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Plantlet regeneration *via* nucellar embryogenesis in *Citrus jambhiri*

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ABSTRACT

To produce true-to-type and rapid multiplication micropropagation technique was utilized to the nucellar tissue with ovular halves of *C. jambhiri.* Nucellar tissues were cultured in a modified MS medium supplemented with different concentrations of 2ip viz. 0.25,0.50 mg l^{-1} alone and in combination of 0.50 mg l^{-1} NAA. Initiation of cell division and differentiation of proembryogenic tissue became apparent in first 80 days.These proembryos developed into embryos through subculturing in fresh medium. Low concentration of 0.25 2ip was found more suitable for the development of embryo producing large number of fully developed embryo in comparision to the embryo produced in the high concentration of 2ip (0.50 mg l^{-1}) and in combination of 0.25 2ip and 0.50 mg l^{-1} NAA. Normally developed embryos in 0.25 2ip showed best germination in the fresh medium supplemented with 0.25 mg l^{-1} IAA, 100 ME mg l^{-1} and 5mg mg l^{-1} amino acids within 30 days as compared to other treatments. These germinated embryos were utilized for producing disease free saplings after hardening and nurturing in laboratory conditions. The disease free saplings thus produced can be used to establish new Citrus orchards within short time.

Figure : 01 References : 21 Table : 00
KEY WORDS : Embryo to embryo proliferation, Nucellar embryogenesis, Regeneration.

Introduction

Citrus jambhiri (rough lemon) species is a rich source of vitamin C, besides a rare and most useful vitamin P and mineral contents, belongs to the family Rutaceae. It is usually grown in tropical and subtropical regions either by cuttings or by seeds. It is now being commercially propagated as rootstocks for the improvement of canopy architecture, food/ or fruit production, quality and tolerance to abiotic and biotic stresses. Improved and uniform production of root stocks of Citrus by conventional method is hampered by polyembryony, sexual incompatibility and male or female sterility[4]. Availability of quality planting material is of utmost importance in the Citrus industry due to limited growing conditions, water resources and high incidence of pest and disease. There is an urgent need to propagate disease free planting material for economic viable sustainable citrus industry and *in vitro* multiplication can be a eco-friendly approach to produce disease free planting material on a large scale in limited space and time[20].

The presence of a complex biology, higher nucellar polyembryony, high heterozygosity, auto-incompatibility and long juvenile period in citrus led the scientists to break these barriers for improvement of the crop through tissue culture[4,13]. Some Indian workers[2,3,18,19] have also carried out work on nucellar embryogeny of *Citrus* species. Workers[5] regenerated plants via embryogenesis using immature ovules of *Citrus* cultivars: *C. sinensis* (sweet oranges), *C. paradisi* (grape fruit), *C. reticulata* (sour mandarin) cv. Owari, *C. paradisi* × *C. reticulata* cv. Orlando, *C. aurantifolia* (lime) cv. Key, and *C. limon* (lemon) cv. Bearss. Some workers[7,15] induced embryogenesis in *C. reticulata* and tried to produce direct *in vitro* plantlet through using epicotyl segment as explants.

Nucellus being a non-vasculated tissue of maternal origin, can be regarded as the juvenile and disease-free tissue of an adult plant. It can be taken to produce somatic embryos to conserve the biodiversity and to eliminate the invasion of micro-organism[1,11]. Therefore in the present investigation nucellar tissue is taken as explants to develop a protocol which can be used to produce large number of disease free, true to type plantlets through micropropogation in *C. jambhiri.*

Materials and Methods

Young fruits at the age of 20-25 days after fruits-set were used for experimenting with nucellar tissue contained in ovular halves of *C. jambhiri* (rough lemon). Fruits of different sizes, *i.e.* measuring ca. 3.5, 4 and

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4.5cm of *C. jambhiri* were plucked and used for dissect out the explants. The fruits were washed in running tap water for 30 min and treated with 5% Labolene (a neutral liquid detergent Glaxo Smithkline Pharmaceuticals Ltd., Mumbai, India), 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 utilization.The surface sterilized fruits were cut longitudinally into two halves. 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 are modifications of medium[10,14]. 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 Murashige and Skoog medium , 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 (Qualigens, Glaxo India, Ltd., Mumbai, India). The medium BM2 differed from BM1 in following respects (concentration in mgl^{-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. Additionally the same concentration was 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. Culture were incubated under 37.5 µ mol $m^{-2}s^{-1}$ quantum flux density for 15 h a day at a temperature of 26° ± 1° C and 75% ± 5% RH. The *in vitro*- raid nucellar plantlets were acclimalized and nurturing them in modified Knop's solution for 30 days and then transferring them to potting mixture comprising farm-yard manure and soil (1:3).

Result and Discussion

First of all nucellar explants drawn from different sizes of fruits have been tested to select the suitable explant for the nucellar embryogenesis. From these experiments, it became clear that the fruit of the different developmental stages of nucellus markedly affect the nucellus embryogenesis. Nucellar polyembryogenic property of citrus was found to enhance regeneration ability and large number of plants were germinated from these nucellar embryonic tissue which were identical to their mother plant. Similar results were also seen in *Citrus reticulata* where seeds show extra ordinary polyembryonic nucellar tissue and further development into many plantlets[8]. Nucellar embryos develop from nucellar wall in citrus seeds along with zygotic embryo are found to have high plant regeneration ability in comparison to zygotic embryos[8]. Nucellus from younger fruits of *C. jambhiri* upon subculture undergoes intensive callus formation instead of embryo-to-embryo formation. The ovular wall and nucellus tissue both took part with equal intensity to produce callus. Therefore, it is clearly indicated that for nucellar embryogenesis, the immature fruits of intermediate or larger size are more suitable.

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.,* showed pluricotyly[3]. The metabolic status of plant greatly influences its response to growth substances present in the medium BM1 as also their preferential requirement for growth hormone for *C. jambhiri* 0.25 mg l^{-1} 2iP found better and nutrient is well-documented *Capsella* embryo culture, nutritional and hormonal requirements of a globular embryo for its growth are different from those of a heart-shaped or torpedo-shaped embryo[16,17]. For the development of embryos and their embryo-to-embryo proliferation, a polyamine 0.50 putrescine was used alongwith the lower concentration of 0.10 2iP and 0.10 NAA to overcome the problem of fasciation coupled with formation cotyledon-like structures (Fig.1A,B).

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 investigated (Fig.1 C, D) In concurrence with the present observations, IBA has been reported to facilitate somatic embryo maturation and germination in larch[21].

The hardening of *in vitro* raised plantlets are 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

Fig. 1 (A-E) : Cultures of *Citrus jambhiri*

A. Differentiating nucellar embryos at the micropylar end, B. Embryo to embryo proliferation, C. Germination of cotyledonary embryos, D. Germinated embryo with plumule , *E. Ex-vitro* growth of *in-vitro* raised plantlets after four month

protected from the attack of microbial as well as the other agents. Direct transfer to sunlight also causes charring of leaves and wilting of the plants[6,12]. In other words, the survival percentage is determined by the hardening of the plantlets. 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.jambhiri* produced by either method, were transplanted *ex vitro* with 100% survival (Fig.1E) following the procedure reported earlier[3].

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