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Research note

Embryogenic protoplast cultures of orange jessamine (*Murraya paniculata*) and their regeneration into plants flowering *in vitro*

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Abstract

Embryogenic callus was induced from the hypocotyl region of seedlings germinated from immature embryos of orange jessamine (*Murraya paniculata* (L.) Jack) on Murashige & Tucker (1969) medium containing 50 g l^{-1} sucrose, 5.0 mg l^{-1} benzyladenine, 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 600 mg l^{-1} malt extract. Isolated protoplasts divided to produce callus on Murashige & Tucker (1969) medium containing 50 g l^{-1} sucrose, 0.01 mg l^{-1} gibberellin A_{4+7} and 600 mg l^{-1} malt extract. Callus developed to plantlets via somatic embryogenesis on Murashige & Tucker (1969) medium with 50 g l^{-1} lactose but no plant growth regulators. These plantlets flowered *in vitro* on half strength Murashige & Tucker (1969) medium containing 50 g l^{-1} sucrose after 2 months culture.

Abbreviations: BA – benzyladenine, 2,4-D – 2,4-dichlorophenoxyacetic acid, FDA – fluorescein diacetate, FM – full strength MT medium, FMG – full strength MT medium + 1 mg l^{-1} GA_3, GA_3 – gibberellin A_3, GA_{4+7} – gibberellin A_{4+7} HM – half strength MT medium, HMG – half strength MT medium + 1 mg l^{-1} GA_3, MT – Murashige & Tucker (1969).

An attempt to use citrus relatives as a source of germplasm appears to be a promising method for rootstock improvement. A wide range of adaptations to climatic and soil conditions as well as other characters may be obtained from citrus and its related genera Aurantioideae. Among the orange subfamily, *Murraya paniculata* may be a source of lime tolerance and resistance to citrus nematode (Bitters *et al.* 1964; Swingle & Reece 1967). 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 (Sykes 1988). Crosses of *M. paniculata* with other genera and species of citrus related genera resulted in failure to obtain hybrids (Iwamasa *et al.* 1988).

Several somatic hybrids via protoplast fusion have been obtained between *Citrus* and some of its wild relatives (Grosser *et al.* 1988; Takayanagi *et al.* 1992; Shinozaki *et al.* 1992). Shinozaki *et al.* (1992) succeeded in producing somatic hybrids between *M. paniculata* and *Citrus sinensis*. In these combinations embryogenic callus with an ability to form somatic embryos was used as a partner in other fusions. The establishment of embryogenic callus of *M. paniculata* will therefore provide a further possibility for making inter-generic hybridization through protoplast fusion. In this paper the establishment of embryogenic callus and the sequence from protoplasts to flowering plant via embryo formation *in vitro* of *M. paniculata* are described.

Fruits of *M. paniculata* were collected 60 days after anthesis. The immature embryos were cultured aseptically on MT medium (Murashige & Tucker 1969) containing 50 g l^{-1} sucrose, 5.0 mg l^{-1} BA, 2.5 mg l^{-1} 2,4-D, 600 mg l^{-1} malt extract and 8.0 g l^{-1} agar. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121°C for 10 min. The cultures were kept under light conditions (35.3 μmol m^{-2} s^{-1} for 16 h daily) at 25°C. After 30 days, embryos developed into plantlets. For callus induction, hypocotyl of plantlets were cut into small segments (2–4 mm), and placed

on fresh MT medium containing the additives listed above. Embryogenic callus induced from hypocotyl segments of *M. paniculata* is friable, soft and pale white in appearance. The callus readily proliferated on the medium containing 50 g l^{-1} sucrose and 10 mg l^{-1} BA and regenerated into embryos after transplanting into medium containing 50 g l^{-1} lactose without plant growth regulators. The frequency of embryo formation from callus pieces cultured for 2 months was 45.8%

About 1 g of embryogenic calluses were pre-cultured in the fresh liquid medium consisting of MT basal medium with 50 g l^{-1} lactose, and incubated on a gyratory shaker at 120 rpm for 6 days at 25°C under 17.7 μmol m^{-2} s^{-1} light for 16 h daily. The callus was mixed with maceration medium consisting of 0.4% macerozyme R-10 (Yakult Pharmaceutical Co., Tokyo), 0.2% cellulase Onozuka (Yakult Pharmaceutical Co., Tokyo) and 0.1% driselase (Kyowa Hakko Kogyo Co., Tokyo). Enzymes were dissolved in a solution composed of half-strength MT inorganic salts and 0.7 M sorbitol as an osmoticum. The maceration solution was adjusted to pH 5.8 and sterilized through a Millipore filter (Millex-HA, 0.45 μm) before use. After 14 h incubation on a reciprocal shaker at 25 rpm in the dark room at 25°C, protoplasts were isolated by filtering through a double layer of Miracloth (Calbiochem, U.S.A), followed by 3 centrifugations at 100 *g* for 5 min in MT inorganic salt solution containing 0.6 M sorbitol. Two ml of protoplast suspension at a density of $3–5 \times 10^4$ ml^{-1} were cultured on MT medium supplemented with 0.0, 200, 400 and 600 mg l^{-1} of malt extract and 0.0, 0.01, 0.10 and 1.00 mg l^{-1} of GA_{4+7}. Cultures were incubated in the dark room at 25°C for 40 days and then kept under light conditions at 25°C. The plating efficiency was evaluated by counting the number of colonies in 20 fields in each petri dish with three replications 40 days after protoplast culture. For the viability test, protoplasts were suspended in 0.01% (w/v) fluorescein diacetate (FDA) and observed under a fluorescent microscope (Olympus model BH-2) at 410 nm wave-length. The cell wall regeneration test was performed by staining with calcofluor white M2R (Nagata & Takebe 1970). Approximately 10^7 protoplasts with diameters of 20 to 50 μm were obtained from 1 g of callus (Fig. 1a).

FDA staining showed that the viability of fresh protoplast was approximately 85%. Seventy % of the surviving protoplasts formed a cell wall within 5 days after culture. The first cell division was observed 13 days after the culture. The formation of small colonies occurred after 32 days of culture (Fig. 1b). The colony formation from protoplasts was improved by the addition of low concentration of GA_{4+7}. For *M. paniculata* the combination of low concentration of GA_{4+7} at 0.01 mg l^{-1} and 600 mg l^{-1} malt extract was the most effective for colony formation (Table 1).

Table 1. Effects of malt extract and GA_{4+7} on percentage of plating efficiency of *M. paniculata* protoplasts.

GA_{4+7} (mg l^{-1})	Malt extract (mg l^{-1})			
	0.0	200	400	600
0.0	13.9a[z]	14.0[a]	15.0a	14.3a
0.01	19.0b	20.0b	19.2b	24.8b
0.10	17.6b	17.5c	18.4b	18.1c
1.00	7.2c	6.ad	7.3c	8.2d

[z] Data were analyzed using the Duncan's multiple range test. Means within a column followed by the same letter are not significantly different at $p = 0.05$.

Calluses derived from protoplasts were transferred onto MT medium containing 50 g l^{-1} lactose and kept under 35.3 μmol m^{-2} s^{-1} light for 16 h daily at 25°C. Protoplast-derived cells developed to form embryos through several stages. Cell colonies became compact and changed into spherical structures, which formed pre-embryos and then developed into embryos. The embryos became heart-shaped forming cotyledon-like structures. The heart-shaped embryos grew into plantlets after 2 months.

When plantlets grew to ca. 2 cm in height, they were used for induction of *in vitro* flowering. Plantlets were transferred onto half-strength MT medium (HM), full-strength MT medium (FM), half-strength MT medium + 1 mg l^{-1} GA_3 (HMG) and full-strength MT medium + 1 mg l^{-1} GA_3 (FMG) containing 50 g l^{-1} sucrose and 8.0 g l^{-1} agar at pH value of 5.6. The plantlets were cultured under 35.3 μmol m^{-2} s^{-1} light for 16 h daily at 25°C or in a dark room at 25°C. Six to ten plantlets were cultured in each treatment in four repeat experiments. After 2 months on the medium, plantlets formed a floral bud on the apical portion. Flowers opened under light conditions (Fig. 1c). Most flowering occurred on half strength MT medium without GA_3 in the presence of light. No flower formation occurred on FMG in the presence of light and on HMG, FM and FMG in the dark (Table 2). Therefore, GA_3 added to the medium suppressed the formation of *in vitro* flowering in cultured plantlets.

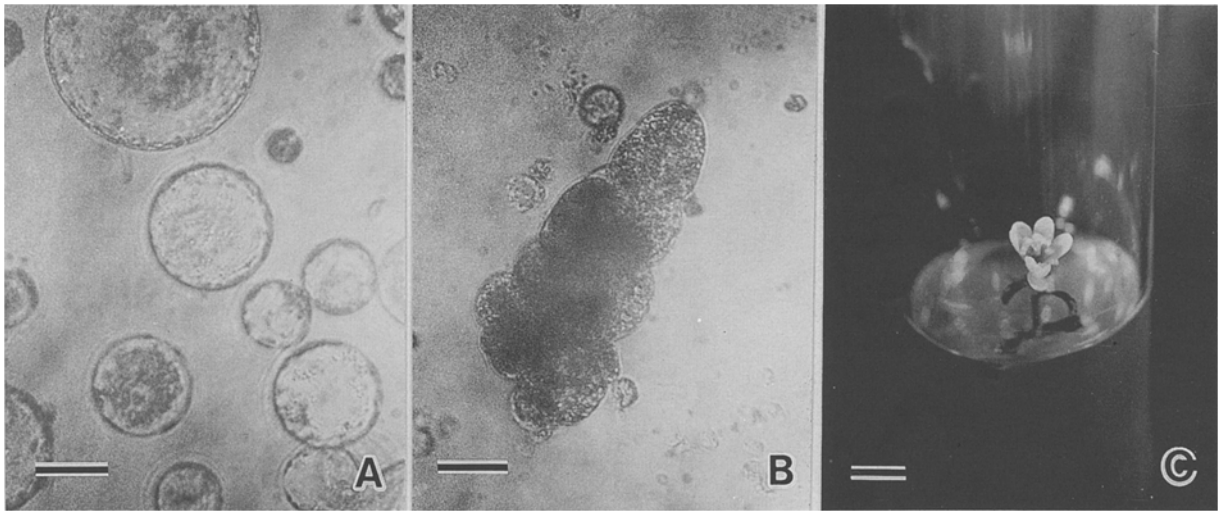

Fig. 1. *(A)* Protoplasts isolated from embryogenic callus (bar = 20 μm). *(B)* Protocolonies composed of several cells after 32 days of protoplast isolation (bar = 30 μm). *(C) In vitro* flowering of plantlet on HM medium containing 50 g l^{-1} sucrose (bar = 0.5 cm).

Table 2. *In vitro* flowering of *M. paniculata* on HM, HMG, FM and FMG media under light and dark conditions.

Medium	Light		Dark	
	No. of plantlet	Plantlet[z] with flower (%)	No. of plantlet	Plantlet[z] with flower (%)
HM	40	60.0±9.1	30	3.1±3.1
HMG	40	10.0±4.1	30	0.0±0.0
FM	40	17.5±4.1	30	0.0±0.0
FMG	40	0.0±0.0	30	0.0±0.0

[z] Values are the mean of four replications (±SE)

The sequence from a protoplast to a whole plant via somatic embryogenesis was established in *M. paniculata* at a high frequency. The mechanism of the precocious flowering on protoplast-derived plants will be examined by this system in future.

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