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In vitro Propagation of *Citrus limonia* Osbeck Through Nucellar Embryo Culture

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Abstract: *Citrus limonia* Osbeck is a promising rootsctock for commercial citrus species with sturdy and disease and drought resistant characters. An efficient and highly reproducible plant regeneration protocol has been developed from nucellar embryo of *Citrus limonia*. Murashige and Skoogs medium was used for plant regeneration from nucellar embryos. It was noted that 6-benzylaminopurine at a concentration of 2.22 mM induced highest number of multiple shoots as 18.26 shoots per explant. On transfer of individual shoots to root inducing MS medium supplied with auxins, Indole-3-butyric acid (2.46 µM) and 6-benzylaminopurine (1.11 µM) proved to be the best combination. This media resulted in 78.80 % rooting and produced plantlets with an average of 5.53 roots/ shoot. Plantlets thus obtained were successfully transferred to green house for further observations. The micropropagated plantlets thus obtained can be used as rootstocks by traditional method or by micrografting.

Key words: *Citrus limonia*, micrografting, propagation, resistant and rootstock

INTRODUCTION

Citrus limonia Osbeck (Family Rutaceae) is native to India and is used as a rootstock to commercially important citrus cultivars of *Citrus reticulata* and *Citrus sinensis* (Singh *et al*., 1994; Karwa and Chikhale, 2004, 2005). Rough lemon and sour orange are the commonly used rootstocks but these are susceptible to many viruses and soil borne fungal diseases. In order to produce citrus trees with high productivity, increased resistance to drought, pests and fungal diseases it has become an urgent necessity to shift to a rootstock, which inculcates all these characters to the tree. On the other hand, Rangpur lime possesses these characters but the seedlings have normally slow growth and majority of them do not attain buddable size within a normal budding period for transplanting and one has to discard such under developed seedlings for budding (Srivastava and Nanaiah, 1972).

The demands for elite rootstock material are continuously increasing. To fulfill such demands, application of *in vitro* propagation technique is the only alternative way. One of the essential requirements for the successful application of plant propagation technology in agriculture is the capacity to regenerate elite plantlets (Bais *et al*., 2002; Karwa and Chikhale, 2002). Micropropagation of commercially important citrus species has been reported by several workers (Hartmann *et al*., 1990; Karwa, 2003) but the *in vitro* studies for *C. limonia* are rarely reported. With above views a protocol for clonal propagation of *C. limonia* has been developed using nucellar embryo as a starting material.

MATERIALS AND METHODS

Large and healthy fruits from disease resistant trees were selected from a local orange orchard. Mature and bold seeds were isolated and washed thoroughly under tap water to remove mucus and sugars present on the seed coat. The seed coats were carefully removed using pointed forceps. Care was also taken not to damage the cotyledons as nucellar embryos are closely embedded with cotyledons. Nucellar embryos were removed and surface sterilized by immersing in 0.1% $HgCl_2$ (w/v) and washed 4 times with sterilized glass double distilled water. The nucellar embryos were placed on surface of a full strength MS (Murashige and Skoog, 1962) basal medium containing macro and micro elements of MS salts and supplemented with (mgl^{-1}): myoinositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), sucrose (30,000) and phytagel (0.25%). Plant growth regulators were added prior to autoclaving at different concentrations and combinations according to the experimental requirements. The pH was adjusted to 5.8 and the medium autoclaved at 121ºC and 15 lb pressure for 15 minutes. Explants were inoculated in culture tubes (20 X150 mm) containing 15 ml culture medium. The tubes were incubated in the culture racks under controlled conditions of temperature (27±2 ºC), humidity (65%) and 16:8 h photoperiod at 1500 lux using fluorescent day light tubes. Treatments were replicated 5 times and 10 plants were used for each replication. Observations were recorded every week for shoot induction and multiple shoot formation. Cultures were examined and observations recorded in the form of statistical data (Panse and Sukhatme, 1985) and photographed using a stereo zoom Pentax camera. The percentage of explants with multiple shoots and the mean number of shoots per explants were determined for each treatment. The shoots thus obtained were transferred independently to culture tubes containing auxin rich MS medium for root induction. Number of roots developed per shoot was noted 30 days after transfer of shoots to the rooting media.

RESULTS AND DISCUSSION

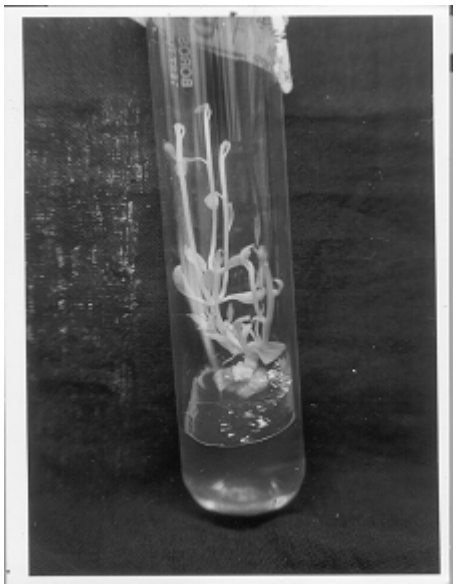
Though *Citrus limonia* is propagated successfully by seeds, the plant developed carries undesirable traits like

inborn virus diseases, soil borne fungi and ununiformity among plants. In the present paper a protocol is established for obtaining healthy, virus free and uniform planting material that could be used as a certified rootstock source.

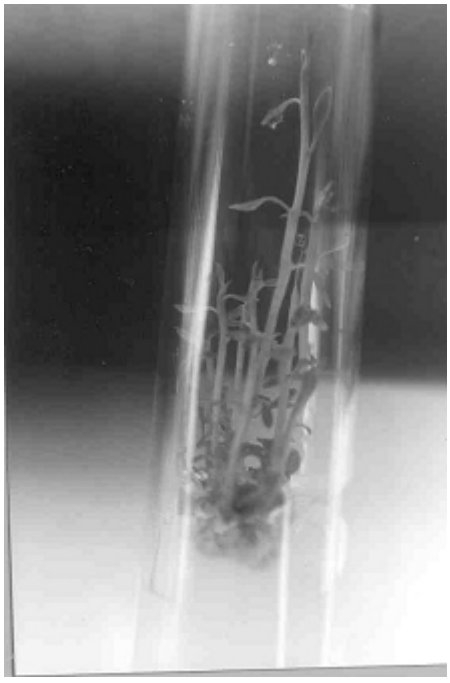
Multiple shoot formation: Two weeks after the nucellar embryos were inoculated, shoot induction was noted in the tubes. However after another six weeks on the same medium without subculture, the maximum multiple shoots were recorded in MS medium fortified with BAP 2.22 µM.

Plant regeneration: All the newly formed shoots developed into plantlets (each with 6-8 leaves and 3-4 roots) after being transferred onto basal medium supplemented with IBA and BAP for 30 days without any subculture. The plants were ready for further use as a rootstock for *in vitro* micrografting technique or to be transferred to green house. In the present study the plantlets thus obtained were transferred to green house for hardenening (Plate 2).

Effects of BAP, NAA and IBA: Without any subculture on hormone free basal medium 13.15 percent of nucellar embryo explants of *Citrus limonia* formed an average of 0.63 shoots in eight weeks (Table 1). However when basal media was fortified with BAP at 2.22 µM concentration, average number of shoots formed was noted to be 18.26 per explant as shown in Plate 1b. This concentration induced a higher percentage of explants with shoots and shoot number per explant than the hormone free treatment. Furthermore when NAA was introduced in the media the number of shoots formed was found to be declining. A concentration of 2.69 µM NAA in combination with BAP 2.22 µM could produce on average 9.6 shoots (Plate 1a). Effect of the cytokinins are said to vary with cultivars however, Mohanty *et al*., (1998) did not find any response for *Citrus sinensis* for shoot proliferation in MS + BAP and 2,4-D. Earlier Gill and Gosal (2002) reported maximum shoot regeneration in MS medium enriched with BAP 1 and GA_3 2 mg L^{-1}. BAP is effective in inducing *in vitro* morphogenesis in shoot regeneration and proliferation of several horticultural crops (Boxus, 1974; Chikhale *et al*., 2002). In this paper it was seen that BAP alone was found to be effective in multiple shoot induction from nucellar embryo explants in *Citrus limonia*. The shoots were transferred to MS medium supplemented with IBA 2.46 µM and BAP 1.11 µM for rooting. A maximum of 78.80% rooting was noted (Table 2). Similar to present investigation, Sandra and Morehart (1998) reported that increasing IBA concentration had no effect on induction of number of roots instead it reduced total root length and also promoted callus production. On the other hand Starrantino and Caruso (1987) rooted citrus rootstocks like Carrizo, Troyer and CPB4475 in MS medium nutrified with NAA 1 mg L^{-1}. The *in vitro* morphogenesis



(a)



(b)

Plate 1. Multiple shooting noted in C.limonia
(a). BAP 2.22 µM +NAA 2.69 µM, (b)BAP 2.22 µM

stimulated by the same explant on different hormone treatments is quiet different. Therefore, the culture requirements and media composition for specific selected plants needs to be identified and optimized.

CONCLUSION

Citrus limonia Osbeck (Rangpur lime) a promising rootstock for commercial citrus species has been

Plate 2: Plantlets of *C. limonia* hardened and transferred to green house.

Table 1: Effect of BAP and NAA on shoot formation from nucellar embryo explants in Citrus limonia Osbeck

BAP (µM)	NAA (µM)	Percentage of explants with shoots	Average number of shoots per explant
00	00	13.15± 0.24	0.63 ±0.03
0.44	00	36.08± 0.30	3.76± 0.42
2.22	00	75.50± 0.33	18.26± 0.49
4.44	00	48.36± 0.90	5.93± 0.42
2.22	2.69	51.00± 0.42	9.6± 00
4.44	2.69	40.35±1.08	6.2± 0.06

Table 2: Effect of BAP and IBA on in vitro rooting in Citrus limonia Osbeck

BAP (µM)	IBA (µM)	Percent rooting	Average number of roots per shoot
00	00	00	00
00	2.46	42.36± 0.54	2.1±0.15
00	4.92	66.28± 0.66	3.26±0.23
1.11	2.46	78.80± 0.75	5.53± 0.14
1.11	4.92	51.72± 0.11	1.06 ± 0.03

Mean±S.D.

successfully raised *in vitro*. A simple efficient and reproducible high frequency plant regeneration protocol has been developed from nucellar embryo of *Citrus limonia*. Enrichment of the culture medium with 6-benzylaminopurine (2.22 µM) resulted in maximum shooting response. These shoots rooted best *in vitro* in MS medium supplemented with Indole-3-butyric acid (2.46 µM) and 6-benzylaminopurine (1.11 µM). Plants with developed roots could be used for *in vitro* micrografting. A reliable protocol via multiple shoot formation and *in vitro* rooting of the shoots for *Citrus limonia* Osbeck was established. Under optimal conditions, 18.26 shoots could be obtained from one nucellar embryo explant after 8 weeks of culture. Profuse rooting was recorded as 5.53 roots per shoot in 4 weeks. The micropropagated plantlets thus obtained can be used as rootstocks for micrografting (Navarro *et al*., 1975; Roistacher, 1977).

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