

Biotechnological Interventions and Prophylactic Studies in Clonal Multiplication through Nodal Stem-segments From Mature Trees

Dubey Rajesh Kumar¹Director, Prakriti Educational & Research Institute, Lucknow, UP, India¹

ABSTRACT: Cultures of proliferating shoots of *C. reticulata* and *C. nobilis* x *C. deliciosa* were raised by culturing nodal stem-segments excised from fresh growth of shoots of field-grown plants. The percentage of success in establishing aseptic cultures depended on the season and the maturity of fresh growth during the period of February-March. Best results were obtained when explants were taken during late February. It is imperative to take effective steps to guard against re-infection of pathogen-free citrus orchards raised through *in vitro* strategies, which may necessitate a thorough sanitation programme, including eradication of alternate hosts from the vicinity of orchards and control of vectors of tristeza virus and greening bacterium in particular, since rest of the viruses are mechanically transmitted. Thus, the sanitation programme has to be complementary with the virus eradication programme. The success of whole programme may also demand enacting a suitable legislation in order to enforce eradication of existing diseased orchards of citrus and plantation of only the certified planting material in new orchards.

KEYWORDS: Shoot Meristem, Clonal Propagation, Micro-Grafting, Germplasm,

1. INTRODUCTION

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 main 3 facets of Plant tissue Culture, which can bring about such a change are: shoot meristem culture and micrografting for virus elimination from scion species besides rapid 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 aurantifolia* will be of significance for producing clonal plants without resorting to grafting and compromising the fruit quality (Chaturvedi and Agnihotri, 2002).

Perhaps 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 increase citrus fruit production by extending the area of cultivation of selected industrially important *Citrus*

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species, e.g., *C. sinensis* in Maharashtra, Andhra Pradesh, Bihar, Uttar Pradesh and Karnataka; *C. aurantifolia* in Gujarat, Maharashtra, Bihar, Uttar Pradesh and Andhra Pradesh, while *C. reticulata* in Maharashtra, Karnataka, Madhya Pradesh, Rajasthan and Northeast region of the country. Particularly in Uttar Pradesh, there is enough scope of extend cultivation of *C. aurantifolia* and *C. limon* 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).

In recognition of the great importance of shoot meristem culture of explants, measuring 500 µm to 1 mm in length, from mature trees of *Citrus*, concerted efforts have been made world over for the past 3 decades but to no avail (Bitters and Murashige, 1967) till the success was achieved by Chaturvedi et al. (2001), Singh (2001). However, such failures paved the way for the success of an alternative method of virus elimination, i.e., micrografting (Navarro, 1992). Excised shoot meristems of *Citrus* are extremely vulnerable to desiccation besides their critical nutritional and hormonal requirements for sustaining growth. That is why, excised shoot meristems of *C. grandis* (L.) Osbeck as long as 1 mm, excised even from the aseptically-grown seedlings could not be grown beyond the size of 1.5 cm in length (Chaturvedi, 1979).

II. MATERIALS AND METHODS

Scion species

C. aurantifolia Christm. Swingle (lime), *C. sinensis* Linn. Osbeck (sweet orange), *C. reticulata* Blanco (orange or mandarin) and *C. nobilis* Lour. x *C. deliciosa* Tenora (Kinnow mandarin).

Explants

Shoot apices, nodal stem-segments taken from field-grown trees and subsequently from established aseptic cultures for obtaining shoot meristems and leafy shoots. Young fruits for obtaining nucellar tissue contained in ovular halves.

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	PDPM3	BM5	BM6	MK
(NH ₄) ₂ SO ₄ ·7H ₂ O	250	250	100	200	—
NH ₄ NO ₃	1500	1650	300	500	500
KNO ₃	1500	1900	500	500	250
KCl	—	—	—	—	—
KH ₂ PO ₄	150	170	150	250	125
NaH ₂ PO ₄ ·H ₂ O	—	—	—	—	—
CaCl ₂ ·2H ₂ O	400	440	100	200	—
Ca(NO ₃) ₂ ·7H ₂ O	—	—	—	—	500
MgSO ₄ ·7H ₂ O	450	450	100	300	250
Na ₂ SO ₄	—	—	—	—	—
Na-Fe-EDTA (ml/l)	5	5	5	5	3

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MnSO ₄ .4H ₂ O	20	20	20	20	22.30
ZnSO ₄ .4H ₂ O	8	8	8	8	8.60
H ₃ BO ₃	6	6	6	6	6.20
KI	0.8	0.8	0.8	0.8	0.83
Na ₂ MoO ₄ .2H ₂ O	0.2	0.2	0.2	0.2	0.25
CuSO ₄ .5H ₂ O	0.02	0.02	0.02	0.02	0.025
CoCl ₂ .6H ₂ O	0.02	0.02	0.02	0.02	0.025
Thiamine-HCl	0.2	0.2	0.02	2.5	–
Pyridoxine-HCl	0.1	0.1	0.1	0.5	–
Nicotinic acid	0.5	0.5	0.5	0.5	–
Riboflavin	–	–	–	0.1	–
Folic acid	–	–	–	0.1	–
d-Biotin	–	–	–	0.1	–
Ascorbic acid	10	10	10	5	–
m-Inositol	300	100	–	–	–
Glycine	3	3	–	3	–
L-Glutamine	–	–	–	–	–
L-Arginine	–	–	–	15	–
L-Asparagine	–	–	–	–	–
DL-Lysine	–	–	–	–	–
L-Cystiene	–	–	–	–	–
Adenine sulphate	15	15	5	5	–
Malt extract	500	–	–	–	–
Sugar (%)	5	3	3	5	–
BAP	0.25	0.25	–	–	–
IAA	0.25	0.25	–	1	–
NAA	–	–	–	0.5	–
IBA	–	–	0.25	–	–
IPA	–	–	–	0.5	–
Chlorogenic acid	–	–	1	1	–
pH	5.8	5.8	5.8	5.8	–
Agar (%)	0.75	0.75	0.75	0.75	–
Phytigel (%)	–	–	–	–	–

III. RESULTS

Cultures of proliferating shoots of *C. reticulata* and *C. nobilis* x *C. deliciosa* were raised by culturing nodal stem-segments excised from fresh growth of shoots of field-grown plants. The percentage of success in establishing aseptic cultures depended on the season and the maturity of fresh growth during the period of February-March. Best results were obtained when explants were taken during late February.

The 2 *Citrus* species varied in their requirements of nutrient media and growth hormones for the initial stage of bud-break. Whilst the medium PM9 and the treatments based on it were effective in *C. reticulata*, the explants of *C. nobilis* x *C. deliciosa* responded better in PDPM3 and treatments based on it.

The 2 *Citrus* species also differed in their responses for bud-break in the respective optimum treatments which was 55% in case of *C. reticulata* and 45% in case of *C. nobilis* x *C. deliciosa*. Also, the growth of regenerated shoots was better in the former than in the latter, while the reverse was true in respect of incidence of contamination (Table 6). It took ca. 20 days for bud-break in both the *Citrus* species and when the regenerated shoots had attained an av. Length of 3 cm (Figs. 21, 24), they were excised from the mother tissue and induced to proliferate in different treatments using

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the same basal medium (PM9), which responses are given in Table 7. In the optimum treatment containing 0.25 mg l⁻¹ each of BAP and 2iP, 0.5 mg l⁻¹ GA and 0.25 mg l⁻¹ each of IAA and CCC, an av. 4 offshoots per shoot were produced in *C. reticulata* and 3 offshoots per shoot in *C. nobilis* x *C. deliciosa* during an incubation of 30 days (Figs. 22, 25). Finally, the shoots along with offshoots grew favourably in the presence of only 0.25 mg l⁻¹ each of BAP and IAA and the data are given in Table 8. Healthy cultures with proliferating and developing shoots were obtained after 2-3 subcultures of 30 days incubation each, where during after 90 of days total incubation an av. 15 offshoots per explants were formed in *C. reticulata* and 13 in *C. nobilis* x *C. deliciosa* (Figs. 23, 26).

Table 6: *In vitro* establishment of *Citrus aurantifolia* and *C. nobilis* x *C. deliciosa* through nodal stem-segments taken from field-grown plants. Incubation period 30 days

Treatment	Citrus species	Contaminated explants (%)	Explants showing bud-break (%)	Growth of regenerated offshoots in length (cm)
0.25 BAP + 0.5 IAA	<i>C. reticulata</i>	32.5	55	2.5±0.42
0.25 BAP + 0.5 IAA	<i>C. nobilis</i> x <i>C. deliciosa</i>	37.5	45	2.2±0.37

Data based on 40 replicates ±SD

Table 7: Effects of different growth hormones used with or without a growth retardant on proliferation of isolated shoots of *Citrus reticulata* and *C. nobilis* x *C. deliciosa*. Incubation period: 30 days

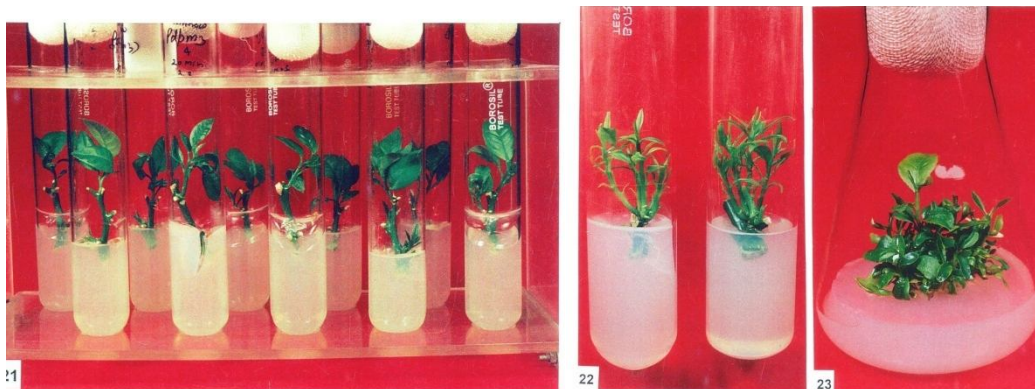
Treatments (0.2 IAA common to all) (conc. in mg l ⁻¹)	<i>Citrus reticulata</i>			<i>C. nobilis</i> x <i>C. deliciosa</i>		
	Explants favourably responded (%)	Number of offshoots per explants	Growth of regenerated shoots: in length (mm)	Explants favourably responded (%)	Number of offshoots per explants	Growth of regenerated shoots: in length (mm)
0.1 BAP + 5 GA + 0.5 CCC	66.67	2.8±0.52	16.2±1.42	73.33	2.1±0.32	14.3±0.82
0.1 2iP + 5 GA + 0.5 CCC	73.33	2.9±0.47	15.8±1.29	66.67	2.3±0.37	13.1±0.79
0.125 BAP + 0.125 2iP + 1 GA + 0.25 CCC	80	3.6±0.63	14.7±0.93	73.33	2.6±0.28	12.8±0.98
0.25 BAP + 0.25 2iP + 0.5 GA + 0.25 CCC	86.67	4.1±0.69	15.6±1.18	80	3.3±0.46	13.4±0.92
0.25 BAP + 0.25 2iP + 0.25 GA + 0.25 CCC	80	3.2±0.48	13.5±0.95	66.67	2.9±0.42	11.9±0.79

Data based on 15 replicates ±SD

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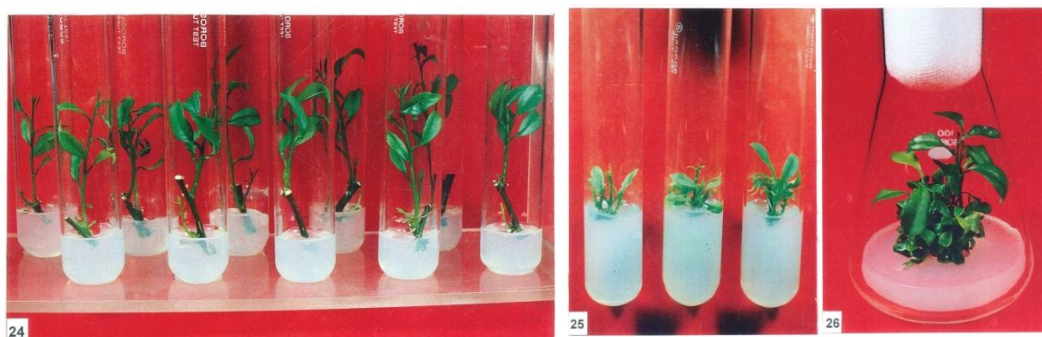
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Figs. 21-23: Cultures of *Citrus reticulata*

- Fig. 21:** Regeneration of healthy shoots from nodal stem explants taken from a field-grown tree
Fig. 22: Initiation of shoot proliferation in excised regenerated shoots
Fig. 23: Cultures of proliferating shoots in a treatment containing 0.25 mg l^{-1} each of BAP and IAA



Figs. 24-26: Cultures of *Citrus nobilis* x *Citrus deliciosa*

- Fig. 24:** Regeneration of axillary shoots from nodal stem-segments taken from a field-grown tree
Fig. 25: Initiation of proliferation of excised regenerated shoots
Fig. 26: A culture of proliferating shoots in a treatment containing 0.25 mg l^{-1} each of BAP and IAA

IV. DISCUSSION

Like, excised shoot meristem of *Citrus*, the success has been eluding to clone it by employing nodal stem-segments, excised from mature trees (Groser, 1994).

Recently, success has been achieved in cloning mature elite trees of *C. aurantifolia* and *C. sinensis* as well as their rootstocks, namely, *C. karna*, *C. jambhiri* and *C. limona* by employing their nodal stem-segments (Singh and Chaturvedi, 1999; Singh, 2001). The crucial aspect of tailoring of rooting of shoots, to be used for micrografting or growing them as such, to a tap root-like system as demonstrated earlier in case of somatic callus-regenerated shoots of *C. grandis* (Chaturvedi, 1979) ensures 100% transplant success.

The proliferating axillary shoots also comprise the source of excised shoot meristems for culture. A very significant advantage in raising plantlets by employing explants from mature twigs is that the juvenile phase of 6 to 20 years is

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circumvented and the clonal plants come to reproductive phase in just 2 to 3 years of ex vitro growth, as the maturity phase is carried over through the functional gene/s in the explants (Peña et al., 2001). The technology developed should be applied to other scion and rootstock *Citrus* species as well.

The low productivity of citrus orchards in India, which may even be less than 7 tonnes per ha in contrast with ca. 23 tonnes per ha in the major citrus producing countries is mainly due to virus infection. In India, a major toll is taken by citrus tristeza closterovirus, citrus ring spot virus, citrus mosaic virus, citrus exocortis viroid and citrus greening caused by *Liberobacter asiaticum* bacterium, which together result into the 'citrus decline'. By an estimate, citrus tristeza virus alone causes a world-wide loss to the tune of ca. £ 9-24 million (Hull and Davies, 1992).

As already stated, the citrus tristeza virus is aphid transmitted, while greening bacterium is transmitted by psyllids. Further, while *Toxoptera citricidus* is the main aphid followed by *Aphis gossypii*, *A. spiraeicola* and *T. aurantii*, *Diaphorina citri* is the main psyllid in India. Both these groups of vectors are controllable mainly by insecticidal spray schedules and insect traps coupled with eradication of alternate hosts. For citrus tristeza virus, *Murraya koenigii*, *Ruta graveolens* and *Evodia hupelensis* are the main alternate hosts, while for citrus greening *Catharanthus roseus* is the only major alternate host in India.

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BIOGRAPHY



Rajesh Kumar Dubey is M. Tech & Ph.D. (Biotechnology), a nationally reputed biotechnologist & sustainable development facilitator, academician, policy maker & administrator. He worked as Visiting Professor & Director, Centre for Technology Development and Research, (CTDR), JRRS University, Jaipur, Rajasthan India. He has rich & versatile experience of more than 2 decades in the field of science & technology and biotechnology for poverty alleviation and sustainability. Associated with the World Bank supported projects on diversification support & micro-credit action research and demonstrated technology based societal transformations through knowledge connectivity. His major contributions are with innovations in rural technology for green growth, eco-livelihood and sustainability. Carrying forward with Council of Science & Technology, Organic Missions, Nature based livelihood development programmes, National Agriculture Technology Project (NATP) & Agriculture Technology Management (ATMA) development, clean energy & climate mitigation programmes etc for different national & international development institutions across the globe, Dr. Dubey is winner of more than a dozen national & international citations and awards.

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