-pair sequences for the DNA extracted from tissues irradiated at the three doses gave similar banding patterns. From these results, it showed that the RAPD was inefficient in detecting the more precise genomic alterations that have occurred due to the gamma rays. In fact, from the use of RAPD, it was expected that polymorphisms resulting from mutations or rearrangements either at or between the primer binding sites could be detected as the presence or absence of amplification products.

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