Eco. Env. & Cons. 22 (4) : 2016; pp. (1727-1734)
Copyright@ EM International
ISSN 0971–765X

Protoplast cultures of *Murraya paniculata* L. Jack and Their regeneration into plant precocious flowering

Hasan Basri Jumin[1]

Department of Agro-technology, Faculty of Agriculture, Islamic University of Riau, Jl. Kaharuddin Nasution No. 113. Perhentian Marpoyan Pekanbaru, 28284, Indonesia.

(Received 12 May, 2016; accepted 30 June, 2016)

ABSTRACT

Protoplasts isolated from embryogenic callus of *Murraya paniculata* (L. Jack.) 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 a cell wall within 6 d of culture and the first cell division was observed 7 days after isolation. The highest plating efficiency was obtained on MT basal medium containing 5% sucrose supplemented with 0.01 mg L^{-1} kinetin 600 mg L^{-1} ME, MT basal medium containing 5% sucrose and supplemented with 0.01 mg L^{-1} Indole-acetic-acid (IAA) was found to be a medium suitable for the development somatic embryos into heart-shaped somatic embryos. The highest percentage of shoot formation was obtained using 0.1 mg L^{-1} Indole-acitic-acid (IAA) 0..1 mg L^{-1} gibberellic acid (GA_3). In this investigation 40 plants were survived and grew normally in the soil. After two months maitained in the soil plants formed flower and flower developed into fruits on the soil treated with BA.

Key words : *Gibberellic-acid, Indole-acetic-acid, Protoplast, Precocious-flowering, Somatic-embryo*

Introduction

Citrus relatives are potensial 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 hybrids purpose (Ceng, 2012; Raveh, 2012; Motoike *et al*., 2005; Tallon *et al*., 2012). Among the orange subfamily, *Murraya paniculata* may be a genetic source of early flowering (Jumin and Nito, 1996c: Swingle and Reece, 1967; Sykes, 1988). Somatic hybridization via protoplast fusion has been used successfully as a method to bypass sexual incompatibilities in some 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 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 (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 a few obtained from monoembryonic

Corresponding author's email : hbjumin@gmail.com or hb_jumin@yahoo.com

types of *Citrus* (Jumin, 1995). We successful to establish embryogenic callus from *M. pniculatai* as a member of *Citrus* relatives. In view of the limited success of plant regeneration from protoplast cultures of *Citrus* relatives, this study succsesful to establish methods in protoplast cultures and their regeneration into plants flowering of *Murraya paniculata.* 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 *Murraya paniculata.*

Material and Methods

Protoplast Sources

Embryogenic callus of *M. paniculata* 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 micro mol.m/l light with a photoperiod of 16 h at 25°C, as described by (Jumin and Nito, 1996a). 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 *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 micro mol.m/l 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% cellulaseOnozuka (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 isolat-ed by filtering through a double layer of Miracloth (Calbiochem; U.S.A) and centri-fuged 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 *M. paniculata* 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 m l^{-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 micro mol.m/l 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).

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.

Chromosome Analysis

Chromosome count was carried out on a small piece callus of the 25 days old. 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) lactopropionicorcein and counted under an inverted microscope.

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 1^{-1} Indole acetic acid 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 upon preliminary dose response trials on stock callus. The number of globular somatic embryos that developed into heart-shaped somatic embryos with cotyledon-like struc-tures was determined after 30 days.

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} IAA and 0.0, 0.01, 0.1, 1.0 or 10.0 mg.1^{-1} Gibberellic Acid (GA3) and solidified with 0.3% Gelrite. The cultures were kept under 52.9 micro mol.m/l 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. 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 acclimated to greenhouse conditions.

Plants Flowering

The plants originated from plantlets after acclimatization at 2-leaf stage were maintained in trays containing 75 % top soil and 25 % sand (v/v) and kept under a green-house 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 transplanted to pots. The plantlets were foliage sprayed with either 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 upon a preliminary dose response trial on stock plantlets.

Results

About 10^6 protoplasts with a diameter of 15-30 micro 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 8 days after isolation. The protoplast plating efficiency obtained after 40 days of protoplast culture was from 16-55% (Table 1).

The formation of colonies (about 55 mm in diameter) occurred after 50 days of protoplast culture. Scanning electron microscope studies of globular somatic embryos revealed large vacuolated parenchymatous cells, highly cytoplasmic, and meristematic cells (Fig. 1B).

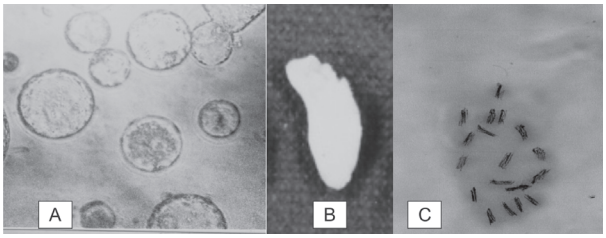

Fig. 1(A). Fresh Protoplast of *M. Paniculata* bar 20 mm.
Fig. 1(B). Heart shaped embryos derived from protoplasts of . *M. paniculatai* in MT basal medium containing 5 % sucrose supplemented with 0.001 mg l-1 BA 90 days after protoplast isolation (bar 1 cm).
Fig. 1(C). Chromosome on the somatic embryo of *M. paniculata* 90 days after protoplast isolation (bar 20 micro m)

The media manipulation could be improved colony formation of the culture. The MT basal medium containing 5% glucose supported cell divisions in protoplast cultures. However, the number of mitotic divisions was increased by the addition of kinetinand 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 1).

Effect of IAA and sucrose on development of protoplast-derived globular somatic embryos of *M. paniculata* 40 days after culture (30 globular somatic embryos tested for each treatment) shown Table 2.

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

Kinetin (mg l^{-1})	Malt Extract (ME) (g l^{-1})		
	0.0	300	600
0.0	17.3a	16.6a	18.0a
0.001	22.6b	27.3b	29.0ab
0.01	27.6c	30.6b	55.0bc
0.1	32.6d	19.0c	37.3cd
1.0	16.3a	17.3c	27.6cd

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 IAA and sucrose on development of protoplast-derived globular somatic embryos (%) *M. paniculata* 40 days after culture (30 globular somatic embryos tested for each treatment)

IAA (mg l^{-1})	Sucrose (g l^{-1})			
	2.5	5/0	7.5	10.0
0.0	63.3a	53.3a	42.0a	36.3a
0.001	68.6a	63.3a	53.0ab	42.3ab
0.01	75.6a	66.6a	73.3c	73.0c
0.1	65.3a	53.0a	63.3cb	41.3ab
1.0	43.3b	33.3b	53.6ab	31.0a

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 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 30 days from protoplast -derived cultures. The number of globular somatic embryos obtained from 5 protoplast isolations was 654 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. Chromosome count originated from callus was ferformed as diploid callus with indicated by 18 number of chromosome (Fig. 1 C), and its no defferences between embryogenic callus with original 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} IAA was 80% (Table 2). About 73% 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 IAA in the culture medium promoted the growth of heart-shaped somatic embryos of *F. hindsii* into plantlets. The highest percentage of shoot formation was obtained using 0.1 mg 1^{-1} IAA. After 30 days of culture on IAA-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.

Requirement for successful plant recovery was the 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 IAA rooted more quickly and readily, while shoots from PGR-free medium formed few roots and were accompanied by hyperhydricity. Consequently, plantlets from medium with IAAsurvived in soil.

The requirement for successful regeneration was the ability of germinated heart--shaped embryos to survive transfer from the tissue culture environment to soil. Plan-tlets 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 its growth survived the transfer to soil. In this study only 4 plants in soil were recovered from PGR-free medium, while 30 plants were survived in the soil from medium with BA (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} kinetin and 600 mg l^{-1} ME.

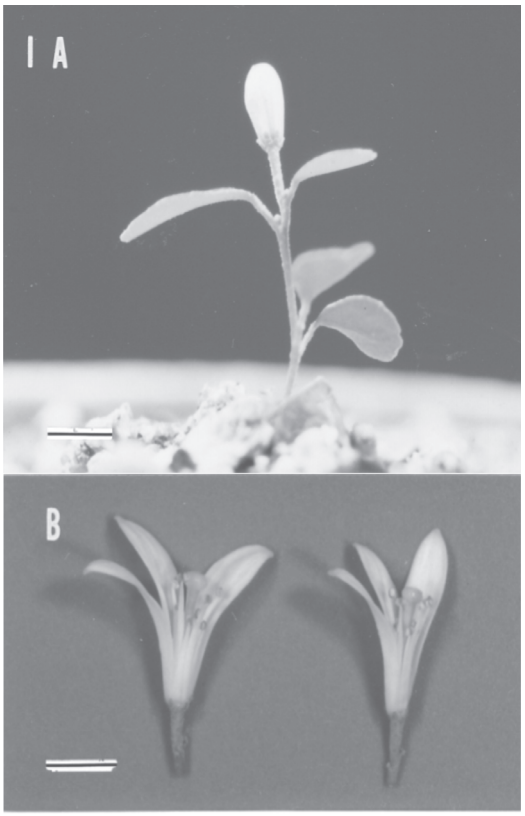

Fig. 2(a). A single terminal precocious flower of *M. paniculata* grown 4 months after acclimatization in the soil under 14 – 16 photoperiod at 25°C with 3 sprays of 0.01 mg 1^{-1} BA solution (bar =1 cm). (B) (right) Flower of *M. paniculata* originated from a a normal plant and (left) Flower of *M. Paniculata* originated from a precocious flowering plant 5 weeks after acclimatisation .

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

The highest number of flower was obtained in the plants with a decline of vegetative growth as was observed in a treatment of 0.1 mg L^{-1} GA_3. Indole-acetic-acid -sprayed *M. Paniculata* seedlings flowered 117 to 135 days after germination, before control seedlings did not flower. Indole-acetic-acid 10.0 mg L^{-1} resulted in a greater net increase in vegetative growth, but inhibited flower initiation. Indole-acetic-acid at 0.1 mg L^{-1} retarded the vegetative growth, but promoted the emergence of floral buds. This treatment was used in all subsequent experiment. (Fig. 3).

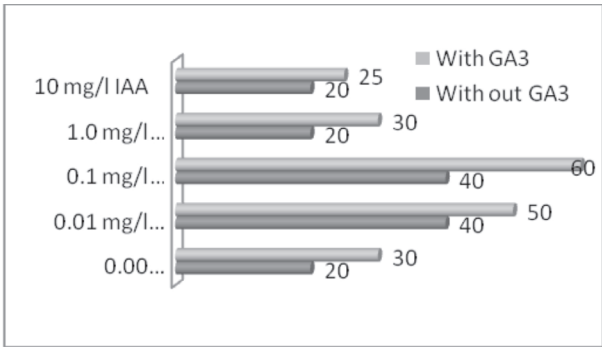

Fig. 3. Number of plants were survived in the soil

Discussion

The sequence from protoplasts to plantlets in *Citrus* relatives was reported by Jumin and Nito, (1996a), that plant growth regulators (PGRs) promoted

Table 3. Effect of IAA and GA_3 on shoot formation of *M. paniculata* 55 days after heart-shaped embryos culture (20 heart-shaped embryos tested for each treatment)

IAA (mg/L)	GA_3 (mg L^{-1})				
	0.0	0.01	0.1	1.0	10.0
0.0	41.3a	48.6a	47.6a	38.0a	31.3a
0.01	48.3ab	50.6a	51.3a	48.6b	49.0ab
0.1	58.6b	69.3b	87.6b	78.3c	72.6b
1.0	53.6b	68.6b	81.3b	74.6c	62.0b
10.0	43.3b	61.6ab	78.3b	65.0bc	63.6b

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

colony formation in six plant species related to *Citrus*. Malt extract added to the protoplast culture medium also promoted colony formation in *Clausena harmandiana* (Jumin, 2013a). 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; Jumin, 2013a).

The promotion of somatic embryo formation by low concen-trations of IAA in this study was consistent with previous studies, where PGRs promoted the initiation and development of embryos *Curcuma attenuate* (Mohanty *et al*., 2008), in *Citrus* and its relatives (Jumin and Nito, 1995; Jumin, 2013a). 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 *Clausena harmandiana* and other *Citrus* relatives (Jumin and Nito, 1996a; Jumin, 2013a). Its results show that GA_3 increased the percentage of germinating somatic embryos. Whereas PGRs 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 was the development of a viable embryo (Kim *et al.,* 2009; Traveres *et al*., 2010). 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 plantets 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 (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 *M. paniculata* survived in soil. Poor root-ing 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 was the ability of germinated heart-shaped somatic embryos to survive transfer from the tissue culture environment to soil. Plantlets were very sensitive to dehy-dration 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 em-bryos 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*, 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 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, 1990; Guo *et al*., 2007).

This kind of 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). Our results indicated that

under inductive 16 h photoperiods, BA trig-gered precociouys flower initiation that precedes flowering. BA exerts a major in-fluence on flower initiation of *Fortunella hindsii* (Jumin, 2013b). In previous studies, cytokinins seemed to be requisite for flower initiation *in* vitro in *Fortunella hindsii* (Jumin 2013b) grapevine (Srinivasan and Mullin 1978), *Passiflora suberosa* (Scorza and Janick, 1980) and bamboo (Nadgauda*et al*., 1990). In the present study, cytokinin enhanced precocious flower formation of *M. Paniculata* 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 that they could not induce subsequent flowering. The authors suggested that another factor 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 *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.

References

Bernier, G., Kinet, J. M., Jacquard, A., Havelange, and Bodson, M. 1977. Cytokininas possible component of the floral stimuli in *Sinapsis alba. Plant Physiology.* 60 : 282-285.

Bernier, G., Kinet, J.M. and Sachs, R.M. 1981. The Physiology of Flowering. CRC Press, Boca Raton, Florida.

Chen, L.S. 2012. Physiological Responses and Tolerance of *Citrus* to Aluminum Toxicity. pp 435-452. In Srivastava, A.K. (Ed.): Advances in *Citrus* Nutrition. Springer Dordrecht Heidelberg New York London.

Das, A., Kesari, V. and Ragan, L. 2010. Plant regeneration in curcuma species and assessment of genetic stability of regenerated plants. *Biologia Plantarum.* 54 : 423-429.

De Baerdemaeker, C. L, Van Huylenbroeck, J. M. and Debergh, P.C. 1994. Influence of paclobutrazol and hotoperiod on growth and flower-ing of *Gardenia jasminoides* Ellis cultivar 'Veitchii'. *Scientia Horticulture.* 315-324.

Deng, X.X., Grosser, J.W. and Gmitter, F.G.Jr. 1992. Intergeneric somatic hybrid plants from protoplast fusion of *Fortunella crassifolia* cultivar 'Meiwa' with *Citrus sinensis. Scientia Horticulture.* 49 : 55-62.

Duquenne, B.T., Eeckhaut., E.S., Werbrouck, E.J. and Van Huylenbroeck, J.M. 2007. Effect of enzyme concentrations on protoplast isolation and protoplast culture of Spathiphyllum and Anthurium. *Plant Cell Tissue and Organ Culture* 191 : 165-173.

Fu, C.H., Guo, W.W., Liu, J.H. and Deng, H.H. 2003. Regeneration of *citrus sinensis* (+) *Clausenna lansium* intergeneric triploid and tetraploid somatic hybrids and their identification by molecular markers. *In vitro cell Development of Plants.* 39 : 360-364.

Garsia, N.F., Garma, J. G. and Olmos, E. 2010. Ros as biomarkers in hyperhydricity pp. 249-274. In Gupta, S.D. (Ed.) Reative Oxygen Species and Antioxidants in Higher Plants. Science Publishers.

Grosser, J.W. and Gmitter, F.G,Jr. 1990. Somatic hybridization of *Citrus* with wild rela-tives for germplasm enhancement and cultivar development. *Hort Science.* 25 : 147-151.

Guo, W.W.,Cheng, Y.J., Grosser, J.W., Deng, X.X. 2007. Protoplast Technology and *Citrus* Improvement. *Biological Sustainable Agriculture.* 121 : 461-464.

Hardy, S., Wilk, P., Viola, J. and Rennie, S. 2010. Growing Australian native finger limes. Frimefact 979: 1-11.

Heller, A., Borochov, A. and Halevy, A. H. 1994. Factor affecting the growth and flowering of *Coleonema aspalathoides. Scientia Hort.* 58 : 329-334.

Jana, S. and Shehawat, G.S. 2010. Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and *in vitro* flowering of *Anethum graveolens*. *Acta Physiologie Plantarum.* 3 : 305-31.

Jumin, H.B. 2015. Somatic embryogenesis derived from protoplast of *Murraya paniculata* L. Jack and their regeneration into plant *in vitro* precocious flowering. *Eco. Env. Cons.* 21 : 1695-1704.

Jumin, H.B. 2013a. Plant regeneration via somatic embryogenesis from protoplasts of *Clausena harmndiana* (Engl.) Swing and M.Kel. Anal.Univ.din Oradea. *Fasc. Bio.* 20 : 23-8.

Jumin, H.B. 2013b. Plant regeneration via protoplast culture of *Fortunella hindsii* (Champ.). *Int. J. Agro. Tech.* 9 : 999-1012.

Jumin, H.B. 1995. Plant regeneration via somatic embryogenesis in citrus and its relatives. *Phytomorphology*. 45 : 1-8.

Jumin, H.B. and Nito, N. 1996a. Plant regeneration via somatic embryogenesis from proto-plasts of six plant species related to *Citrus.* Plant Cell Reports 15: 332-336.

Jumin, H. B. and Nito, N. 1996b. *In vitro* flowering of *Fortunella hindsii (*Champ.). *Plant Cell Reports.* 15: 484-488.

Jumin, H.B and Nito, N. 1996c. *In vitro* flowering of *Murraya paniculata (*L.*). Jack. Experientia.* 52:268-272.

Jumin, H.B. and Nito, N. 1995. Embryogenic protoplast cultures of orange jessamine *(Murraya paniculata)* and their regeneration into plants flowering *in vitro. Plant Cell, Tissue and Organ Culture.* 41 : 277-279.

Kim, S.W.,Oh, M.J. and Liu, J.K. 2009. Somatic embryogen-

esis and plant regeneration in zygotic embryo explants cultures of rugosa rose. *Plant Biotechnology Reports* 3 : 199-203.

Kou,Y., Guohua, M.A., Jaime, A., Da-Silva, T. and Liu, N. 2013. Callus induction and shoot organogenesis from anther cultures of *Curcuma attenuata* Wall. Plant Cell, Tissue Organ Culture 112: 1-7.

Lal, D. and Singh, N. 2010. Mass Multiplication of *Celastrus paniculatus* Willd; An Important Medicinal Plant Under *in vitro* Conditions using Nodal Segments. *Journal of American Science.* 6 : 55-61.

Lin, C.C.,Lin, C.S. and Chang, W.S. 2003. *In vitro* flowering of *Bambusa edulis* and subsequent plantlet survival. Plant Cell, Tissue and Organ Culture 72 : 71-78.

Ling, J,T. and Iwamasa, M. 1994. Somatic hybridization between *Citrus reticulata* and *Citropsis gabunensis* through electrofusion. *Plant Cell Reports.* 13 : 493-497.

Louzada, E.S., Grosser, J.W. and Gmitter, F.G.Jr. 1993. Intergeneric somatic hybridization of sexually incompatible parents. *Citrus sinensis* and *Atalantia ceylanica. Plant Cell Reports.* 12 : 687-690.

Mohanty, S., Panda., M.K., Subudhi, E. and Nayak, S. 2008. Plant regeneration from callus culture of *Curcuma aromatica* and *in vitro* detection of somaclonal variation through cytophotometric analysis. *Biologia Plantarum.* 52 : 783-786.

Moiseeva, N.A.,Serebryokova, V.N., Lucretti, S. and Nosov, A.M. 2010. Morphogenetic status of somatic embryos of *Citrus sinensis* from mature polyembryonic seeds and those produced *in vitro*. *Russian Journal of Plant Physiology.* 57 : 720-731.

Moiseeva, N.A., Serebryokova, V.N., Nardi, L., Lucretti, S. and Butenko, R.G. 2006. Organization of initial stages of somatic embryogenesis in tissue culture of *Citrus sinensis* cv. Tarocco at the organisma level. *Russian Journal of Plant Physiology.* 53 : 548-555.

Motoike, S.,Skirvin, R.M., Norton, M.A. and Mulwa, R.M. 2005. Somatic embryogenesis in American grapes pp. 59-68. In Jain, S.M., Gupta, P.K. (Eds.): Protocol of Somatic Embryogenesis in Woody Plants. Springer Printed in The Netherland.

Murashige, T. and Tucker, D.P.H. 1969. Growth factor requirements of citrus tissue culture. *Proceeding lst International Citrus Symposium.* 3: 115-116.

Nadgauda, R. S., Parasharami, V. A. and Mascarenhas, A. F. 1990. Preco-cious flowering and seedling behavior in tissue-cultured bamboos. *Nature* 344 : 335-356.

Nagata, T. and Takebe, I. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. *Planta.* 92 : 301-308.

Ralker, S.V., Braun, R.H., Bryant, C., Conner, A.J. and Christey, M.C. 2008. Efficient isolation, culture and regeneration of *Lotus carniculatus* protoplasts. *Plant Biotechnology Reports.* 2 : 171-177.

Raveh, E. 2012. Assessing Salinity Tolerance in *Citrus*: Latest Developments. pp. 425-433. In Srivastava, A.K (Ed.) Advances in *Citrus* Nutrition. Springer Dordrecht Heidelberg New York London.

Scorza, R. and Janick, J. 1980. *In vitro* flowering of *Passiflora suberosa*L. *Jornal of American Society for Horticulture Science* 105 : 982 : 897.

Skiada, F.G., Grigoriadou, K. and Eleftheriou, E.P. 2010. Micropropagation of *Vitis vinefera* L. cv. 'Malagouzia' and Xinamovra, *Central European Journal of Biology.* 6: 839-852. 29

Sreedhar, R.V., Venkatachalam. and Neelwarne, B. 2009. Hyperhidricity-related morphologic and biochemical changes in vanila (*Vanilla planifolia*). *Journal of Plant Growth Regulator* 28 : 46-57.

Srinivasan, C. and Mullins, M.G. 1978. Control of flowering in the gra-pevine (*Vitis vinifera*). Formation of inflorescences in vitro by isolat-ed tendrils. *Plant Physiology*. 61 : 127-130.

Sykes, S.R. 1988. An overview of the family Rutaceae. pp. 93-100. In Walker, R.R. (eds.): *Citrus* Breeding Workshop. CSIRO Australia Melbourne.

Tallon, C.I., Porras, I. and Perez-Tornero, O. 2012. Efficient propagation and rooting of three citrus rootstocks using different plant growth regulators. *In vitro Cell Development of Biology Plant.* 48 : 488-499.

Travares, A.C., Salgueiro, L.R. and Canhoto, J.M. 2010. In vitro propagation of the wild carrot *Daucus carota* L. subsp. *halophilus* (Brot.) A. pujadas for conservation purposes. *In Vitro Cellular & Developmental Biology Plant.* 46 : 47-56.

Tremblay, F.M.,Iraqi, D. and El-Maskaoui, A. 2005. Protocol of somatic embryogenesis: Black spruce (*Picea mariana* (Mill) B.S.P). pp. 59-68. In Jain, S.M.,Gupta, P.K. (Eds.): Protocol of Somatic Embryogenesis in Woody Plants. Springer Printed in The Netherland.

Xu, Q.L., Xie,Y.H., Ru, H., Hu, Y. and Wang, Ch.Y. 2009. Efficient plant regeneration in vitro from red leaf beet via organogenesis. *Russian Journal of Plant Physiology*. 56 : 546-550.