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# Plant regeneration via somatic embryogenesis from protoplasts of Uganda cherry orange (*Citropsis schweinfurthii*)

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**Abstract.** Protoplasts isolated from embryogenic callus of *Citropsis schweinfurthii* (Engl.) Swing. & M. Kell 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, 0, 300, 600 or 900 mg $l^{-1}$ malt extract 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 $l^{-1}$ malt extract. MT basal medium containing 5% sucrose and supplemented with 0.01 mg $l^{-1}$ kinetin 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 $l^{-1}$ $GA_3$. A complete protoplast-to-plant system was developed for *C. schweinfurthii*, which could facilitate the transfer of nuclear and cytoplasmic genes from this 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 $A_3$; ME, malt extract

## 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). Among the orange subfamily, *C. schweinfurthii* may be a source of resistant to *Citrus* nematode (Swingle and Reece 1967), burrowing nematode (Hearn et al. 1974) and *Phytophthora* spp (Sykes 1988). 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, 1990b; 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, Feronia limonia, Clausena lansium, Citropsis gilletiana* and *Atalantia ceylanica*) of a second parent (Louzada and Grosser 1994).

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; Jumin and Nito 1995a, 1995b). In general, citrus plants cannot be regenerated from leaf mesophyll protoplasts (Grosser and Chandler 1987). Although Grosser et al. (1992) have reported plant regeneration from leaf mesophyll protoplasts used in fusion experiments. 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 *C. schweinfurthii*. This system has potential as an additional method to be used for making wide hybridizations through protoplast fusion for rootstock improvement. A regeneration sequence via somatic embryogenesis from protoplasts of *C. schweinfurthii* is described in this paper.

## Materials and Methods

***Protoplast sources.*** Embryogenic callus of *Citropsis schweinfurthii* (Engl.) Swing. & M. Kell. 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 $l^{-1}$ ME and maintained under 52.9 μmol $m^{-2}$ $s^{-1}$ light with a photoperiod of 16 h at

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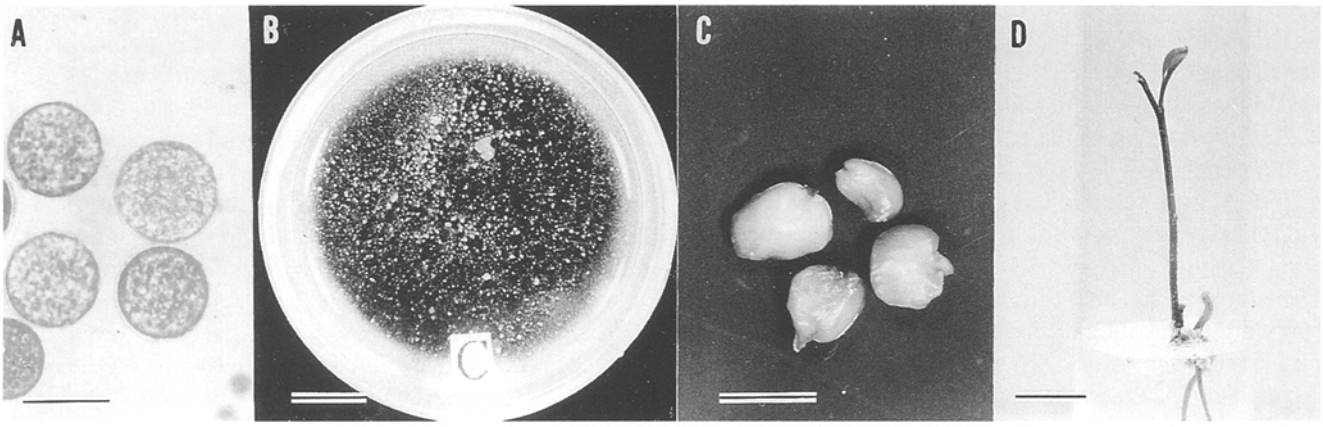


**Fig. 1.** A. Fresh protoplasts of *C. schweinfurthii* (bar = 20 um). B. Colonies derived from protoplasts of *C. Schweinfurthii* in MT basal medium containing 5% sucrose supplemented with 0.01 mg $l^{-1}$ BA, 60 d after protoplast isolation (bar = 1 cm). C. Heart-shaped somatic embryo with cotyledon-like structures differentiated from protoplasts, 30 d after transfer to MT basal medium containing 5% sucrose supplemented with 0.01 mg $l^{-1}$ kinetin (bar = 0.5 cm). D. *C. schweinfurthii* plantlet derived from protoplasts, 8 weeks after heart-shaped embryos were cultured on half-strength MT basal medium containing 0.1 mg $l^{-1}$ $GA_3$ (bar = 1 cm).

25°C, as described by Jumin and Nito (1995a). 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 pelleted protoplasts.

***Protoplast culture.*** Protoplasts of *C. schweinfurthii* were resuspended in MT basal medium containing 5% sucrose, supplemented with 0.0, 0.001, 0.01, 0.1 or 1.0 mg $l^{-1}$ BA, 0, 300, 600 or 900 mg $l^{-1}$ ME, 0.6 M sorbitol, and solidified with 0.1% Gelrite (Kelco, Division of Merck & Co. Inc., San Diego, California). The protoplasts were cultured at a density of 3 to 5 x $10^4$ cells $ml^{-1}$ in 60 x 15 mm plastic petri dishes containing 2 ml of culture medium. For embedding the protoplasts in Gelrite, the liquid medium containing the protoplasts was mixed with an equal amount of Gelrite medium to obtain a final concentration of 0.1% Gelrite. All dishes were sealed with Parafilm and maintained at 25°C in the dark for 40 d, and then kept 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 (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 *C. schweinfurthii* 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 $l^{-1}$ kinetin 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 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, 0.01, 0.1, 1.0 or 10.0 mg $l^{-1}$ $GA_3$ 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 wasrecorded 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 30 d. Plantlets were subsequently transferred to larger pots and acclimated to greenhouse conditions.

## Results and Discussion

About $10^7$ 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 8 d after isolation. The protoplast plating efficiency obtained after 40 d of protoplast culture was from 10–40% (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 media 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 and ME to the medium. 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 $l^{-1}$ ME, higher plating efficiency was obtained (Fig. 2). Jumin and Nito (1995b) reported that BA promoted colony formation in six plant species related to *Citrus*. ME added to the protoplast culture medium also promoted colony formation in *Murraya paniculata* (Jumin and Nito 1995a).

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. The number of globular somatic embryos obtained from 5 protoplast isolations was 540. 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 structures (Fig. 1c). This is similar to the previous reports for *Citrus* and its relatives (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; Jumin and Nito 1995a, 1995b).

Protoplast-derived globular somatic embryos were cultured on MT basal medium supplemented with 2.5 -10% sucrose and 0.0-1.0 mg $l^{-1}$ kinetin 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 $l^{-1}$ kinetin was 75% (Fig. 3). About 65% 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. The promotion of somatic embryo formation by low concentrations of kinetin in this study was consistent with previous studies, where cytokinin promoted the initiation and development of embryos in *Citrus* and its relatives (Vardi and Raveh 1976; Gmitter and Moore 1986; Jumin and Nito 1995b). When the sucrose concentration was varied from 2.5 to 10 %, the optimal concentration was 5 % (Fig. 3). The commonly used carbohydrate for *Citrus* tissue culture is sucrose (Grosser and Gmitter 1990a). In nature, carbohydrate is transported within plant tissues as sucrose and tissue may have an inherent capacity for uptake, transport and utilization of sucrose (Eapen and George 1993; Ashburner et al. 1993).

The beneficial effect of $GA_3$ on shoot formation has been reported in *Citrus* (Kochba et al. 1974; Gmitter and Moore 1986) and *Citrus* relatives (Jumin and Nito 1995b). Our results show that $GA_3$ increased the percentage of germinating somatic embryos (Fig. 4). A low level of $GA_3$ in the culture medium promoted the growth of heart-shaped somatic embryos of *C. schweinfurthii* into plantlets. The highest percentage of shoot formation 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 and many of these developed into plantlets. $GA_3$ promoted shoot formation and the

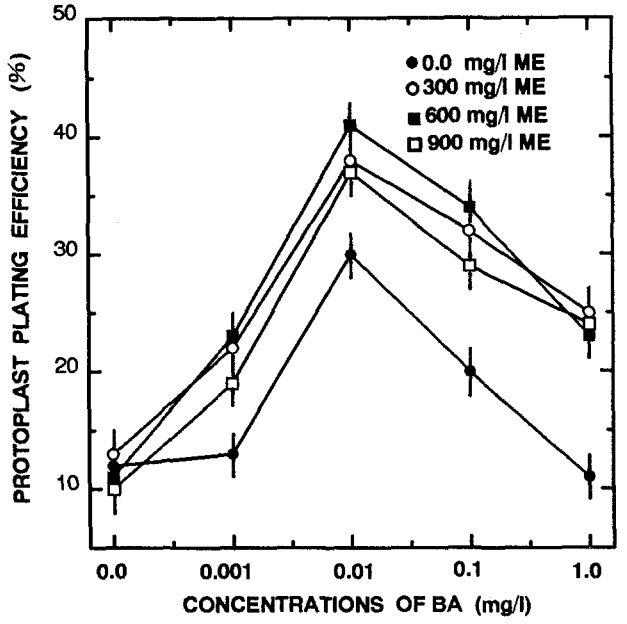


Fig. 2. Effect of BA and ME on protoplast plating efficiency of *C. schweinfurthii*, 40 d after protoplast culture. Bars indicate means ± SE of five independent replications.

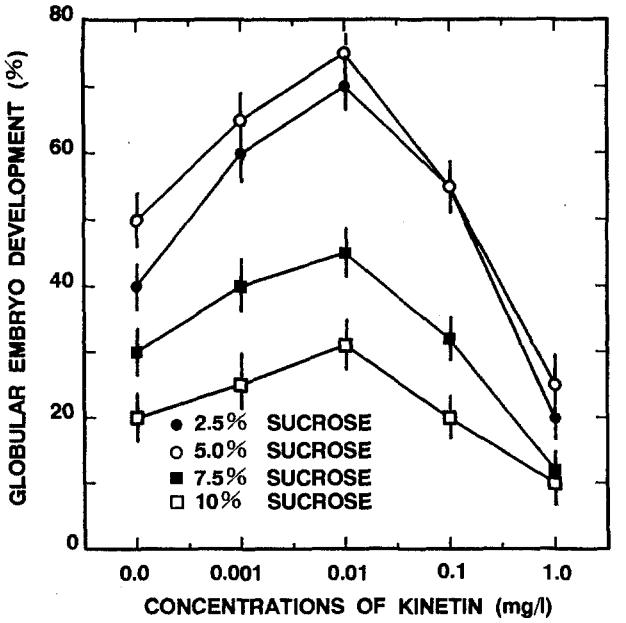


Fig. 3. Effect of kinetin and sucrose on development of protoplast-derived globular somatic embryos of *C. schweinfurthii*, 30 d after culture. Bars indicate means ± SE of five independent replications (25 globular somatic embryos tested for each treatment).

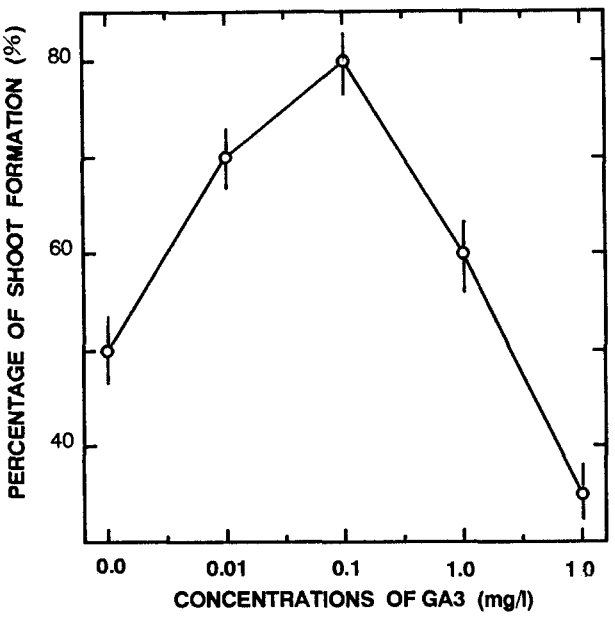


**Fig. 4.** Effect of $GA_3$ on shoot formation of *C. schweinfurthii*, 60 d after heart-shaped somatic embryo culture. Bars indicate means ± of five independent replications (25 heart-shaped somatic embryos tested for each treatment).

subsequent ability to develop plantlets (Fig. 1d).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.

A sequence from protoplast to a plant via somatic embryogenesis was established for *C. schweinfurthii*. The highest plating efficiency was obtained on MT basal medium containing 5% sucrose supplemented with 0.01mg $l^{-1}$ BA and 600 mg $l^{-1}$ ME. Cell colonies changed into gloular somatic embryos on MT medium containing 5% lactose without plant growth regulators. MT basal medium containing 5% sucrose and supplemented with 0.01 mg $l^{-1}$ kinetin 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 $l^{-1}$ $GA_3$. This efficient protoplast-to-plant system for this species could facilitate the transfer of nucellar and cytoplasmic genes of a source of nematode and *Phytophthora* resistances 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 et al. 1988a; Grosser et al. 1990, 1992; Grosser and Gmitter 1990a, 1990b).

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