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Direct somatic embryogenesis from protoplasts of *Citrus mitis* Blanco

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ABSTRACT

Protoplasts isolated from embryogenic suspension cultures of Citrus mitis were cultured in a medium without any plant growth substances. Somatic embryos developed directly from protoplasts without an obvious intervening callus phase. As many as 1,800 somatic embryos developed from 4 ml of protoplast suspension (density 2 x 10^6/ml) cultured for 35 days. Upon transferring the embryoids to medium with 1 mgl^{-1} GA_3, they developed into plantlets. Rooted plantlets were obtained in 3 months after protoplast isolation.

ABBREVIATIONS

BAP: Benzylaminopurine; GA_3: Gibberellic acid; MT: Murashige and Tucker medium (1969); FDA: Fluorescein diacetate.

INTRODUCTION

Protoplast culture of crop plants is of great importance because of the potential for genetic improvement such as somatic hybridization or transformation. In Citrus, a genus of economic importance, plant regeneration from protoplasts of C. sinensis, C. aurantum, C. reticulata, C. limon, C. paradisi and C. yuko has been reported (Vardi et al., 1975; Vardi and Spiegel-Roy, 1982; Kobayashi et al., 1985; Hidaka and Kajiura, 1988). In addition, somatic hybridization of C. sinensis and Poncirus trifoliata was achieved (Ohgawara et al., 1985; Grosser et al., 1988). However, there has been no report on protoplast culture of other Citrus species such as C. mitis, C. grandis or C. nobilis, which are of regional importance, particularly in South and South East Asia. We have conducted a comprehensive tissue culture programme on Citrus mitis, a commercial lime also known as C. microcarpa Bunge. Methods of micropropagation with explants from seedling and mature plant tissues will be reported elsewhere. In this paper, direct somatic embryogenesis from protoplasts derived from nucellar suspension cultures is described.

MATERIALS AND METHODS

Establishment of embryogenic cell suspension cultures: Ovules of Citrus mitis were excised from young fruits (1-month-old, 10 mm in diameter) and placed on MT medium consisting of Murashige and Tucker (1969) nutrient medium supplemented with 5% sucrose, 500 mgl^{-1} malt extract and gelled with 0.2% gelrite. The resulting nucellar callus was subcultured on MT medium at monthly intervals for 1 year.

To establish the suspension culture, 300-500 mg of callus was transferred to 100 ml Erlenmeyer flasks, each containing 20 ml of liquid MT medium supplemented with 5% sucrose, and 10 mgl^{-1} BAP. The cultures were incubated at 24 ± 1°C under light (30 $\mu Em^{-2}s^{-1}$) on a reciprocal shaker at 120 rpm. The cell suspension was subcultured every 2 weeks. Packed-cell-volume of cell suspension was determined by pipetting 10 ml of the suspension and centrifuging at 190 g for 2 min.

Protoplast isolation and culture: Cells pelleted from 10 ml of 8-day-old suspension cultures were used for protoplast isolation. The cell pellet was incubated with 15 ml enzyme solution containing 0.3% cellulase R10 (Kinki Yakult, Japan), 0.3% macerozyme R10 (Kinki Yakult, Japan), 0.1% Driselase (Kyowa Hakko Kogyo, Japan), 0.7 M mannitol and half-strength macroelements of MT medium for 16 h at 30°C in the dark on a rotary shaker at 50 rpm. The protoplast preparations were sieved through miracloth (Calbiochem, USA) and then 94 µm nylon sieve. The protoplasts were washed 4 times by centrifugation (90 g; for 4 min) in culture medium.

A layer of protoplast suspension (4 ml at a density of 2 x 10^6 protoplast/ml) in liquid medium was dispersed over 25 ml of gelrite (0.2%) solidified MT medium in 9 cm petri dish.

The culture medium used was based on MT containing 0.15 M sucrose and 0.45 M glucose. The pH of the medium was adjusted to 5.7 before sterilization through membrane filtration (0.2 µm, Gelman Science). Cultures were then incubated under diffused light (10 $\mu Em^{-2}s^{-1}$) for 16 h per day at 24 ± 1°C.

To detect the presence of cell wall, protoplasts were stained with Calcoflour White according to the method of Nagata and Takebe (1970). FDA was used to determine the viability of protoplasts (Widholm, 1972).

Regeneration of plantlets: Cotyledonary stage embryoids developed from protoplasts were transferred to MT medium supplemented with 5% sucrose, 1 mgl^{-1} GA_3 and solidified with 0.2% gelrite. They were

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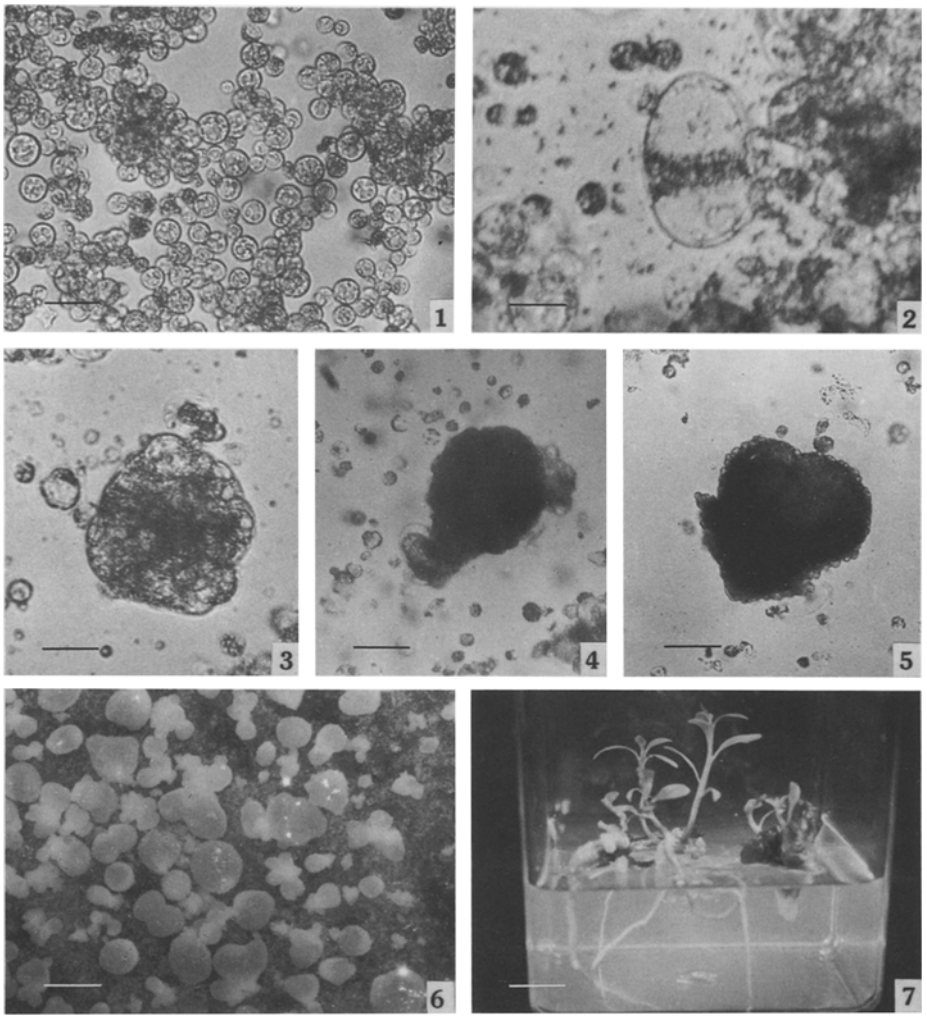

Fig. 1. Freshly isolated protoplasts from suspension culture of C. mitis Blanco, bar = 100 µm. **Fig. 2.** First cell division after 3 days of culture, bar = 54 µm. **Fig. 3.** A microcolony observed after 15 days of culture, bar = 36 µm. **Fig. 4.** A globular embryoid, bar = 80 µm. **Fig. 5.** A heart-shaped embryoid, bar = 80 µm. **Fig. 6.** Abundance of embryoids at different developmental stages, bar = 2 mm. **Fig. 7.** Complete plantlets regenerated from protoplasts 3 months after isolation, bar = 1 cm.

cultured either in GA7 (Magenta Corpn., USA) containers containing 50 ml medium or individually in boiling tubes (25 x 150 mm), each containing 15 ml medium.

RESULTS

The suspension culture comprised of creamy coloured, non-vacuolated and densely cytoplasmic oval shaped cells. The cells developed into globular somatic embryos 9-10 days upon plating on MT medium supplemented with 5% sucrose, 500 mgl^{-1} malt extract and 0.2% gelrite. The globular embryoids continued to grow and reached cotyledonary stage about 20 days after subculture. Complete plantlets with roots were obtained in about 3 weeks after transferring the cotyledonary stage embryoids to MT medium supplemented with 5% sucrose, 1 mgl^{-1} GA_3 and 0.2% gelrite.

The embryogenic potential of the suspension culture was maintained for over a year without diminution by regular subculturing of the suspension. Eight-day-old cultures at the exponential phase of growth period were used for protoplast isolation.

About 4 x 10^7 protoplasts were harvested from 1.0 ml packed-cell-volume of cells after enzyme treatment. Cell wall was totally digested after the 16 h enzyme incubation period as shown by the lack of Calcoflour White fluorescence. FDA staining of protoplasts immediately after enzyme incubation revealed that 86.4 ± 3.06% of the protoplasts remained viable. The size of isolated protoplasts ranged from 20 µm to 94 µm, with the majority in 40-60 µm range (Fig. 1).

Within 24 h after culture, about 80% of the viable protoplasts became slightly elongated, indicating cell wall formation. This was confirmed by Calcoflour White fluorescence. Nearly all viable protoplasts regenerated cell walls within 48 h after culture.

First cell divisions were observed amongst cultured cells as early as 3 days after culture (Fig. 2). Divided cells went through the second division within the next 2-3 days and microcolonies of 98-100 µm were observed in 12 days (Fig. 3).

These microcolonies increased in size, became globular in shape and were white in colour. The globular embryoids turned light green at about 20 days of culture (Fig. 4) and at the same time still more microcolonies appeared. The plating efficiency was 0.0052% on the 21st day of culture.

Green heart-shaped embryoids, measuring about 400 μm in length, developed in 25-day-old cultures (Fig. 5). The plating efficiency was 0.013% after 28 days of culture. In one experiment, 905 embryoids at various stages were observed in one petri dish cultured with 4 ml of 2 x 10^6 protoplasts/ml suspension (Fig. 6). The total number of embryoids increased to 1,828 on the 35th day when some cotyledonary stage embryoids were observed, at the same time, 255 newly formed microcolonies were counted. The plating efficiency was 0.026%.

Upon transferring cotyledonary embryoids to MT medium supplemented with GA_3 (1 mgl^{-1}) and gelrite (0.2%), the embryoids developed further. The roots and first pair of leaves were observed 20-40 days following transfer and complete plantlets with well developed roots were obtained in about 3 months after protoplast isolation (Fig. 7).

About 25-30% of the embryoids, however, grew abnormally with the production of secondary embryoids on their surfaces.

The experiments have been repeated 3 times with similar results.

DISCUSSION

The present study demonstrates that protoplasts prepared from embryogenic suspension cultures of Citrus mitis are capable of developing into somatic embryos without an intervening callus stage. This is similar to earlier reports on Citrus sinensis (Kobayashi et al., 1985) and Foeniculum vulgare (Miura and Tabata, 1986). It is also of interest to note that direct formation of somatic embryos from protoplasts of Citrus mitis was achieved in a medium without any plant growth substances. The absence of plant growth substances in protoplast culture media is also considered to be related to stability of diploidy (Vardi and Spiegel-Roy, 1982).

In our experiments, a higher density of 2 x 10^6 protoplasts/ml was used as compared to 10^4 to 10^5 protoplasts/ml in experiments conducted with C. sinensis (Kobayashi et al., 1985). Although the plating efficiency appeared low as a result of initial high density of protoplasts, the actual number of embryoids obtained was very high. A culture dish with 4 ml of protoplast preparation yielded more than 1,000 somatic embryos in 35 days of culture. The highest yield obtained was 1,828 embryoids. This high yield is rather exceptional not only in Citrus protoplast culture but also amongst other plants (Li and Kohlenbach, 1982; Kobayashi et al., 1985; Miura and Tabata, 1986; Hidaka and Kajiura, 1988). Together with the relatively short regeneration cycle of only 3-4 months (from protoplast to plantlets), the present system is ideal for genetic manipulation experiments and unconventional breeding programmes. Indeed, phenotypic stability found in certain Citrus protoclones is considered to be the result of regeneration through somatic embryogenesis (Kobayashi, 1987).

Further studies to define more precisely conditions for promoting or inhibiting direct somatic embryogenesis from protoplasts of Citrus mitis as well as fusion experiments with related species are now in progress.

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