Agric. Rev., 23 (1) : 1 - 13, 2002

MICROPROPAGATION IN CITRUS - A REVIEW

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ABSTRACT

Citrus trees are propagated both by seed and by vegetative means. Vegetative propagation is preferred because it ensures true to type plants, uniform quality, regular bearing etc. Tissue culture technique particularly micropropagation, is now gaining popularity due to many reasons. Micropropagation has many advantages over conventional methods of vegetative propagation and its application in citrus is currently expanding world wide. The goal of micropropagation is to obtain a large number of genetically identical, physiologically uniform and developmentally normal plantlets preferably with a high photosynthetic potential to survive the harsh *ex vitro* conditions, in a reduced time period and at a lowered cost. The performance of micropropagated plantlets should be at par in comparison to plants raised by conventional method.

Citrus is the most important fruit crop in the world and is produced in over 100 countries in all six continents, and it is often regarded as golden fruit or queen of all fruits (Nito, 1996). In India, citrus fruits rank third in area and production after mango and banana with an estimated production of 37.0 Lakh tonnes and an area of 4.4 Lakh hectares (Chadha, 1997) and contributes 4.78% towards world's total citrus. Total cultivation worldwide exceeds $2x10^6$ hectares and production at over 63 million metric tonnes, far surpasses that of all deciduous fruit crops.

The important commercial citrus fruits in India are the mandarin orange (*Citrus reticulata* Blanco), followed by sweet orange (*C. sinensis* Osbeck) and acid lime (*C.aurantifolia* Swingle) and lemon (*C.limon* Burm). In India, mandarins constitute about 41%, sweet orange 23% and limes and lemons about 23% of total citrus produced (Ghosh, 1997). *Citrus* trees are propagated both by seed and by vegetative means. There is huge demand of planting material. Non availability of scientifically propagated planting material from elite clones for plantation are the main constraints in citrus cultivation. In recent years, tissue culture techniques (micropropagation) are increasingly used for rapid clonal propagation of several economic plants, restoration of vigour and yield due to infection and preservation of germplasm. Hence micropropagation is a very useful tool for a production of large number of planting materials. Besides, this technique is also useful for saving the citrus species which are facing extinction.

Tissue culture in citrus

The importance of tissue culture in citrus research was recognised long back, and amply emphasized by Bitters and Murashige (1967) and Kochba and Spiegel-Roy (1976). The far reaching significance of tissue culture in citrus breeding for improvement and augmenting production was discussed by Kochba and Spiegel-Roy (1977) and various other aspects of citrus tissue culture by Button and Kochba (1977) and Spiegel-Roy and Kochba (1980).

Micropropagation

Regeneration of plantlets can be achieved from a wide range of explants such as stem sections (Grinblat 1972, Chaturvedi and Mitra 1974, Raj Bhansali and Arya 1978, 1979; Barlass and Skene 1982), root sections (Raj Bhansali and Arya 1978, Sim *et al.*,1989 and Duran-villa *et al.*, 1989), root meristems (Sauton *et al.*,1982), shoot and shoot tips (Sim *et al.*, 1989, Singh *et al.*, 1994, Kitto and Young 1981 & Barlass and Skene 1982) and leaf sections (Chaturvedi and Mitra

1974, Yelenosky 1987). Protocols have been standardised for mass multiplication of different citrus species of NEH Region in ICAR Res. Complex for NEH Region, Meghalaya (Parthasarathy *et al.* 1996, Parthasarathy, 1993, Parthasarathy and Parthasarathy, 1993, Parthasarathy and Nagaraju, 1992, 1994a, 1994b,1996a 1996b).

In the field of micropropagation in citrus, it was the efforts of Murashige, *et al.* (1972) that triggered the advent of shoot tip culture in citrus varieties. They developed the technique mainly for obtaining virus-free propagative budwood in order to lower the economic losses caused by citrus virus and virus-like diseases. Micropropagation has been the most successful out come of plant tissue culture research with profound commercial application mostly in herbaceous plants (Murashige, 1974, 1978; Vasil and Vasil, 1980). The spectacular success achieved have been mostly in ornamental and fruit crops which have revolutionized the tissue culture (Hussey, 1997; Monaco *et al.* 1977; Chaturvedi 1979; Chaturvedi and Sharma, 1979; George and Sherrington, 1984).

Micropropagation has many advantages over conventional methods of vegetative propagation (cutting or seed) and its application in Horticulture, Agriculture and Forestry is currently expanding world wide (Jeong *et al.*, 1995). The goal of micropropagation is to obtain a large number of genetically identical, physiologically uniform and developmentally normal plantlets preferably with a high photosynthetic potential to survive the harsh *ex vitro* conditions, in a reduced time period and at a lowered cost.

In recent years micropropagation is increasingly used for rapid clonal multiplication, restoration of vigor, yield and conservation of germplasms. Tissue culture method of plant propagation is useful alternative in plant propagation when conventional methods permit only slow increase in clonal multiplication.

A summary of results obtained from micropropagation of citrus are given in Table 1.

Bud establishment and shoot proliferation

Successful shoot proliferation has been achieved in 33 citrus types belonging to 19 species by Parthasarathy *et al.* (1998). Among cytokinins they found BAP is the most suitable cytokinin for proliferation of shoots. More number of shoot, leaves and nodes were found with BAP than kinetin. BAP at 0.75 mg/l produced maximum number of shoots, they were shorter than the shoots produced by lower concentration of BAP (0.1 mg/l). One cm long *in vitro* shoot tips of 33 citrus types/cultivars belonging to 19 species of citrus when cultured on MS media supplemented with 0.75 mg/l BAP revealed that proliferating ability of endemic species like *C. indica*, *C. assamensis* and *C. latipes* was low (Table 2).

The shoot meristem culture of citrus provides an important means of virus elimination (Chaturvedi and Sharma, 1987). They also suggested an alternate procedure for meristem culture by culturing single node segments obtained from field grown plants. Chaturvedi and Mitra (1974) established from callus culture of *Citrus grandis* maximum number of shoot buds using 0.25mg/l of BAP with 0.1mg of NAA. This was probably one of the first cases of success with citrus. Barlass and Skene (1982) produced successfully micropropagated plantlets using 10mg/l of BAP for shoot proliferation and same concentration of NAA for rooting in case of Carrizo citrange, trifoliate orange, Cleopatra mandarin, Rangpur lime and sweet orange. Starrantino and Caruso (1988) obtained success in citrange with BAP (0.5 mg/l) with IBA supplementation (0.5 mg/l). Edriss and Burger (1984) also obtained success with epicotyle segment using BAP (0.25 mg/l) and NAA (1 mg/l) in case of Troyer citrange. Sim *et al.* (1989) presented protocol for

Table 1. Summary of literature on micropropagation of citrus.

Species	Common name	Age of material	Explant source	Shoot regeneration medium		Root induction medium		References
				Basal medium (and % source)	Growth component (mgl^{-1})	Basal medium (and % source)	Growth component (mgl^{-1})	
1	2	3	4	5	6	7	8	9
Citrus aurantifolia	West Indian acid lime	S	Stem, root	Mod. MS (5%) (C/M)	BA (0.5%)+ ME (1000)	Mod. MS(2%) (C/M)	NAA (1-2) IAA(2), or IBA (1-2)	Raj Bhansali and Arya (1978a,b)
	PKM.1	S	Stem	MS (3%)	BA (0.25)+ NAA (0.1)	MS 3/4 (3%) -	NAA (3) -	Thirumalai & Thamburaj, 1997
	Kagzilime	S	Stem	MS	BAP (0.75)	-		Parthasarathy & Nagaraju, 1996
C. jambhiri	Sohmyndong	S	Stem	MS	BAP (.75)	-	-	Parthasarathy & Nagaraju 1996
C. aurantium	Sour orange	S,M	Stem	MS (3%)	K(1)+NAA(1)	No Change	No change	Bouzid (1975)
	cv. Brezilia	S	Stem	MS(3%)	BAP (2)	MS (3%)	IBA (1)+NAA	Can *et al.*, 1992
P. trifoliate x C. lemon	Citremon 1452	M	Stem	MS (5%)	BA (0.5)+NAA(0.5)	MS1/2 (2.5%)	Thiamine (0.2) & NAA(0.15)	Mas *et al.*, 1994
C. grandis	Shaddock	S	Stem, leaf	Mod. MS (5%)	BA (0.25), NAA (0.1), +ME(500)	No change	NAA (0.1-0.5)	Chaturvedi and Mitra (1974)
	Pummelo	S	Stem	MS	BAP (0.75)	- -	-	Parthasarathy & Nagaraju, 1996
C. volkameriana	Volkamer lemon	S	Stem	MS	BAP 0.75			Parthasarathy & Nagaraju 1996
C. limettioides	Sweet lime	S	Stem	Mod. MS (3%) (C/M)	BA(0.5), K(0.25),NAA (0.2)+ME(500)	Mod. MS (2%) (C/M)	NAA (1-2-5)+ K(0.1)	Raj Bhansali and Arya (1979)
C. limon	Lemon	S,M	Stem	MS (3%)	K(1)+NAA(1)	No change	No change	Bouzid (1975)
		S	Root	Mod. MS (3%)	BA(1)+2,4-(D (0.1)	MS (3%)	-	Sauton *et al.* (1982)
	Assam lemon	M	Shoot tips	MS (3%)	BA (1)+Kinetin (0.5)+NAA (0.5)	MS (3%)	BAP (0.25)+NA A(0.5)+IBA(0.5)	Singh *et al.*, 1994
C. madurensis	Calamondin	S	Stem	MS (5%)	BA(0.1-10) NAA (0.1), +ME (500)	MS (5%)	NAA(0.1) +ME (500)	Grinblat (1972)

(Contd.

1	2	3	4	5	6	7	8	9
		S	Stem	White 1943 (2%) Tukey, 1938 (0.5% glucose)	Barbitone (5) or ME (50)	No change	No change	Rangaswamy (1975)
		S	Shoot tip	White 1943 (2%)	Adenine (40) or CCM (40%v/v)	No change	No change	Rangaswamy (1975)
C. paradisi	Grape fruit	S	Stem, leaf	Mod. MS (5%) (MIT)	BA (0.5), NAA (0.15), +ME (1000)	No change	No change	Raj Bhansali & Arya (1978c)
C. reticulata	Mandarin	S,M	Stem	MS (3%)	K (1)+NAA(1)	No change	No change	Bouzid (1975)
	Cleopatra Mandarin	S,M	Stem	MS (3%)	BA (2)	No change	No change	Barlass and Skene (1982)
		M	Shoot apex	1/2 MS(1.5%) or MS (3%)	BA (0.5) or BA (2)	No change	No change	Barlass and Skene (1982)
	Khasi mandarin	M	Shoot tips	MS (3%)	BAP(1)+ Kinetin (0.5)+NA(0.5)	MS (3%)	BAP 0.25+ NAA (0.5)+ IBA(0.5)	Singh *et al.*, 1994
C. limonia	Rangpur lime	S,M	Stem	MS (3%)	BA (2)	White 1943 (2%)	NAA (2-5)	Barlass and Skene (1982)
C. sinesis	Sweet Orange	S	Stem, leaf	Mod. MS (5%)	BA (0.25) NAA (0.1)+ME (500)	No change	NAA (0.1-0.5)	Chaturvedi and Mitra (1974)
		M	Gimary Bud	Mod. MS (5%)	BA(0.2-2)	No change	No change	Altman and Gore (1974)
		S,M	Stem	MS+Helles, 1953 (3%)	BA (0.5)+IBA (1)	No change	No change	Bouzid (1975)
		S	Stem	Mod. MS (5%) (C/M)	BA (0.5)+ME (500)	Mod. MS (2%)	NAA (1-2)IAA (2), +IBA (1-2)	Raj Bhansali and Arya (1978b)
		S	Root	Mod. MS (3%)	BA (1)+2,4-D(0.1)	MS (3%)	-	Sauton *et al.* (1982)
		S,M	Stem	MS (3%)	BA (2)	White, 1943 (2%)	NAA (2-5)	Barlass and Skene (1982)
		S	Shoot apex	1/2 MS (1.5%) or MS (3%)	BA (0.5) or (2)	White 1943 (2%)	NAA (2-5)	Barlass and Skene (1982)
	cv. Mosambi	S	Nodal segment	MS	BAP(0.25)+ IAA (1.00)	MS (3%)	NAA (1)+IBA (2)	Mohanty *et al.*, 1978

(Contd.

1	2	3	4	5	6	7	8	9
C. sinesis x P. trifoliate	Citrange	S	Stem	Mst Nitschand Nitsch, 1965 (5%)	BA (10)+ NAA (10)	No change	NAA (1)	Primo Millo and Harada (1976)
		S	Root	Mod.MS(3%)	BA(1)+2,4-D (0.1)	MS (3%)	-	Sauton *et al.* (1982)
		S,M	Stem	MS (3%)	BA (2)	White, 1943 (2%)	NAA (1-2)	Barlass and Skene (1982)
		S,M	Shoot apex	1/2MS(1-5%) or MS (3%)	BA (0.5) or BA (2)	White, 1943 (2%)	NAA (1-2)	Barlass and Skene (1982)
	Carrizo citrange	S	Stem	MS (5%)	BA (22 um)	MS1/2(2.5%)	NAA (5.4µm)	Moore, 1986
P. trifoliate x C. sinensis	Troyer citrange	S	Stem	MS (3%)	BAP (0.08)	--	--	Lukman *et al.* (1990)
	Troyer citrange	S	Epicotyl Segments	MS (5%)	BAP (1)+ NAA (1)	MS	NAA (2)	Edriss and Burger, (1984)
Poncirus trifoliate	Trifoliate orange	S	Root	Mod.MS (3%)	BA (1)+2,4-D ().1)	MS (3%)	-	Sauton *et al.* (1982)
		S	Stem	MS (3%)	BA (2)	White, 1943 (2%)	NAA (2)	Barlass and Skene (1982)
C. indica	Indian wild Orange	S	Stem	MS (3%)	BAP 0.75	-	-	Parthasarathy *et al.* 1997
		S	Stem	MS (3%)	BAP 0.5	-	Soil rite	Baruah *et al.*, 1996
C. latipes	Khasi papeda	S	Stem	MS (3%)	BAP 0.75	-	-	Parthasarathy *et al.* 1997
	Khasi Papeda	S	Stem	MS (3%)	BAP (0.5)	-	Soil rite	Baruah *et al.*, 1997
C. assamensis	Ada Jamir	S	Stem	MS (3%)	BAP(0.5)	-	Soil rite	Baruah *et al.*, 1997.
C. unshiu	cv. Aoshima Unshiu	M	Shoot tips	MS (3%)	GA 3(50µm)+ 1 µm BA+ 0.1µm NAA	MS	NAA (0.1µm) or IBA (10µm)	Omura and Hidaka 1992.

Source: (Button *et al.*, 1977 and Singh, 2000)

Table 2. Proliferating ability of shoot tips of certain citrus species *in vitro*

Spp. No.	Species/Cultivars	Mean shoot No.	Mean shoot length (cm)	No. of nodes	Fresh culture weight (gm)
1.	*C. reticulata* Blanco Cv. Khasi mandarin	5.9	7.7	3	0.1
2.	*C. reticulata* Blanco Cv. Shuntala	9.5	1.1	4	0.144
3.	*C. sinensis* Osbeck Cv. Malta	7.5	6.0	3.1	0.16
4.	*C. sinensis* Osbeck Cv. Soh bitara	2.2	7.4	2.7	0.1
5.	*C. limon* Burn Cv. Assam lemon	2.9	19.5	4.11	0.12
6.	*C. limon* Burn Cv. Jaintia lemon	5.09	5.97	2.45	0.1
7.	*C. limon* Burn Cv. Gol Neembu	3.0	6.3	3.2	0.07
8.	*C. limmettiodes* Tanaka Cv. Sweet lime	2.7	3.9	2.17	0.09
9.	*C. limmettiodes* Tanaka Cv. Sweet lime sour	2.7	5.1	4.00	0.155
10.	*C. medica* Linn Cv. Citron Seeded	3.2	13.7	38.7	0.165
11.	*C. medica* Linn Cv. Citron Gandharaj	2.2	11.4	2.5	0.07
12.	*C. medica* Linn Cv. Soh mad	3.7	12.6	3.1	0.16
13.	*C. aurantifolia* Swingle Cv. Kagzi lime	5.5	8.8	2.6	0.07
14.	*C. meyerii* Y. Tan Cv. Lemon major	4.0	1.1	3.5	0.167
15.	*C. jambhiri* Lush Cv. Soh myndong	4.0	6.3	2.2	0.14
16.	*C. aurantium* Linn Cv. Karun jamir	2.9	7.8	2.6	0.155
17.	*C. paradisi* Macf Cv. Grape fruit	2.7	12.1	2.6	0.17
18.	*C. macroptera* Mont Cv. Satkara	3.7	4.3	4.4	0.09
19.	*C. karna* RP CV. Soh Sarkar	2.7	13.1	3.1	0.167
20.	*C. volkameriana* Pasq Cv. Volkameriana	3.8	6.5	3.0	0.12
21.	*C. grandis* Osbeck Cv. Pummelo	3.5	10.9	3.2	0.11
22.	*C. reshni* Tanaka Cv. Cleopatra mandarin	6.6	9.1	3.2	0.12
23.	*C. taiwanica* Tanaka Cv. Taiwanica	4.7	11.2	3.1	0.13
24.	*C. madurensis* Lour Cv. Calamondin	8.3	12.1	3.2	0.2
25.	*C. latipes* Tanaka Cv. Khasi Papeda	3	1.8	6	0.297
26.	*C. assamensis* Dutt & Bhattacharya Cv. Ada jamir	3.5	1.2	6	0.297
27.	*C. indica* Tanaka Cv. Indian wild orange	2	1.0	4.5	0.11
28.	*C. hybrid* Cv. Kara mandarin	3.2	4.1	2.6	0.076
29.	*C. hybrid* Cv. Kinnow (*C. nobilis* x *C. deliciosa*)	2.3	2.0	5.2	0.13
30.	*Carrizo citrange* (*P. trifoliata* x *C. sinensis*)	7.5	10.1	4.0	0.56
31.	*Troyer citrange* (*P. trifoliata* x *C. sinensis*)	13.0	2.1	13.5	0.683
32.	*Tangelo Dancy* (*C. reticulata* x *C. paradisi*)	6.7	7.1	4.5	0.26
33.	*Citremon* (*P. trifoliata* x *C. lemon*)	5.7	13.3	4.6	0.22

(Source, Parthasarathy, *et al.*,1998)

micropropagation of calamondin. Duran Villa *et al.* (1989) defined tissue culture techniques and cultures conditions for morphogenesis, callus culture and plantlet of sweet orange (*Citrus sinensis* Osb.), citron (*Citrus medica* l.) and lime (*C. aurantifolia* Christm.Swing.). A medium containing 22 µM BA with or without 5.4 µM NAP was optimum for shoots initiation in case of Carrizo citrange, Cleopatra mandarin and sour orange seedling explants, but the 3 genotypes varied greatly in number of shoots produced (Moore, 1986). Bhatt *et al.* (1992) regenerated plants from long term root culture of lime, *Citrus aurantifolia* (Christm.) Swing. Can *et al.* (1992) standardized the *in vitro* clonal propagation of sour orange (*C. aurantium* var. Brezilia) by using epicotyl segments. MS medium containing 2mg/l BAP with or without 4mg/l GA_3 was optimum for shoot initiation. The optimum rooting of in vitro regenerated shoots was observed with 1mg/l of each IBA & NAA. In *Poncirus trifoliata* and Carrizo citrange, the highest rate of induction of adventitious shoots

and globular embryoids was obtained on a culture medium supplemented with 5mg/l BAP (Beloualy, 1991). Malt extract has been found to be an ideal supplementaion for induction of adventive embryogenesis in citrus (Parthasarathy and Nagaraju,1994). Parthasarathy (1993) found that MS medium was the best for inducing and increasing the weight of embryoides without any supplementation. Lukman *et al.* (1990) produced multiple shoots of Troyer citrange *in vitro* from shoot apices. BAP at 0.08mg/l associated with 1.0mg /l of GA_3 induced the proliferation of the apices. Multiple shoots were obtained from shoot tip (2-3mm) derived from mature plants (5 to 6 years old) of *Citrus reticulata* Blanco cv. Khasi mandarin and *C. limon* Burm. cv. Assam lemon when cultured on MS medium supplemented with 1.0 BAP, 0.5 kinetin and 0.5 NAA (mg/l) (Singh *et al.*, 1994). Grosser and Chandler (1986), obtained multiple shoots of Swingle citrumelo rootstock with cumarin at 90-150 μM.

In Vitro Rooting

Number of factors have been observed to be associated with rooting of microshoot. That includes nature of cuttings, rooting co-factor, synergistic role of exogenously applied growth hormone and endogeneously present co factors in the rooting, the relative efficiency of different auxins, their combination and methods of application (Audus, 1972; Weaver, 1972; Haissig, 1974). Existence of certain co-factors for rooting or auxin synergist in tissues of cutting have been demonstrated by Van Overbeek *et al.* (1946). Certain easy to root varieties can be rooted each in absence of leaves cutting in a combination of IBA with Arginine HCL, NH_4SO_4 and Sucrose which is cofactor for rooting in addition to IAA and is reviewed (Hackett, 1970, Haissig, 1974). Role of different nitrogen source have been reported to be important in combination with auxin(s).

Many factors do play very important role for root induction. Murashige (1977) emphasised that high light intensity also induces better rooting and causes hardening of plants which renders them more tolerant to moisture stress and diseases. One fourth salt concentration as MS medium help in induction of roots (Skirvin and Chu, 1979). James and co-workers have found the importance of phlorogluci-nol (PG), a phenolic compound found in xylem sap of apple for rooting of number of rosaceous fruit cultivars. A low salt medium is found satisfactory for rooting of shoots in a large number of plant species. Often where shoot multiplication induced on full strength MS medium, the salt concentration was reduced to half (Garland and Stoltz, 1981) for better rooting. Addition of activated charcol in root expression medium improved the overall rooting capacity in *Pinus panaster* (Elizabeth and Oliver, 1995). Elizabeth and Oliver (1995) further reported that 98% of Juvenile explant to *Pinus pinaster* rooted easily while only 49% mature explant rooted.

In the micropropagation practice, usually natural auxin IAA and synthetic auxins NAA and IBA are used for rooting (Nemeth, 1986). Jones *et al.* (1977) used filter sterilized IBA in their experiments. Auxins alone or with cytokinins, GA3, ABA and phenolics exert their effect mainly during the root induction and initiation phase. Crabapple (*Malus* spp.) rooting of *in vitro* produced shoots was obtained on basal medium containing 5 or 10mg/l IBA (Norton and Noe, 1982). Excellent rooting of *in vitro* derived shoots was obtained on 1/2 strength MS medium containing 0.1 or 0.2mg/l NAA. An increasing percentage of rooted shoots and more roots/shoots were obtained with a concentration range of NAA from 0 to 1mg/l in Quince (*Cydonia oblonga* Mill). (Al Marri *et al.*, 1986). At the optimal NAA concentration of 0.5mg/l, the shoots reached 90% of rooting. The average root length was maximum in control medium without auxin and

decreased with increasing NAA concentrations. On the contrary, the root number per shoot increased with the auxin concentration.

A combination of 0.5mg NAA, 0.5mg IBA and 0.25mg BAP/liter was found best for root development in microshoot of Assam lemon (*Citrus limon*) and Khasi mandarin (*Citrus reticulata*)cv. of citrus (Singh *et al.*, 1994). Duran-Villa (1989) tested different concentration (0-50mg/l) of NAA for rooting of microshoots of 3 *Citrus* species. The optimal concentrations of NAA to induce root formation on stem segments were 10mg/l for sweet orange and lime and 3mg/l for citron. Beloualy (1991), tried NAA and GA_3 for rooting in *Poncirus trifoliata*, Carrizo citrange and *Citrus aurantium* microshoots developed through embyrogenesis. Rooting was promoted by a supplement of 1mg/l GA_3 or 1mg/l NAA. GA_3 enhanced stem elongation and rooting in embryoids and NAA stimulated adventitious root formation.

Can *et al.*, (1992) obtained best rooting of microshoots of sour orange (*Citrus aurantium* var. Brezilia) in the medium contained 1mg/l IBA and 1mg/l NAA. NAA alone was also effective on the rooting but to a lesser extent than the combination with IBA. In another experiment Lukman *et al.* (1990) reported NAA at various levels (0, 0.1, 0.3, 0.5, 0.7 and 1.0mg/l) did not promote rooting in Troyer citrange microcutting. On the other hand transferred to vermiculite provided approximately 17% rooting. Juvenile shoots of trifoliate orange rooted readily in media containing 5 µM NAA (60%) and 10 µM NAA (90%) (Barlass and Skene, 1982). In this case, roots were produced after 3 weeks exposure to 10 µM NAA (as for both Juvenile and mature citrange shoots at all NAA concentrations applied). 2mg NAA/l found best for rooting of Troyer citrange microshoots (Edriss & Burger, 1984). Similarly Parthasarathy and Nagaraju (1996 a) found NAA supplementation at the rate of 0.05 mg/l induced good rooting of microcuttings on three citrus species while for *C.sinesis* cv. Musambi NAA at 0.2 mg/l was best.

Acclimatization

The survival and growth of *in-vitro*-propagated plants after removal from culture has been a major problem in the micropropagation of citrus. Parthasarathy *et al.* (1999 b) developed efficient and reproducible procedure for successful *in-vitro* rooting and acclimatization of micropropagated shoots of important citrus species in a single step with very high establishment in the soil. The protocol involved direct planting of 6-8 week old microshoots(2-2.5 cm long) in sterile soil rite topped over FYM Better rooting (80-90 %) and and very high *ex-vitro* survival (90-97%) was achieved using this protocol. The present study describes an efficient and reproducible procedure for successful *in-vitro* rooting and acclimatization of micropropagated shoots of important citrus species in a single-step procedure with very high establishment in the soil.Transfer of rooted plantlets from sterile to non-sterile conditions with reduced humidity lead to very high mortality (Brainerd and Fuchigami, 1981; Sutter and Langhans, 1982) and poor growth and delay in the attainment of completely acclimatized plants (Wardle *et al.*, 1983; Ziv *et al.*, 1983). *In vitro* rooted plants often lack root hairs and die shortly after transplanting (Donnelly *et al.*, 1985). The process of acclimatizing *in-vitro*-rooted plants involves various steps of acclimatization and extended periods (Cao, 1990). Debergh and Maene (1981) reported that the entire process of rooting *in vitro* and hardening has been estimated to account for approximately 35 to 70% of the total cost of micropropagation. However, in citrus the success rate has been reported to range from 22% (Fred *et al.*, 1986) to 60% (Singh *et al.*, 1994). Hidaka and Kajiura (1989) achieved 61.4-91.1% survival

in a mist house but the carnies used in their study are not commonly available. In addition, serveral months have to elapse before the plants can be transferred to the soil.

Performance of micropropagated plantlets

It is very important to evaluate micropropagated plantlets with conventionally propagated plants for recommending this planting material to farmers. However, very little work have been done on this important aspect of micropropagation. Singh (2000) evaluated micropropagated plant with conventionally grown seedling at Umaim Meghalaya. Studies revealed that seedlings showed comparatively higher shoot and root length as well as higher plant weight, shoot and root weight over micro propagated plantlets during initial period (upto 2 months) (Singh *et al.*, 1997). Later on a reverse growth trend was observed. Micropropagated exhibited better growth over seedling plantlets in terms of plant height, stem girth, total canopy, numbers of branches and numbers of leaves. The reason may be attributed to root system. The micropropagated plantlets showed more fibrous roots, which helps in absorption of more nutrients from the soil than seedling. Palma *et al.* (1997) found better secondary roots in microcutting of *C. macrophylla* than seedlings.

Table 3. Comparative growth of micropropagated and seedling plants of citrus sp. (1 year)

Sl.No.	Species/ cultivar	Method of propagation	Plant height (cm)	Root length (cm)	No.of leaves	No. of secondary roots	Shoot wt. (g)	Root wt. (g)	Plant wt. (g)
1	SAT	S	10.88	14.72	15.45	46.5	1.06	0.51	1.57
		M	16.53	18.72	20.27	64.75	1.36	0.64	1.99
2	LAT	S	27.98	29.95	26.4	63.75	2.90	1.78	4.68
		M	28.78	30.72	27.42	75.55	2.94	1.88	4.82
3	SLS	S	20.13	22.97	20.95	52.97	1.43	0.76	2.18
		M	32.05	26.07	33.32	66.30	2.36	1.41	3.77
4	SOB	S	22.83	23.40	25.95	62.00	2.70	1.72	4.25
		M	31.75	27.40	29.95	83.00	3.37	1.03	5.37
5	I	S	14.00	19.55	19.05	45.00	0.54	1.38	0.77
		M	17.58	20.75	21.22	67.00	0.62	2.30	0.95
6	ADA	S	19.38	22.67	21.2	73.25	3.78	2.34	5.66
		M	19.78	22.97	21.82	78.00	3.81	0.54	5.70
7	KM	S	14.68	20.87	22.92	45.00	0.86	0.79	1.29
		M	17.80	21.70	24.02	60.00	0.93	0.96	1.43
8	SM	S	26.58	23.27	29.9	89.50	4.65	1.27	6.77
		M	34.73	24.9	37.32	102.75	4.98	1.99	7.45
9	JL	S	38.10	32.07	28.07	86.37	5.00	2.23	7.23
		M	41.90	31.82	29.37	89.82	5.24	2.33	7.57
10	P	S	25.78	24.27	22.8	86.92	2.71	2.30	5.01
		M	27.85	24.35	23.75	96.75	2.77	2.34	5.11
11	CV	S	24.98	27.32	24.57	56.85	1.17	0.43	1.71
		M	29.85	27.00	26.47	72.87	1.39	0.5	2.18
12	AL	S	25.85	21.62	22.07	87.5	2.77	2.11	3.73
		M	34.95	26.62	26.35	97.0	3.14	2.47	4.41

S= Seedling M= Micropropagated

Satkara (*Citrus macroptera* Mont.) SAT, Khasi papeda (*Citrus latipes* Tanaka) LAT, Sweet lime (*Citrus limettioides* Tanaka)SLS, Soh Bitara (*Citrus sinensis* osbeck) SOB, Indian wild orange (*Citrus indica* Tanaka) I, Ada Jamir (*Citrus assamensis* Dutta & Bhattacharya) ADA, Khasi mandarin (*Citrus reticulata* Blanco)KM, Soh myndong (*Citrus Jambhiri* Lush)SM, Jaintia lemon (*Citrus limon* Burm)JL, Pummelo (*Citrus grandis* Osbeck)P, Assam lemon (*Citrus limon* Burm)AL, Volkamer Lemon (*Citrus volkameriana* Pasq.) CV

(Source : Singh, 2000)

Micrografting

The development of shoot tip grafting (STG) as an *in vitro* multiplication technique was a consequence of the high economic losses caused by citrus virus and virus like diseases, which made the use of virus free propagative bud wood necessary. It is a technique that potentially can combine the advantage of rapid *in vitro* multiplication with the increased productivity that results from *in vitro* graft between superior scion and rootstock combination (Gebhardt and Goldbach, 1988). The method most widely used in the past to obtain virus free citrus plants was the selection of nucellar seedlings of polyembryonic cultivars (Weather and Calavan, 1959). The limitation of this method was the long period required for nucellar seedlings to proceed from juvenile to adult phase before becoming commercially productive (Roistacher, 1977).

The *in vitro* micrografting technique has proved to be very useful in the regeneration of whole orchards of citrus infected by viruses (Jonard, 1986). This technique was first developed by Murashige *et al.* (1972) with a view to obtaining healthy citrus trees. Later Navarro *et al.* (1975), Navarro and Juarez (1977), Roistacher *et al.* (1976) and Roistacher and Kitto (1977) extended micrografting technique to diverse species of citrus. The efficiency of the technique in eradication of virus infected citrus stocks has been well demonstrated (Russo and Starrantino 1975 and Youtsy 1978). In India work on shoot tip grafting in citrus continuing at NRCC, Nagpur, Biotech lab ICAR Research Complex for NEH region, Assam Ag. University, Jorhat and BCKV, West Bengal. Vijaya Kumari *et al.* (1994) have followed a modified procedure of Navarro for Nagpur mandarin and standardized the STG technique for Nagpur mandarin. Mukhopadhyay *et al.* (1997) defined the STG technique for Darjeeling oranges. Parthasarathy *et al.* (1996) and Singh *et al.* (1997) have also standardize the STG technique for Khasi mandarin. Singh (1999) standardize the STG technique of Sweet orange at NRCC, Nagpur.

Several factors influence the rate of success of grafting or virus elimination. Successful grafts have been obtained using many different scion cultivars of the commercially grown citrus species, and significant differences in grafting success among three different types have not been noted when appropriate rootstocks were used. Both good and poor rootstocks scion combinations have been identified but it is not known if grafting success *in vitro* is related to graft compatibility in vivo (Navarro 1981). The frequency of successful graft increases with the size of shoot tip, while the percentage of virus free plants declines. Murashige *et al.* (1972) obtained new citrus plants by grafting shoot tip on rootstock seedlings grown *in vitro*. Navarro *et al.* (1975) developed a routine procedure of shoot tip grafting to obtain 30 to 50% successful grafts which were transplanted to soil with over 95% survival. Most of the micrografted plants were free of the citrus virus and virus like diseases which were present in the source plant and they did not have juvenile characters (Roistacher *et al.*, 1976 and Murashige *et al.*, 1972). Edriss and Burger (1984), reported that the placement of apical meristem on the seedling rootstock is critical. They showed that placement of the shoot tip in contact with the vascular ring or in the cortical surface in an inverted T incision have been the most successful treatment. Navarro *et al.* (1975) and Singh (1999), studied the age effect of growing rootstock seedlings. The greatest success was achieved using seedlings two weeks after sowing. They also reported an inverse relationship between the size of shoot tip and the micrografting success rate. Pretreatment of the shoot tip and/or seedling rootstcok have been shown to increase the micrografting success rate. Jonard *et al.* (1983) treated peach shoot tip in 0.1 mg zeatin/l and increased the success rate by 300%. Starrantino and Caruso (1988) observed that

by dipping the apex and decapited seedlings for ten minutes before micrografting in a solution of BAP (0.5 ppm) the percentage of sprouting increased from 73% to 91%. The standard procedure of micrografting developed by Navarro was modified by Su and Chu (1984) who used rectangular triangle hole instead of inverted cut for insertion of scion. With the modified technique more than 60% successful grafts were obtained while the inverted T cut method yielded less than 20%.

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