

Study of the Comparative Performance of *Citrus Aurantifolia* Plants Grown on Their Own Roots and Raised by Micro-Grafting of Nucellar Plantlets

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ABSTRACT: Micrografting provides cloned plants, which are also largely free from pathogens, including viruses and virus-like pathogens. Though micrografting is a tedious and time consuming technique requiring also exceptive dexterity, it provides a practicable method, whereby very minute shoot meristems (less than 1 mm in length) can be regenerated even in those plant species where such small excised shoot meristems cannot be regenerated as has been the case in *Citrus*. Micrografting has been most extensively and usefully applied to *Citrus* amongst all the fruit trees. The most glaring example of improvement of citrus orchards leading to many-fold increase in fruit production has been demonstrated in Spain by producing about 31 million healthy plants originally recovered by micrografting. Despite the fact that shoot meristems of all the scion species, viz., *C. sinensis*, *C. aurantifolia*, *C. reticulata* and *C. nobilis* x *C. deliciosa* could be regenerated and proliferated without the support of rootstock seedlings already having developed root systems, it had been necessary to see the performance of micrografted shoot meristems of these species, using 3 important citrus rootstocks, namely, *C. limonia*, *C. karna* and *C. jambhiri*. Except *C. aurantifolia*, which could be profitably grown on its own root system under field conditions, rest of the scion species required rootstocks for their *ex vitro* survival and flourish in order to be productive.

KEYWORDS: Nucellar Plantlets, Nucellar Polyembryony, Micropropagation

1. INTRODUCTION

Although citrus is classified as number three fruit of the world, but in the national context, it may be considered as the top priority fruit of India, considering the huge orchard area and its commercial applications. 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 (Chaturvey 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,

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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* 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).

Rootstock species: *C. limonia* Osbeck (Rangpur lime), *C. karna* Raft. (karna Khatta) and *C. jambhiri* Lush. (rough lemon).

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	CMC	PN	BM5	BM6	MK
(NH ₄) ₂ SO ₄ ·7H ₂ O	250	250	250	100	200	–
NH ₄ NO ₃	1500	1500	500	300	500	500
KNO ₃	1500	1500	500	500	500	250
KCl	–	–	–	–	–	–
KH ₂ PO ₄	150	150	150	150	250	125

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NaH ₂ PO ₄ .H ₂ O	–	–	–	–	–	–
CaCl ₂ .2H ₂ O	400	400	200	100	200	–
Ca(NO ₃) ₂ .7H ₂ O	–	–	–	–	–	500
MgSO ₄ .7H ₂ O	450	450	450	100	300	250
Na ₂ SO ₄	–	–	–	–	–	–
Na-Fe-EDTA (ml/l)	5	5	5	5	5	3
MnSO ₄ .4H ₂ O	20	20	20	20	20	22.30
ZnSO ₄ .4H ₂ O	8	8	8	8	8	8.60
H ₃ BO ₃	6	6	6	6	6	6.20
KI	0.8	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	0.2	0.25
CuSO ₄ .5H ₂ O	0.02	0.02	0.02	0.02	0.02	0.025
CoCl ₂ .6H ₂ O	0.02	0.02	0.02	0.02	0.02	0.025
Thiamine-HCl	0.2	0.2	0.5	0.02	2.5	–
Pyridoxine-HCl	0.1	0.1	0.1	0.1	0.5	–
Nicotinic acid	0.5	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	–	3	–
L-Glutamine	–	25	–	–	–	–
L-Arginine	–	15	–	–	15	–
L-Asparagine	–	10	–	–	–	–
DL-Lysine	–	10	–	–	–	–
L-Cystiene	–	10	–	–	–	–
Adenine sulphate	15	15	25	5	5	–
Malt extract	500	–	200	–	–	–
Sugar (%)	5	3	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	5.8	5.8	–
Agar (%)	0.75	0.75	–	0.75	0.75	–
Phytigel (%)	–	–	0.2	–	–	–

III. RESULTS

Shoot meristems, measuring ca. 2 mm in length, of all the 4 *Citrus* species were excised from cultures of proliferating shoots raised from their respective shoot meristems; and hence, pathogen-free including viruses. Survival percentage of shoot meristems of size bigger, i.e., ca. 2 mm in length than required for elimination of viruses, i.e. ca. 0.5 mm in length had been better. Whereas, shoot meristems bigger than 2 mm in length showed poor union of tissues with the epicotyls of the rootstock seedling. Major emphasis was given to micrografting of *C. sinensis* meristems, while meristems of all of them were micrografted using seedlings of all the 3 rootstocks. There was strikingly better suitability of *C. limonia* for *C. sinensis*, *C. aurantifolia* and *C. reticulata* than other 2 rootstocks. However,

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micrografted shoot meristems of *C. nobilis* x *C. deliciosa* gave slightly better survival percentage on *C. karna* than the other 2 rootstocks, albeit *C. limonia* proved better than *C. jambhiri* in this case also (Table 10 and Figs. 38-42). The union of micrografted shoot took ca. 10 days by which time the maximum number of meristems dried out. However, the process of drying out continued till 40 days of incubation, after which most of the meristems showed regeneration, but many remained quiescent and remained so up to 60th day of micrografting and even beyond that period.

In view of the limitations imposed by availability of seeds of rootstocks all the year round, an alternative procedure was adopted employing rooted shoots having elongated internodes and at the most 3 nodes, from which the axillary shoot buds were scooped out. The practical utility of conventional procedure, where the epicotyl of seedlings were used and that of the alternative procedure of rooted shoots was studied in detail in case of *C. sinensis*, results in which respect are given in Table 11 and Fig. 39. Initially, survival of micrografted meristems appeared more on rooted shoots of *C. limonia* after 10 days of planting, but in due course of time, i.e., 60 days of planting the survival of meristems and their growth was better in case where epicotyls were used, i.e. 48% than the rooted shoots, i.e., 44%. However, percentage of quiescent meristems was less in case of rooted shoots, i.e., 28% than in case of epicotyls. However, this advantage with rooted shoots was nullified with the incidence of much higher percentage of adventitious shoot bud formation in rooted shoots than the epicotyls of seedlings (Fig. 43-36). Also, the relative growth of grafted shoot meristem was more in the latter case than the former (Fig. 39). However, through the rooted shoots were no better than plantlets, epicotyls of plantlets for grafting shoot meristems, they may be used to supplement the conventional process of micrografting. Similar results were obtained with micrografting experiments using shoot meristems of *C. aurantifolia*, *C. reticulata* and *C. nobilis* x *C. deliciosa* (Table 12).

Once, the shoot meristem of scion got properly established on the epicotyls or rooted shoot, its growth had been satisfactory and after hardening and transplantation of micrografts-raised plantlets, there was no further mortality, in any of the *Citrus* species used (Figs. 47-50).

Table 10: Effect of different combinations of scions and rootstocks (seedling) on percentage of successful micrografting of *Citrus sinensis*, *C. aurantifolia*, *C. reticulata* and *C. nobilis* x *C. deliciosa* as affected in 3 different rootstocks of *Citrus* species. Scion: Shoot meristem (size 0.5 mm), Incubation period: 30 days.

Scions	Rootstocks	Successful micrografts (%)*
<i>C. sinensis</i>	<i>C. limonia</i>	25
	<i>C. karna</i>	15
	<i>C. jambhiri</i>	15
<i>C. aurantifolia</i>	<i>C. limonia</i>	46
	<i>C. karna</i>	26
	<i>C. jambhiri</i>	20
<i>C. reticulata</i>	<i>C. limonia</i>	40
	<i>C. karna</i>	33
	<i>C. jambhiri</i>	26
<i>C. nobilis</i> x <i>C. deliciosa</i>	<i>C. limonia</i>	26
	<i>C. karna</i>	33
	<i>C. jambhiri</i>	20

*Data based on 50 micrografts on each rootstock

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Table 11: Comparative percentage of abortive micrografts of *Citrus sinensis* during an incubation of 60 days employing seedlings and rooted shoots of *C. limonia*. Scion: Shoot meristem (size 0.5 mm)

Sl. No.	Incubation period (days)	Seedlings			Rooted shoots		
		Plantlets survived (%)	Quiescent micrografts (%)	Adventitious shoot buds formed (%)	Plantlets survived (%)	Quiescent micrografts (%)	Adventitious shoot buds formed (%)
1	10	68	20	-	76	16	-
2	20	60	36	-	52	32	28
3	30	52	28	12	48	24	64
4	40	48	32	28	48	24	74
5	50	48	32	28	44	28	74
6	60	48	32	28	44	28	74

Data based on 25 replicates

Table 12: Comparative efficacy of epicotyls of seedlings and rooted shoots of *C. aurantifolia*, *C. reticulata* and *C. nobilis* x *C. deliciosa*. Incubation period: 30 days

Scions	Seedlings		Rooted shoots	
	Successful micrografts (%)	Growth of grafted meristem in length (mm)	Successful micrografts (%)	Growth of grafted meristem in length (mm)
<i>C. aurantifolia</i>	60	4.3±0.44	52	3.8±0.32
<i>C. reticulata</i>	48	3.1±0.36	36	2.9±0.34
<i>C. nobilis</i> x <i>C. deliciosa</i> .	44	3.6±0.42	40	3.4±0.40

Data based on 20 replicates ±SD



Figs. 38-42: Micrografting in *Citrus* spp.

Fig. 38: A growing shoot meristem of *C. sinensis* after ca. 10 days of micrografting over *C. minonia*

Fig. 39: Growing shoot meristems of *C. sinensis* micrografted over the epicotyls of seedling and over rooted shoot of *C. limonia*, respectively, as seen after 1 month of grafting

Figs. 40-42: Successful micrografts of *C. reticulata*, *C. nobilis* x *C. deliciosa*, and *C. aurantifolia*, respectively, grafted over rooted shoots of *C. limonia* showing perfect union of scion and rootstock tissues

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Figs. 43-46: Anomalies associated with micrografting

Fig. 43: Not so perfect union of meristem with the cortical tissue of the rootstock

Fig. 44: Necrosis of a grafted shoot meristem of *C. sinensis* over *C. jambhiri*, while an adventitious shoot is developing in its close proximity

Fig. 45: A micrograft of *C. reticulata* over *C. jambhiri* showing fast growing axillary shoot from the rootstock

Fig. 46: Micrograft of shoot meristem of *C. aurantifolia* over rooted shoot of *C. limonia* showing callusing at the juncture of micrograft and regeneration of an axillary shoot from the rootstock



Figs. 47-48: Cultures of *Citrus sinensis*

Figs. 47-48: Hardening of successful micrografts of *C. sinensis* in inorganic salt solution and *ex vitro* growth of one of them as seen after 2 months of transplantation



Figs. 49-50: Potted plants *Citrus reticulata* and *Citrus nobilis* x *Citrus deliciosa*

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Figs. 49-50: Plantlets raised by micrografting shoot meristems of *C. reticulata* and *C. nobilis* x *C. deliciosa*, over the seedlings of *C. limonia* and *C. karna*, respectively

IV. DISCUSSION

Substantial work has been done on micrografting in *Citrus* and equally well it has been covered in exhaustive reviews on the subject (Navarro and Juárez, 1977; Navarro, 1992). The most noteworthy result of micrografting is demonstration of how the citrus production has been revolutionized by its application in Spain (Navarro, 1992).

Nearer home, a successful beginning for elimination of tristeza and greening from *C. reticulata* through micrografting has been made by Mukhopadhyay et al. (1997). The relative merit of various modes of grafting of shoot meristem, the maturity of rootstock seedling, length of epicotyls, its etiolation before grafting, comparative merit of zygotic seedlings vs. nucellar seedlings of rootstocks and advantages of using rooted shoots taken from cultures of proliferating shoots raised from shoot meristems of a rootstock (*C. limonia*) instead of zygotic or nucellar seedlings have been critically examined in this Laboratory (Chaturvedi et al., 2001, 2007; Singh, 2001; Jain et al., 2005; Singh et al., 2005). Micrografted plants of *C. aurantifolia* and *C. sinensis* showed early flowering under field conditions.

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. jambhri* 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 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).

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