

PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS FROM PROTOPLAST OF *Clausena harmandiana* (Engl.) Swing and M. Kell

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Abstract. Protoplasts isolated from embryogenic callus of *Clausena harmandiana* (Engl.) 'Swing and M. Kell' were cultured in MT (Murashige and Tucker 1969) basal medium containing 5% sucrose supplemented with bezyladenine (BA), malt extract (ME) and 0.6 M sorbitol. The highest plating efficiency was obtained on MT basal medium containing 5% sucrose supplemented with 0.01 mg l⁻¹ BA and 600 mg l⁻¹ ME, MT basal medium containing 5% sucrose and supplemented with 0.01 mg l⁻¹ 6-(γ , γ -dimethylallylamino)-purine 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 gibberellic acid (GA₃) + 0.1 mg l⁻¹ zeatin. In this investigation 25 plants were survived and grew normally in the soil.

Key Words: *Clausena harmandiana*, cytokinin, protoplast, somatic embryo, sucrose

Abbreviations: BA - N⁶-benzyladenine, 2,4-D - 2, 4-dichlorophenoxyacetic acid, DAP - 6-(γ , γ -dimethylallylamino)-purine, FDA - fluorescein diacetate, GA₃ - gibberellic acid, ME - malt extract,

INTRODUCTION

Citrus relatives are potential sources of useful resistance traits for citrus genetic improvement [5, 9, 12, 26, 29]. 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 [1, 26, 22, 30]. Among the orange subfamily, *C. harmandiana* may be a genetic source of lime tolerance [30, 31]. Somatic hybridization via protoplast fusion has been used successfully as a method to bypass sexual incompatibilities in some cases [5, 7, 17]. Intra- and intergeneric somatic hybrids have been obtained between *Citrus* and some of its relatives [4, 5, 30]. 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 gillettiana* or *Atalantia ceyanica* of a second parent [18].

Plant regeneration from cultured protoplasts in *Citrus* has been reported for a number of species [8]. However, there have been few reports of successful plant regeneration from protoplast cultures of *Citrus* relatives [12]. In general, citrus plants cannot be regenerated from leaf mesophyll protoplasts [5]. Although Deng *et al.* [4] 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* [11]. We successful to establish embryogenic callus from *C. harmandiana* as a member of *Citrus* relatives. 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 of *C. harmandiana*. 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 of *C. harmandiana*.

MATERIAL AND METHODS

Embryogenic callus of *Clausena harmandiana* (Engl.) Swing and M. Kell. was induced from the hypocotyl region of seedlings on MT basal medium [23] 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 μ mol m⁻² s⁻¹ light with a photoperiod of 16 h at 25°C. Seedlings used for hypocotyl excision were germinated from immature nucellar embryo explants and cultured on MT medium containing 5% sucrose, 5.0 mg l⁻¹ BA, 2.5 mg l⁻¹ 2, 4-D and 600 mg l⁻¹ ME and maintained at a temperature of 25°C with a 16/8 h light/dark photoperiod under an illumination of 52.9 μ mol m⁻² s⁻¹ photosynthetic photon flux intensity provided by cool-white fluorescent light.

Prior to protoplast isolation, about 1 g of *C. harmandiana* callus was transferred to fresh liquid medium consisting of MT basal medium containing 5% lactose, and incubated on Gyrotory shaker at 120 rpm for 6 d under 17.7 μ 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 medium consisting of 0.4% macerozyme R-10, 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 with 0.45 μ 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 and re-suspension of the pellet protoplasts with MT inorganic salt solution containing 0.6 M sorbitol by centrifugation at 100 x g for 2 min.

Protoplasts of *C. harmandiana* were re-suspended in MT basal medium containing 5% sucrose, supplemented with various concentrations of BA, and various concentrations of 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 10⁴ 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 d of culture [2, 24]. 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 [24].

Calli derived from protoplasts had been subcultured three times at 30 d 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.

Somatic embryo development of *C. harmandiana* was studied by culturing globular somatic embryos onto MT basal medium supplemented with various concentrations of DAP and 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 structures was determined after 30 d. Specimen 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 with 15 KV.

Heart-shaped somatic embryos were cultured individually to form shoot on half-strength MT basal medium containing 5% sucrose supplemented with various concentrations of gibberellic acid (GA₃) and various concentrations of zeatin and solidified with 0.3% Gelrite. Cultures were maintained at a temperature of 25°C with a 16/8 h light/dark photoperiod under an illumination of 52.9 µmol. m⁻² s⁻¹ photosynthetic photon flux intensity provided by cool-white fluorescent light. Shoot formation was recorded as the percentage of cultured heart-shaped somatic embryos which formed shoots after 2 months.

Regenerated shoots were transferred to half-strength MT basal medium containing 5% sucrose without PGR. When root length reached 4-5 cm and, the plantlets were transplanted to covered glass pots

with hyponex solution. Transferred plantlets were held in the growth chamber for 2 months. Plantlets were subsequently transferred to larger pots and acclimated to greenhouse conditions.

RESULTS

About 10⁶ protoplasts with a diameter of 10-30 µm 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 d after isolation. The protoplast plating efficiency obtained after 40 d of protoplast culture was from 10-45% (Table 1).

The formation of colonies (about 70 µm in diameter) occurred after 60 d 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 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 BA and ME to the medium. A low concentration of BA stimulated colony formation. When protoplasts were cultured on MT basal medium containing 5% sucrose without BA and ME, the protoplast plating efficiency was low. However, when protoplasts were cultured in the medium supplemented with 0.01 mg l⁻¹ BA and 600 mg l⁻¹ malt extract, higher plating efficiency was obtained (Table 2).

After 60 d, 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 d from protoplast derived cultures. The number of globular somatic embryos obtained from 5 protoplast isolations was 460. 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 (Fig. 1C).

Globular somatic embryos derived from protoplasts were cultured on MT basal medium supplemented with 2.5-10% sucrose and 0.0-1.0 mg l⁻¹ DAP for 30 d. The frequency of globular somatic embryos that developed into heart-shaped somatic embryos in the medium supplemented with 5 % sucrose and 0.01 mg l⁻¹ DAP was 75% (Table 2). About 65% of the hear-shaped embryos were 0.4 to 0.8 mm in diameter, while others were elliptical in shape and = 0.9 mm long. The heart-shaped somatic embryos averaged 1.0-2.0 mm in diameter after 2 months.

Table 1. Effect of BA and ME on protoplast plating efficiency (%) of *C. harmandiana*, 40 days after protoplast culture

BA (mg l ⁻¹)	Malt Extract (ME) (g l ⁻¹)		
	0.0	300	600
0.0	10.3a	9.6a	11.0a
0.001	15.6a	20.3b	22.0b
0.01	20.3b	23.3b	45.0c
0.1	25.3b	12.0c	30.3d
1.0	9.6a	10.3a	20.6b

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.

Table 2. Effect of DAP and sucrose on development of protoplast-derived globular somatic embryos (%) *C. harmandiana* 30 days after culture (25 globular somatic embryos tested for each treatment)

DAP (mg l ⁻¹)	Sucrose (g l ⁻¹)			
	2.5	5.0	7.5	10.0
0.0	60.3a	50.3a	40.0a	33.3a
0.001	65.6a	60.3a	50.0ab	40.3ab
0.01	72.3a	63.3a	70.3c	70.0c
0.1	65.3a	50.0a	60.3cb	40.3ab
1.0	40.3b	30.3b	50.6ab	28.0a

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

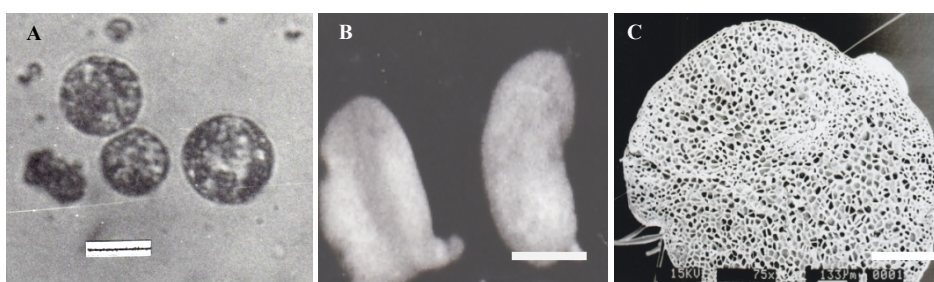


Figure 1. (A), Fresh protoplasts of *C. harmandiana* (bar 20 mm). (B), Heart-shaped embryos derived from protoplasts of *C. harmandiana* in MT basal medium containing 5% sucrose supplemented with 0.001 mg l⁻¹ BA 90 days after protoplast isolation (bar 0.5 cm). (C), Scanning electron micrograph showing meristematic cells of globular somatic embryos derived from protoplasts four weeks after transplanting to MT basal medium containing 50 g l⁻¹ lactose supplemented with 0.01 mg l⁻¹ BA (bar 266 μm)

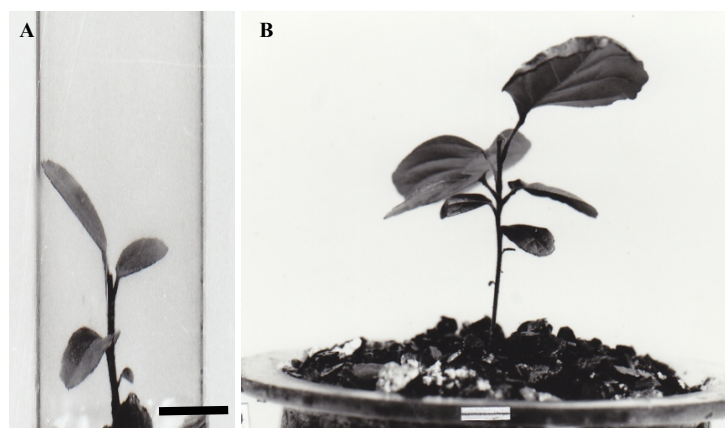


Figure 2. (A), Plantlet differentiated from protoplasts 60 days after transfer to MT basal medium containing 5% sucrose supplemented with 0.01 mg l⁻¹ DAP (bar = 1 cm). (B), *C. harmandiana* plant derived from protoplasts 2 months after acclimatization in the soil (bar = 1 cm)

A low level of GA₃ + zeatin in the culture medium promoted the growth of heart-shaped somatic embryos of *C. harmandiana* into plantlets. The highest percentage of shoot formation was obtained using 0.1 mg l⁻¹ GA₃ and 0.1 mg l⁻¹ Zeatin. After 30 d of culture on GA₃ + 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. 2A).

Second 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 GA₃ + Zeatin rooted more quickly and readily, while shoots from PGR-free medium formed few roots and were accompanied by hyperhydricity. Consequently,

plantlets from medium with GA₃ + Zeatin survived in soil (Fig. 2B).

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 its growth survived the transfer to soil. In this study only 2 plants in soil were recovered from PGR-free medium, while 23 plants were survived in the soil from medium with GA₃ + zeatin. (Fig. 3). 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 l⁻¹ BA and 600 mg l⁻¹ 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 l⁻¹ DAP 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 of shoot formation was obtained using 0.1 mg l⁻¹ GA₃ + 0.1 mg l⁻¹ zeatin (Table 3).

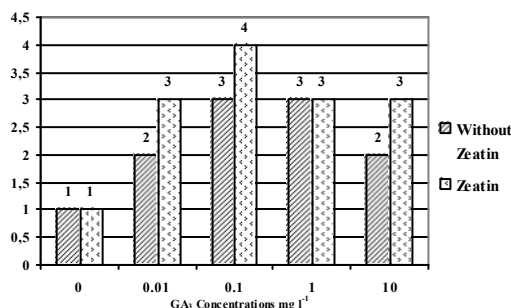


Figure 3. Number of plants were survived in the soil

DISCUSSIONS

The sequence from protoplasts to plantlets in *Citrus* relatives was reported by Jumin and Nito [15], 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 *Citrus* and its relatives [11]. The colony formation obtained from this study is similar to the previous reports for bamboo [16] and *Citrus* relatives [12].

The promotion of somatic embryo formation by low concentrations of DAP in this study was consistent with previous studies, where cytokinin promoted the initiation and development of embryos *Curcuma attenuate* [19], in *Citrus* and its relatives [11]. 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 [7]. In nature, carbohydrate is transported within plant tissues as sucrose and tissue may have an inherent capacity for uptake, transport and utilization of sucrose [10].

The beneficial effect of GA₃ on shoot formation has been reported in *Citrus* relatives [12]. Its results show that GA₃ increased the percentage of germinating somatic embryos (Table 3). Whereas cytokinin was found to promote shoot formation from callus cultures in *Citrus* and other woody species [8, 25, 27]. Several hundred globular embryos were produced from protoplast cultures, but fewer plants were obtained. There were several steps in the regeneration process [3, 20, 21].

The first requirement for plant regeneration was the development of a viable embryo [13, 22, 31]. 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 [12]. Most of the shoots rooted more quickly and readily, while others formed few roots and were accompanied by hyperhydricity. Hyperhydricity 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 [6, 28]. Consequently, plantlets of *C. harmandiana* 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* [12].

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 dehydration when transferred from *in vitro* to natural conditions [22, 32]. 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

Table 3. Effect of GA3 and Zeatin on shoot formation of *C. harmandiana* 60 days after heart-shaped embryos culture (25 heart-shaped embryos tested for each treatment)

GA3 (mg l ⁻¹)	Zeatin (mg l ⁻¹)				
	0.0	0.01	0.1	1.0	10.0
0.0	30.3a	40.3a	39.3a	30.6a	24.0a
0.01	40.6ab	42.3ab	43.6a	40.3b	41.6a
0.1	50.3b	61.6b	79.6b	70.3c	64.6b
1.0	45.3ab	60.3b	73.3b	66.3c	55.6b
10.0	35.6ab	53.6ab	70.6b	58.0c	55.3b

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

number of globular somatic embryos which then developed into heart-shaped somatic embryos.

The success of plant regeneration via somatic embryogenesis from protoplasts of *C. harmandiana* is strongly depend on BA and DAP. Cytokinin was necessary to recover organs and plants from protoplasts to plantlets [1, 10, 14, 15, 30, 33]. 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 [7, 11].

A sequence from protoplast to a plant via somatic embryogenesis was established for *C. harmandiana* 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* through protoplast fusion [12].

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Received: 11 January 2013

Accepted: 11 April 2013

Published Online: 14 April 2013

Analele Universității din Oradea – Fascicula Biologie

<http://www.bioresearch.ro/revistaen.html>

Print-ISSN: 1224-5119

e-ISSN: 1844-7589

CD-ISSN: 1842-6433