

Biotechnological Methods and Tissue Culture Techniques for Mass Production of Nucellar Plants of *Citrus limonia*

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ABSTRACT: The present experimentation is mainly aimed to resolve this problem and produce sufficient number of nucellar plantlets per culture. The significance of nucellar embryogenesis, particularly in case of rootstocks can hardly be over emphasized. However, it is also significant in case of *C. limonia*, where commercial use of its nucellars for raising orchards is in practice. Nucellus is a somatic tissue having a genetic makeup similarly to the vegetative plant body. The culture of nucellar tissue and augmentation and induction of nucellar polyembryony have initially been the main focus of research. However, the associated extended juvenile characteristics of nucellar plantlets, if not slightly poor quality of fruit, restrict the application of *in vitro* nucellar embryogenesis to rootstocks only, since both such characteristics will not be of any consequence while virus-free rootstocks will be produced at a rapid rate throughout the year, which in turn is imperative for all the year round production of micrografts. When critically examined, the nucellar embryonal proliferation, i.e., embryo-to-embryo multiplication is an uncommon phenomenon. Instead, there is cotyledonary proliferation forming resette-like structures having a single radicle with pluricotyly, which is a big snag in production of nucellar plantlets.

KEYWORDS: Nucellar Plantlets, Nucellar Polyembryony, Micropropagation

I. INTRODUCTION

The nutritional value, the magnitude of fruit production and an array of commercial products made, the citrus may be considered as a number one fruit of the world. Besides being a rich source of vitamin C and of minerals, it is a rare source of vitamin P, the anti-haemorrhage vitamin. However, paradoxically enough, while the citrus orchard area in the country is comparable to that of USA and Brazil, and it has a very rich heritage of *Citrus* species as also the Northeast region of India is considered to be the place of citrus origin as its production is not comparable to these countries and is even less than that of Spain or Italy (FAO, 2002), with virtually nil export. On the top of it, the citrus production is persistently declining mainly because of spread of incidence of virus infection in orchards (Chaturvedi et al., 2001). At the same time, there is a glaring example that with the application of Tissue Culture Technology, the Citrus Industry has been revolutionarized in Spain (Navarro and Juarez, 1977; Navarro, 1992), by virtue of which has become the biggest exporter of citrus fruits (FAO, 2002). Thus, there exists a great scope for ameliorating such a deplorable state of citrus fruit production in the country with the application of Plant Tissue Culture to not only boost the Citrus Industry for home consumption but also for export promotion. The Plant tissue Culture, which can bring about such a change are clonal propagation and nucellar embryogenesis, particularly in citrus rootstocks for rapid production of clean and clonal plants. Besides, micropropagation through nodal stem-segments taken from healthy field-grown trees, particularly of citrus rootstocks and tailoring of root system in regenerated shoots, as propounded earlier for *Citrus* spp. (Chaturvedi, 1979) may also promote the same cause. Like citrus rootstocks, accelerated production of nucellars in at least one of the scion species, *Citrus limonia* will be of significance for producing clonal plants without resorting to grafting and compromising the fruit quality (Chaturvedi and Agnihotri, 2002).

The maximum tissue culture research amongst the fruit trees has been done in Citrus along the various aspects useful for boosting the Citrus Industry. Besides improving the existing citrus orchards in the country, there is enough scope to

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increase citrus fruit production by extending the area of cultivation of selected industrially important *C. limonia* in Gujarat, Maharashtra, Bihar, Uttar Pradesh and Andhra Pradesh. Particularly in Uttar Pradesh, there is enough scope of extend cultivation of *C. limonia* in the areas, like, Agra, Aligarh, Etah, Mainpuri, Mathura and lower foot hills of Western Uttar Pradesh. This goal can be realized by implementing whatever new achievements in the citrus tissue culture technology have already been made, such as, production of cloned and cleaned plants through shoot meristem culture and micrografting, cloning of mature trees through nodal stem segment culture and production of clonal and disease-free prized rootstocks through nucellar embryogenesis, albeit only to some extent. Coupled with these aspects, an equally important proposition is the germplasm preservation through normal as well as slow growth culture of shoots raised from shoot meristem and nodal stem segments of elite mature trees. All these aspects are also covered in detail in a number of exhaustive reviews on Citrus Tissue Culture, mainly by Button and Kochba (1977), Spiegel-Roy and Kochba (1980), Barlass and Skene (1986), Chaturvedi and Sharma (1988), Navarro (1992), Grosser (1994), Parthasarthy et al. (2001) and Chaturvedi et al. (2001).

II. MATERIALS AND METHODS

The various nutrient formulations used in the present investigation were derived mostly by variously modifying Murashige and Skoog (1962) medium; except the hardening medium of inorganic salts, which was a modification of Knop (1865) medium. Compositions of these media are given in Table 1.

Table 1: Compositions of nutrient media used (mg l⁻¹)

Constituents	PM9	BM5	BM6	MK
(NH ₄) ₂ SO ₄ ·7H ₂ O	250	100	200	–
NH ₄ NO ₃	1500	300	500	500
KNO ₃	1500	500	500	250
KCl	–	–	–	–
KH ₂ PO ₄	150	150	250	125
NaH ₂ PO ₄ ·H ₂ O	–	–	–	–
CaCl ₂ ·2H ₂ O	400	100	200	–
Ca(NO ₃) ₂ ·7H ₂ O	–	–	–	500
MgSO ₄ ·7H ₂ O	450	100	300	250
Na ₂ SO ₄	–	–	–	–
Na-Fe-EDTA (ml/l)	5	5	5	3
MnSO ₄ ·4H ₂ O	20	20	20	22.30
ZnSO ₄ ·4H ₂ O	8	8	8	8.60
H ₃ BO ₃	6	6	6	6.20
KI	0.8	0.8	0.8	0.83
Na ₂ MoO ₄ ·2H ₂ O	0.2	0.2	0.2	0.25
CuSO ₄ ·5H ₂ O	0.02	0.02	0.02	0.025
CoCl ₂ ·6H ₂ O	0.02	0.02	0.02	0.025
Thiamine-HCl	0.2	0.02	2.5	–
Pyridoxine-HCl	0.1	0.1	0.5	–
Nicotinic acid	0.5	0.5	0.5	–
Riboflavin	–	–	0.1	–
Folic acid	–	–	0.1	–
d-Biotin	–	–	0.1	–
Ascorbic acid	10	10	5	–
m-Inositol	300	–	–	–
Glycine	3	–	3	–
L-Glutamine	–	–	–	–
L-Arginine	–	–	15	–

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L-Asparagine	–	–	–	–
DL-Lysine	–	–	–	–
L-Cystiene	–	–	–	–
Adenine sulphate	15	5	5	–
Malt extract	500	–	–	–
Sugar (%)	5	3	5	–
BAP	0.25	–	–	–
IAA	0.25	–	1	–
NAA	–	–	0.5	–
IBA	–	0.25	–	–
IPA	–	–	0.5	–
Chlorogenic acid	–	1	1	–
pH	5.8	5.8	5.8	–
Agar (%)	0.75	0.75	0.75	–
Phytigel (%)	–	–	–	–

Experiments with old cultures: When the work of the present project was taken up, there already existed prolonged cultures of proliferating shoots raised from shoot meristems, micrografts and nodal stem-segments taken from field-grown healthy plants of *C. limonia*. Production of plants of respective *Citrus limonia* was taken up by utilizing such cultures.

In order to increase the number of proliferating shoots, groups of regenerated shoots originated from different explants were cultured on the same medium PM9. Besides the medium composition, the duration between 2 subcultures was crucial for raising healthy and rapidly proliferating shoots. The duration of 20-25 days of subculture was found to be optimum in all the cases, whereby vigorously proliferating cultures raised from shoot meristems, micrografts and nodal stem-segments were realised.

During prolonged culture there had been a little decline in the rate of proliferation as well as growth of shoots. Cultures initiated from shoot apices after rooting of isolated shoots taken from old cultures showed improvement in proliferation as well as growth of proliferated shoots, which were at par with those of initial cultures. It is an interesting observation similar to that reported in case of shoot regeneration of *Citrus* species from stem callus obtained from declining cultures of proliferating shoots, where through reinitiation of shoot cultures from callus regenerated shoots, original vigour of growth has been restored (Chaturvedi, 1979). In certain other cases restoration of so called lost regeneration potentiality of somatic callus tissue has been reported to be effected by change in cytokinin from 6-benzylaminopurine (BAP) to zeatin as in citrus (Chaturvedi and Mitra, 1974) and substitution of gibberellic acid (GA) from BAP in the medium in case of *Digitalis purpurea* (Chaturvedi and Jain, 1994).

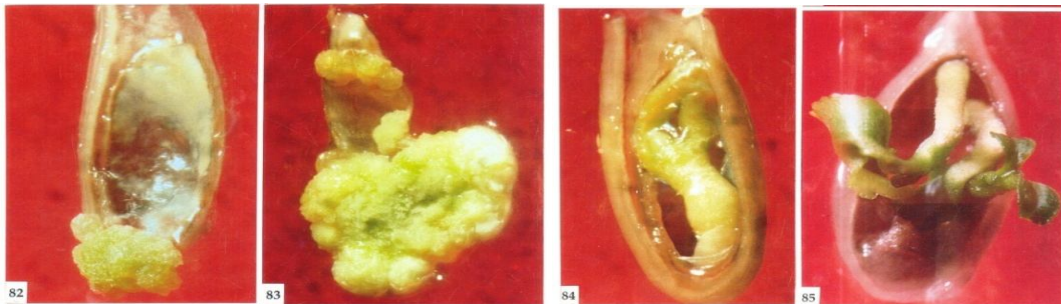
The microshoots isolated from the aforesaid 3 kinds of cultures of proliferating shoots rooted 100% within 15 days in the earlier formulated rooting medium, BM5. There was no difference in the rooting behaviour of isolated shoots taken from any of the 3 kinds of cultures. Significance of nucellar embryogenesis, particularly in case of rootstocks can hardly be over emphasized. However, it is also significant in case of *C. limonia*, where commercial use of its nucellars for raising orchards is in practice.

III. RESULTS

The young fruits *C. limonia* of the size of ca. 2.5 to 3.5 cm in diameter contained nucellar tissue of desirable maturity, so that in an optimum treatment containing 0.5 mg l⁻¹ putrescine with 0.1 mg l⁻¹ NAA produced several nucellar embryos at the micropylar region devoid of zygotic embryo. Favourably responded nucellar tissue with nucellar embryos on transfer to the same treatment or slightly different treatment 0.5 mg l⁻¹ putrescine + 0.25 mg l⁻¹ 2iP + 0.1 mg l⁻¹ NAA resulted in profuse multiplication of globular embryos, which further differentiated into preheart shaped structures intermixed with globular embryos in treatment containing 0.25 mg l⁻¹ putrescine + 0.1 mg l⁻¹ each of 2iP and NAA. Besides, the most suitable cytokinin, i.e., 2iP, putrescine had a crucial role to play in alleviating the proliferating

cotyledon-like-structures and their fasciation taking over the normal differentiation and proliferation of embryos. The point became more explicit when the bicotyledonary structures were taken out from such cultures and subjected to germination in comparison to a few normal embryos. BAP along with putrescine deteriorated the response of putrescine used alone in respect of normal embryo formation per culture, while Z at any of the 3 concentrations used inhibited the process of normal differentiation of embryos. Details of responses evidenced by proliferating globular nucellar embryos to different concentrations of putrescine used alone and along with 3 cytokinins, viz., 2iP, BAP and Z. In the optimum treatment containing 0.1 mg l^{-1} 2iP, 0.5 mg l^{-1} putrescine and 0.1 mg l^{-1} NAA, mostly the proliferating embryos remained isolated as if there had been embryo-to-embryo proliferation. Occasionally, the embryos were also seen in groups, but did maintain their individual entity with cotyledonary and radicular ends. Besides, embryo-to-embryo proliferation, there was another mode of multiplication of embryos, in which the secondary embryos differentiated from the hypocotyls region of earlier formed developed embryos. The deceptive impression given by the cultures of cotyledon-like-structures as if the 2 proximally united cotyledonary structures were destined to produce plantlets was obvious when they were isolated and cultured in the embryo germination medium. From such aberrant embryos, radical like structures were formed but their was no formation of shoots from the so called plumular ends. On the other hand, the normal embryos picked up from amongst proliferating and developing normal embryos readily germinated giving rise to root and shoot from opposite poles.

The maturity of nucellus tissue in the ovule was more critical in *C. limonia* than in *C. aurantifolia* and there had been a propensity of more callusing of young ovular halves, while in older ones, individual nucellar embryos developed into plantlets without proliferation (Figs. 82-86). The nucellar tissue of optimum maturity produced mass of proliferating nucellar embryos sometimes associated with callus tissue (Figs. 87-89). But in general, the proliferating nucellar embryos could be sustained to proliferate, albeit at a slow rate where embryos at different developmental stages from globular to heart to cotyledonary were seen intermixed together (Figs. 90-92). Occasionally, *in situ* germination of proliferating nucellar embryos was also witnessed where radicle development remained suppressed (Fig. 93).



Figs. 82-85: Cultures of *Citrus limonia*

Figs. 82-83: Excessive callusing from the nucellus tissue and the ovular wall tissue in ovular halves (explants) taken from fruits younger than of desirable maturity

Fig. 84-85: Formation of individual nucellar embryos from halves of ovules taken from fruits of older than of desirable maturity



Figs. 86-89: Cultures of *Citrus limonia*

Fig. 86: Regeneration of multiple shoots from a developed nucellar embryo contained in an ovular half

Fig. 87: Culture of isolated nucellus tissue with differentiating nucellar embryos

Fig. 88-89: Multiplication of nucellar embryos at the micropylar end of an ovular half with desirable nucellar maturity (Fig. 88); proliferation of nucellar embryos along with the nucellar tissue (Fig. 89)



Figs. 90-93: Cultures of *Citrus limonia*

Fig. 90-91: Nucellar embryos of different developmental stages (Fig. 90), picked up from cultures of proliferating embryos (Fig. 91)

Fig. 92: Culture of proliferating nucellar embryos without any fasciation seen at different developmental stages

Fig. 93: Regeneration of multiple shoots developed from embryos still contained in an ovular half

IV. DISCUSSION

The paradoxically enough, though the main emphasis of tissue culture studies in *Citrus* has been on nucellar embryogenesis (Button and Kochba, 1977; Rangaswamy, 1981), the success in the real sense of the term has not been achieved (Chaturvedi et al., 2001, 2007, Jain et al., 2005; Singh et al., 2005). Induction of nucellar embryogenesis has also been reported in monoembryonic *Citrus* species, as in *C. grandis*, *C. limon* and *C. reticulata* x *C. sinensis* (temple orange) (Rangan et al., 1968; Chaturvedi, 1979). Also, the *in vitro*-raised nucellar plants have been grown *ex vitro* in case of *C. sinensis* (Button and Bornman, 1971), *C. aurantifolia* and *C. sinensis* (Mitra and Chaturvedi, 1972; Gmitter and Moore, 1986) and *C. paradisi* and *C. reticulata* (Gmitter and Moore, 1986). But the main drawback in all the studies carried out so far has been embryonal fasciation and pluricotily instead of embryo-to-embryo multiplication with the result that though multiple embryos may appear to have been formed, but in fact, it has been more often than not a single embryo with a single radical, or else, cotyledon-like-structures proliferate without any plumule or radical differentiation. As such, production of nucellar plantlets per culture is extremely poor. Nucellar embryogenic tissue provides the best system for genetic transformation studies at a single cell level avoiding chimeric problems.

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International Journal of Innovative Research in Science, Engineering and Technology

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Vol. 3, Issue 7, July 2014

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