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In Vitro Propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm.f.

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Abstract. **Multiple shoots were obtained from shoot tips (2 to 3 mm) derived from mature plants (5 to 6 years old) of *Citrus reticulata* Blanco CV. Khasi mandarin and *C. limon* Burm.f. CV. Assam lemon when cultured on Murashige and Skoog (MS) medium, supplemented with (mg·liter^{-1}) 1.0 BAP, 0.5 kinetin, and 0.5 NAA. Root induction was observed when 7-week-old single shoots (≈ 2 cm long) of both Citrus species were cultured on MS medium supplemented with (mg·liter^{-1}) 0.25 BAP, 0.5 NAA, and 0.5 IBA. These plantlets were successfully established in the soil. Chemical names used: naphthalene acetic acid (NAA), indole 3-butyric acid (IBA), and benzylamino purine (BAP).**

Citrus reticulate CV. Khasi mandarin and *Citrus limon CV.* Assam lemon are the two most commercially important *Citrus* species of northeastern India. 'Khasi' mandarin is polyembtyonic, whereas 'Assam' lemon is monoembryonic. Both species are generally cross-pollinated. They are highly heterozygous, and zygotic embryos, even if produced by selfing, would differ from the maternal parents. In 'Khasi' mandarin, seedlings genetically identical to the maternal parents are derived from nucellar embtyos. However, nucellar and zygotic seedlings are difficult to distinguish by morphology at early growth stages. The methods available (several biochemical methods, including isozyme analysis) for distinguishing nucelksr from zygotic plants are either not sufficiently reliable or are laborious. They also require somewhat sophisticated laboratory equipment not usually available to nursery operators (Ashari et al., 1988; Furs and Reece, 1946; Pieringer and Edwards, 1965; Weinbaum et al., 1982). Further, plants grown from seeds exhibit extended juvenility. One method that can be used to overcome these problems is micropropagation of shoot tips derived from mature plants. This technique allows the establishment of true-to-type sexually mature plants for both species mentioned above. Although this can be achieved by conventional grafting, lack of suftlcient promising rootstock for 'Khasi' mandarin and 'Assam' lemon limits the scope of grafting.

Growing shoot tips (5 to 6 cm long) of 'Khasi' mandarin (2n = 18) and 'Assam' lemon (2n = 18) were collected from 5- to 6-year-old plants from the orchard of the Assam Agricultural Univ., Jorhat. The expanded leaves were removed and washed thoroughly with a 1% solution of the detergent Teepol (Sigma, St. Louis) and then washed thoroughly with distilled water. Explants were surface-sterilized with 2% (w/v) calcium hypochlorite solution for 15 min and rinsed three times in sterile distilled water. Shoot tips (2 to 3 mm long) were isolated under aseptic conditions and inoculated into culture tubes (25 × 150 mm) (Fig. 1a) containing 20 ml of nutrient media. To study the effect of BAP, kinetin, and NAA on shoot proliferation, they were supplemented alone or in various combinations and concentrations in basal Murashige and Skoog (MS) (1962) medium with 0.8% agar (Himedia, India). Only those media (Ml, M2, M3, and M4) where positive results were obtained are given (Table 1). The pH of the media was 5.8 before autoclaving. The culture tubes were maintained at 25 ~ 2C and 12-h light (3000 lx, Phillips TL 40W/54 fluorescent tubes)/12-h dark cycles. The data were tested by analysis of variance and least significant difference.

Applying BAP, kinetin, and NAA at 0.5 or 1 mg·liter^{-1} did not allow shoot proliferation. A combination of 1.0 mg BAP, 0.50 mg kinetin, and 0.50 mgNAA/liter (M2 medium) gave the highest percentage of shoot proliferation in both species (Table 2, Fig. lb). After 7 weeks of culture, the number of multiple shoots, average length of shoots, and number of leaves per shoot were significantly higher in M2 medium compared to other media tested (Table 2, Fig. lc). Altman and Goren (1974) reported that BAP and kinetin caused the development of multiple shoots in cultures of citrus lateral buds collected from a 40-year-old Shamouti

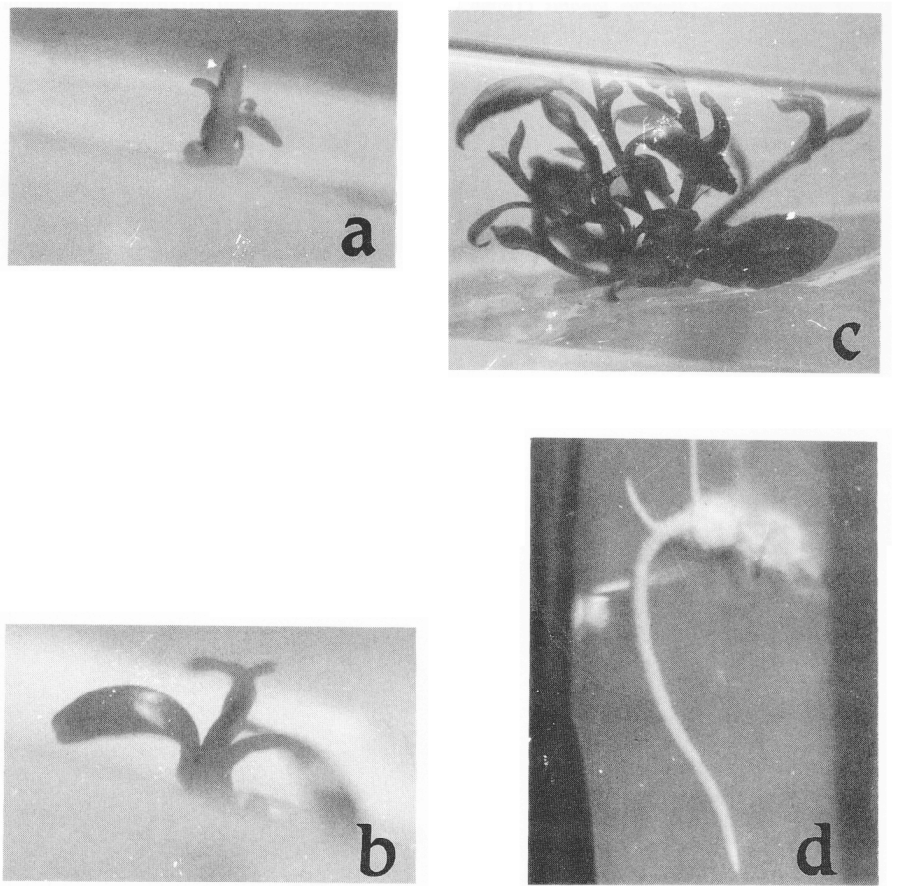

Fig. 1. (a) *Citrus reticulate* shoot tip (2 to 3 mm) with two leaf primordia cultured in M2 medium. (b) Regeneration of *C. limon* shoot tip in M2 medium after 17 days of culture. (e) Multiple shoots of *C. reticulate* developed after 7 weeks in M2 medium. (d) Root developed from single shoots of *C. limon* after 4 weeks in M5 medium.

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Table 1. Composition (in mg·liter^{-1}) of various modified Murashige and Skoog media tested for shoot-tip culture of *Citrus reticulata* and *C. limon.*

Growth regulators	Medium						
	M0	M1	M2	M3	M4	M5	M6
			(mg·liter^{-1})				
BAP	0.0	0.5	1.0	0.5	1.0	0.25	0.25
Kinetin	0.0	0.5	0.5	1.0	1.0	---	---
NAA	0.0	0.5	0.5	0.5	0.5	0.5	1.00
IBA	0.0	---	---	---	---	0.5	1.00

Table 2. Shoot-tip culture of *Citrus reticulata* and *C. limon* on modified MS media.[z]

Medium	Cultures with multiple shoots[y] (%)	Time elapsed to initiation (days)		Characteristics after 7 weeks		
		Shoot	Leaf	Shoots developed/explant (no. ± SE)	Shoot length/culture (cm ± SE)	Leaves developed/shoot (no. ± SE)
C. reticulata						
M0	00.0[x]	---	---	---	---	---
M1	30.0	21	24	2.3 ± 0.17	0.95 ± 0.14	1.25 ± 0.18
M2	70.0	17	19	6.1 ± 0.11	2.60 ± 0.09	2.40 ± 0.12
M3	52.5	19	21	3.4 ± 0.13	1.20 ± 0.11	1.35 ± 0.14
M4	50.0	19	21	3.2 ± 0.13	1.15 ± 0.11	1.40 ± 0.14
$LSD_{0.05}$				0.690	0.587	0.410
C. limon						
M0	00.0	---	---	---	---	---
M1	40.0	22	24	2.4 ± 0.17	1.00 ± 0.12	1.30 ± 0.18
M2	75.0	18	20	6.7 ± 0.11	2.50 ± 0.09	2.60 ± 0.12
M3	50.0	20	22	2.5 ± 0.13	1.30 ± 0.11	1.30 ± 0.13
M4	30.0	19	21	3.3 ± 0.13	1.20 ± 0.11	1.35 ± 0.14
$LSD_{0.05}$				0.610	0.593	0.497

[z]There were 40 shoot tips per medium.
[y]Values refer to the percentage of explants that produced multiple shoots.
[x]Zero values indicate absent; of multiple shoots.

orange *(C. sinensis)* tree. Barlass and Skene (1982) reported development of adventitious buds from shoot apex collected from mature plants of *C. reticulate* CV. Cleopatra mandarin in MS medium with BAP (0.50 mg·liter^{-1}). Pasqual and Audo (1989) reported axillary bud multiplication (juvenile explants) of *Poncirus trifoliata* in MS medium with 1 mg NAA and 2 mg BAP/liter. Thus, the combination of BAP, NAA, and kinetin appeared to be essential for multiple-shoot formation in different *Citrus* species.

For root induction, several combinations and concentrations of BAP, NAA, and IBA were tried, but only those media (M5 and M6) that elicited favorable root induction are shown (Table 1). Root induction was possible only when IRA was added to the medium. Roots were initiated and developed when excised single shoots were transferred to M5 and M6 media (Table 3). A combination of 0.50 mg NAA, 0.50 mg IBA, and 0.25 mg BAP/liter (M5 medium) was best for root development in both *Citrus* species.

Root initiation generally took place within 17 to 18 days of inoculation on M5 medium. The average number and length of roots were found to be significantly higher in M5 medium for both *Citrus* species after 4 weeks of culture (Table 3, Fig. id). Root initiation usually occurred near the cut surface of the shoot explant's basal ends. Nel (1987) reported root development from shoot meristems of in vitro-grown seedlings of *Citrus* species after 2 months in half-strength Murashige and Tucker (1969) medium supplemented with NAA only. Starrantino and Caruso (1988) reported rooting from excised shoots of citranges and trifoliate orange *(Poncirus triloliata)* cv. Flying Dragon on MS medium without cytokinin or IBA but with NAA (1 mg·liter^{-1}). However, in our investigation, a combination of NAA, IBA, and BAP was found to be essential for root development. Rooted shoots of both *Citrus* species were transferred to MS inorganic + 1% agar (Himedia, India) after 5 weeks. After 8 weeks, in vitro-grown plantlets were thoroughly washed with tap water to remove agar from the roots, which were then soaked in 0.2% Bavistin (BASF, India), a fungicide, for 5 to 10 min. The plantlets were potted in a sterilized mixture of 1 sand: 1 soil. They were initially irrigated with half-strength MS solution for 1 week, and subsequently with water. They were acclimatized at 27 ± 2C with a 16-h photoperiod for 3 to 4 weeks. Polyethylene covers were used to ensure high humidity around plants. About 60% of the plantlets were established in soil. Established plantlets were then transplanted to the field and watered regularly. Based on morphological characters such as thorniness, growth habit, internode length and leaf size, the established plants did not appear to be juvenile. However, the final assessment of maturity can be made only when the plants reach the flowering stage.

Table 3. Rooting of *Citrus reticulata* and *C. limon* shoots on modified MS media.[z]

Medium	Shoot explants formed roots[y] (%)	Avg days to root initiation	Characteristics after 4 weeks	
			Roots/explant (no. ± SE)	Length of roots (cm ± SE)
C. reticulata				
M5	70	18	4.3 ± 0.09	1.6 ± 0.64
M6	40	21	2.4 ± 0.13	0.7 ± 0.08
$LSD_{0.05}$			0.688	0.426
C. limon				
M5	80	17	4.9 ± 0.09	1.65 ± 0.03
M6	50	22	2.6 ± 0.13	0.80 ± 0.04
$LSD_{0.05}$			0.594	0.253

[z]There were 40 shoots per treatment.
[y]Vatues refer to the percentage of explants that produced roots.

In conclusion, in most reports on *Citrus* micropropagation, juvenile explants derived from seedling tissues have been used. We have been able to micropropagate explants derived from mature plants of two *Citrus* species where this has not been previously accomplished.

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