Annals of Botany **81**: 775–777, 1998

SHORT COMMUNICATION

Factors Affecting Protoplast Culture of *Cucumis melo* 'Green Delica'

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Received: 12 November 1997 Returned for revision: 8 January 1998 Accepted: 13 March 1998

Hypocotyls, cotyledons and etiolated half-expanded leaves of *Cucumis melo* 'Green Delica' were used as explants for protoplast isolation and culture. Protoplasts isolated from cotyledons and etiolated half-expanded leaves cultured in Durand, Potrykus and Donn (DPD) medium supplemented with 0·9 μM benzylaminopurine (BAP), 3·6 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1% sucrose, using the agarose bead culture method, were able to form cell walls and subsequently go through cell division. Pretreatment of half-expanded leaf explants in the dark for 14 d provided the best material for protoplast isolation and cell division. Approximately one third of protoplasts from etiolated half-expanded leaves formed microcolonies. For hypocotyl protoplasts, none of the treatments used were suitable to induce cell division. There was no significant difference between sucrose, glucose, and sucrose plus glucose, in culture media on the plating efficiency of leaf protoplasts of *C. melo* 'Green Delica'; however, bigger colonies were formed in media supplemented with 1% sucrose. No shoot or whole plant regeneration was achieved. However, the methods reported here provide further information on *C. melo* protoplast culture.

Key words: *Cucumis melo*, protoplast culture, 2,4-D, BAP, yeast extract, casein hydrolysate.

INTRODUCTION

Cucumis melo is a valuable commercial vegetable crop which could benefit substantially from characters found in other *Cucumis* species. However, cross breeding through conventional methods e.g. between *C. melo* and *Cucumis metuliferus* (African horned cucumber) is not possible due to a number of incompatibility barriers (Fassuliotis, 1977).

Somatic hybridization can enable gene flow between species which are unable to breed normally. This is dependent on the ability of the protoplasts to regenerate cell walls, undergo cell division and to regenerate into plants. Before protoplasts from two species are fused, factors which influence protoplast survival and division of each partner should be optimized. Some studies on the protoplast culture of *C. melo* have been reported e.g. Moreno, Zubeldia and Roig, 1984; Tabei, 1989; Li, Sun and Zhang, 1990; Debeaujon and Branchard, 1991. However, favourable conditions vary widely with variety. This paper examines some of the variables affecting cell division in *C. melo* 'Green Delica' and 'Fastoso'; these include source of explant, culture method, effect of dark pretreatment, hormone concentration and addition of sugars, yeast extract and casein hydrolysate.

MATERIALS AND METHODS

Plant materials

Seeds of *Cucumis melo* 'Green Delica' (New World Seeds Co., Australia) and 'Fastoso' (Minter Excelgrow Seeds Co., Australia) were surface sterilized with 15% sodium hypochlorite solution (4% w/v available chlorine) for 10 min, and then rinsed three times with sterile distilled water. They were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0·8% (w/v) agar and 2% (w/v) sucrose, and germinated in the dark at 28 °C. Three kinds of explants were used in this experiment: (1) hypocotyls excised from 8-d-old seedlings; (2) cotyledons removed from 8-d-old seedlings and cultured in the dark for 3 d on solid MS medium containing 3% (w/v) sucrose, 0·8% (w/v) agar and supplemented with 1·1 μM NAA and 4·6 μM kinetin; (3) etiolated half-expanded leaves. Excised hypocotyls with their cotyledons attached were transferred to a 16 h photoperiod and cultured on MS medium until the first leaves appeared. These plants were then incubated in the dark for a further 0, 2, 4, 6, 8, 10, 12, 14 and 16 d. Protoplasts isolated from these explants were cultured using the agarose bead method.

Protoplast isolation

Explants (hypocotyls, cotyledons or etiolated half-expanded leaves) were cut into strips approximately 2 mm wide. These tissues were then incubated in the dark with enzyme solution (1 ml 0·2 g^{-1} tissue) consisting of 2% (w/v) cellulase 'Onozuka' R-10 (Yakult Honsha Co. Ltd, Japan) and 0·5% (w/v) macerozyme R-10 (Yakult Honsha Co. Ltd, Japan) in 0·6 M sorbitol–DPD salt medium (Durand, Potrykus and Donn, 1973) (pH 5·8). The flasks were agitated on a shaker for 3 h at a speed of 100 rpm. The

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0305-7364/98/060775+03 $25.00/0 bo980635

crude protoplast extract was filtered through stainless steel mesh (200 μm), and then centrifuged at approx. 100 *g* for 5 min. The supernatant was discarded and the residue resuspended in washing medium (0·6 M sorbitol in DPD medium). The protoplasts were washed three times. Protoplasts were resuspended in 0·5 or 0·6 M sorbitol in liquid DPD medium.

Protoplast culture

Cells were cultured using three methods, namely the agarose bead, hanging drop and thin layer methods. For the agarose bead method, protoplasts (2×10^5 protoplasts ml^{-1} in DPD medium) were mixed with an equal volume of agarose (Sigma) to give a final agarose concentration of 0·6 % (w/v) at 36 °C. The protoplast suspension was pipetted in small droplets into a Petri dish (35×10 mm). After gelling of the agarose, liquid DPD medium was added. In the hanging drop method, protoplasts in liquid DPD medium with a density of 1×10^5 protoplasts ml^{-1} were suspended in small drops (using a pipette) on the inner sides of the lids of Petri dishes. For the thin-layer method, protoplasts in liquid DPD medium at a density of 1×10^5 protoplasts ml^{-1} were placed in Petri dishes (approx. 1 mm depth). Plates were then sealed with Parafilm to reduce the loss of water from the culture medium. The protoplasts were cultured at 25 °C in the dark on a gyratory shaker (Lab-Line) at 50 rpm. To prevent evaporation, Petri dishes in all experiments were surrounded with sterile distilled water.

The agarose bead method was used to investigate the effect of a number of different media components. The DPD medium was supplemented with different carbon sources: either 1 % sucrose, 1 % glucose, 1 % sucrose plus 0·5 % glucose, or 1 % lactose. In addition, seven concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) were assayed (0, 0·9, 1·8, 3·6, 6·3, 9·0 and 13·6 μM), with benzylaminopurine (BAP) kept constant at 1·8 μM. Similarly, seven BAP concentrations were tested (0, 0·9, 1·8, 3·5, 6·2, 8·9 and 13·3 μM), with 2,4-D kept constant at 6·3 μM. The effects of adding yeast extract and casein hydrolysate at a concentration of 0·1 % (w/v) were also tested.

Cell wall formation was determined using the Calcofluor White staining technique (Constabel, 1982) and observations made with an Olympus fluorescent microscope. After 2 weeks, cells were observed under a light microscope, and counted as the number of cells which were able to undergo cell division.

RESULTS AND DISCUSSION

Culturing method

The correct choice of culturing method was the most important factor in the culture of protoplasts. Aggregation of protoplasts was observed when using the hanging drop and thin layer methods. In contrast, the agarose bead method was found to be suitable for cotyledon and leaf protoplasts of 'Green Delica' to form cell walls and divide. This method was used for all subsequent experiments.

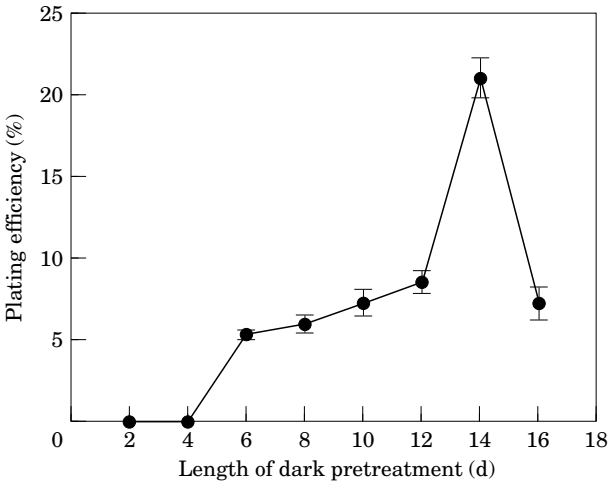

FIG. 1. The effect of dark pretreatment of leaf explants of *C. melo* 'Green Delica' on protoplast plating efficiency. Data are presented as the number of cells having undergone division, expressed as a percentage of the total. Results represent the mean of three separate experiments with ten readings in each ± s.e. Protoplasts were cultured in agarose beads containing DPD medium supplemented with 1 % sucrose and 3·6 μM 2,4-D plus 0·9 μM BAP.

Effect of light prior to isolation on the plating efficiency of leaf protoplasts

Protoplasts from untreated leaves or those pretreated in the dark for only 2 d were not able to divide. It tended to be more difficult to isolate protoplasts of these leaves. After several days of culture these protoplasts normally did not survive (Fig. 1). Cells from leaf tissue had a greater capacity to divide as the tissue was kept in the dark for longer (between 4 and 14 d). Dark pretreatment also increased protoplast yield. There was a significant enhancement ($P < 0{\cdot}05$) of plating efficiency as the period of dark incubation of the explant increased (Fig. 1). The highest plating efficiency (21 %) was found in protoplasts from explants pretreated for 14 d.

Source of protoplasts

The source of explant of *C. melo* 'Green Delica' strongly influenced the ability of protoplasts to divide. Around 35 and 33 %, respectively, of protoplasts from cotyledon and leaf (14 d pretreatment) tissue of 'Green Delica' underwent cell division, but none of the hypocotyl protoplasts cultured under various conditions could divide, or even form a cell wall. In a separate experiment, fine calli were obtained from hypocotyl tissue by culturing directly on MS medium supplemented with auxin and cytokinin. It is therefore clear that intact cells within tissue have the capacity for cell division but removing cell walls caused this ability to be lost.

The first division of protoplasts isolated from cotyledon explants occurred after 8 to 9 d of culture in the dark, while etiolated leaf protoplasts took 5 to 6 d to divide. Furthermore, colonies derived from leaf protoplasts tended to be bigger. Microcolonies derived from protoplasts were

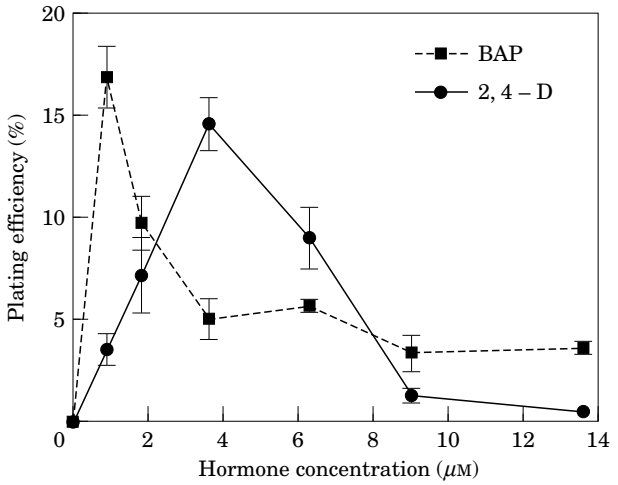

FIG. 2. The effect of different concentrations of BAP and 2,4-D on the plating efficiency of leaf protoplasts of *C. melo* 'Green Delica'. During testing of the effect of 2,4-D concentration BAP was held at 1·8 μM. When testing the response to BAP, the 2,4-D concentration was held at 6·3 μM. The media were also supplemented with 1% sucrose, 0·5% glucose and 0·5 M sorbitol. Data are presented as the number of cells having undergone division, expressed as a percentage of the total. Results represent the mean of three separate experiments with ten readings in each ± s.e.

visible after 14 d of culture. The different lengths of time required by the protoplasts to undergo cell division may relate to the observation that protoplasts isolated from cotyledon explants tended to have less cytoplasm and more vacuoles than leaf protoplasts.

None of the protoplasts isolated from hypocotyl, cotyledon or leaf explants of the variety 'Fastoso' were able to undergo cell division under the conditions tested. Budding occurred on these protoplasts. High rates of seedling growth did not seem to be correlated with protoplast division, since 'Fastoso' seeds can grow faster *in vitro* or *in vivo* than 'Green Delica'.

Effects of sugars, yeast extract and casein hydrolysate on the plating efficiency of leaf protoplasts

The effects of using different sugars as a carbon source were tested; no significant differences in plating efficiencies were seen between the sucrose, glucose, and sucrose plus glucose treatments. However, leaf protoplasts divided faster and formed bigger colonies on media supplemented with 1% sucrose. It is suggested that sucrose may be taken up and used more easily by the etiolated leaf protoplasts. Increasing the concentrations of sucrose or glucose did not enhance cell division. Using lactose resulted in low plating efficiencies of leaf protoplasts of 'Green Delica', although cell division still occurred.

Although preculture with media supplemented with 0·1% yeast extract was found by Moreno *et al.* (1984) to be essential for the culture of *C. melo* leaf protoplasts, our results did not show this. Furthermore, addition of yeast extract to the culture medium resulted in a greatly decreased plating efficiency (2·6%). Addition of 0·1% casein hydrolysate to the culture media had no effect on plating efficiency.

Effect of 2,4-D and BAP on the plating efficiency of leaf protoplasts

Both an auxin and a cytokinin are commonly required to induce cell division and callus formation. A concentration of 3·6 μM 2,4-D appeared to be best for the division of leaf protoplasts of 'Green Delica', in the presence of 1·8 μM BAP (Fig. 2). The ability of cells to divide decreased at 2,4-D concentrations of 6·3 μM and higher. At concentrations of 2,4-D greater than 9·0 μM protoplasts enlarged.

When 6·3 μM 2,4-D was present in the media, a concentration of 0·9 μM BAP was found to be optimal for the culture of leaf protoplasts of 'Green Delica'. At high concentrations of BAP (more than 3·5 μM) protoplast survival decreased due to the formation of disorganized enlargements in the protoplasts and plasma membrane disruption.

CONCLUSION

In this experiment on *C. melo*, etiolated half-expanded leaves of 'Green Delica', which had been pretreated in the dark for 14 d, provided the best source of explants for successful protoplast culture. They were able to divide and form microcolonies in the DPD medium using agarose bead culture. Optimum results were obtained by supplementing the medium with 0·9 μM BAP, 3·6 μM 2,4-D and 1% sucrose (one third of protoplasts formed microcolonies). Conditions were dependent on variety. Protoplasts derived from the cultivar 'Fastoso' failed to divide under any of the conditions used. Further research needs to be carried out to define suitable environments in which microcolonies can grow and develop into plantlets.

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