IN VITRO PROPAGATION OF *Citrus maxima* (Burm) Merr.

Marilyn M. Belarmino and Darlyn B. Posas

Department of Horticulture, Visayas State College of Agriculture, Baybay, Leyte 6521-A, Philippines

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ABSTRACT

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A system for *in vitro* propagation of pummelo or *Citrus maxima* (Burm) Merr. was established using shoot-tip and single-node stem segment taken from young seedlings. The procedure involved the initiation of shoots from explant tissue, followed by shoot proliferation, *in vitro* rooting, and finally, hardening of plantlets and potting out in soil. The agar-solidified Murashige and Skoog (MS) medium containing 30 g/L sucrose and supplemented with 0.2 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine (MSI medium) was optimum for shoot initiation from both explants. For rapid shoot proliferation, the MS medium containing 0.5 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine (MSP medium) was optimum. Eight-week-old shoots efficiently produced roots in agar-solidified MS medium containing 0.5 to 1.0 mg/L IBA compared with 4-wk-old shoots, indicating the importance of an adequate shoot growth prior to root induction. Using this system, 126 transplantable plantlets and 494 6-wk-old shoots can be produced from one nodal explant in a 4-month micropropagation cycle. One shoot tip explant can produce 26 transplantable plantlets and 50 6-wk-old shoots after 4 months.

KEYWORDS: Citrus. Micropropagation Nodal explant Plantlet. Pumello. Shoot tip.

INTRODUCTION

Citrus is one of the commercially important fruit crops in the Philippines. Most citrus species are cross-pollinated and are thus heterozygous and polyembryonic. Reproduction of true-to-type plants is

possible by marcots or via nucellar embryony. However, depending upon the cultivar, there can be 1 to 40% zygotic seedlings produced which must be culled from the nursery seedbeds to maintain clonal uniformity. If citrus could be propagated by tissue culture techniques, production of uniform and healthy planting materials would be substantially increased to meet the demand of growers.

Numerous studies have been conducted to improve genotype and propagation of selected citrus species and cultivars. However, efforts to enhance the efficiency of tissue culture methods for propagation have been largely confined to sweet orange, *C. sinensis* L. (Barlass and Skene, 1982); grapefruit, *C. paradisi* Macf. (Bhansali and Arya, 1982); mandarins, *C. reticulata* Blanco; and true lemons, *C. limon* L. (Sauton et al., 1982). Other species such as pummelo, *C. maxima* (Burm) Merr. and calamondin, *C. mitis* Blanco have attracted relatively less attention.

C. maxima or pummelo is a commercial species known for outstanding eating qualities. The common methods of propagation is by seeds, marcotting and grafting to selected rootstocks (Gardner, 1993). These traditional methods are relatively slow and inefficient thereby limiting the volume of planting materials subsequently produced. Moreover, the use and distribution of asexually propagated plants often lead to the endemic spread of diseases and insect-pests. It is in these areas where tissue culture technique becomes a sound alternative to the conventional methods of propagation (Murashige, 1987). The increasing use of tissue culture technique for citrus propagation has been due to the following reasons: (1) economic pressure to reduce production costs, particularly for rootstocks that are difficult to propagate; (2) mass propagation of cultivars or hybrids that may be seedless, produce few seeds, or require several years before sufficient seeds can be produced (Kitto and Young, 1981); and (3) production of disease-free plants that serve as a nuclear stock for the delivery of high quality planting materials (Pierre, 1994). The *in vitro* propagation technique relies largely on two developmental processes: enhanced axillary branching of shoot segments and adventitious shoot

formation in diverse explants. The present paper outlined a successful *in vitro* propagation system for *C. maxima* through adventitious shoot formation from shoot tip and single-node stem segments taken from young seedlings grown *in vitro*.

MATERIALS AND METHODS

Plant material

Pummelo was used in this study for the reason, that its fruits have good eating quality and are sold at a relatively high price in local markets. Seeds from a mature fruit were used as initial materials and were germinated *in vitro*. Prior to germination, the seeds (10 seeds/vial) were decontaminated by quick immersion in 70% ethyl alcohol. Inside the laminar flow hood, this solution was decanted and replaced with 5% sodium hypochlorite solution (added with Tween 40). The seeds were soaked for 20 min. The solution was again decanted and the seeds were rinsed three times with sterile distilled water. Decontaminated seeds were transferred to sterile Petri dishes lined with sterile paper to absorb excess water. To facilitate germination of seeds, the seed coats were removed before inoculation on cooled autoclaved medium containing 9 g/L agar. The seed cultures were maintained in a culture room provided with dim light from cool white fluorescent tube at 25± 1 °C.

Preparation of explants and inoculation

Apical shoot tips (~0.5 cm long) and single-node stem segments (~0.5-1.0 cm long) were the two types of explants used in this study. The explants were taken from 2-wk old aseptically produced seedlings to ensure the use of contaminant-free and physiologically uniform explants. After excision, explants were individually inoculated into vials (95 x 28 mm in size) containing 10 mL of autoclaved culture medium. Cultures were incubated under 16 h daylight provided by 40 W cool white fluorescent tubes at 25± 1 °C.

Regeneration of shoots by shoot tip and nodal cultures

A. Initial culture of shoot tip and nodal explants

Shoot tip and nodal explants were induced to regenerate shoots on agar-solidified (7 g/L agar-agar, Japan) MS medium containing 30 g/L sucrose, 50 mg/L folic acid, 40 mg/L adenine and supplemented with combinations of 0.5-2.0 mg/L benzylamino purine (BAP) and 0.2-0.5 mg/L indole butyric acid (IBA) in screw-capped micro sample vial (American Scientific Products, McGraw Park, IL, USA). The hormone-free MS medium served as control. The experiment was laid out following 2 x 9 factorial in Completely Randomized Design (CRD) with type of explant and culture media as factors. Each treatment was evaluated using 10 samples and were replicated three times. Shoot regenerating capacity of explants was evaluated after 4 wks of incubation based on the following parameters: (1) percent shoot formation - percentage of explants that successfully produced shoots at 4 wks after inoculation; (2) number of days to shoot initiation - recorded when the first shoot bud appeared from the explant; (3) number of shoots per explant - average number of shoots produced from 30 explants; (4) shoot length (cm); (5) number of nodes; and (6) number of leaves. The average values in parameters 4-6 were obtained per shoot, taking into consideration all the shoots produced from 30 explants.

B. Subculturing shoot tip- and nodal-derived shoots

After 4 wks of incubation in shoot proliferation media, shoot tip- and nodal-derived shoots were subcultured into freshly prepared culture media of the same plant growth regulator (PGR) supplements. Subculturing was done aseptically by removing shoot cluster from culture medium followed by careful separation of individual shoot. A single shoot was subcultured into a screw-capped micro sample vial containing 20 mL of sterile medium. Newly subcultured shoots were incubated under the same conditions previously mentioned. The same parameters as those given in Section A were measured.

In vitro root formation of plantlets

Shoots containing at least 4 leaves were transferred into the rooting medium composed of agar-solidified half-strength MS medium containing 30 g/L sucrose and 0.5-1.0 mg/L IBA. The half-strength MS medium without IBA supplement served as control. A single shoot was inoculated into 100-mL capacity tin-capped round glass jar (San Miguel Corp., Phil.) containing 20 mL of rooting medium. Cultures were incubated under 12 h light at 25 ± 1 °C. Rooting experiment was laid out using simple CRD with 10 shoots/treatment and replicated three times. Rooting capacity of shoots was evaluated after 4 wks of incubation based on the following parameters: (1) percent shoots forming roots; (2) number of days to root initiation; (3) number of roots per shoot; and (4) length of roots (cm).

Acclimatization and soil establishment of plantlets

In vitro-propagated plantlets with developed shoot and root systems were hardened (while still inside the culture bottle) by placing them at ambient room condition (28~32 °C) for 1 wk prior to potting. The plantlets were then taken out of culture jars and the roots were washed with tap water to remove adhering agar. Roots were quickly dipped in weak fungicide solution to prevent fungal infection. Plantlets were individually transplanted into two types of sterile soil media, namely: garden soil, and garden soil and compost mixture (1:1, v/v). Potted plantlets were covered with transparent plastic to maintain high relative humidity, and placed inside the greenhouse. Hand mistingwas provided everyday for the first week of potting and at one day interval thereafter. Percent survival of plantlets was assessed 4 wks after potting.

RESULTS AND DISCUSSION

Regeneration of shoots by shoot tip and nodal cultures

The production of plants from axillary shoots has proved to be the most generally applicable and reliable method of*in vitro* propagation

(Murashige, 1987). In citrus, two methods were adopted, namely: apical shoot tip culture and single-node stem culture. Both methods depend on stimulating axillary shoot growth by overcoming the dominance of the shoot apical meristem (Kitto and Young, 1981; Singh et al., 1994). In the present experiment, production of multiple shoots directly from shoot tip and nodal explants of *C. maxima* was induced through the outgrowth of the pre-existing apical or axillary bud and by the formation of adventitious shoot meristems without passing a callus phase (Figure 1A). The wounded basal portion of shoot tip and node thickened prior to the emergence of bud primordia which later on differentiated into shoot buds. These shoot buds when subcultured into fresh medium became woody and developed into plantlets.

Interestingly, 50 to 100% of nodal explants produced multiple shoots on PGR-enriched medium while only 30% on PGR-free medium (Table 1). The addition of 0.5 mg/L IBA into the culture medium seemed to be critical for 100% shoot formation from nodal explants. Generally, PGR-enriched medium hastened the initiation of shoots from nodal explants by 9-16 days earlier than those in PGR-free medium (27 days). Specifically, the medium containing 0.2 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine produced greater number of shoots (4.2) per nodal explant at shorter incubation period (11 days) compared with the other PGR combinations hence, designated as the optimum medium for shoot initiation from explant tissue of *C. maxima*. For discussion purposes, this medium is referred to as shoot initiation medium or MSI medium.

Increasing the concentration of BAP from 0.5 mg/L to 0.2 mg/L was no longer beneficial. It appears that there was a concentration effect of BAP and IBA similar to those reported in *C. mitis* Blanco (Sim et al., 1989). Using MSI medium, nodal-derived shoots with an average length of 1.14 cm containing an average of 6.8 leaves were obtained which was significantly longer/greater than those shoots cultured on PGR-free medium and PGR-enriched media, except on medium containing 0.5 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine (Table 1). The average number of nodes per shoot did not differ significantly among treatments.

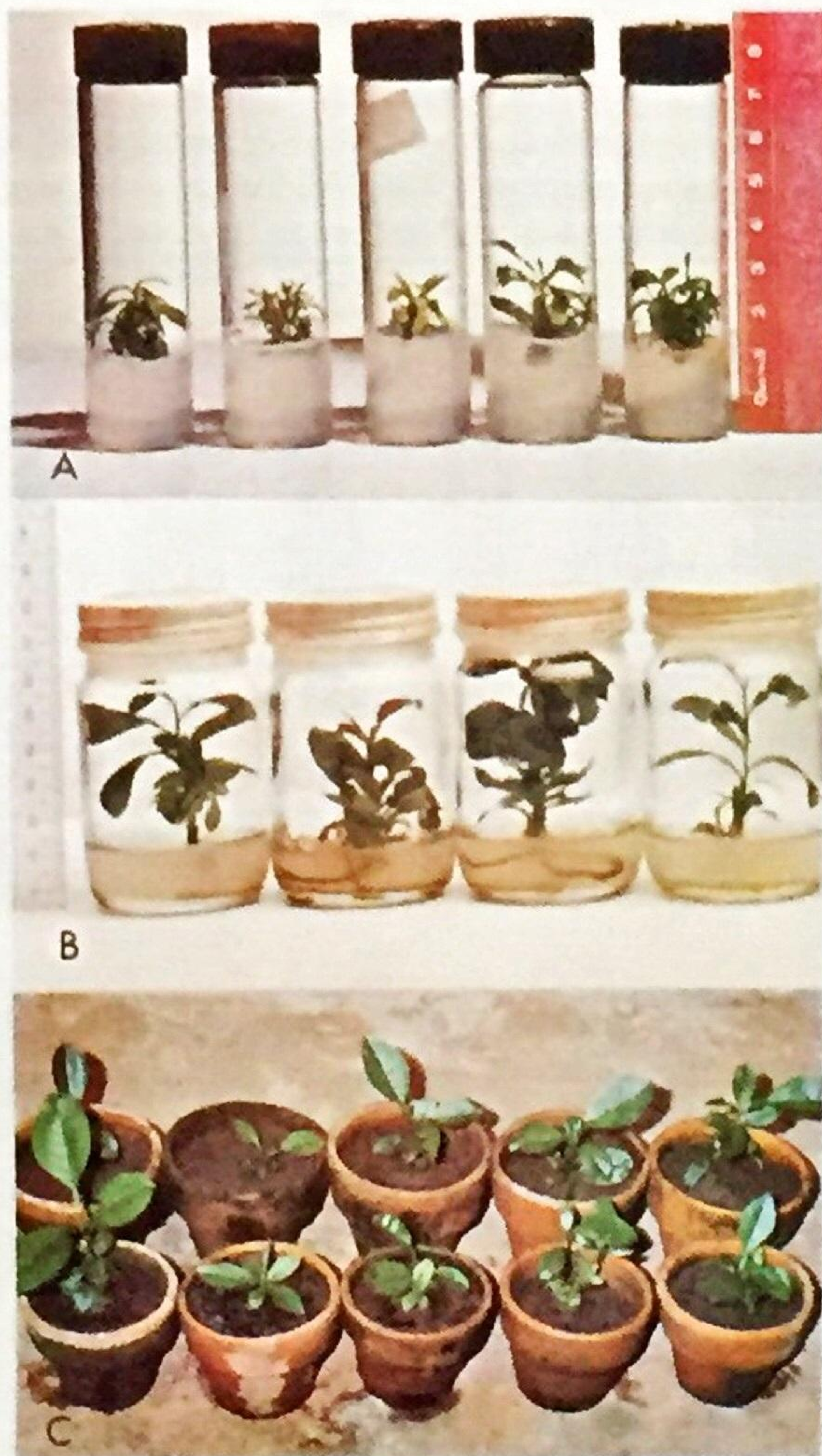

Figure 1. In vitro propagation of *Citrus maxima* (Burm) Merr. using single-node stem segment, showing (A) proliferation of shoots from nodal cultures at 4 wks of incubation on agar-solidified MS medium containing 0.5 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine (MSP medium); (B) 8-wk-old shoots showing well-developed roots after 3 wks of incubation in agar-solidified half-strength MS medium containing 1.0 mg/L IBA; and (C) in vitro propagated plantlets after 4 wks of establishment in soil.

Table 1. Production of multiple shoots from nodal cultures of *C. maxima* at 4 wks of incubation on agar-solidified MS medium containing 30 g/L sucrose, 40 mg/L adenine and combinations of BAP and IBA.[1]

PGR supplement	Multiple shoot formation from explant, %	Ave. no. of days to shoot initiation	Ave. no. of shoots/ explant	Ave. shoot length, cm	Ave. no. of nodes/ shoot	Ave.no. of leaves/ shoot
PGR-free medium	30.0 d	27 c	1.4 c	0.82 bc	4.5 a	4.0 bc
0.2 BAP + 0.2 IBA	50.0 c	16 ab	1.6 bc	0.77 cd	4.5 a	4.2 bc
0.5 BAP + 0.2 IBA	60.0 c	18 b	1.7 bc	0.82 bc	4.4 a	4.7 bc
1.0 BAP + 0.2 IBA	50.0 c	18 b	1.6 bc	0.82 bc	4.5 a	4.0 bc
2.0 BAP + 0.2 IBA	83.3 b	18 b	1.9 bc	0.41 e	3.3 a	3.3 c
0.2 BAP + 0.5 IBA	100.0 a	11 a	4.2 a	1.14 a	5.0 a	6.8 a
0.5 BAP + 0.5 IBA	100.0 a	14 ab	2.8 b	1.10 ab	4.9 a	5.7 ab
1.0 BAP + 0.5 IBA	100.0 a	16 ab	1.9 bc	0.49 de	3.5 a	2.9 c
2.0 BAP + 0.5 IBA	100.0 a	16 ab	1.8 bc	0.51	3.8 a	2.8 c

[1]Means in a column having a common letter are not significantly different at 0.05 level according to Duncan's Multiple Range Test (DMRT).

The MSI medium also induced 100% shoot initiation from shoot tip explants after 14 days of incubation (Table 2). Deviations from MSI medium significantly decreased percentage shoot formation and extended the duration of shoot bud formation. For example, increasing the BAP level from 0.2 to 0.5 mg/L decreased the percentage of shoot tip explants that produced multiple shoots from 100 to 86.7%, and extended the number of days to shoot initiation by 2 days. Likewise, decreasing IBA level from 0.5 to 0.2 mg/L correspondingly decreased shoot formation from 100 to 6.7%, and required an additional 6 days to initiate shoots (Table 2). The PGR-free medium and other PGR-enriched media containing combinations of 0.2-0.5 mg/L BAP and 0.2 mg/L IBA, or 1.0-2.0 mg/L BAP and 0.5 mg/L IBA did not stimulate multiple shoot formation from shoot tip explants except for the continued growth of the apical shoot meristem that resulted in the development of a single plantlet. Thus, zero percent multiple shoot formation in Table 2 means that each of the 30 shoot tip explants continued to grow as single shoot without proliferation.

Table 2. Production of multiple shoots from shoot tip cultures of *C. maxima* at 4 wks of incubation on agar-solidified MS medium containing 30 g/L sucrose, 40 mg/L adenine and combinations of BAP and IBA.[1]

PGR supplement	Multiple shoot formation from explant, %	Ave. no. of days to shoot initiation	Ave. no. of shoots/ explant	Ave. shoot length, cm	Ave. no. of nodes/ shoot	Ave. no. of leaves/ shoot
PGR-free medium	0.0 d	28 c	1.0 b	0.75 d	2.89 b	2.9 bc
0.2 BAP + 0.2 IBA	6.7 c	20 b	1.1 b	0.91 abcd	4.10 ab	2.8 bc
0.5 BAP + 0.2 IBA	0.0 d	18 ab	1.0 b	1.20 abc	4.90 a	4.0 abc
1.0 BAP + 0.2 IBA	0.0 d	18 ab	1.0 b	0.99 abcd	4.70 ab	3.1 bc
2.0 BAP + 0.2 IBA	0.0 d	18 ab	1.0 b	0.88 bcd	4.00 ab	2.4 c
0.2 BAP + 0.5 IBA	100.0 a	14 a	2.3 a	1.34 a	4.70 ab	5.2 a
0.5 BAP + 0.5 IBA	86.7 b	16 a	1.9 a	1.29 ab	5.50 a	4.5 ab
1.0 BAP + 0.5 IBA	0.0 d	16 a	1.0 b	0.74 d	3.50 a	3.1 c
2.0 BAP + 0.5 IBA	0.0 d	18 ab	1.0 b	0.70 d	4.40 ab	3.3 c

[1]Means in a column having a common letter are not significantly different at 0.05 level according to Duncan's Multiple Range Test (DMRT).

Using the MSI medium, a shoot tip explant produced an average of 2.3 shoots which was significantly greater than those incubated in PGR-free medium which formed a single shoot (Table 2). Shoots with an average length of 1.34 cm and containing an average of 4.7 nodes and 5.2 leaves were also produced from shoot tip explants cultured in MSI medium.

Comparatively, the nodal explant has a greater capacity (74.8%) to produce multiple shoots compared with shoot tip (21.5%) (Table 3). Both explants, however, did not differ significantly in the average number of days to initiate shoots and in the number of nodes per shoot. Although the MSI medium induced shoot bud formation from both explants, roots were not formed. It seems that BAP played a key role in the initiation of shoots from explant tissue (Belarmino et al., 1994; Hasegawa, 1980) but may have inhibited the formation of roots (George and Sherrington, 1984).

Table 3. Comparative performance of shoot tip and nodal explants of *C. maxima* at 4 wks of culture on agar-solidified MS medium containing 30 g/L sucrose, 40 mg/L adenine and combinations of BAP and IBA.[1]

Parameters	Shoot tip explant	Single-node stem segment
Multiple shoot formation from explant tissue, %	21.49 b	74.81 a
Average number of days to shoot initiation	18.44 a	17.11 a
Average number of shoots/explant	1.04 b	2.11 a
Average shoot length, cm	0.98 a	0.76 b
Average number of nodes/shoot	4.31 a	4.26 a
Average number of leaves/shoot	3.49 b	4.25 a

[1]Means in a row having a common letter are not significantly different at 0.05 level according to Duncan's Multiple Range Test (DMRT).

Subculturing shoot tip- and nodal-derived shoots

Adventitious shoots need to be subcultlured into fresh medium every 3-4 wks or as required, to renew essential nutrients for further growth or differentiation (George and Sherrington, 1984). In the present study, 4-wk-old shoots were subcultured to fresh media containing the same PGR concentrations used for the initiation of shoots from shoot tip and nodal explants. The agar-solidified MS medium supplemented with 0.5 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine supported further growth and proliferation of shoots from both explants (Figure 1). Thus, this medium is designated as an optimum medium for the proliferation of *C. maxima* shoots, and is referred to as MSP medium.

Using MSP medium, the average number of shoots per nodal explant increased from 2.8 (Table 1) to 4.6 (Table 4) and 1.9 (Table 2) to 2.6 shoots per shoot tip (Table 5). Increasing the concentration of BAP to 1.0-2.0 mg/L was no longer beneficial, indicating an optimum concentration

of 0.5 mg/L (Belarmino et al., 1992). The result also implies that *C. maxima* requires a low concentration of BAP (0.2 mg/L) to induce shoot initiation from explant tissue and increased BAP level (0.5 mg/L) to stimulate further proliferation of shoots during the subculture phase.

A similar trend was observed on shoot-tip derived shoots after subculture phase. The average number of shoots increased from 1.9 (Table 2) to 2.6 (Table 5) after subculture in MSP medium. Increased shoot length, number of nodes and leaves were also observed in this medium. Generally, the nodal explant showed a high capability for shoot production compared with shoot tip explant (Table 6). This implies the potential of using nodal explants for micropropagation of *C. maxima*. Using this type of explant, the agar-solidified MS medium containing 3% sucrose, 0.5 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine can be recommended for *in vitro* propagation of *C. maxima* since it induced rapid shoot proliferation from explant tissue.

Table 4 Production of multiple shoots from nodal cultures of *C. maxima* after 4 wks of subculture on agar-solidified MS medium containing 30 g/L sucrose, 40 mg/L adenine and combinations of BAP and IBA [1]

PGR supplement	Ave. no of shoots/ explant	Ave. shoot length, cm	Ave. no of nodes/ shoot	Ave. no of leaves/ shoot
PGR-free medium	1.6 b	1.3 bcd	5.5 bc	5.3 bcd
0.2 BAP + 0.2 IBA	1.8 b	1.3 bcd	6.0 abc	5.2 bcd
0.5 BAP + 0.2 IBA	2.6 b	1.5 bcd	5.8 bc	6.1 b
1.0 BAP + 0.2 IBA	1.7 b	1.8 abc	6.4 ab	5.6 bc
2.0 BAP + 0.2 IBA	1.9 b	0.99 d	4.7 c	4.8 cd
0.2 BAP + 0.5 IBA	4.9 a	2.21 a	7.7 a	8.9 a
0.5 BAP + 0.5 IBA	4.6 a	1.9 ab	7.3 a	8.6 a
1.0 BAP + 0.5 IBA	2.4 b	1.2 cd	4.9 bc	4.3 d
2.0 BAP + 0.5 IBA	2.4 b	1.2 cd	5.2 bc	4.4 cd

[1]Means in a column having a common letter are not significantly different at 0.05 level according to Duncan's Multiple Range Test (DMRT)

Table 5. Production of multiple shoots from shoot tip cultures of *C. maxima* after 4 wks of subculture on agar-solidified MS medium containing 30 g/L sucrose, 40 mg/L adenine and combinations of BAP and IBA.[1]

PGR supplement	Ave. no. of shoots/ explant	Ave. shoot length, cm	Ave. no. of nodes/ shoot	Ave. no of leaves/ shoot
PGR-free medium	1.0 b	1.2 a	4.9 c	4.3 c
0.2 BAP + 0.2 IBA	1.1 b	1.7 a	5.5 c	4.3 c
0.5 BAP + 0.2 IBA	1.0 b	2.2 a	6.3 bc	5.6 abc
1.0 BAP + 0.2 IBA	1.0 b	2.0 a	6.2 bc	4.6 c
2.0 BAP + 0.2 IBA	1.0 b	1.7 a	5.3 c	4.1 c
0.2 BAP + 0.5 IBA	2.8 a	3.1 a	7.9 ab	7.4 a
0.5 BAP + 0.5 IBA	2.6 a	3.1 a	8.1 a	1.0 ab
1.0 BAP + 0.5 IBA	1.0 b	1.2 a	5.1 c	5.1 abc
2.0 BAP + 0.5 IBA	1.0 b	3.1 a	5.5 c	5.1 abc

[1]Means in a column having a common letter are not significantly different at 0.05 level according to Duncan's Multiple Range Test (DMRT).

Table 6. Comparative performance of shoot tip and nodal explants of *Citrus maxima* after 4 wks of subculture on agar-solidified MS medium containing 30 g/L sucrose, 40 mg/L adenine and combinations of BAP and IBA.[1]

Parameters	Shoot tip Explant	Single-node stem segment
Ave. no. of shoots/explant	1.39 b	2.65 a
Ave. shoot length, cm	2.16 a	1.49 b
Ave. no. of nodes/shoot	6.08 a	6.00 a
Ave. no. of leaves/shoot	5.30 a	5.90 a

[1]Means in a row having a common letter are not significantly different at 0.05 level according to Duncan's Multiple Range Test (DMRT).

In vitro root formation of plantlets

It is well-known that root initiation depends on a low cytokinin to auxin ratio (Hu and Wang, 1983). Usually, there is sufficient residual

cytokinin in shoots from the regeneration medium thus, less or no cytokinin is needed in rooting medium. In most instances however, the presence of cytokinins in regeneration medium inhibits root formation thus a separate root-inducing medium has to be used (George and Sherrington, 1984). In the present experiment, the combination of half-strength MS medium and 0.5-1.0 mg/L IBA were stimulatory for rooting of citrus plantlets regardless of explant origin (Figure 1B). The beneficial effect of reduced salt strength on rooting of various plants was reviewed by George and Sherrington (1984). Similarly, the stimulatory effect of IBA on adventitious root primordium initiation is supported by the findings in *C. reticulata* and *C. limon* (Singh et al., 1994), pot roses (De Vries and Dubois, 1988) and in difficult-to-root *Pinus banksiana* cuttings (Haissig, 1979).

In *C. maxima*, best result was obtained by inoculating 8-wk-old shoots in half-strength MS medium enriched with 1.0 mg/L IBA, i.e. 100% of the inoculated shoots initiated an average of 4 roots of 3.8 cm length after 14 days (Table 7). Reduction of IBA level from 1.0 mg/L to 0.5 mg/L produced the same result except for an extended root initiation period (10 days later) and decreased number of roots (an average of 2 roots/shoot). Likewise, 8-wk-old shoots needed 35 days to initiate an average of 2 roots 2.4 cm long in IBA-free medium.

Table 7. In vitro formation of 4- and 8-wk-old *Citrus maxima* shoots at 1 mo of incubation on IBA-containing MS medium.[1]

Rooting media, mg/L	Age of shoots used for rooting, wk	Shoots forming roots, %	Ave. no. of days to root initiation	Ave. no. of roots/ shoot	Ave. length of roots, cm	Plantlets established in soil, %
IBA-free 1/2 MS	4	0	-	-	-	0
1/2 MS + 0.5 IBA	4	10	60	1.0	0.5	0
1/2 MS + 1.0 IBA	4	10	48	1.0	0.5	0
IBA-free 1/2 MS	8	15	35	2.0	2.4	15
1/2 MS + 0.5 IBA	8	100	24	2.0	3.8	100
1/2 MS + 1.0 IBA	8	100	14	4.0	3.8	100

[1]Total number of shoots inoculated per rooting medium is 30.

Failure of shoots to initiate roots *in vitro* is often due to inadequate shoot elongation at the previous stage (Mott, 1981 as cited in George and Sherrington, 1984). This was demonstrated in this study, using 4-wk-old *C. maxima* shoots for rooting; 4-wk-old shoots were still very small that only 10% initiated an average of one root (0.5 cm long) per shoot after 48-60 days of incubation in IBA-containing medium (Table 7). The rooting medium lacking IBA did not induce rooting of shoots. Apparently, rooting of woody plantlets requires more time compared with herbaceous species like *Brassica*. This is confirmed by Nel (1987) who reported root development from shoot meristems of *in vitro*-grown citrus seedlings after 2 mos in half-strength Murashige and Tucker (1969) medium.

One noticeable feature of *C. maxima* roots was the absence of lateral roots, which may suggest that IBA was critical for root initiation (Spiegel-Roy and Vardi, 1984) but was not stimulatory for lateral root formation. The phenomena of delayed rooting and absence of lateral roots in *C. maxima* has yet to be explained and will require further experimentation.

Acclimatization and soil establishment of plantlets

Adequate hardening procedures, a well-proportioned shoot and root systems, as well as a suitable soil medium are essential to ensure survival of *in vitro*-propagated plants in soil. In *C. maxima*, the process of gradual acclimatization involved the exposure of *in vitro* plants (while still inside culture bottle) to ordinary room condition (28-32°C) for 1 wk, covering potted plantlets with transparent plastic, and hand misting. This acclimatization procedure was done to prevent substantial water loss and desiccation of plantlets upon immediate exposure to ordinary conditions which may lead to irreparable damage or death of the transplanted plant (Grout, 1975).

All of the *C. maxima* plantlets derived from rooting of 8-wk old shoots were easily hardened and survived after potting out in soil (Figure 1C). This implies the importance of a balance between the shoot and root systems of plantlets for a successful post vitrum growth.

From the results of the study, transplantable *C. maxima* plantlets can be produced after four sequential culture phases and using respective optimum culture medium/condition, namely: (1) shoot initiation phase using MSI medium; (2) shoot proliferation phase using MSP medium; (3) *in vitro* rooting phase using half-strength MS medium containing 1.0 mg/L IBA; and (4) acclimatization and soil establishment. By adopting the presented *in vitro* propagation system, and given the 4.9 rate of shoot proliferation from nodal explant, a total of 126 transplantable plantlets plus 494 6-wk old shoots can be produced in a 4-mo micropropagation cycle (Table 8). Another option is to use the 6-wk old shoots as source of nodal cuttings for further micropropagation. Given an average of 7.7 nodes per shoot, a projected total of 3,804 shoots can be produced after 4 mos.

Utilizing shoot tip explant, on the other hand, can produce 26 transplantable plantlets plus 50 6-wk old shoots after 4 mos of *in vitro* propagation. The 50 shoots can be used as a source of nodal cuttings for further micropropagation. Given an average of 7.9 nodes per shoot, a projected total of 398 shoots can be produced after 4 mos. The use of nodal stem segments as starting materials for micropropagation of *C. maxima* is therefore recommended. The shoot tip, on the other hand, can be used as a source of meristems, for the recovery of disease-free plantlets from severely infected stock plants (Nauer et al., 1983.)

Table 8. Comparative multiplication rate of nodal and shoot tip explants using agar-solidified MS medium supplemented with 0.2 mg/L BAP, 0.5 mg/L IBA and 40 mg/L adenine.

Type of Explant	No. of shoots	Proliferation rate	No. of plantlets in one micropropagation cycle
Nodal stem	147/30[1]	4.9	4,438.89
Shoot tip	84/30	2.8	485.57

[1]Number of plantlets after 8 wks of culture/number of explants used at initial culture.

The present study demonstrates a system for *in vitro* propagation of *C. maxima* using shoot tip and nodal segments taken from young seedlings. Micropropagation of citrus from vegetative tissues, e.g., shoot tip and stem node, could be beneficial because several years are required before sufficient seeds can be produced from a newly-introduced root stock, especially cultivars with few seeds (Kitto and Young, 1981). Moreover, scion cultivars might fruit more precociously in root stocks derived from vegetatively-propagated shoots than on those produced from seedlings (Sim et al., 1989). *In vitro*-propagated plants are also uniform and disease-free and are thus preferable for use as nuclear stock for multiplication of planting materials.

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