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Induction of nucellar embryogenesis in nucellar tissue of *Citrus aurantifolia* for their clonal multiplication

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ABSTRACT

Citrus aurantifolia (lime) has been selected as explant for nucellar embryogenesis. Nucellus is a non-vascularized tissue being true-to-type same as mother plant, meristematic cells have no plasmodesmata connection, no virus can pass through nucellus, thus it seems to be a good material for production of virus freeplantlet.Putrescine at 0.25 or 0.5 $mg1^{-1}$ and a-napthaleneacetic acid at 0.10 $mg1^{-1}$ supplemented to nutrient formulation were most effective in alleviating cotyledonary proliferation and fasciation while promoting embryo-to-embryo proliferation producing numerous whitish globular embryos were formed. For further development of globular embryos to well-differentiated cotyledonary embryos, additional presence of 2-isopentenyladenine at concentrations of 0.10 or 0.25 mg 1^{-1} was essential, contrary to incorporation of 0.10 or 0.25 mg 1^{-1} 6-benzylaminopurine, which promoted excessive proliferation of cotyledonary structures and their fasciation while zeatin at the same concentrations produced intermediate response. In the optimum treatment containing 0.25 mg l^{-1} putrescine, 0.10 mg 1^{-1} isopentenyladenine, 0.10 mg 1^{-1} indole-3-acetic acid and 100 mg l^{-1} malt extract, an average 10 well-developed embryos per culture were formed, besides some abnormal cotyledonary structures. Well-developed embryos measuring ca. 2 cm. in length (leaving the root) germinated 100% into plantlets, during 60 days, in the additional presence of amino acid supplement comprising, 5 mg 1^{-1} each of L-arginine, L-asparagine, L-histidine, L-cysteine, L-lysine and 10 mg l^{-1} L-glutamine. Such plantlets nurtured in a different medium attained a height of ca. 4 cm in 45 days before they were taken out for *ex vitro* growth. There was 100% transplant success and the plants grew normally.

Figure : 01 References : 25 Table : 01
KEY WORDS : *C.aurantifolia,* Cloning, Embryo-to-embryo proliferation, Nucellarembryogenesis.

Introduction

Citrus aurantifolia is the third commercially important fruit tree of the world and belongs to the family Rutaceae. It is commonly known as Kagzi Nimbu and is now being commercially propagated on its root system. For processing clean as well as genetically uniform plants to be used as root stocks and the nucellar plants can serve as a good option. However, nucellar embryogenesis in this species is not only scanty, but also associated with the serious problem of fasciation of such embryos and as a consequence still fewer plantlets are formed per *in vitro* culture.Embryogenic cells are unique, although superficially resemble meristematic cells, these are generally smaller, more isodiametric in shape, have larger,more densely stained nuclei and have a denser cytoplasmic[2,24].

Nucellar embryogenesis in this species is not only scanty but also associated with the serious problem of fasciation of such embryos and as a consequence still fewer plantlets are formed per *in vitro* culture. In order to alleviate these problems,experiments have been conducted to enhance multiple embryo formation in *C. aurantifolia* by culturing nucellus tissue in the modified MS medium supplemented with different concentrations of BAP, 2ip and Zeatin.

Somatic embryogenesis plays an important role in the improvement of commercial crops by incorporating molecular techniques during clonal propagation[13,19,21,25] successfully produced somatic embryos. Somatic embryogenesis forn is the basis of cellular totipotency, a unique phenomenon in higher plants. Differing from its zygotic counterpart, somatic embryos are easily traceable. Culture conditions can be controlled and lack of material is not a limiting factor for experimentation[11].

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TABLE-1 : *C. aurantifolia* : Effect of different concentrations of putrescine, used alongwith different concentrations of BAP and 2ip on development of globular embryos differentiated from nucellus

Treatment 0.10 NAA common top all (Conc. in mg l^{-1})	Response* Incubation : Total 90 days 30 — 30 + 30 — 30 + 30 + 30				Remarks
	Embryo to embryo Proliferation	Proliferation of cotyledon-like Structures	Fasciation of cotyledon-like Structures	Number of normal embryos produced per culture	
0.05 BAP + 0.25 Putrescine	±	+ +	+ +	-	embryonal
0.05 BAP + 0.50 Putrescine	±	+ + + +	+ + +	-	Fasciation
0.05 BAP + 0.75 Putrescine	±	+ +	+ + + +	-	-
0.10 BAP + 0.25 Putrescine	±	+ +	+ +	-	Fasciation
0.10 BAP + 0.50 Putrescine	±	+ + + +	+ + +	-	-
0.10 BAP + 0.75 Putrescine	±	+ +	+ + + +	-	-
0.25 BAP + 0.25 Putrescine	±	+ + +	+ + + +	-	Excessive
0.25 BAP + 0.50 Putrescine	±	+ + + +	+ + + + +	2	Embryonal
0.25 BAP + 0.75 Putrescine	±	+ + + +	+ + + + + +	-	Fasciation
0.05 2iP + 0.25 Putrescine	+ +	+ +	+ +	6	Individual bipolar
0.05 2iP + 0.50 Putrescine	+ +	+ + +	+ +	5	Embryos have
0.05 2iP + 0.75 Putrescine	+	+ +	+ +	4	been formed
0.10 2iP + 0.25 Putrescine	+ + +	+ +	+	10	been formed
0.10 2iP + 0.50 Putrescine	+ +	+ + +	+ +	9	-
0.10 2iP + 0.75 Putrescine	+	+ +	+ +	4	-
0.25 2iP + 0.25 Putrescine	+ +	+ + +	+ +	8	been formed
0.25 BAP + 0.50 Putrescine	+ +	+ +	+ +	6	-
0.25 BAP + 0.75 Putrescine	±	+ + +	+ + +	4	-

* Data based on 5 replicates

Number of + marks connotes the extent of response (the value of ± mark is less than + mark)

These characteristics have made somatic embryogenes is a model for the study of morphological, physiological, molecular and biochemical events that occur during the onset and development of embryogenesis in higher plants. It has potentially rich biotechnological applications such as production of artificial seeds, micropropagation and raising transgenic plants, *etc.*

Materials and Methods

Young fruits at the age of 20-25 days after fruit-sets were used for experimenting with nucellar tissue contained in ovular halves of *C. aurantifolia* (lime). Fruits of different sizes, *i.e.* measuring ca. 2.5, 3 and 3.5 cm were plucked and used to dissect out the explants. The fruits were washed in running tap water for 30 min and treated with 5% Labolene (a neutral liquid detergent), along with 2 drops of tween 20 per 100 ml solution for 30 min and rinsed with single distilled water. Further process was gone through in a Laminar air Flow Cabinet. The fruits were given a quick dip in the rectified spirit for surface sterilization. Afterwards, these were transferred in chlorine saturated water for 30 min. Subsequently, after a thorough wash with sterilized distilled water, these were ready for fertilization. The surface sterilized fruits were cut longitudinally into two halves and after removing the zygotic embryo, the ovular halves containing nucellar tissue were scooped out from the pericarp and cultured with the cut face away from agarified nutrient medium. Different media used were modifications[15,17]. BM1 and BM2 were used for induction of nucellar embryogenesis, whereas BM3 was employed for germination of well-developed cotyledonary embryos. These germinated embryos were transferred in BM4 for further development of the plantlets.

The medium BM1 comprised (concentration in mg l^{-1}): 250 $(NH_4)_2SO_4$. $7H_2O$, 1500 NH_4NO_3, 1500 KNO_3, 150 KH_2PO_4, 400$CaCl_2$. $2H_2O$, 450 $MgSO_4$. $7H_2O$, 5ml l^{-1}Na-Fe-EDTA (ml l^{-1}), (prepared by dissolving 557 mg $FeSO_4.7H_2O$ in 100ml of warm solution containing 745 mg of Na-Fe-EDTA in double distilled water), 1 ml l^{-1} trace elements of medium[15,17], 1Thiamine-HCl, 0.1 Pyridoxine-HCl, 0.5Nicotinic acid, 0.1Folic acid, 0.1 d-biotin, 0.1Rioboflavin, 100m-Inositol, 3Glycine, 10 DL-tryptophan, 10 Larginine, 25 L-glutamine, 10L-asparagine, 10 Ascorbic acid, 25Adenine sulphate (AdS), 200Malt extact (ME), 50,000 Sucrose and 7,500Agar powder. The medium BM2 differed from BM1 in following respects (concentration in mg l^{-1}): Reduced concentrations of $NH_4NO_3$from 1500 to 500, KNO_3 from 1500 to 500, $CaCl_2.2H_2O$ from 400 to 200, Thiamine-HCl from 1to 0.5, deletion of Folic acid, d-biotin, Rioboflavin, m-Inositol, DL-tryptophan, Larginine, L-glutamine, Lasparagine, Ascorbic acid and use of 1800 phytagel powder (Sigma Aldrich, USA) in place of agar. The medium BM1 was supplemented with different concentrations (0.25, 0.50) of each 6-benzylaminopurine (BAP) and 2-isopentanyladenine (2iP) used singly or in combination with 0.50 mg l^{-1}a naphthaleneacetic acid (NAA), while BM2 was supplemented with BAP, 2iP or zeatin (Z) at concentrations, *viz.,* 0.25, mg l^{-1} plus different concentrations, *viz.,* 0.25, 0.5, mg $l^{-1}$of putrescine along with 0.1 mg l^{-1} NAA. Additionallythe same concentration were also used without cytokininin combination with 0.1 mg l^{-1} NAA alone.

For further development of isolated embryos, the medium used was BM2 supplemented with 0.1, 0.25 or 0.50 mg l^{-1} 2iP, 0.25, 0.5 or 0.75 mg l^{-1}putrescine 0.1mg l^{-1} NAA or IAA. The medium BM3 was used for germination of well-developed cotyledonary embryos, which differed from BM2 in following respects: Addition of 5 mg l^{-1} of each DL-tryptophan,L-arginine, L-asparagine, L-lysine, L-histidine, L-cystiene, 10 mg l^{-1} L-glutamine 0.1, 0.25mg l^{-1} IAA and reduction in the concentration from 200 to 100mg l^{-1} ME.

For germinated embryos nucellar embryos and growth of plantlets, medium BM4 was employed. The medium BM4 differed from BM2 in following respect increased concentrations (mg l^{-1}) of NH_4NO_3 and KNO_3 from 500 to 1500 each, $CaCl_2.2H_2O$ from 200 to 400 and ME 200 to 500, reduced concentration of Thiamine-HCl from 0.5 to 0.2, AdS from 25 to 15 and additional of 10 Ascorbic acid and 300 m-Inositol along with 0.01 2iP and 0.1 IAA and substitution of agar for phytagel.

All the media were adjusted to pH 5.8, before adding agar or phytagel and sterilized by autoclaving at 1.08 kg /cm^2 for 15 min. Cultures were incubated under 37.5 µ mol $m^{-2}s^{-1}$ quantum flux density for 15 h a day at a temperature of 25° ± 1° C and 75% ± 5% RH. The *in vitro* raised nucellar plantlets were acclimatized and nurtured them in modified Knop's solution for 30 days and then transferred them to potting mixture comprising farm-yard manure and soil(1:3).

Results and Discussion

Nucellar tissue contained in ovular halves of *C. aurantifolia* remained fresh without necrosis in the nutrient formulation employed for and after 10 days of incubation, meristematic activities in discrete sectors, but mainly proximal to micropylar region were ensued which transformed into visible proembryogenic tissuewithin 15-20 days(Fig.1A). Explants from fruits of intermediate size (3 diameter in cm) showed highest meristematic activation of nucellus, which was observed to be 50% in 0.25 2iP+0.5 mg l^{-1} NAA.This negates the earlier report[18] that nucellus from immature, younger fruits showed better response to embryo formation in *Mangifera indica.*

Embryos formed under different treatment

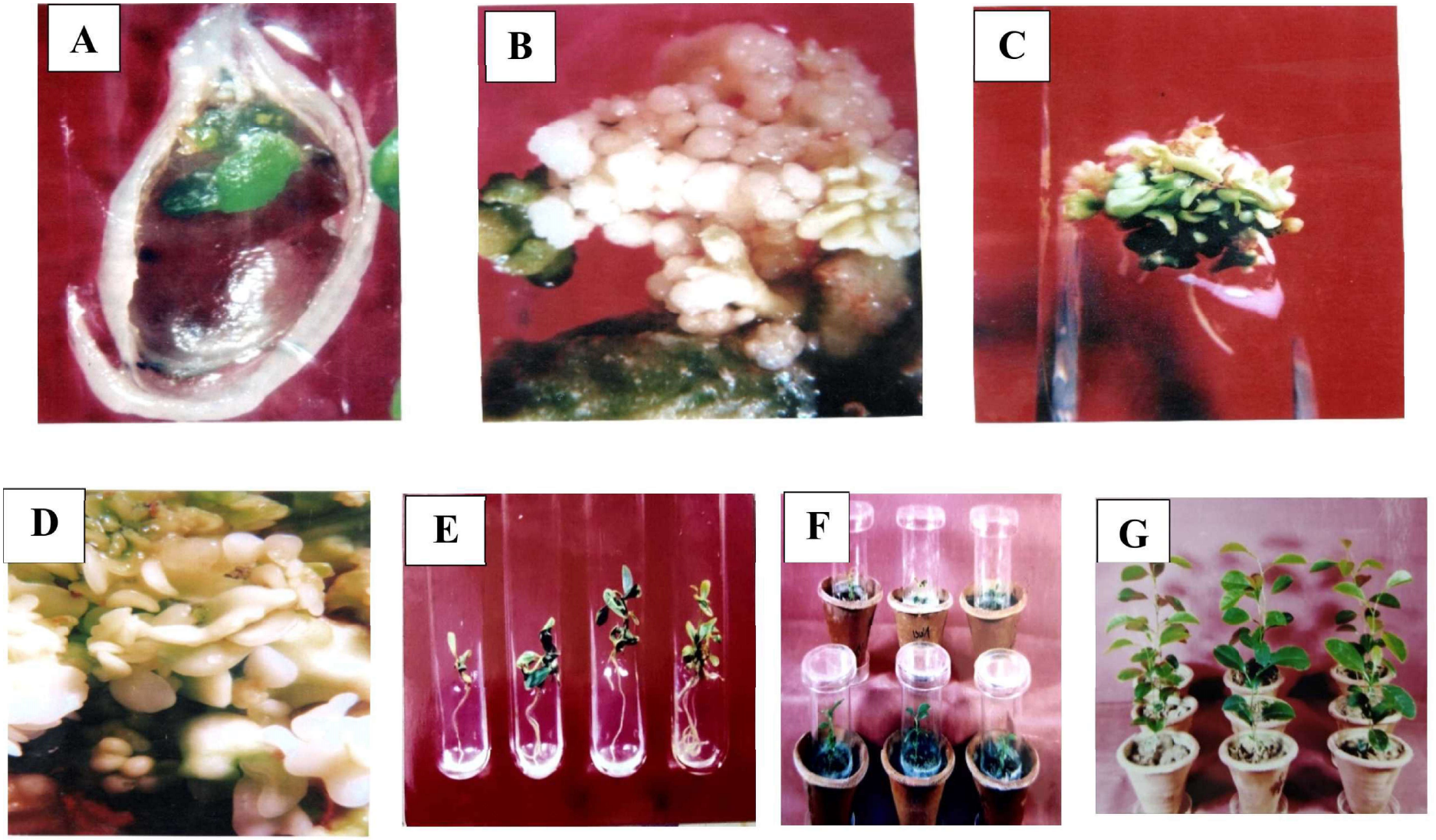

Fig. 1 (A-G) : Culture of *Citrus aurantifolia*

A. Differentiation of globular embryos at micropylar end. B. Proembryogenic tissue differentiated from nucellus showing formation of numerous globular embryos. C.Fasciated mass of nucellar embryos D. Anomalous proliferation of cotyledon-like structures and their fasciation E. Nurturing of nucellar plantlets before shifting them for hardening F. Hardened plantlets transplanted into potted soil for acclimatization G. Nucellar plantlet in potted soil

developed normally and invariably germinated to form individual plantlets. Thus, in the absence of proliferation of embryos before germination resulted in the formation of limited number of plants per culture. This was further reduced since all the germinated embryos did not develop into plantlets. Earlier worker[14] also tried to produce direct *in vitro* plantlets through using epicotyl segment as explants in *C. reticulata.*

Embryogenesis once introduced to form the embryo continued even upon a change in basal medium BM1 to BM_2, containing 0.25 2iP + 0.10 mg l^{-1} NAA which also showed embryo-to-embryo proliferation. Similar synergistic effect of two cytokinin has also been observed in combination as compared to their individual effect during tissue culture of some other taxa[1,20]. Further embryo proliferation was not pronounced as expected. But slightly increased embryo proliferation (7.8 embryo) per culture was observed.

The effects of 2ip and NAA on nucellar embryogenesis were not upto the mark since the fasciation of proembryos resulted in the formation of limited number of developed embryos. To minimize fasciation, 0.25, 0.50 mg l^{-1} putrescine was added along with 0.25 2iP and 0.10 mg l^{-1} NAA which differentiated and proliferated into a mass of globular embryos and these embryos multiply continuously producing a prolific number without individually developing to further advanced stages of embryos and few cotyledonary embryos were witnessed which converted into a mass of proliferating green cotyledonary structures without forming any plumular or radicular organs, ultimately drowning in cotyledonary structures (Fig.1 B, C). Similar effects of NAA had been observed earlier[22].

This is the desirable pattern of *in vitro* nucellar embryogenesis, where individual embryos proliferated through budding mainly from the micropylar region of preformed embryos, which is totally different from the pattern of nucellar embryo "proliferation" reported earlier in *Citrus*, where the deceptive embryonal mass actually comprised proliferation of cotyledons, *i.e.*, other showed pluricotyly[3]. Thus, in the latter instance, there is a very poor yield of embryos, which could be developed into plantlets.In the present study, the proliferation of nucellar embryos resembled the pattern reported for several varieties of mango[3-6]. Even when the embryos proliferation was in progress,these showed a tendency of fasciation of cotyledonary structures during subsequent subcultures instead of embryo-to-embryo proliferation. Such abnormalities have been checked successfully by the modification of nutrient medium in respect of *C. aurantifolia* and properly formed nucellar embryos were produced, which on germination led to normal plantlets development.

In order to further enhance the number of embryo differentiation per culture, the role of 3 cytokinin *viz.* BAP, 2iP and Z used individually with three different concentrations (0.1, 0.25, 0.5 mg l^{-1})of NAA have been analyzed. The latter response might have been due to presence of BAP and Z, whereas in the 2iP containing treatments, the number of normally differentiated embryos in any culture was distinctly higher, albeit only 8 such embryos were formed in *C. aurantifolia*.

In an optimum treatment, comprising BM_2 supplemented with 0.10 2iP, 0.25 putrescine, 0.10 NAA and 200 mg l^{-1} ME, an average of 10 well developed embryos were formed from each inoculated of globular embryos in*C.aurantifolia* (Table-1). Besides the formation of cotyledonary embryos, a proliferation of globular embryos was also variable from the white, friable proembryogenic tissue formed from dedifferentiated inoculated embryos. A few cotyledonary embryos thus formed also drifted to give rise to abnormal proliferated cotyledonary masses (Fig.1 D). The role of putrescine in channelizing embryonal differentiation if not augmenting the number of developed embryos formed per inoculated globular embryos is in conformity with the general role of polyamines in somatic embryogenesis[7] and precisely demonstrated during *in vitro* nucellar embryogenesis in carrot[8] and mango[4,16].

However, the presence of IAA in place of NAA, all five young embryos grew into developed embryos from average length of 4mm of the embryos to 8-10mm without any proliferation of the cotyledons.Addition of amino acid, IAA and ME promote embryo germination (Fig.1 E, F). In concurrence with the present observations, IBA has also been reported to facilitate somatic embryo maturation and germination in larch[23].

The hardening of *in vitro* raised plantlets is essential for their normal growth, survival and successful establishment. Direct transfer of tissue culture raised plants into the field/wild is not possible due to high rate of mortality, as the regenerates in the culture condition have been in cosseted environment, with a very high humidity, varied light and temperature conditions and being protected from the attack of microbial as well as the other agents. Direct transfer to sunlight also caused charring of leaves and wilting of the plants[9,10]. It is, therefore, necessary to accustom the plants to a drier or natural atmosphere by a process called acclimatization or hardening.

The *in vitro* raised plantlets of *C.aurantifolia* produced by this method were transplanted *ex vitro* with 100% survival (Fig.1G) has also been reported[3].

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