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Plant regeneration via somatic embryogenesis from protoplasts of six plant species related to *Citrus*

Hasan Basri Jumin and Nobumasa Nito

Department of Applied Biological Sciences, Saga University, Saga 840, Japan

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Abstract. Protoplasts isolated from embryogenic callus of *Fortunella polyandra* (Ridl.), *Atalantia bilocularis* (Pieree ex Guill.), *Hesperethusa crenulata* (Roxb.), *Glycosmis pentaphylla* (Retz.) Corr., *Triphasia trifolia* (Burm. f.) P. Wils. and *Murraya koenigii* (L.) Spreng. were cultured in MT (Murashige and Tucker 1969) basal medium containing 5% sucrose supplemented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg l^{-1} BA and 0.6 M sorbitol. The highest plating efficiencies for all species were obtained on MT basal medium containing 5% sucrose supplemented with 0.001 mg l^{-1} BA. *F. polyandra* produced higher percentages of globular somatic embryo development, while *A. bilocularis* consistently showed a lower percentage of globular somatic embryo development in all 5 concentrations of BA. MT basal medium containing 5% sucrose and supplemented with 0.001 mg l^{-1} BA 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 percentages of shoot formation for all 6 species were obtained using 0.1 mg l^{-1} GA_3. A complete protoplast-to-plant system was developed for *F. polyandra, A. bilocularis* and *T. trifolia*, which could facilitate the transfer of nuclear and cytoplasmic genes from these species into cultivated *Citrus* through protoplast fusion.

Abbreviations: BA: N^6-benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; FDA: fluorescein diacetate; GA_3: gibberellin A_3

Introduction

For improvement of *Citrus* rootstocks, citrus relatives hold much promise as a germplasm source for some traits of agronomic value. Plant material tolerant to diseases and environmental stresses may be selected from indigenous citrus cultivars and relatives (Bitters et al. 1964; Swingle and Reece 1967; Sykes 1988; Grosser and Gmitter 1990b).

Somatic hybridization via protoplast fusion has been used successfully as a method to bypass sexual incompatibilities in some cases. Intra- and intergeneric somatic hybrids have been obtained between *Citrus* and some of its relatives (Ohgawara et al. 1985; Grosser et al. 1988a, 1988b; Deng et al. 1992; Louzada et al. 1993; Ling and Iwamasa 1994). In these combinations, *Citrus* somatic embryogenic protoplasts are used as one partner in the protoplast fusion with leaf-derived protoplasts (*Fortunella crassifolia* and *Atalantia ceylanica*) of a second parent.

Plant regeneration from cultured protoplasts in *Citrus* has been reported for a number of species (Vardi et al. 1982; Kobayashi et al. 1983, 1985; Hidaka and Kajiura 1988; Sim et al. 1988; Ling et al. 1989, 1990; Kunitake et al. 1991). However, there have been few reports of successful plant regeneration from protoplast cultures of *Citrus* relatives. Vardi et al. (1986) reported plant regeneration from protoplasts isolated from a *Microcitrus* embryogenic culture. In general, citrus plants can not be regenerated from leaf mesophyll protoplasts (Grosser and Chandler 1987). Grosser et al. (1992) have reported plant regeneration from leaf mesophyll protoplasts used in fusion experiments, but it does not appear to be repeatable. Embryogenic callus has not been obtained from monoembryonic types of citrus (Button and Kochba 1977; Kobayashi et al. 1982; Moore 1985). In view of the limited success of plant regeneration from protoplast cultures of *Citrus* relatives, we conducted a study on protoplast cultures of six *Citrus* relatives. This method has potential to be used for making wide hybridizations through protoplast fusion for rootstock improvement. A regeneration sequence via somatic embryogenesis from protoplasts of three plant species related to *Citrus* is described in this paper.

Materials and Methods

Protoplast sources. Embryogenic callus of *Fortunella polyandra* Ridl., *Atalantia bilocularis* Pieree ex Guill., *Hesperethusa crenulata* Roxb., *Glycosmis pentaphylla* Retz. Corr., *Triphasia trifolia* Burm. f. P. Wils.

Correspondence to: H. B. Jumin

and *Murraya koenigii* L. Spreng. was induced from the hypocotyl region of seedlings on MT (Murashige and Tucker 1969) basal medium containing 5% sucrose, 5.0 mg l^{-1} BA, 2.5 mg l^{-1} 2,4-D and 600 mg l^{-1} malt extract and kept under 35.3 µmol m^{-2} s^{-1} light with a photoperiod of 16 h at 25°C, as described by Jumin and Nito (1995). Seedlings used for hypocotyl excision were germinated from immature nucellar embryo explants using the same medium as mentioned above.

Protoplast isolation. Protoplast isolation was performed following the method described by Ling et al. (1989) with slight modification. The resulting protoplasts were washed twice with MT inorganic salt solution containing 0.6 M sorbitol by centrifugation at 100 x g for 2 min and resuspension of the collected protoplasts.

Protoplast culture. Protoplasts of all species were resuspended in MT basal medium containing 5 % sucrose, supplemented with 0.0, 0.001, 0.01, 0.1 or 1.00 mg l^{-1} BA, 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 at 25°C under 35.3 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 (Grosser and Gmitter 1990a; Kunitake et al. 1991). The viability of the protoplasts was checked by FDA staining (Widholm 1972; Larkin 1976). 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 subcultured three times at 30 d intervals using MT basal medium containing 5% sucrose without plant growth regulators. For somatic embryo induction, the calli were transferred onto MT basal medium containing 5% lactose without plant growth regulators and solidified with 0.25% Gelrite.

Globular embryo development. Somatic embryo development of the six species was studied by culturing globular embryos onto MT basal medium supplemented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg l^{-1} BA and 5% sucrose in 90 x 20 mm petri dishes. The concentrations of plant growth regulators was chosen for each species 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.

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, 0.001, 0.01, 0.1, 1.0 or 10.0 mg l^{-1} GA_3 and solidified with 0.3% Gelrite. The cultures were kept under 35.3 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.

Plant regeneration from shoots. Regenerated shoots were transferred to half-strength MT basal medium containing 5% sucrose without plant growth regulators. When root length reached 4–5 cm and some amount of shoot elongation had occurred, the plantlets were transplanted to covered glass pots with sterile vermiculite, watered with a 0.1% Hyponex solution and kept in a growth chamber for 2 months. Plantlets were subsequently transferred to larger pots and acclimated to greenhouse conditions.

Results and Discussion

About 10^7 protoplasts with a diameter of 10–20 µm were obtained from 1 g of callus (Fig. 1a). FDA staining showed that the viability of fresh protoplasts was 75–90%. About 75% of the surviving protoplasts formed a cell wall within 5 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 3.5–35% (Fig. 2). The formation of colonies (about 80 µm in diameter) occurred after 60 d of protoplast culture (Fig. 1b). Colony formation was improved by medium manipulation. 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 in the medium. A low concentration of BA stimulated colony formation in all six species tested. Ling et al. (1989) reported that BA inhibited colony formation in *Citrus*. This difference between the present study and their study may be due to a number of factors including protoplast isolation methods, genetic variability between species, culture medium and environmental conditions, etc.

Two main factors were essential for embryogenic protoplast culture of *F. polyandra, A. bilocularis, H. crenulata, G. pentaphylla, T. trifolia* and *M. koenigii*. First, a suitable culture media with optimal concentration of BA was essential to stimulate high frequencies of cell division and colony formation from protoplasts. When protoplasts were cultured on MT basal medium containing 5% sucrose without BA, the protoplast plating efficiencies were low in all six species. However, when protoplasts were cultured in the medium supplemented with 0.001 mg l^{-1} BA, higher plating efficiencies were obtained in all six species (Fig. 2). Second, the potential for callus formation from protoplasts was apparently species-dependent. *A. bilocularis* showed a low plating efficiency even though this species originated from a good callus source, which was similar to the other species.

After 60 d, protoplast-derived colonies were transferred to hormone-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 (Table 1). Cell colonies became compact and changed into spherical structures, which formed proembryos and then developed into globular somatic embryos. The globular somatic embryos then became heart-shaped forming cotyledon-like struc-

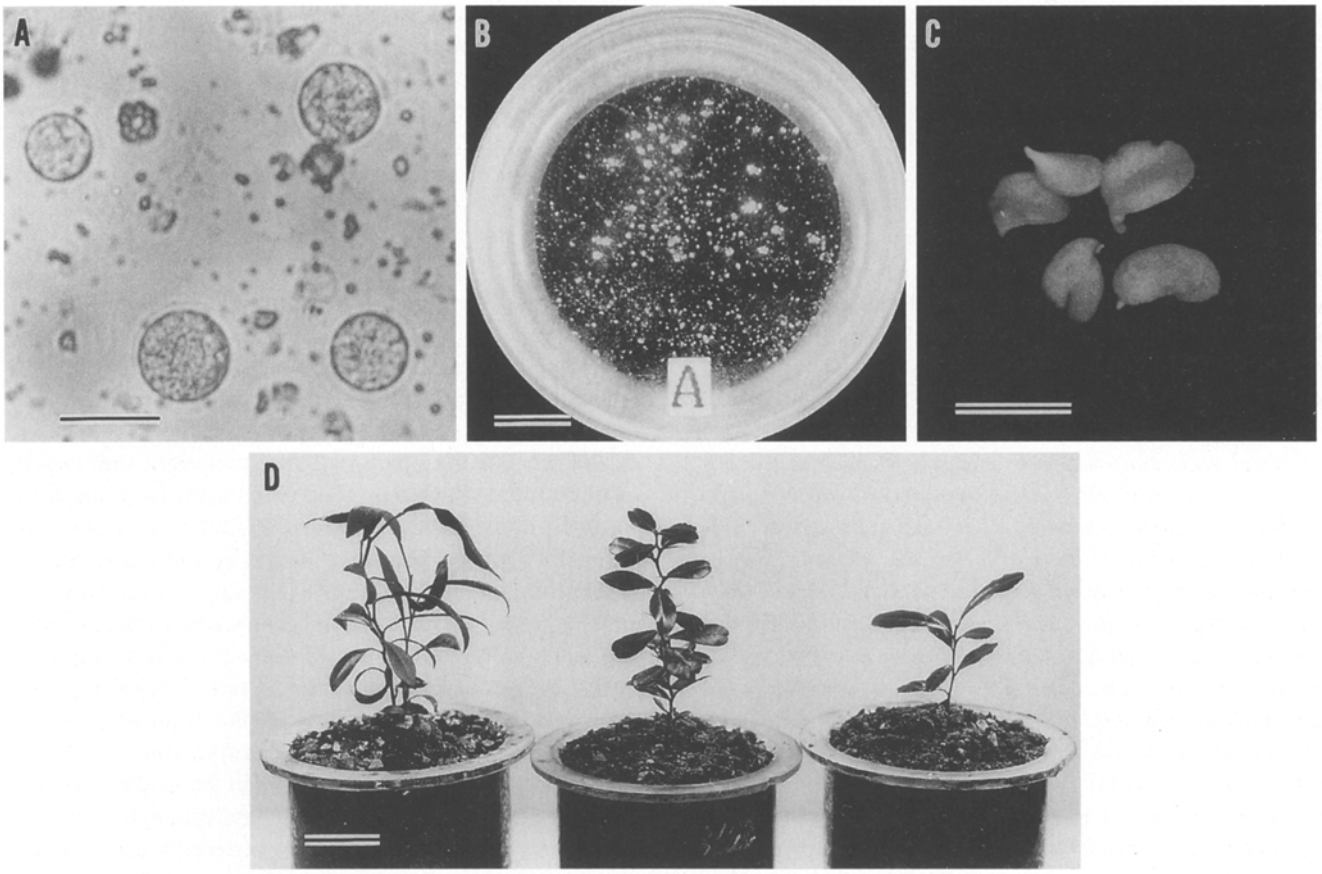

Fig. 1. A. Fresh protoplasts of *T. trifolia* (bar = 20 μm). B. Colonies derived from protoplasts of *F. polyandra* in MT medium containing 5% sucrose supplemented with 0.001 mg l^{-1} BA, 60 d after protoplast isolation (bar = 1 cm). C. Heart-shaped somatic embryo with cotyledon-like structures of *A. bilocularis* differentiated from protoplasts, 60 d after transfer to MT basal medium containing 5% sucrose supplemented with 0.001 mg l^{-1} BA (bar = 0.5 cm). D. *F. polyandra, A. bilocularis* and *T. trifolia* plants (from left to right) regenerated from protoplasts, 4 months after transfer to soil (bar = 2 cm).

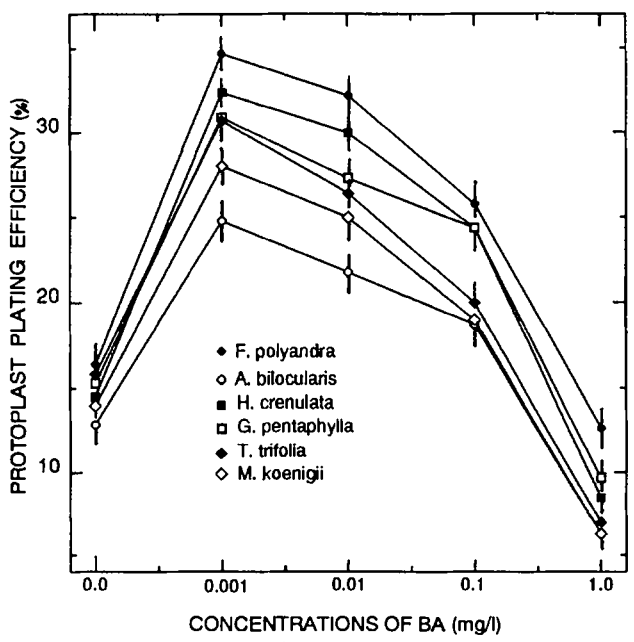

Fig. 2. Effect of BA on protoplast plating efficiency of six plant species related to *Citrus*, 40 d after protoplast culture.

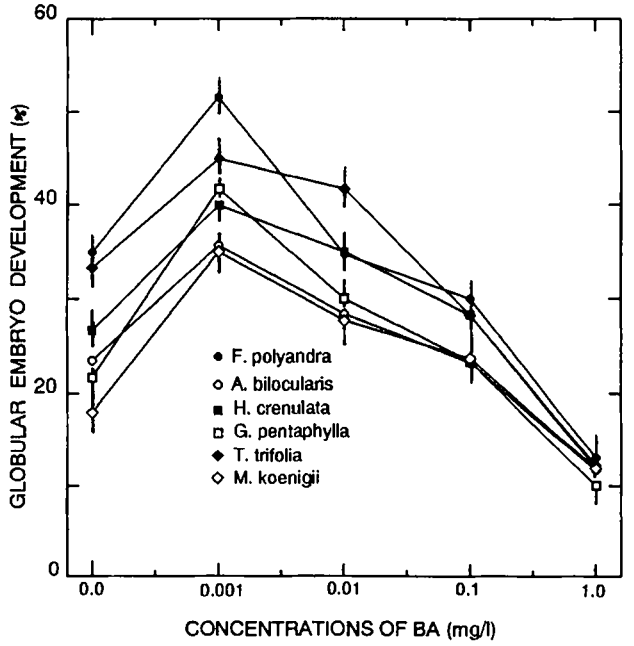

Fig. 3. Effect of BA on development of protoplast-derived globular somatic embryos of six plant species related to *Citrus*, 30 d after culture.

tures (Fig. 1c). This is similar to the previous reports for *Citrus* (Vardi et al. 1982; Kobayashi et al. 1983, 1985; Hidaka and Kajiura 1988; Sim et al. 1988; Ling et al. 1989, 1990; Kunitake et al. 1991).

Protoplast-derived globular somatic embryos were cultured on MT basal medium containing 5% sucrose supplemented with BA in the range of 0.0–1.0 mg l^{-1} for 30 d. The frequency of globular somatic embryos which developed into heart-shaped somatic embryos in the medium supplemented with 0.001 mg l^{-1} BA ranged from 35 to 51% (Fig. 3). About 70% of the embryos were 0.4 to 0.8 mm in diameter, while others were elliptical in shape and 0.9 mm long. The globular somatic embryos averaged 1.0–2.0 mm in diameter after 2 months. On the MT basal medium containing 5% sucrose supplemented with 1.0 mg l^{-1} BA, 80% of the globular somatic embryos formed callus. Differences in response of somatic embryo development due to species were found in this study. *F. polyandra* produced a higher percentage of heart-shaped somatic embryos, while *A. bilocularis* consistently showed a lower percentage of heart-shaped somatic embryo development over all 5 concentrations of BA. The promotion of somatic embryo formation by low concentrations of BA in this study contrasted with certain previous studies, where cytokinin inhibited embryo formation from undeveloped ovules of 'Marsh' grapefruit (Moore 1985) and from protoplasts of 'Calamondin' (Ling et al. 1989). However, Vardi and Raveh (1976) and Gmitter and Moore (1986) reported that cytokinin promoted the initiation and development of embryos in *Citrus*.

The beneficial effect of GA_3 on shoot formation has been reported in *Citrus* (Kochba et al. 1974; Gmitter and Moore 1986). Our results show that GA_3 increased the percentage of germinated somatic embryos (Fig. 4). A low level of GA_3 in the culture medium promoted the growth of heart-shaped somatic embryos of all species to form plantlets. The highest percentage of shoot formation for all six species was obtained using 0.1 mg l^{-1} GA_3. After 30 d of culture on GA_3-containing medium, the formation of adventitious shoot buds was observed. Many of these shoot buds developed into shoots after 25 d. GA_3 promoted shoot formation and the subsequent ability to develop plantlets. Shoot induction also was dependent upon the species. *F. polyandra* had a higher regeneration capacity via somatic embryogenesis than did the other five species. Almost 90% of the *F. polyandra* somatic embryos cultured on 0.1 mg l^{-1} GA_3 formed shoots, compared to less than 40% of the *A. bilocularis* somatic embryos.

Several hundred globular somatic embryos were produced from protoplasts of most of the species studied, but fewer plants were obtained (Table 1). There were several steps in the regeneration process (Gmitter and Moore 1986). The first requirement for plant regeneration was the development of a viable mature embryo. The development of many globular somatic embryos was halted as a result of abnormality (pluricotyly, multiple shoot meristems, fused embryos and fasciation).

The second requirement for successful plant recovery was the balanced germination of somatic embryos. About 90% of *F. polyandra*, 80% of *A. bilocularis* and 75% of *T. trifolia* heart-shaped somatic embryos underwent normal shoot elongation, while 80% of heart-shaped somatic embryos from *H. crenulata, G. pentaphylla* and *M. koenigii* underwent abnormal shoot elongation. Shoots from *F. polyandra, A. bilocularis* and *T. trifolia* rooted more quickly and readily, while shoots from *H. crenulata, G. pentaphylla* and *M. koenigii* formed few roots and were accompanied by hyperhydricity. Consequently, plantlets from *F. polyandra, A. bilocularis* and *T. trifolia* survived in soil (Table 1).

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. In general, only those heart-shaped somatic embryos that had balanced root and shoot growth survived the transfer to soil (Table 1). In this study, no plants in soil were recovered from *H. crenulata, G. pentaphylla* and *M. koenigii*, even though these species 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. However, few plantlets were obtained and these died within 1 month of transfer to soil. In contrast, *A. bilocularis* produced a low number of globular and heart-shaped somatic embryos as well as a low number of shoots, but regenerated plantlets were successfully grown in soil under greenhouse conditions, as was the case with *F. polyandra* and *T. trifolia* (Fig. 1d).

Regenerated plantlets grew normally and no differences were noticed in growth habits and leaf characters such as

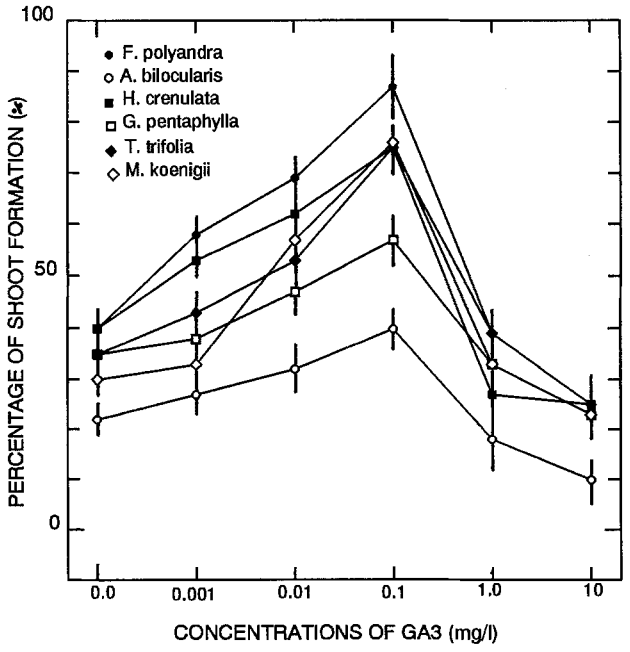

Fig. 4. Effect of GA_3 on shoot formation of six plant species related to *Citrus*, 60 d after heart-shaped somatic embryo culture.

shape, thickness and color between protoplast-derived plants and nucellar seedlings.

Table 1. Plant regeneration from protoplasts via somatic embryogenesis of six plant species related to *Citrus*

Species	No. globular embryos obtained*	No. plantlets with shoots and roots	No. plants in soil
F. polyandra	682	103	48
A. bilocularis	390	43	13
H. crenulata	504	6	0
G. pentaphylla	440	7	0
T. trifolia	472	56	24
M. koenigii	560	9	0

*Total from 5 protoplast isolations of each species

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