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Somatic embryogenesis via protoplast cultures of *Fortunella hindsii* (Champ.) and their regeneration into precocious flowering plants

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Protoplasts isolated from embryogenic callus of *Fortunella hindsii* (Champ.) were cultured in MT (Murashige and Tucker, 1969) basal medium containing 5% sucrose supplemented with kinetin, malt extract (ME) and 0.6 M sorbitol. About 85% of the surviving protoplasts formed cell wall within 6 d of culture and the first cell division was observed 7 days after isolation. The highest plating efficiency was obtained in MT basal medium containing 5% sucrose supplemented with 0.01 mg 1^{-1} kinetin 600 mg 1^{-1} ME, MT basal medium containing 5% sucrose and supplemented with 0.01 mg 1^{-1} zeatin was found to be a medium suitable for the development somatic embryos into heart-shaped somatic embryos. The highest percentage shoot formation was obtained using 0.1 mg 1^{-1} benzyladenine (BA) + 0.1 mg 1^{-1} gibberellic acid (GA_3). In this investigation 40 plants survived and grew normally in the soil. After two months of being maintained in the soil, the plants formed flowers and the flowers developed into fruits in the soil treated with BA.

Key words: cytokinin, *Fortunella hindsii*, flowering, protoplast, somatic-embryo, sucrose

Introduction

Citrus relatives are potential sources of useful resistance traits for citrus genetic improvement (Fu *et al*., 2003; Ceng, 2012; Raveh, 2012; Jumin and Nito, 1996). Successful somatic embryogenesis and subsequent maintenance *in vitro* and plant conversion in fruit plant coud be used as a genetic resources for somatic hybrid purposes (Ceng, 2012; Raveh, 2012; Motoike *et al*., 2005; Tallon *et al*., 2012). Among the orange subfamily, *Fortunella hindsii* may be a genetic source of early flowering plant (Jumin and Nito, 1996b: Swingle and Reece, 1967; Sykes, 1988). Somatic hybridization via protoplast fusion has been used successfully as a method to bypass sexual incompatibilities in some

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cases (Fu *et al*., 2003). Intra and intergeneric somatic hybrids have been obtained between *Citrus* and some of its relatives (Deng *et al*., 1992; Fu *et al.*, 2003; Tallon *et al*., 2012). Embryogenic protoplasts of *Citrus* are used as one partner in the protoplast fusion to obtain inter-intrageneric somatic hybrids with leaf-derived protoplasts of *Fortunella crassifolia, Feronia limonia, Clausena lansium, Citropsis gilletiana or Atalantia ceylanica* of a second parent (Louzada *et al*., 1993).

Plant regeneration from cultured protoplasts in *Citrus* has been reported for a number of species (Jumin, 1995). However, there has been few reports of successful plant regeneration from protoplast cultures of *Citrus* relatives (Jumin and Nito, 1996a). In general, citrus plants cannot be regenerated from leaf mesophyll protoplasts (Fu *et al*., 2003) although Deng *et al*. (1992) have reported plant regeneration from leaf mesophyll protoplasts used in fusion experiments. The Establishment of embryogenic callus has not been obtained from monoembryonic types of *Citrus* (Jumin, 1995). The study was successful in establishing embryogenic callus from *F. hindsii* as a member of *Citrus* relatives. In view of the limited success of plant regeneration from protoplast cultures of *Citrus* relatives, this study successfully established methods in protoplast cultures and their regeneration into plants. This system has potential as an additional method to be used for making wide hybridizations through protoplast fusion for rootstock improvement. The objective of this paper is to describe the regeneration sequence via somatic embryogenesis from protoplasts and precocious flowering of *F. hindsii*

Materials and methods

Protoplast sources

Embryogenic callus of *Fortunella hindsii* was induced from the hypocotyl region of seedlings on MT basal medium (1969) containing 5% sucrose, 5.0 mg 1^{-1} benzyladenine (BA), 2.5 mg 1^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D) and 600 mg 1^{-1} malt extract (ME) and maintained under 52.9 □mol m^{-2} s^{-1} light with a photoperiod of 16 h at 25°C, as described by (Jumin and Nito, 1996a). The seedlings used for hypocotyl excision were germinated from immature nucellar embryo explants using the same medium as mentioned above.

Protoplast isolation

Prior to protoplast isolation, about 1 g of *F. hindsii* callus was transferred to fresh liquid medium consisting of MT basal medium containing

5% lactose, and incubated on gyratory shaker at 120 rpm for 6 d under 17.7 □mol m^{-2} s^{-1} light with a photoperiod of 16 h at 25°C. Callus tissue was placed in 50 ml Erlenmeyer flasks and mixed with 5 ml of filter-sterilized maceration medium consisting of 0.4% macerozyme R-10 (Yakult Phamaceutical Co., Tokyo), 0.2% cellulase Onozuka (Yakult Pharmaceutical Co., Tokyo), 0.1% driselase (Kyowa Hakko Kogyo Co., Tokyo), half-strength MT inorganic salts, 0.7 M sorbitol, and the pH was adjusted to 5.8. The enzyme solution was sterilized through a Millipore filter (Millex-HA, 0.45 □m) before use. After 14 h incubation on a reciprocal shaker at 25 rpm in the dark at 25°C, protoplasts were isolated by filtering through a double layer of Miracloth (Calbiochem; U.S.A) and centrifuged at 100 x g for 5 min. The protoplasts were then washed twice with MT inorganic salt solution containing 0.6 M sorbitol by centrifugation at 100 x g for 2 min and re-suspension of the pellet protoplasts.

Protoplast culture

Protoplasts of *F. hindsii* were re-suspended in MT basal medium containing 5% sucrose, supplemented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg 1^{-1} kinetin, 0, 300, 600 or 900 mg 1^{-1} ME, 0.6 M sorbitol, and solidified with 0.1% Gelrite (Kelco, Division of Merck & Co. Inc., San Diego, California). The protoplasts were cultured at a density of 3 to 5 x 10^4 cells ml^{-1} in 60 x 15 mm plastic petri dishes containing 2 ml of culture medium. For embedding the protoplasts in Gelrite, the liquid medium containing the protoplasts was mixed with an equal amount of Gelrite medium to obtain a final concentration of 0.1% Gelrite. All dishes were sealed with Parafilm and maintained at 25°C in the dark for 40 d, and then kept under 52.9 □mol m^{-2} s^{-1} light with a photoperiod of 16 h at 25°C. The plating efficiency was recorded as the percentage of plated protoplasts which formed colonies after 40 days of culture. The viability of the protoplasts was checked by fluorescein diacetate (FDA) staining. The cell wall regeneration test was performed by staining with Calcofluor white M2R (Nagata and Takebe, 1970).

Embryo induction

Calli derived from protoplasts used in this experiment had been sub-cultured three times at 30 days intervals using MT basal medium containing 5% sucrose without plant growth regulators (PGR). For somatic embryo induction, the calli were transferred into MT basal medium containing 5% lactose without PGR and solidified with 0.25% Gelrite.

Chromosome analysis

Chromosome count was carried out on a small piece of 25 days old callus. The callus was pretreated with 0.02M 8-hydroxiquinone for 6 h at room temperature, fixed in ethanol-acetic acid (3:1, v/w) solution for 16 h, and then stained with 1% (v/w) lactopropionic orcein and counted under an inverted microscope.

Globular embryo development

Somatic embryo development of *F. hindsii* was studied by culturing globular somatic embryos onto MT basal medium supplemented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg 1^{-1} zeatin and 2.5, 5.0, 7.5 or 10.0 % sucrose and solidified with 0.25 % Gelrite in 90 x 20 mm petri dishes. The concentrations of kinetin and sucrose were chosen based on preliminary dose response trials on stock callus. The number of globular somatic embryos that developed into heart-shaped somatic embryos with cotyledon-like structures was determined after 30 days. Specimens were prepared for scanning electron microscopy as described. Tissue samples were affixed on aluminum stubs with silver paint and coated with gold palladium in a fine coat Ion Sputter Topcon ABT-3. The tissue samples were examined under a scanning electron microscope, Topcon ABT-3, at accelerating voltage of 15 KV.

Shoot formation

For shoot formation, heart-shaped somatic embryos were cultured individually on half strength MT basal medium containing 5% sucrose supplemented with 0.0, 0.01, 0.1, 1.0 or 10.0 mg 1^{-1} BA and 0.0, 0.01, 0.1, 1.0 or 10.0 mg.1^{-1} GA_3 and solidified with 0.3% Gelrite. The cultures were kept under 52.9 □mol m^{-2} s^{-1} light with a photoperiod of 16 h at 25°C. Shoot formation was recorded as the percentage of cultured heart-shaped somatic embryos which formed shoots after 2 months.

Plant regeneration from shoots

Regenerated shoots were transferred to half-strength MT basal medium containing 5% sucrose without PGR. When root length reached 4-5 cm and some amount of shoot elongation had occurred, the plantlets were transplanted to covered glass pots with hyponex solution. The transferred plantlets were held in the growth chamber for 2 months under the same conditions of temperature and light as cultured embryos. Humidity was

maintained by covering the plantlets with transparent glass containers. Plantlets were subsequently transferred to larger pots and acclimatized to greenhouse conditions.

Plants flowering

The plants originated from plantlets after acclimatization at 2-leaf stage were maintained in trays containing 75 % peat and 25 % sand (v/v) and kept under a greenhouse condition for 4 weeks. Seedlings were selected for uniformity in growth habit and size at the 2-leaf stage and transplanted into 0.5 per pots containing 50 % peat and 50 % sand. BA treatment was carried out immediately after the plantlets were transplanted to pots. The plantlets were foliage sprayed with distilled water or 0.01, 0.1, 1.0 and 10.0 mg 1^{-1} BA once a week for 3 weeks. The concentrations of BA were chosen based on a preliminary dose response trial on stock plantlets.

Results

About 10^6 protoplasts with a diameter of 10-30 □m tin were obtained from 1 g of callus (Fig. 1A). FDA staining showed that the viability of fresh protoplasts was 80 %. About 70% of the surviving protoplasts formed a cell wall within 6 d of culture as judged by Calcofluor white M2R staining. First cell division was observed 7 days after isolation. The protoplast plating efficiency obtained after 40 days of protoplast culture was from 10-45% (Table 1).

Table 1. Effect of kinetin and ME on protoplast plating efficiency (%) of *F. hindsii*, 40 days after protoplast culture

Kinetin (mg l^{-1})	**Malt Extract (ME) (g l^{-1})**		
	0.0	300	600
0.0	15.3^{a}	14.6^{a}	16.0^{a}
0.001	20.6^{b}	25.3^{b}	27.0^{ab}
0.01	25.3^{c}	28.3^{b}	50.0^{bc}
0.1	30.3^{d}	17.0^{c}	35.3^{cd}
1.0	14.6^{a}	15.3^{c}	25.6^{cd}

Mean value followed by different alphabet/s within a column do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test.

The formation of colonies (about 70 □m in diameter) occurred after 60 days of protoplast culture. Scanning electron microscope studies of globular

somatic embryos revealed large vacuolated parenchymatous cells, highly cytoplasmic, and meristematic cells (Fig. 1B).

The media manipulation could improved colony formation of the culture. The MT basal medium containing 5% sucrose supported cell divisions in protoplast cultures. However, the number of mitotic divisions was increased by the addition of kinetin and ME to the medium. A low concentration of kinetin stimulated colony formation. When protoplasts were cultured on MT basal medium containing 5% sucrose without kinetin and ME, the protoplast plating efficiency was low. However, when protoplasts were cultured in the medium supplemented with 0.01 mg l^{-1} kinetin and 600 mg 1^{-1} malt extract, higher plating efficiency was obtained (Table 2).

Table 2. Effect of zeatin and sucrose on development of protoplast-derived globular somatic embryos (%) *F. hindsii* 30 days after culture (30 globular somatic embryos tested for each treatment)

Zeatin (mg l^{-1})	**Sucrose1(g l^{-1})**			
	2.5	5.0	7.5	10.0
0.0	65.3[a]	55.3[a]	45.0[a]	38.3[a]
0.001	70.6[a]	65.3[a]	55.0[ab]	45.3[ab]
0.01	77.3[a]	68.3[a]	75.3[c]	75.0[c]
0.1	67.3[a]	55.0[a]	65.3[cb]	45.3[ab]
1.0	45.3[b]	35.3[b]	55.6[ab]	33.0[a]

Mean value followed by different alphabet/s within a column do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test

After 60 days, protoplast-derived colonies were transferred to PGR-free MT basal medium containing 5% lactose (embryo induction medium). The number of globular somatic embryos <0.5 mm in diameter was determined after 30 days from protoplast derived cultures. The number of globular somatic embryos obtained from 5 protoplast isolations was 530. Cell colonies became compact and changed into spherical structures, which formed pro-embryos and then developed into globular somatic embryos. The globular somatic embryos then became heart-shaped forming cotyledon-like structures and developed to plantlets (Fig. 1C). Chromosome count originated from callus was diploid with indicated 18 chromosome (Fig. 1 D), and there was no difference between embryogenic callus original and plants.

Globular somatic embryos derived from protoplasts were cultured on MT basal medium supplemented with 2.5-10% sucrose and 0.0-1.0 mg 1^{-1} zeatin for 30 days. The frequency of globular somatic embryos that developed into heart-shaped somatic embryos in the medium supplemented

with 5 % sucrose and 0.01 mg 1^{-1} zeatin was 80% (Table 2). About 70% of the hear-shaped embryos were 0.5 to 0.9 mm in diameter, while others were elliptical in shape and = 1.0 mm long. The heart-shaped somatic embryos averaged 1.2 - 2.2 mm in diameter after 2 months.

A low level of BA + GA_3 in the culture medium promoted the growth of heart-shaped somatic embryos of *F. hindsii* into plantlets. The highest percentage shoot formation was obtained using 0.1 mg 1^{-1} BA and 1.0 mg 1^{-1} GA_3. After 30 days of culture on BA + GA_3 containing medium, the formation of adventitious shoot buds was observed and many of these developed into plantlets, and then promoted shoot formation and then subsequently developed into plantlets.

The requirement for successful plant recovery is balanced germination of the embryos. About 70% of embryos on PGR-free medium underwent normal shoot elongation. It was evident that there was no correlation between embryo induction and subsequent shoot differentiation. When shoots were transferred onto half-strength MT basal medium containing 5% sucrose with or without PGR, there were differences in rooting ability among the plantlets. Shoots from medium with BA + GA_3 rooted more quickly and readily, while shoots from PGR-free medium formed few roots and were accompanied by hyperhydricity. Consequently, plantlets from medium with BA + GA_3 survived in soil.

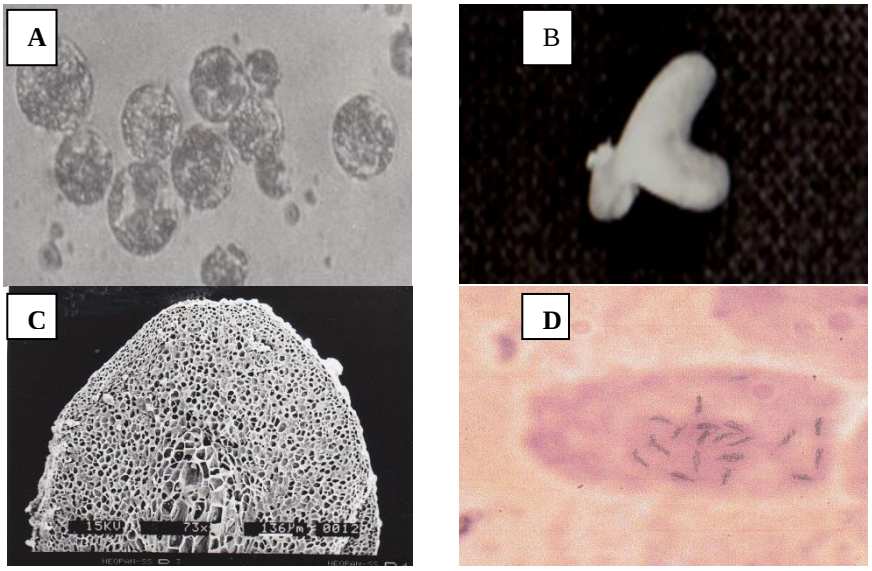

Fig. 1. (A), Fresh protoplasts of *F. hindsii* (bar 20 □m). (B), Heart-shaped embryos derived from protoplasts of . *F. hindsii* in MT basal medium containing 5 % sucrose supplemented with 0.001 mg l-1 BA 90 days after protoplast isolation (bar 1 cm). (C), Scanning electron micrograph showing meristematic cells of globular somatic embryos derived from protoplasts four weeks after transplanting to MT basal medium containing 50 g 1^{-1} lactose supplemented with 0.01 mg 1^{-1} BA (bar 156 µm), (D). Chromosome on the somatic embryo of *F. hindsii* 90 days after protoplast isolation (bar 20 □m)

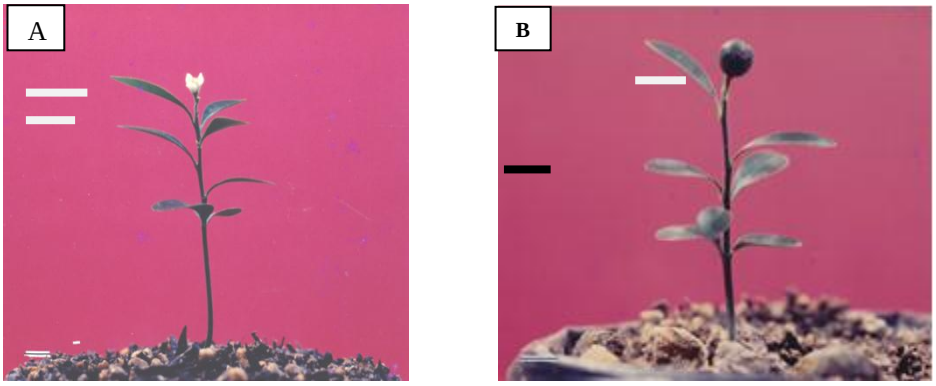

Fig. 2. (A). A single terminal flower of *F. hindsii* grown 4 months after acclimatization in the soil under 14 – 16 photoperiod at 25°C with 3 sprays of 0.01 mg 1^{-1} BA solution (bar =1 cm). (B) Flower of *F. hindsii* developed to fruit after 5 month of planting.

The requirement for successful regeneration was the ability of germinated heart-shaped embryos to survive transfer from the tissue culture environment to soil. Plantlets were very sensitive to dehydration and extreme temperatures when transferred from *in vitro* to natural conditions. In the present investigation, only those heart-shaped embryos had balanced root and shoot survived the transfer to the soil. In this study, only 4 plants in the soil were recovered from PGR-free medium, while 30 plants survived in the soil from medium with BA + GA_3. (Fig. 2). After 30 days, regenerated plantlets grew normally and no differences were noticed in growth habits and leaf characters such as shape, thickness and color between protoplast-derived plants and nucellar seedlings.

The highest plating efficiency was obtained on MT basal medium containing 5% sucrose supplemented with 0.01 mg 1^{-1} zeatin and 600 mg l^{-l} ME. Cell colonies changed into globular somatic embryos on MT medium containing 5% lactose without plant growth regulators. MT basal medium containing 5% sucrose and supplemented with 0.01 mg 1^{-1} zeatin was found to be a medium suitable for the development of globular somatic embryos derived from protoplasts into heart-shaped somatic embryos with cotyledon-like structures. The highest percentage shoot formation was obtained using 0.1 mg 1^{-1} BA + 0.1 mg 1^{-1} GA_3 (Table 3).

Table 3. Effect of BA and GA_3 on shoot formation of *F. hindsii* 60 days after heart-shaped embryos culture (25 heart-shaped embryos tested for each treatment)

BA (mg l^{-1})	GA_3 (mg l^{-1})				
	0.0	0.01	0.1	1.0	10.0
0.0	38.3[a]	45.3[a]	44.3[a]	35.6[a]	29.0[a]
0.01	45.6[ab]	47.3[a]	48.6[a]	45.3[b]	46.6[ab]
0.1	55.3[b]	66.6[b]	84.6[b]	75.3[c]	69.6[b]
1.0	50.3[b]	65.3[b]	78.3[b]	71.3[c]	60.6[b]
10.0	40.6[b]	58.6[ab]	75.6[b]	63.0[bc]	60.3[b]

Mean value followed by different alphabet/s within a column do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test

The highest number of flowers was obtained in the plants with a decline in vegetative growth as was observed in treatment with 0.1 mg 1^{-1} BA. BA-sprayed *F. hindsii* seedlings flowered 128 to 140 days after germination, control seedlings did not flower (Table 5-5). BA at 10.0 mg 1^{-1} resulted to a greater net increase in vegetative growth, but inhibited flower initiation. BA at 0.1 mg 1^{-1} retarded the vegetative growth but promoted the emergence of floral buds. This treatment was used in all subsequent experiments. (Fig. 3).

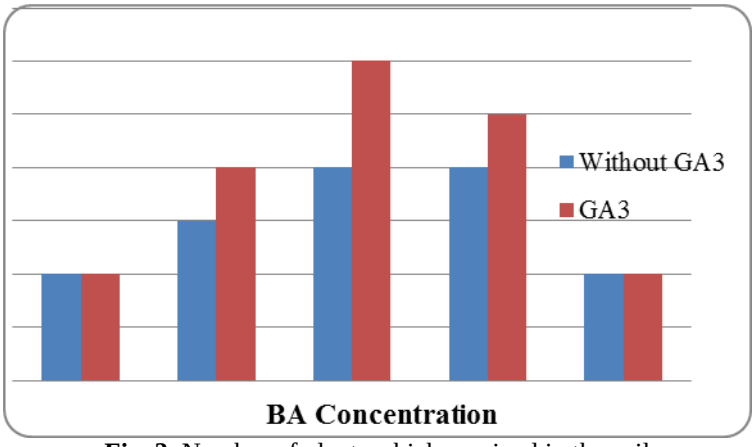

Fig. 3. Number of plants which survived in the soil

Discussion

The sequence from protoplasts to plantlets in *Citrus* relatives reported by Jumin and Nito (1996a) that BA promoted colony formation in six plant species related to *Citrus*. Malt extract added to the protoplast culture medium

also promoted colony formation in *Murraya paniculata* (Jumin and Nito, 1995). The colony formation obtained from this study is similar to the previous reports for bamboo (Lin *et al*., 2003) and *Citrus* relatives (Jumin and Nito, 1996a).

The promotion of somatic embryo formation by low concentrations of zeatin in this study was consistent with previous studies, where cytokinin promoted the initiation and development of embryos *Curcuma attenuate* (Mohanty *et al*., 2008), in *Citrus* and its relatives (Jumin and Nito, 1995). When the sucrose concentration was varied from 2.5 to 10%, the optimal concentration was 5 %. The commonly used carbohydrate for *Citrus* tissue culture is sucrose (Grosser and Gmitter, 1990). In nature, carbohydrate is transported within plant tissues as sucrose and tissue may have an inherent capacity for uptake, transport and utilization of sucrose (Jana and Shehawat, 2010).

The beneficial effect of GA_3 on shoot formation has been reported in *Citrus* relatives (Jumin and Nito, 1996a). The results show that GA_3 increased the percentage of germinating somatic embryos. Whereas cytokinin was found to promote shoot formation from callus cultures in *Citrus* and other woody species (Guo *et al*., 2007; Ralder *et al*., 2008; Skiada *et al*., 2010). Several hundred globular embryos were produced from protoplast cultures but fewer plants were obtained. There were several steps in the regeneration process (Moiseva *et al*., 2006; Moiseva *et al*., 2010).

The first requirement for plant regeneration is the development of a viable embryo (Kim *et al*., 2009; Traveres *et al*., 2010). The development of many globular embryos may be halted as a result of abnormality (multiple shoot meristem, fused embryos and fasciation).

The second requirement for successful plant regeneration from protoplasts depends on the balance and sufficiently of nutrition in the medium during germination of somatic embryos. About 80% of the heart-shaped embryos were successful to became normal plantets with shoots. Meanwhile, about 20 % of heart-shaped somatic embryos grew abnormality and resulted to plantlets without shoots. Development of heart-shaped somatic embryos to plantlets growth depends on balance and sufficient culture media and it was evident that there was no correlation between somatic embryo induction and subsequent shoot growth. When shoots were transferred onto half-strength MT basal medium containing 5 % sucrose without plant growth regulators, there were great differences in rooting ability among shoot (Jumin and Nito , 1996a). Most of the shoots rooted more quickly and readily, while others formed few roots and were accompanied by hyperhydricity. Hyperhidricity has been linked to various metabolic disorders, metabolic alterations, changed

array of protein and altered stress responsive pathways and it can lead to irreversible loss of multiplication and regenerative potensial (Garsia *et al*. 2010; Sreedhar *et al*. 2009). Consequently, plantlets of *F. hindsii* survived in soil. Poor rooting ability of plantlets produced *in vitro* was also reported in *Fortunella polyandra, Atalantia bilocularis, Hesperethusa crenulata, Glycosmis pentaphylla, Triphasia trifolia* and *Murraya koenigii* (Jumin and Nito, 1996a).

The third requirement for successful regeneration is the ability of germinated heart-shaped somatic embryos to survive transfer from the tissue culture environment to soil. Plantlets were very sensitive to dehydration when transferred from *in vitro* to natural conditions. In general, only those heart-shaped somatic embryos that had balanced root and shoot growth survived the transfer to soil. In this study, little plantlets grew successfully in soil even though the protoplasts showed a higher frequency of cell division in protoplast culture. They produced higher number of globular somatic embryos which then developed into heart-shaped somatic embryos.

The success of plant regeneration via somatic embryogenesis from protoplasts of *F. hindsii* is strongly depended on kinetin and zeatin. Cytokinin was necessary to recover organs and plants from protoplasts to plantlets (Das *et al.*, 2010; Kou *et al*., 2013; Lal and Singh, 2010; Xu *et al.*, 2009; Tallon *et al*., 2012). This efficient protoplast-to-plant system for this species could facilitate the transfer of nucellar and cytoplasmic genes into cultivated *Citrus* through protoplast fusion since *Citrus* relatives have been difficult or impossible to hybridize by conventional methods (Grosser and Gmitter, 1990; Guo *et al*., 2007).

Antagonism between vegetative growth and flowering is widely observed in woody plants (Bernier *et al*., 1981; Heller *et al*., 1994; De Baerdemaeker *et al*., 1994). The results of this study indicated that under inductive 16 h photoperiods, BA triggered flower initiation that precedes flowering. BA exerts a major influence on flower initiation of *F. hindsii*. In previous studies, cytokinins seemed to be a requisite for flower initiation *in* vitro in *F. hindsii* (Jumin and Nito, 1996b), *Murraya paniculata* (Jumin and Nito, 1996c), grapevine (Srinivasan and Mullin, 1978), *Passiflora suberosa* (Scorza and Janick, 1980) and bamboo (Nadgauda *et al*., 1990). In the present study, cytokinin enhanced flower formation of *F. hindsii* plants. Bernier *et al*. (1977) found that cytokinin applied to an apical meristem of *Sinapsis alba* triggered the mitotic cycles that commonly preceded flowering, but could not induce subsequent flowering. The authors suggested that another factors present in the plants could initiate flowering in conjunction with BA and it could induce subsequent flowering and fruiting.

A sequence from protoplast to a plant via somatic embryogenesis and flowering was established for *F. hindsii* at a high frequency. This efficient protoplast-to-plant system for this species could facilitate the transfer of nucellar and cytoplasmic genes from this species into cultivated *Citrus* though protoplast fusion.

Conclusion

The highest plating efficiency was obtained in MT basal medium containing 5% sucrose supplemented with 0.01 mg 1^{-1} kinetin 600 mg 1^{-1} ME, MT basal medium containing 5% sucrose and supplemented with 0.01 mg 1^{-1} zeatin was found to be a medium suitable for the development somatic embryos into heart-shaped somatic embryos. The highest percentage shoot formation was obtained using 0.1 mg 1^{-1} benzyladenine (BA) + 0.1 mg 1^{-1} gibberellic acid (GA_3). In this investigation 40 plants survived and grew normally in the soil. After two months of being maintained in the soil, the plants formed flowers and the flowers developed into fruits in the soil treated with BA.

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