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Preliminary studies on direct plant regeneration from stem segments of pummelo *(Citrus grandis)* cv. Melomas

[Kajian awal penjanaan pokok secara langsung dari segmen batang pummelo *(Citrus grandis)* cv. Melomas]

S. Rogayah*, J. Mohd. Shaib* and H. Marzukhi*

Key words: *Citrus grandis*, stem explant, shoot regeneration, in vitro

Abstract

A direct plant regeneration system for *Citrus grandis* cv. Melomas was established using stem segments of one-month-old in vitro seedlings. Multiple shoots were proliferated on Murashige and Skoog (MS) medium supplemented with 6-(gamma, gamma-Dimethylallylamino) purine (2-ip) and indole-3-acetic acid (IAA). The highest number of shoots, 6 shoots per explant were produced on medium containing 1 mg/litre each of 2-ip and IAA. Roots developed well when regenerated shoots were excised and cultured on half strength MS medium supplemented with naphthaleneacetic acid (NAA). The highest rooting percentage was produced on MS medium supplemented with 0.1 mg/litre NAA and the highest number of roots per plantlet was produced on MS medium supplemented with 1 mg/litre NAA. In vitro pummelo plantlets exhibited normal growth in soil under greenhouse conditions.

Introduction

Pummelo *(Citrus grandis)* is a tropical fruit of the Rutaceae family. The major commercial producers of pummelo are Thailand, Malaysia, Indonesia, China, Taiwan and Japan. In Malaysia, 39% (5,055 ha, 1996) of the total hectares of citrus planted is pummelo, which is widely grown in the states of Perak, Kedah, Melaka, Kelantan and Johor. The commercially important pummelo varieties are Tambun Manis, Tambun Masam, Shatian-yu and Melomas, with the latter as the most potential. Pummelo, a popular fruit, especially during Chinese festive season, is consumed fresh or as processed juice.

As with other citrus species, pummelo is characterised by a long juvenile period and high heterozygosity causing serious obstacles in conventional improvement programmes (Soost 1965). The majority of commercial citrus varieties require only improvement in one or a few traits, mainly disease resistance. The integration of modern biotechnology (genetic engineering), can provide an alternative approach in the improvement of pummelo cv. Melomas. A critical prerequisite in the incorporation of genetic engineering to improve this cultivar is to establish an efficient and reproducible plant regeneration system.

Tissue culture in the genus citrus has been extensively studied, where plant regeneration was obtained both through somatic embryogenesis (Vardi and Spiegel-Roy 1982; De Pasquale et al. 1994) and organogenesis from various explants (Barlass and Skene 1982; Sauton et al. 1982;

*Biotechnology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
Authors' full names: Rogayah Sekeli, Mohamad Shaib Jaafar and Marzukhi Hashim
E-mail: lynn@mardi.my
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Moore 1986; Singh et al. 1994). However, only a few reports of direct in vitro plant regeneration of pummelo seedlings are available in the literature, where regeneration was obtained from cotyledonary nodes, nodal and shoot tip segments (Amin and Akhtar 1993). Cotyledonary nodal explants was the most responsive (5–7 times greater) when compared to nodal and shoot tip explants.

The earlier work was carried out using different parts of the pummelo explants including leaf, root and stem. Shoots were induced using various combinations of plant growth regulator (PGR) involving 6-(gamma, gamma-Dimethylallylamino) purine (2-ip), indole-3-acetic acid (IAA), 6-furfurylamino purine (Kinetin), 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA). The combination of 2-ip and IAA showed a good shoot induction as compared to the other treatment combinations. This paper describes further results on the shoot regeneration for stem segment of *Citrus grandis* by using various concentration combinations of 2-ip and IAA.

Materials and methods

Plant material

The seeds of *Citrus grandis* cv. Melomas were obtained from mature fruit harvested at MARDI Station, Jelebu, Negeri Sembilan. Only the large seeds, 8–10 mm in length, were germinated because they were well developed. Stem segments, 10 mm in length, were excised from 4-week-old germinated seedling and placed horizontally into the culture medium for shoot regeneration studies. The plant growth regulators were supplemented according to experimental stage.

Medium composition

MS basal medium (Murashige and Skoog 1962) supplemented with full strength vitamins, 100 mg/litre myo-inositol, 3% (w/v) sucrose (Sigma) and solidified with 0.2% (w/v) gelrite (Sigma) were used throughout the study. Seeds were germinated on hormone free MS basal medium and direct shoot induction was assessed on medium supplemented with various concentrations of 2-ip and IAA ranging from 0.0, 0.1, 1.0 and 10.0 mg/litre. Rooting of shoots obtained was carried out on half MS medium, supplemented with different levels of NAA at 0.1, 0.5 and 1.0 mg/litre. The pH of all media was adjusted to 5.8 prior to autoclaving. Seeds were germinated in (150 ml) conical flasks and direct regeneration was initially carried out in 9 cm petri dishes before transferring to conical flasks for further shoot and root development.

Culture condition

The seeds were surface-sterilised under aseptic conditions with 1% (v/v) *Virkon* for 10 min, followed by 80% (v/v) absolute ethanol for 30 s, 10% (v/v) domestic Chlorox (3.5% sodium hypoclorite) for 5 min, and then rinsed 3 times with sterile distilled water. The seed coat was removed, cultured on MS basal medium without plant growth regulator (PGR). In vitro stem segments from 4-week-old cultures were used as explants for direct regeneration studies. The experiment was replicated thrice with 15 explants per replicate. All cultures were kept at 26 ± 2 ºC under white fluorescent light at 25 mMol photons/m^2/s in 12/12 hours of light/dark regime. Cultures were sub-cultured at 4-week intervals. Observations were recorded on the number of explants that produced shoots. Individual shoots approximately 2–3 cm in length were then transferred to MS basal medium containing different levels of NAA for rooting. The percentage of shoots that rooted was recorded after two months with a 4-week interval subculture. Rooted shoots were then transferred into polybags containing standard soil mixture for 2 months after which the acclimatized plantlets were placed in the glasshouse.

Results and discussion

Direct shoot induction from stem explants

Combinations of BAP and NAA have been used to induce shoot formation in numerous species. This hormonal combination has also been shown effective to induce direct adventitious shoot from various citrus species (Moore 1986; Tripepi 1997; Huang et al. 2000). But in the current study, different types and combinations of hormone were used for the induction of adventitious shoots from stem explants of pummelo. Various combinations of 2-ip and IAA were evaluated and it showed a good shoot induction compared to combination of BAP and NAA.

Shoot formation was affected by the interaction between 2-ip and IAA concentration *(Table 1)*. Shoot formation was less in the absence of 2-ip. However, when 2-ip was added to the culture medium, the percentages of shoot formation were increased. Stem segments on medium with IAA alone, produced roots with single to several roots per explant, whereas 51.1% of stem segments cultured on medium with 2-ip at 0.1 mg/litre alone, produced 2–3 shoots per stem segment with fully expanded leaves *(Plate 1)*. With an increase in the concentration of 2-ip from 0.1–1.0 mg/litre, the number of stem segments that produced shoots also increased by 60%. The number of shoots produced per stem segment at a concentration of 2-ip at 10.0 mg/litre increased, but the shoots were stunted and the leaves did not expand fully. These results suggested that pummelo requires a higher concentration of cytokinin to produce adventitious shoots from stem segments. Although all combinations of 2-ip and IAA used produced shoots, the number of shoots per explant varied considerably according to the PGR combinations used. The highest frequency of stem segment responsiveness (95.6%) and normal shoot formation (6 shoots per stem segment) was produced on medium containing a combination of 1 mg/litre each of 2-ip and IAA.

In vitro rooting of shoots

Shoots 2–3 cm in length were separated from shoot clusters and transferred to half strength MS basal medium supplemented with different levels of NAA. In vitro rooting is normally induced in reduced strength basal media containing an auxin (Hu and Wang 1983). Roots started to appear at the base of the separated shoots at about 20 days after being transferred to the rooting media. All treatments induced root development but differed in the number of roots developed per shoot and the frequency of shoots producing roots. Shoots (62.22%) on MS basal medium without NAA produced 1–2 roots of 1–4 cm in length. The roots were thin, well elongated and devoid of any hairy roots.

In the current study, the addition of NAA increased the number of roots as well as the number of shoots that produced roots. Half MS containing 0.1 mg/litre NAA with 91.1% rooting efficiency produced 2–4 roots of 2–4 cm in length. The roots were thicker than on MS medium without NAA and moderately elongated with no hairy roots. The highest rooting percentage was obtained on a medium containing 0.1 mg/litre NAA whereas the highest number of roots was

Table 1. The effect of different concentrations of 2-ip and IAA on direct shoot regeneration on pummelo (*Citrus grandis* cv. Melomas)

IAA \ 2-ip	0.0 mg/litre	0.1 mg/litre	1.0 mg/litre	10.0 mg/litre
0.0 mg/litre	20.00h	51.11f	60.00e	73.33d
0.1 mg/litre	17.78h	40.00g	80.00c	88.89b
1.0 mg/litre	13.33i	82.23c	95.55a	80.00c
10.0 mg/litre	11.11i	80.00c	80.00c	86.67b

Means with the same letter are not significantly different at $p < 0.05$ using the LSD

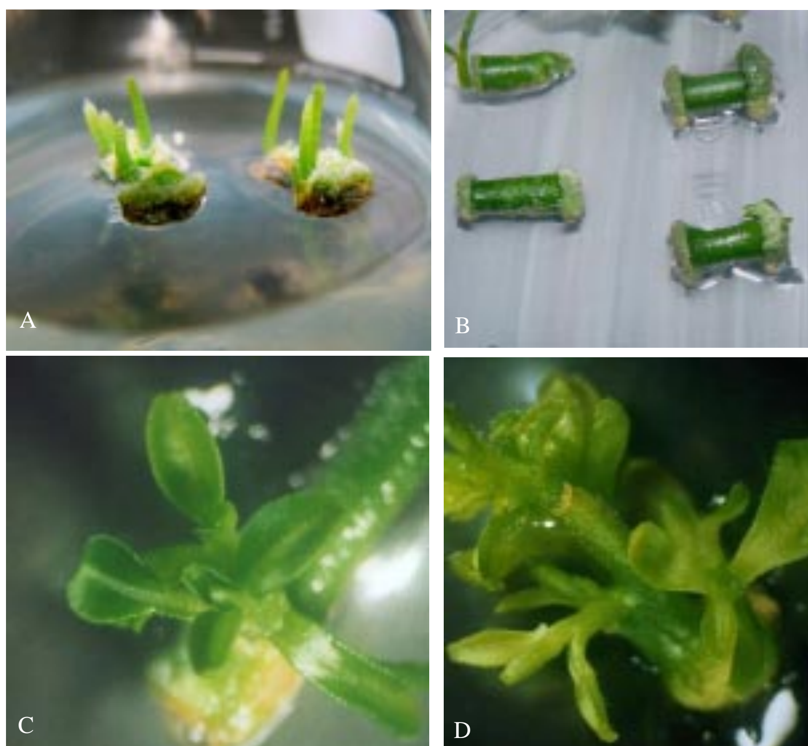

Plate 1. Direct shoot regeneration from stem explants of pummelo. (A). Stem segment cultured on medium containing 0.1 mg/litre IAA. (B). Induction and development of adventitious shoots from stem segments cultured on MS medium supplemented with 0.1 mg/litre 2-ip. (C). Shoot formation on medium supplemented with 2-ip and IAA and (D). Multiple shoot formation from stem segments on 1 mg/litre 2-ip and 1 mg/litre IAA

produced on a medium containing 1 mg/litre NAA *(Table 2)*.

NAA has also been reported for inducing root in *Citrus sinensis* and *Citrus limonia* (Barlass and Skene 1982). From this experiment it was observed that without the addition of NAA or at very low concentrations (0.1 mg/litre), the plantlets still rooted *(Plate 2)*. A possible explanation could be that endogenous auxins are synthesized or naturally present in the shoots and substantially translocated to the roots through basipetal movement. When vigorously growing, healthy plantlets with well-expanded leaves and roots reached 6 cm in height, they were transferred to soil.

Table 2. Effect of NAA concentration on the rooting of shoots of pummelo

Culture medium	No. of roots/shoot	% of rooting
½ MS (Control)	2	62.22d
½ MS + 0.1 mg/litre NAA	4	91.11a
½ MS + 0.5 mg/litre NAA	5	80.00b
½ MS + 1.0 mg/litre NAA	8	71.11c

Means with the same letter are not significantly different at $p < 0.05$ using the LSD

Conclusion

Based on the results obtained from this preliminary study, direct shoot regeneration from stem segments was best achieved in MS medium supplemented with 1 mg/litre each of 2-ip and IAA. Although plantlets rooted on half MS medium without any

A

B

C

Plate 2. (A) Complete plantlet with root in a MS medium with 0.1 mg/litre NAA. (B) Pummelo plantlet after being removed from the rooting medium showing root and shoot growth. (C) In vitro plantlets were transferred into the soil

PGRs, the frequency was higher at low concentrations of NAA (0.1 mg/litre) where 91.1% of the shoots rooted. This protocol developed could be used in subsequent genetic engineering experiments for the improvement of pummelo cv. Melomas and also for the mass propagation of uniform planting material.

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Abstrak

Satu sistem penjanaan pokok secara terus telah diwujudkan untuk tanaman limau bali cv. Melomas dengan menggunakan segmen batang dari biji benih in vitro yang berusia satu bulan. Pucuk-pucuk telah dihasilkan di atas medium Murashige and Skoog (MS) yang telah ditambah dengan hormon 6-(gamma, gamma-Dimethylallylamino) purine (2-ip) dan asid indola-3-asetik (IAA). Bilangan pucuk terbanyak iaitu 6 pucuk per eksplan telah terhasil di atas medium yang mengandungi hormon 2-ip dan IAA dengan kepekatan 1 mg/liter. Akar telah terbentuk apabila pucuk-pucuk dipindahkan ke medium MS separa kepekatan yang ditambah dengan asid naftalenaasetik (NAA). Peratusan pengakaran tertinggi diperoleh apabila pucuk diletakkan di dalam medium MS yang mengandungi 0.1 mg/liter NAA. Walau bagaimanapun bilangan akar terbanyak bagi setiap pucuk terhasil di atas medium MS yang mengandungi 1 mg/liter NAA. Pokok-pokok limau bali yang dibiak secara in vitro telah menunjukkan pertumbuhan yang normal apabila dipindahkan ke tanah dan diletakkan di dalam rumah kaca.

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