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Micropropagation of citrus rootstock

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Abstract
Tissue culture is most suitable for *in vitro* propagation of citrus rootstock owing to difficulty in conventional method of propagation. Micropropagation rootstock development is an unending process and success depends on several factors viz. genotype, explant age, culture medium and PGR's affecting regeneration capacity. Efforts have been made to review the status of efficient protocol for micro propagation in citrus rootstocks in various parts of the world.

Keywords: micropropagation, citrus rootstock, tissue

Introduction
Triclosan Citrus rootstocks plays important role in crop husbandry and provides varied effects on scion vigour, yield and quality, tolerance to biotic and abiotic stress. Citrus rootstocks are generally propagated for commercial use by growing open pollinated nucellar seeds. Depending upon the rootstock, there can be from 1-40% zygotic seedlings produced which must be culled from nursery seed beds to maintain clonal uniformity. Some potentially valuable rootstocks type produces few or no seeds and periodic seed shortages of popular rootstock occur. Tissue culture offers an advantage over conventional methods of propagation in producing large number of genetically uniform healthy plants within a short period. *In vitro* propagation ensures the availability of disease free plant material throughout the year avoiding the necessity to import both seeds and rootstock seedling. In this review, attempt has been made to compile the research work on various aspects of *in vitro* propagation of citrus rootstocks in India and abroad.

Juvenility
The best results in terms of rapid rates of proliferation are normally obtained using stock material in the juvenile phase as an explant source. Many protocols for *in vitro* organogenesis of citrus rootstocks have been developed for epicotyls or internode segments from juvenile explants (Molina *et al.*, 2007; Tong *et al.*, 2009; Germanà *et al.*, 2011; Marques *et al.*, 2011) [14, 32, 9, 13]. Sim *et al.* (1989) [28] and Cervera *et al.* (1998) [4] have reported that explants collected from juvenile citrus plantsgive the best regeneration frequency in plant tissue culture as compared to explants collected from adult plants. D'Onghia *et al.* (2001) [6] reported that plants regenerated by somatic embryogenesis from style and stigma cultures exhibited initial juvenile growth, characterized by the presence of thorns on stems and branches and the absence of flowers.

Explant
Various explants such as nodal segments (Kumar *et al.*, 2014) [12], axillary shoots (Tallón *et al.*, 2012) [31], shoot tip (Sharma *et al.*, 2009) [27], mature stem buds (Kanwar *et al.*, 2013) [10] root sections (Sim *et al.*, 1989) [28] and leaf sections (Yelenosky, 1987) [34] has been used for micropropagation of citrus. The explant giving better results varies from species to species.
The explant used greatly affects the survival percentage in Carrizo citrange as suggested by Kaur *et al.* (2015) [11]. Kanwar *et al.* (2013) [10] reported that nodal segments needed longer culture periods for shoot regeneration as compared to bud culture in Carrizo citrange. Higher bud proliferation was obtained when nodal segments were used than that obtained from shoot tips in case of Cleopatra mandarin, pectinifera, and rough lemon (Sharma *et al.*, 2009) [27]. Samaan *et al.* (2009) [20] showed the superiority of epicotyls to the shoot tip ones on average leaves and shoots number in sour orange. Saini *et al.* (2010) [19] used five explants viz. epicotyl, root segments, cotyledons, petiole, leaf segments of rough lemon and found that out of the five explants the mean percent of bud induction from epicotyl segments was significantly higher than others irrespective of culture media.

Organogenesis

Phytohormones

Phytohormones or plant growth regulators play a very vital role in regeneration, multiplication and rooting of *in vitro* grown plants. Cytokinins viz. BAP, Kinetin, TDZ and 2ip mainly used at multiplication stage for shoot induction and auxins (NAA, IBA, IAA, and 2, 4-D) are mostly used for root initiation. BAP is more stable, less expensive cytokinin than the others and is the most commonly used cytokinin in tissue culture for direct organogenesis of citrus rootstocks (Costa *et al.*, 2004; Germanà *et al.*,2008) [5, 8], but the optimum concentration for maximum proliferation varies among species. Apart from auxins, gibberellins also have been found to affect the growth of explant as suggested by Kanwar *et al.,* 2013 [10]. In nodal explants from mature trees of Carrizo cultivars the number of shoots was dependent on the BAP and GA3 concentrations and the best results were obtained with BAP 1 mg /l + GA3 1 mg/l. Tallón *et al.* (2012) [31] found that the highest productivity for Cleopatra mandarin was obtained when the culture medium was supplemented with 2 mg/l BA and 1 mg/l GA3 or 4 mg/l adenine. High concentration of cytokinin and low concentration of auxin is required for shoot multiplication (Sharma *et al.*, 2012) BAP and Kinetin either singly or in combination has been reported to be the most effective cytokinins for shoot regeneration and development. However their concentrations vary from species to species.

In rooting stage, combination of IBA & NAA gave high rooting percentages. 1 mg/l IBA in combination with 1 or 2 mg/l NAA produced the best results. to be the most effective cytokinins for shoot regeneration and development. However their concentrations vary from species to species.

Shoot regeneration

Explant establishment and regeneration is affected by a number of factors like cut modes, photoperiod, culture vessel, explant orientation, season of collection of explant, hormone combination, the plant part used as explant and age of explant etc. Rattanpal *et al.* (2011) [18] studied the effect cut modes and photoperiod on shoot regeneration parameters in rough lemon (*Citrus jambhiri* Lush.) and found that longitudinal cut on an average gave higher number of buds and shoots per explant as compared to transverse cut. Longitudinal cut could increase the wood area of epicotyl explants, resulting in more shoot regeneration than transversal cut. The culture vessel in which the explants are cultured may also affect the shoot regeneration. The air volume capacity of the culture vessels has been suggested to possibly influence the number of shoots formed on each explant.

Bordoân *et al* (2000) [2] found that epicotyl explants of Troyer citrange (*C. sinensis ×Poncirus trifoliata*) when planted vertically regenerated shoots at the apical end by a process of direct organogenesis without callus formation while when explants were incubated horizontally, regeneration at the apical end occurred by an indirect organogenesis pathway after callus formation. Kaur *et al.*(2015) [11] found that shoot tip and nodal segment explants of Carrizo citrange collected during April- May showed maximum establishment (96.60%) followed by explants collected during August-September.

Root regeneration

The incubation of adventitious shoots on an NAA (Naphthalene acetic acid) or IBA- supplemented medium induces the formation of roots in many different Citrus species (Carimi and De Pasquale, 2003) [3]. Kumar *et al.* (2014) [12] worked on *in vitro* propagation of virus tolerant citrus rootstock *Citrus reshni* and found that the maximum survival of micro-shoot for rooting and length of root (6.28 cm) were recorded on M S medium modified with NAA 0.5 mg/l + IBA 0.5 mg/l. The minimum time taken to root induction on NAA 0.5mg/l + IBA0.1 mg/l and maximum number of root on NAA 0.5 mg/l + IBA 1.0 mg/l were observed. Sen and Dhawan (2010) [26] observed that growth regulators viz. IAA and IBA, when used individually on microshoots of Swingle citrumelo gave low rooting percentages. However, when these two auxins were used in combinations, cent percent rooting was observed on half MS medium with IAA (1.42 µM), IBA (0.49 µM), and 3% sucrose. A list showing effect of genotype, explants type, age of explants, media formulation on organogenesis in citrus rootstocks (Table 1).

Table 1: Influence of explant, media and growth regulators on organogenesis in citrus rootstock

Species/ Common Name	Explant	Age of explant	Shoot parameters		Root parameters		Reference
			Regeneration	No of shoots	Rooting	No of roots	
Carrizo citrange (*C. sinensis* Osbeck. x *Poncirus trifoliata Raf.*)	Single nodal	From green house raised plants	MS medium + BA 1mg/l + IBA 0.5mg/l + Adenine 40mg/l	-	-	MS medium + 1mg/l NAA	Starrantino and Caruso (1987) [29]
	Nodal segments	3 year old plant	MS medium + 2 µM BAP +1 µM NAA	MS medium + 2 µM BAP +1 µM NAA	MS medium + 10 µM IBA + 500 mg/l malt extract	half- strength MS medium + 2.5 µM IBA + 2.5µM NAA	Kaur *et al.* (2015) [11]
	Nodal segments	From mature plants	MS medium + 1.0 mg/l BAP + 1.0 mg/l GA3 or 0.5 mg/l BAP + 1.0 mg/l GA3	MS medium + 1.0 mg/l BAP + 1.0 mg/l GA3	-	-	Kanwar *et al.* (2013) [10]
	Epicotyl segments	From *in vitro* raised seedling	MT medium+ 2.5 mg/l BAP		MT medium+ 1 mg/l IBA		Almeida *et al.* (2002)
Citrus aurantium L. (Sour orange)	Nodal segments	From mature plants	DKW medium + 2 mg/l BA	-	MS +1 mg/l IBA + 1 or 2 mg/l NAA	MS +2 NAA + 1 mg/l IBA	Tallón *et al.* (2012) [31]
Citrus jambhiri Lush.(Rough lemon)	Nodal segments	From mature plants	MS+ 3 mg/l BAP	MS+ 3 mg/l BAP	MS +0.5 mg/l NAA		Savita *et al.* (2012) [22]
	Leaf segments,	From in	nodal segment 3 mg/l BA		MS +0.5 mg/l		Savita *et*

	Nodal segments Root segments	vitro raised seedling	and 0.5 mg/l NAA	-	NAA	-	*al.* (2010) [21]
	Epicotyl segment	From in vitro raised seedling	MS+ 0.5 mg/l BA	MS +0.5 mg/l BA + 1.0 mg/l GA3	MS +1.0 mg/l NAA + 1.0 mg/l IBA	-	Saini *et al.* (2010) [19]
Citrus karna Raf.	Nodal segments	From in vitro raised seedling	BAP 0.5mg/ NAA and 40 mg/l adenine sulphate	BAP 0.5mg/ NAA and 40 mg/l adenine sulphate	MS 0.5 mg/l IBA+ 0.5mg/l NAA	-	Murkute *et al.* (2008) [15]
Citrus limettioides L. (Sweet lime)	Nodal segments	From in vitro raised seedling	MS + 0.1 mg/l NAA + Malt extract (500 mg/l) + 10 mg/l BA	MS +1.0 mg/l BA	MS +10 mg/l NAA	MS +10 mg/l NAA	Usman *et al.* (2005)
Citrus limonia Osbeck. (Rangpur Lime)	Epicotyl segments	From in vitro raised seedling	MT medium + BAP 0 mg/l	MS +1.5 mg/l BAP	-	-	Schinor *et al.* (2011) [24]
Citrus macrophylla Wester (Alemow)	Nodal segments	From mature plants	DKW medium + 1 mg/l BA	-	MS +1 mg/l IBA + 1 mg/l IAA	-	Tallón *et al.* (2012) [31]
Citrus reshni Tanaka (Cleopatra mandarin)	Nodal segments	From in vitro raised seedling	MS medium +BAP 1.0 mg/l + kin 0.5 mg/l	MS +2.0 mg/l BAP + 1.0 mg/l Kin	MS +0.5 mg/l NAA + 0.5 mg/l IBA	MS +0.5 mg/l NAA + 1.0 mg/l IBA	Kumar *et al.* (2014) [12]
	Nodal segments	From mature plants	DKW and MS +1 or 2 mg/l BA	-	MS +1 mg/l IBA + 1 or 2 mg/l NAA	MS +2 mg/l NAA or 2 mg/l NAA + 1mg/l IBA	Tallón *et al.* (2012) [31]
	Nodal segments	From	MS medium +BAP	MS medium	Half strength	Full	Prakash *et al.*,
		mature plants	0.5 mg/l + kin 0.5 mg/l	+BAP 1 mg/l + kin 0.5 mg/l	MS medium + 20 mg/l IBA	strength MS medium + 20 mg/l IBA	2017
Citrus volkameriana Tene Pasq.	Epicotyl segments	From in vitro raised seedling	MT+ 0.0 mg/l & 0.5 mg/l BAP	MS +0.5 mg/l & 1.0 mg/l BAP	-	-	Schinor *et al.* (2011) [24]
Poncirus trifoilata Raf. (Flying Dragon)	Single nodal cutting	From green house raised plants	MS medium + BA 1mg/l + IBA 0.5mg/l + Adenine 40mg/l	-	-	MS medium + 1mg/l NAA	Starrantino and Caruso (1987) [29]
Poncirus trifoilata Raf. (Trifoliate Orange)	Single nodal cutting	From green house raised plants	MS medium + BA 1mg/l + IBA 0.5mg/l + Adenine 40mg/l	-	-	MS medium + 1mg/l NAA	Starrantino and Caruso (1987) [29]
Swingle Citrumelo(Poncirus trifoilata Raf. x C. paradise Macf.)	Node segment	From mature plants	MS+ BA 1.2 μM	MS+ Kinetin 1.25-2.5μM	half-strength MS medium + 1.42μM IAA + 0.49μM IBA		Sen and Dhawan (2010) [26]
Troyer citrange (C. sinensis Osbeck. x Poncirus Trifoliata Raf.)	Single nodal	From field grown	MS medium + BA 1.11 μM+1.1625μMKinetin	-	-	half- strength MS medium + 0.5 μM NAA	Sen and Dhawan (2009)
	Single nodal cutting	From green house raised plants	MS medium + BA 1mg/l + IBA 0.5mg/l + Adenine 40mg/l	-	-	MS medium + 1mg/l NAA	Starrantino and Caruso (1988) [30]

Acclimatization

The benefit of any micro propagation system can only be fully realized by the successful transfer of plantlets from tissue culture vessels to ambient conditions found *ex vitro*. Starrantino and Caruso (1987) [29] observed that transplanting the plants in plastic pots filled with sterilized soil and growing in a glass house with high humidity and temperature 25-27 0 C was successful. Murkute *et al.* (2008) [15] reported that the hardening medium consisted of 2 coco-peats: 1 solrite in which the survival rates of were 94.7% in *C. jambhiri* and 96.7% in *C. karna* after one month of transplanting. Sen and Dhawan (2009) observed 100% survival of *in-vitro* Troyer citrange plantlets during hardening in a mixture of soil and agrope. Sharma *et al.* (2009) [27] also found maximum survival rate of hardened plants six weeks after transferring to the pots (potting media consisting of soil, sand and FYM in the ratio of 1:1:1) under greenhouse conditions over the rootstocks Rough lemon followed by Pectinifera and Cleopatra mandarin rootstocks

Genetic fidelity

A major problem associated with micropropagation is the occurrence of somaclonal variations among the sub clones of the parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissue, and organs. The frequency of these variations varies with the source of explant and their pattern of regeneration, media composition and cultural conditions (Savita *et al.*, 2015) [23]. This approach for shoot regeneration is correlated with an increasing risk of genetic instability. Navarro *et al.* (1985) [16] observed that 29% of plants obtained by somatic embryogenesis of nucelli of monoembryonic *Citrus* presented abnormal phenotypic

characteristics. A significant genetic variation existed in nucellar seedling populations of *Citrus*. This necessitates verification of the clonal fidelity of *in vitro*-generated plants and an assessment of protocol reliability. This process is essential when production of uniform planting material is the main consideration. Duran-Vila *et al.* (1992) found that none of the plants regenerated from *in vitro* bud cultures or internode stem segments showed phenotypic abnormalities and that no differences, as determined by isoenzyme analysis, were observed among regenerated plants. Over the last two decades, genetic polymorphisms such as RAPD markers have been extensively used to determine clonal fidelity of micropropagated plants (Savita *et al.*, 2012) [22].
Savita *et al.* (2012) [22] worked on development of efficient protocol for micropropagation of *citrus jambhiri* and finally to confirm the fidelity of regenerated plants with that of donar plant used RAPD analysis. Randomly amplified polymorphic DNA analysis confirmed that all the regenerated plants were genetically identical to their donor plant, suggesting absence of detectable genetic variation in the regenerated plantlets. Sen and Dhawan (2010) [26] used seven ISSR markers for genetic fidelity of tissue culture raised plants. These six ISSR primers produced 35 distinct and scorable bands in the micropropagated progenies along with the mother plant of Swingle Citrumelo. All micropropagated plantlets gave exactly identical ISSR profiles matching with the parent profile confirming the genetic uniformity of the micropropagated progeny. All the plantlets were morphologically similar to the mother plant, indicating no polymorphism.

Conclusion
The success has been achieved to develop efficient protocol for *in vitro* propagation in several citrus rootstocks. However, still lot to be done in areas like appropriate, substantial modifications in order to reduce the high cost of production and decrease the risk of variability and production of off-type individuals. A critical economic analysis of the benefits of the use of citrus micropropagated rootstocks tissue culture versus the traditional propagation systems is not found in the literature. The process of rooting *in vitro* has been estimated to account for approximately 35 to 75% of the total cost of micropropagation. Therefore, the preference should be given to those systems that use a single, simultaneous step for both rooting and acclimatization. It would substantially decrease the costs of micropropagation.

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