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Somatic embryogenesis derived from protoplast of *Murraya paniculata* L. Jack and their regeneration in to plant flowering *In vitro*

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ABSTRACT

The *in vitro* flowering of orange jessamine plantlets derived from protoplast was affected by the manipulation of plant growth regulators, sugar and light conditions. MT basal medium containing 5% sucrose and supplemented with 0.001 mg L^{-1} indole-acetic-acid was found to be a suitable medium for development of globular somatic embryos derived from protoplasts to form heart-shaped somatic embryos with cotyledon-like structures. The highest percentage (85 %) of flowering was achieved with plantlet on half-strength MT basal medium containing 5% sucrose and 0.001 mg L^{-1} indole-acetic-acid in light. Exposure to darkness for more than 3 weeks followed by re-exposure to light reduced flowering. Flowering required a 10-day exposure to indole-acetic-acid. Photoperiod with 18 h and 79.4 µmolm^{-2} s^{-1} light intensity promoted *in vitro* flowering in high frequencies. The sucrose treatment affected the flower bud size distribution. Flower buds originating from plantlet derived from protoplasts developed into normal flowers.

Key words : *Indole-acetc-acid, Light-intensity, Murraya-paniculata, Photoperiod, Plantlet, Zeatin*

Introduction

An attempt to use citrus relatives as a source of germplasm appears to be a promising method for rootstock improvement Deng *et al*., (1992, Chang and Chang, 2003; Chen, 2012; Nadgauda *et al*. 1990; Nagata and Takebe, 1970; Swingle and Reece, 1967). Successful somatic embryogenesis and subsequent maintenance *in vitro* and plant conversion in fruit plant could be used as a genetic resources for somatic hybrids purpose (Chang and Chang, 2003; Hardy *et al*., 2010; Raveh, 2012; Moiseeva *et al*., 2010; Sykes, 1988; Travares, 2010). Among the orange subfamily, *Murraya paniculata* may be a genetic source of early flowering (Jumin and Nito, 1996b; Srinivasan and Mullins, 1978), may be a source of lime tolerance and resistance to citrus nematode Bitters, *et al*., Srinivasan and Mulin, 1978). The wood of genus *Murraya* is valued and the tree has potential as an ornamental due to its large white flowers and attractive red fruits (Swingle and Reece, 1967).

Somatic hybridization via protoplast fusion has been used successfully as a method to bypass sexual incompatibilities insome cases Grosser and Gmitter (1990a). Intra-and inter-generic somatic hybrids have been obtained between Citrus and some of its relatives (De Baerdemaeker *et al*., 1994; Grosserand Gmitter, 1990; Grosser and Gmitter, 1990. Embryogenic protoplasts of Citrus are used as one partner in the protoplast fusion to obtain inter-intrageneric somatic hybrids with leaf-derived, *Feronia* protoplasts of citrus relatives; *Murrayap aniculata, Fortunella*

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crassifolia, *Feronia limonia*, *Clausen alansium, Citropsis gilletiana* or *Atalantia ceylanica* of a second parent (Ling and Iwamasa, 1994; Grosser and Gmitter, 1990a; Grosserand Gmitter. 1990b).

Plant regeneration from cultured protoplasts in Citrus has been reported for a number of species (Jana and Shekhawat, 2011). However, there have 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 (Deng *et al*., 1992). 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 a few obtained from monoembryonic types of Citrus (Jana and Shekhawat, 2011). This experiment successful to establish embryo-genic callus from *M. paniculata* and subsequence regeneration from protoplasts into plant flowering *in vitro*

Flowering is a complex process which is regulated by several intricate external and internal factors that make its induction under *in vitro* culture highly sensitive (Sim *et al*., 2007; Skiada *et al*., 2010; Scorza and Janick, 1980; Sreedhar *et al*., 2009). Recently, *in vitro* flowering has been extensively investigated for many plant species and significant advances in the understanding of this phenomenon have been made. *In vitro* flowering have successfully induced in several plants and methods (Raveh, 2012; Jumin, 2013b). *Dendrobium* Madame Thong-in (*Orchidaceae*) have successfully developed a method to induce early *in vitro* flowering of the self-pollinated seedlings of a tropical orchid hybrid (Raveh, 2012). Jumin and Ahmad (1999) was successfully obtained in inducing flowering *in vitro* at high frequency (95%) from seedlings, stem internodes of seedling, and root segments derived from seedlings of *M. paniculata.* The effect of auxin on flowering of plantlets of *Capsicum annuum* L. ev. Sweet Banana regenerated via somatic embryogenesis from immature zygotic embryos was dependent with concentration of auxin. Plantlets were capable of producing flower, fruit, and seed when cultured in small tissue culture containers. *In vitro* floral buds were first formed on plantlets that grew on plantlet development medium Murashige and Skoog (MS) basal medium containing 1 mgL^{-1} (5.3 µM) NAA in a growth chamber at 22°C and continuous illumination (Bodhipafatma and Leung,2003).

In view of the limited success of plant regeneration from protoplast cultures of Citrus relatives, this study successful to establish methods in protoplast cultures and their regeneration into plants flowering *in vitro* of *M. paniculata*. The objective of this research is to describe the regeneration sequence via somatic embryogenesis from protoplasts into plant flowering *in vitro* of *M. paniculata.*

Material and Methods

A. Protoplast Sources

Embryogenic callus of *Murraya paniculata* was induced from the hypocotyl region of seedlings on MT basal medium (Jumin, 1995; Motoike *et al*., 2005) containing 5% sucrose, 5.0 mg L^{-1}benzyladenine (BA), 2.5 mg L^{-1} 2,4¬dichlorophenoxyacetic acid (2,4-D) and 600 mg L^{-1} malt extract (ME) and maintained under 52.9 µmol m^{-2} s^{-1} light with a photoperiod of 16 h at 25°C, as described by (Jumin and Nito, 1996). Seedlings used for hypocotyl excision were germinated from immature nucellar embryo explants using the same medium as mentioned above.

B. Protoplast Isolation

Prior to protoplast isolation, about 1 g of *M. paniculata* 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 µmol 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 mm) 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.

C. Protoplast Culture

Protoplasts of *M. paniculata* were re-suspended in MT basal medium containing 5% sucrose, supple-

mented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg L^{-1} Zeatin, and 0.0, 0.3, 0.6, 09M 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 at 25°C under 52.9 µmol m^{-2} s^{-1} light with a photoperiod of 16 h. 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).

D. 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 onto MT basal medium containing 5% lactose without PGR and solidified with 0.25% Gelrite.

E. Globular Embryo Development

Somatic embryo development of *M.paniculata* 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 L^{-1}Zeatin, 5.0%sucrose and solidified with 0.25 % Gelrite in 90 x 20 mm petri dishes. The concentrations of Zeatin and sucrose were chosen based upon 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.

For shoot formation (plantlets), heart-shaped somatic embryos were cultured individually on half strength MT basal medium containing 5% sucrose supplemented with 0.0, 0.001, 0.01, 0.1, or 1.0 mg L^{-1}IAA and solidified with 0.3% Gelrite. The cultures were kept under 52.9 µmol 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.

The effect plant growth regulator on flowering was investigated by culturing seeds in MT basal medium containing 1, 3, 5, and 7% 5% sucrose, solidified with 0.25 Gelrite plus 0.001 mg L^{-1} IAA, and photoperiod was adjusted with 12, 14, 16,18 hours daily, pH medium was 5.7 (as measured before autoclaving).

Specimens were prepared for scanning electron microscopy as described by Jumin and Ahmad (1999). Floral bud samples were affixed on aluminum stubs with silver paint and coatedwith 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.

Results

About 10^6 protoplasts with a diameter of 10-30 mm tin were obtained from 1 g of callus (Fig. 1). FDA staining showed that the viability of fresh protoplasts was 80 %. About 90% 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 50 days of protoplast culture was from 18-54% (Table 1).

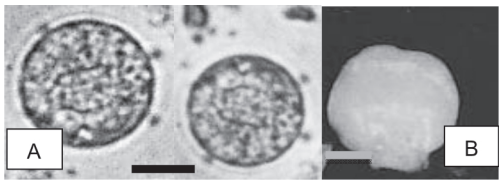

Fig. 1(A). Fresh protoplasts of *M. paniculata* (bar 20 mm).
Fig. 1(B). Embryos derived from protoplasts 2 months protoplast cultured to half-strength MT basal medium without plant growth regulators.

The formation of colonies (about 70 µm in diameter) occurred after 60 days of protoplast culture. The media manipulation could be 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 Zeatin and sorbitol to the medium. A low concentration of Zeatin stimulated colony formation. When protoplasts were cultured on MT basal medium contain-

Table 1. Effect of Zeatin and Sorbitol on Protoplast Plating Efficiency (%) of *M. paniculata* 40 Days after Protoplast Culture

Zatin (l^{-1})	Sorbitol (M)			
	0.0	0.3	0.6	09
0.0	19.3a	18.6a	20.0a	20.9.1a
0.001	24.6b	29.3b	31.0ab	26.3b
0.01	29.3c	31.3b	54.0bc	31.3c
0.1	34.3d	21.0c	39.3cd	41.3d
1.0	18.6a	19.3c	29.6cd	19.6a

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.

Table 2. Effect of Zeatin on globular somatic embryo of *M. paniculata*, 40 Days after Protoplast Culture

Zeatin (L^{-1})	Number of Embryos obtained	
	No shoot	With shoot
0.0	605a	66a
0.001	654ab	143b
0.01	517c	65a
0.1	604a	47a
1.0	363d	28a

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.

ing 5% sucrose without Zeatin and sorbitol the protoplast plating efficiency was low. However, when protoplasts were cultured in the medium supplemented with 0.001 mg L^{-1} Zeatin and 0.6 M sorbitol, higher plating efficiency was obtained.

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 0.001 mg L^{-1}Zeatin 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.

After 55 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 40 days from protoplast-derived cultures. 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 plantlet.The highest percentage of shoot formation was obtained using 0.01 mg 1^{-1}Zeatin (Table 3).

Globular somatic embryos derived from protoplasts were cultured on MT basal medium supplemented with 5.0% sucrose and 0.0-1.0 mg L^{-1} Zeatin for 40 days. The number of globular somatic embryos that developed into heart-shaped somatic embryos in the medium supplemented with 5 % sucrose and 0.001 mg L^{-1} Zeatin was 654 (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 µm in diameter after 55 days.

A low level of Zeatin in the culture medium promoted the growth of heart-shaped somatic embryos of *M. paniculata* into plantlets. The highest percentage of plantlet was obtained using 0.001 mg L^{-1}Zeatin. After 30 days of culture on Zeatin-containing medium, the formation of adventitious shoot buds was observed and many of these developed into plantlets, and then promoted shoot formation and the subsequent ability to develop plantlets (Fig. 3).

Requirement for successful plant recovery was the balanced germination of the embryos. About 70% of embryos on Zeatin-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 Zeatin there were differences in rooting ability among the plantlets. Shoots from medium with Zeatin rooted more quickly and readily, while shoots from Zeatin-free medium formed few roots and were accompanied by hyperhydricity. Consequently, plantlets from medium with plant growth regulator survived in soil

After 2 months on medium, the plantlets formed flower buds on the apical portion and flower opened under light conditions (Fig. 2). One flowers opened from a plantlet. The flower buds generally appeared after at least one pair of small leaves were formed. Not all of the flower buds matured into characteristic bright white flowers. In some cases the flowers possessed a few petals and were pale white. Some flowers were lacked stamens and pistils, but a few were large.

Fig. 2. *In vitro* of a plantlet on half strength medium containing 5 % sucrose supplemented with 0.001 mg l^{-1} IAA (bar = 1cm),

Most flowering occurred on half-strength MT basal medium with 0.001 mg L^{-1} IAA in the presence of light. *In vitro* flower could be occurred in the dark condition without IAA and 0.001 mgL^{-1} IAA and No flower formation occurred on medium contained 0.01 mg L^{-1} until 1.0 mg.L^{-1} IAA in the dark condition. IAA strongly formed plantlets, however not affected the flower bud initiation. Maximum percentage of flowering (85%) was obtained on half strength MT medium containing IAA (0.001 mg.L^{-1}) after 100 days of culture in the presence of light. The highest number of flower was obtained in the plantlets with a decline of vegetative growth as was observed in a treatment of 0.0 to 1.0 mg L^{-1} IAA. When IAA supplemented to medium, *M. paniculata* plantlets flowered 45 to 60 days after culture, however in IAA-free medium, the plantlets did not flower. Exposure to light 0 to 5 weeks event with NAA did not flower. IAA at 1.0 mg L^{-1} resulted in a greater net increase in vegetative growth, but inhibited flower initiation. IAA at 0.001 mg L^{-1} and exposure to light until less than 10 weeks retarded the vegetative growth, but promoted the emergence of floral buds (Fig. 5).

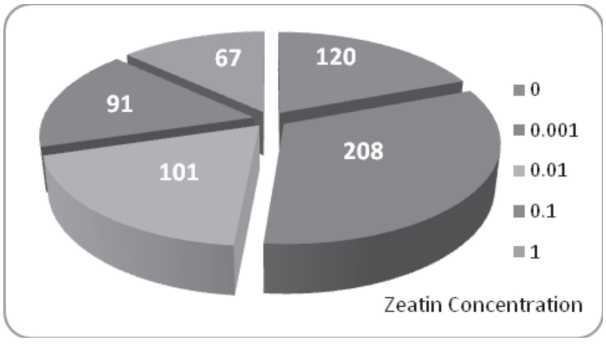

Fig. 3. Number of plantlets obtained from culture of protoplast of orange jessamine on MT basal medium containing several concentration of Zeatin

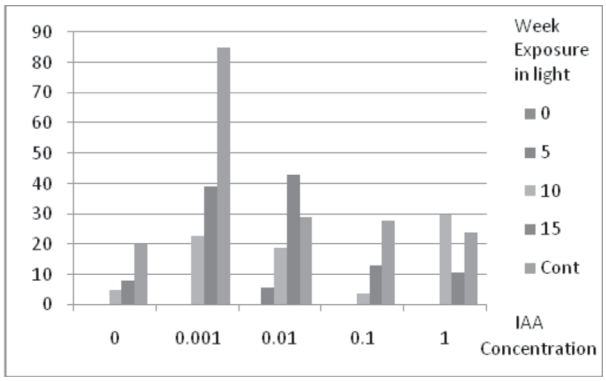

Fig. 4. Effect of IAA on Flower Formation from plantlets Derived from Protoplasts Cultured on half-strength MT basal medium 60 days supplemented to IAA

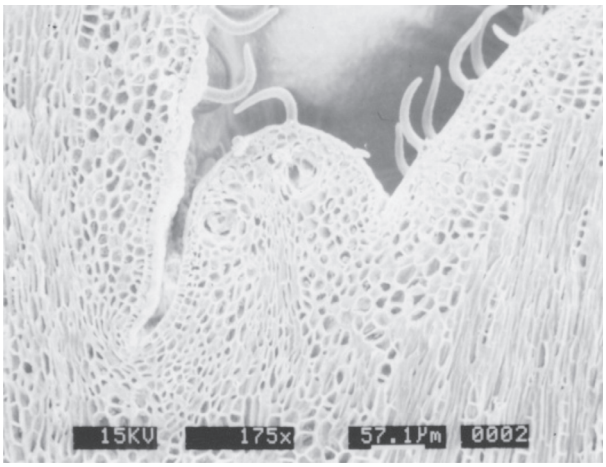

Fig. 5. Scanning electron micrographs of floral bud formation of *M. paniculata.*

Percentage of the plantlets which formed floral buds was dependent on the kinds and concentration of sugars and IAA that were added to the medium (Table 4). Sugars are necessary carbon source for reliable induction and development of flowers. Addition of sugar to the medium is necessary for induction of floral stimulus. In the present investiga-

Table 4. The effect of sucrose concentrationon orange jessamine flower bud size distribution from the plantlets cultured on half-strength mt basal medium plus 0.001 mg.L^{-1} IAA.

Sucrose Concentrations (%)	Bud length (mm)		
	➢5	3 - 5	1 - 3
1	0±0.6	0	0
3	3±0.6	3±0.6	3±0.6
5	9±0.6	13±0.6	5±0.6
7	3±0.6	7±0.6	1±0.6

*) ±values indicated standard error of the mean.

tion, flower bud differentiation in high frequency was observed by keeping the light condition, sucrose concentration at 5 % and combined to 0.001 mg L^{-1} IAA.

Discussion

The sequence from protoplasts to plantlets in Citrus relatives was reported by Jumin and Nito (1995); Jumin and Ahmad (1999); Jumin and Nito (1996b); 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 *M. paniculata* (Jumin, 1995). The colony formation obtained from this study is similar to the previous reports for bamboo (Louzada *et al*., 1993), *Spathiphyllum* and *Anthurium* and (Duquenne *et al*., 2007).

Length of photoperiod, recorded after 10 weeks of heart-shaped culture, showed no significant effect on shoot multiplication but did influence flowering *in vitro*. The percentage of flowering was 79.5% indicating that the flowering stimulus did occur (Table 5).

Table 5. Effect of photoperiod and light intensity to in vitro flowering of orane jessamine cultured to half strength mt basal medium containing 5% sucrose Plus 0.001 Mg L^{-1} IAA 90 Days of culture

Photoperiods (h)	Light Intensity (mmol.m^{-2}.s^{-1})			
	26.5	52.9	79.4	105.8
12	6.0a	33.5a	17.0a	24.5a
14	17.5b	44.0b	28.5b	41.5b
16	24.5bc	59.5c	79.0c	59.0c
18	31.5c	79.0d	79.5c	59.5c

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

In the present study, it was observed that the exposure of plantlets to light (18/4 h photoperiod) was most effective for flowering which showed highest cultures producing *in vitro* flower buds (Table 5).

The promotion of somatic embryo formation by low concentrations of BA in this study was consistent with previous studies, where cytokinin promoted the initiation and development of embryos *Curcuma attenuate* (Louzada *et al*., 1993), 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 (Grosserand Gmitter, 1990b). In nature, carbohydrate is transported within plant tissues as sucrose and tissue may have an inherent capacity for uptake, transport and utilization of Sucrose.

The beneficial effect of cytikinin on shoot formation has been reported in Citrus relatives

(Jumin, 1995). Its results show that cytokinin 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; Qiao *et al*., 2009; Singh *et al*., 2006; Travares *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 (Mohanty *et al*., 2008; Moiseeva *et al*., 2006).

The first requirement for plant regeneration was the development of a viable embryo Das *et al*., 2010; Kim *et al*., 2009; Ling and Iwamasa, 1994; Travares *et al*., 2010; Tremblay *et al*., 2005). The development of many globular embryos was halted as a result of abnormality (multiple shoot meristem, fused embryos and fasciation).

The second requirement for successful plant regeneration from protoplasts depend on the balance and sufficiently of nutrition in the medium during germination of somatic embryos. About 80% of heart-shaped embryos was successful to promote became normal plantlets with shoots. Meanwhile, about 20 % of heart-shaped somatic embryos was grown abnormality and resulted plantlets without shoots. Development of heart-shaped somatic embryos to plantlets growth depend on balance and sufficiently of 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 (Louzada *et al*., 1993; Jumin, 2013a). 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 potential (Garsia *et al*., 2010; Sin *et al*., 2007). Consequently, plantlets of *Murraya paniculata* survived in soil. Poor rooting ability of plantlets produced in vitro was also reported in *Fortunell apolyandra, Atalantia bilocularis, Hesperethusa crenulata, Glycosmis pentaphylla, Triphasi atrifolia* and *Murraya koenigii* (Jumin and Nito, 1996a).

The third requirement for successful regeneration was the ability of germinated heart-shaped somatic embryos to survive transfer from the tissue culture environment to soil (Jumin, 2013b; Kou *et al*., 2013; Tremblay *et al.,* 2005). 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, a little plantlets successful to soil even though this protoplasts showed a higher frequency of cell division in protoplast culture, and produced a high number of globular somatic embryos which then developed into heart-shaped somatic embryos.

The success of plant regeneration via somatic embryogenesis from protoplasts of *M. paniculata* is strongly depend on BA. Cytokinin was necessary to recover organs and plants from protoplasts to plantlets (Das *et al*., 2009; Lang, 1987; Sykes, 1988; Taylor *et al*., 2005; Xu *et al*., 2009). This efficient protoplast-to-plant system for this species could facilitate the transfer of nucellar and cytoplasmic genes of a source of time tolerance from this species into cultivated Citrus through protoplast fusion. While Citrus relatives have been difficult or impossible to hybridize with Citrus by conventional methods (Grosser and Gmitter, 1990b; Guo *et al*., 2007)

This kind of antagonism between vegetative growth and flowering is widely observed in woody plants (Bodhipafatma *et al*., 2003; De Baerdemaeker *et al*., 1994; Guo *et al*., (2007). Our results indicated that under inductive 18 h photoperiods, BA triggered flower initiation that precedes flowering. BA exerts a major influence on flower initiation of *M. paniculata*. In previous studies, auxin to be requisite for flower initiation *in vitro* in *Dendrocal amuslatiflorus* (Raveh, 2012), combination of NAA and BA in *Anethum graveolens*) (Jana and Shekhawat, 2011). In the present study, auxin enhanced flower formation of *M. paniculata* plants. Chang and Chang (2003) found that, callus-derived rhizomes of *Cymbidium ensifolium* var. *misericors* produced flowers precociously on a defined half-strength basal Murashige Skoog medium containing of NAA with thidiazuron, N^6-(2-isopentenyl) adenine or N^6-benzyladenine BA within 100 days of culturing. Combination of cytokinin and NAA were the most effective combinations for achieving flower induction *in vitro* and these undersized flowers were physically normal and bloomed for two weeks *in vitro*.

The highest number of floral buds was obtained in the plantlets with a decline in vegetative growth (Fig. 5). These were also widely observed in woody plants (Bernier *et al.,* 1981; De Baerdemaeker *et al*., 1994; Franklin *et al*., 2000; Heller *et al*., 1994; Lin *et al*., 2003; Lin *et al.,* 2007). However, a decline in vegetative growth was also observed in the plantlets exposed to 8 hphotoperiod, in which no flower formation occurred even supplemented to 0.01 mg L^{-1} IAA. This result has indicated that under inductive 18 h photoperiod, Zeatin triggered flower initiation in advance of flowering could be considerably modified by IAA and their interaction 18 h photoperiod.

Lang (1987), mentioned that, after passing a juvenile phase, the plants may require a period of photoinductive condition, to produce a sufficient amount of a hormone-like (florigen), or flower-inducing substances, which causes flower formation quite soon. There is no indication, however, that a signal of second factor was involved in the attainment of the floral state. Flower-ing could be delayed by an early transfer of plants from inductive photo period to non inductive photoperiod (Bernier *et al*., 1981; Heller *et al*., 1994; Lal and Singh, 2010).

The effects Auxin and cytokinin to flowering were several report from many researchers. Some reports we shown that a single kind of PGRs for any plants promoted the flowering, however, for any case combination of more kind of PGRs is important to induce flowering. Factors implicated in the promotion of floral transition of the *C. attenuata* have been identified which are 4-amino-3, 5, 6-trichloropicolinic acid (picloram), 6-

benzylaminopurine, sucrose and photoperiod. The highest frequency of flowering (100%) was obtained when axillary shoot explants were transferred to MS medium supplemented with picloram (4.14 µM) within 4 weeks of culture. Transfer of *in vitro* regenerated shoots to half strength MS medium with 2.46 µM indole-3-butyric acid (IBA) showed maximum root induction (Heller *et al.*, 1994). Rapid shoot multiplication from nodal explants was established using varying concentrations of cytokinins and auxins either alone or in combinations. The highest frequency of shoot induction was achieved when nodal explants were inoculated on Murashige and Skoog (MS) medium supplemented with 13.31 µM 6-benzylaminopurine with a mean of 12.9±0.5 shoots per explant (Heller *et al*., 1994).

Sugars are essential carbon sources in culture media for reliable induction and development of flowers (Jumin and Nito, 1995; Jumin and Nito, 1996a). Sucrose is most commonly used, because it could promote flowering. An optimal sugar concentration for flowering differs in species (Qiao *et al*., 2009). Five % sucrose was found to be a suitable concen-tration for flower initiation in *M. paniculata* (Jumin and Ahmad, 1999). Sucrose maintains suitable osmotic potential of nutrient media. According to Franklin (Franklin and Ignacimuthu, 2000), added sugars were not effective in controlling osmotic condition, but they activated the penthose phosphate pathway Franklin and Ignacimuthu, (2000). Root, internode, and leaf explants of *Saposhnikoviadivaricata* formed in vitro flowering and fruiting when plantlets were sub-cultured for over 15 months on MS with 4–5% sucrose, 2.26 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and subsequently developed into somatic embryos on MS medium containing 4–5% sucrose, 1.74 µM NAA, 4.44 µM BA, and 1.90 µM abscisic acid (Nguyen *et al.*, 2006).

Nguyen *et al*. (2006), mentioned that indicate that sucrose is the key factor in floral morphogenesis while cytokinin increases the flowering percentage and helps the normal development of floral buds. From the three cytokinins that were used BA and zeatin were considered to be more suitable as inductive flowering agents than thidiazuron. The morphology of shoots bearing floral buds varied with different cytokinin treatments. The highest percentage (45%) of flowering was obtained on MS medium supplemented with 3.0 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 30 g L^{-1} sucrose. From our results, the effect of sugars to promote flowering was shown only in the light. It indicates that the added sucrose interacts with light in expressing the effect of sugars.

A sequence from protoplast to a plant via somatic embryogenesis and flowering was established for *M. paniculata* 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.

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