

Plant regeneration from protoplasts of *Capsicum annuum* L. cv. California Wonder

A H PRAKASH*, K SANKARA RAO[†] and M UDAYA KUMAR**

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

*Central Institute for Cotton Research, P O Box No. 225, GPO, Nagpur 440001, India

**Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore 560065, India

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Abstract. Plant regeneration from mesophyll protoplasts of pepper, *Capsicum annuum* L. cv. California Wonder has been demonstrated via shoot organogenesis. Protoplasts isolated from fully expanded leaves of 3-week-old axenic shoots when cultured in TM medium supplemented with 1 mg l⁻¹ NAA, 1 mg l⁻¹ 2,4-D, 0.5 mg l⁻¹ BAP (CM I) resulted in divisions with a frequency ranging from 20-25%. Antioxidant ascorbic acid and polyvinylpyrrolidone (PVP) in the medium and incubation in the dark helped overcome browning of protoplasts. Microcalli and macrocalli were formed in TM medium containing 2 mg l⁻¹ NAA and 0.5 mg l⁻¹ BAP (CM II) and MS gelled medium containing 2 mg l⁻¹ NAA and 0.5 mg l⁻¹ BAP (CM III), respectively. Regeneration of plantlets was possible via caulogenesis. Microshoots, 2-5 per callus appeared on MS gelled medium enriched with 0.5 mg l⁻¹ IAA, 2 mg l⁻¹ GA and 10 mg l⁻¹ BAP (CM IVc). Rooting of microshoots was obtained on half strength gelled medium containing 1 mg l⁻¹ NAA and 0.5 mg l⁻¹ BAP. Protoplasts isolated from cotyledons failed to divide and degenerated eventually.

Keywords. Leaf protoplasts; *Capsicum annuum* L. cv. California Wonder; regeneration; organogenesis.

1. Introduction

The genus *Capsicum* includes five cultivated and twenty six wild species. *Capsicum annuum* L. (pepper) is the most extensively cultivated species of *Capsicum* and constitutes an important economic crop of India. Improvement of peppers through transfer of morphological traits from wild to cultivated species by conventional hybridization has yielded some success (Tanksley and Iglesias-Olivas 1984). Traits of agronomical and economic importance such as increased yield by multiple flowering and disease resistance have been successfully transferred from *C. chinensis* to *C. annuum* (Berkmortel 1977), whereas in most other cases, interspecific incompatibility and/or F₁ hybrid sterility are blocking further improvement. In very few cases, these incompatibility barriers have been overcome by embryo rescue methods (Pundeva and Zagorska 1984). Somatic hybridization provides an alternative method to conventional hybridization for recombining desirable traits among species. Somatic hybridization, however, depends largely on the success of regeneration of plants from protoplasts. *Capsicum* has been a difficult genus from which to isolate and culture protoplasts. Reports indicate that even the calli obtained from either the cultured protoplasts (Niedz *et al* 1987;

[†]Corresponding author (Fax, 091-80-3341814; Email, baradwaj@biochem.iisc.ernet.in).

Donato *et al* 1989; Nishio *et al* 1989) or directly from tissue explants (Ochoia-Alijo and Ireta-Mereno 1990; Christopher *et al* 1991) are recalcitrant. To date, only two reports of whole plant regeneration from protoplasts of peppers have been published (Saxena *et al* 1981; Diaz *et al* 1988). In the present study, plant regeneration from mesophyll protoplasts of *C. annuum* cv. California Wonder has been demonstrated and the regenerability of protoplasts derived from different explants tested.

2. Materials and methods

Seeds were surface sterilized by soaking in bavistin solution for 2 h, followed by 0.1% (w/v) mercuric chloride treatment for 1 min and rinsed thoroughly in autoclaved double distilled water. They were incubated for germination on filter paper discs. The shoot portion of the seedlings was excised and maintained on half-strength MS medium (Murashige and Skoog 1962) containing 0.2 mg l⁻¹ kinetin. Cultures were incubated at 25 ± 1°C under a 14 h photoperiod with a fluorescent light of intensity 200 µEm⁻² s⁻¹. Hypocotyl and cotyledonary explants from 2-week-old axenic shoots and fully expanded leaves from 3-week-old shoot cultures were used for protoplast isolation.

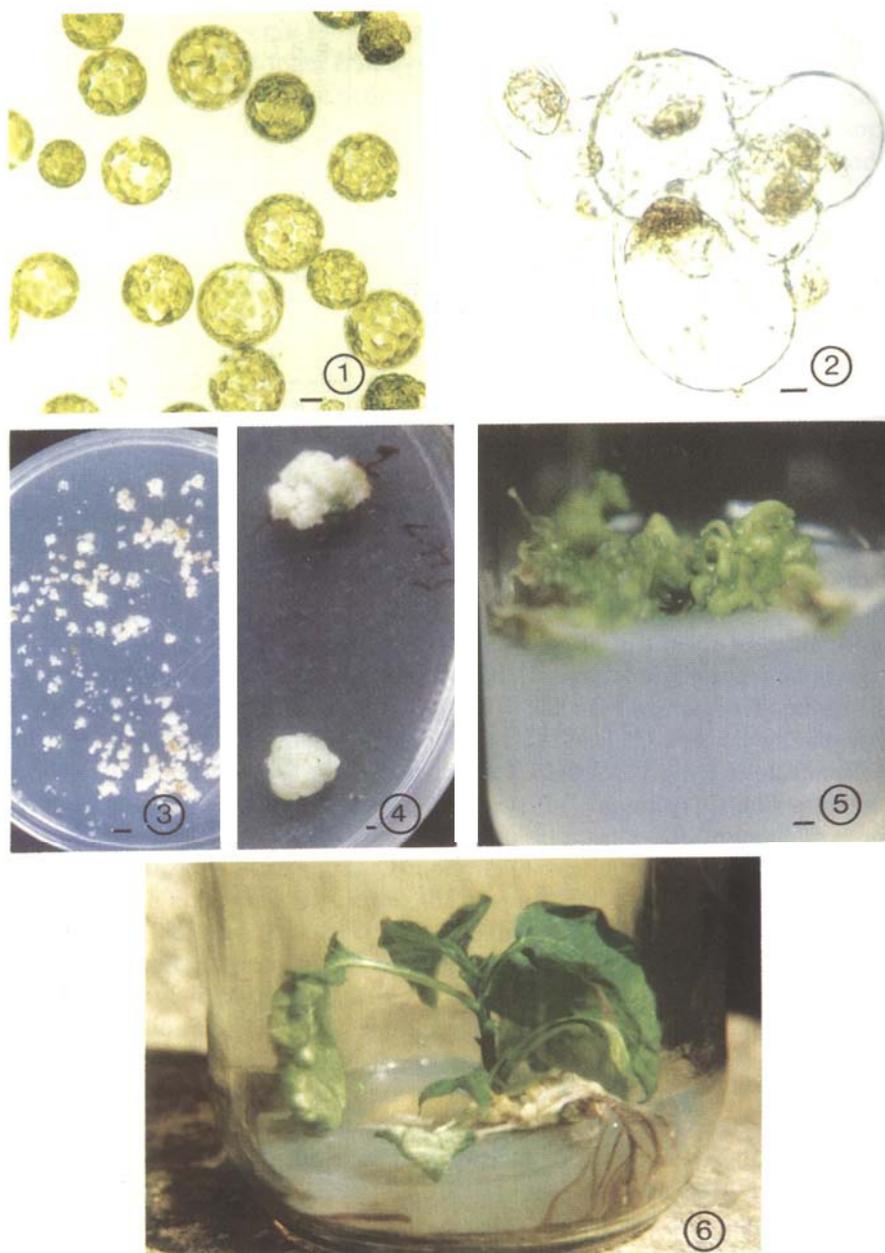
The plant material was dark-preincubated at 4°C for 18 h prior to explanting for enzymatic digestion. Explants were cut into small (2-3 mm long) pieces and incubated overnight in digestion medium containing CPW salts (Power and Chapman 1985), mannitol (9.0%), kinetin (0.2 mg l⁻¹), ascorbic acid (1 mg l⁻¹) and cell wall degrading enzymes, cellulase R10 (2.0%) and macerozyme R10 (0.5%) from Yakult Honsha Co Ltd., Japan, with and without agitation. Earlier, the amount (%) and ratio of the enzymes to be used for effective protoplast release were determined. Protoplast isolation was carried out at 25 ± 1°C. Osmolarity of the digestion mixture was adjusted to 550 mOsmols. Following digestion, the protoplasts were purified as described earlier (Sankara Rao and Prakash 1995). The different culture media (CM) used for protoplast culture were designed as detailed in table 1. Growth regulators, indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxy-acetic acid (2,4-D), gibberellic acid (GA₃), 6-benzylaminopurine (BAP), kinetin (kin) and zeatin (zea) were added to the medium prior to autoclaving at 1.1 kg cm² and 121°C for 15 min. Antioxidant ascorbic acid (1 mg l⁻¹) and polyvinylpyrrolidone (PVP) at 0.1% were added to culture media. Osmotic concentration of different media was gradually reduced (table 1) at different stages as protoplast culture progressed. Osmolarity was adjusted using an osmometer (Osmomat 030, Gonotec, Germany). All experiments conducted were repeated thrice with five replicates maintained each time. The purified protoplasts were transferred to CM I at a plating density of 10⁴ protoplasts per ml in Petri plates. The protoplasts were dark-incubated for 25 days and later exposed to diffuse light. Before plating, the viability of freshly isolated protoplasts was tested using fluorescein diacetate (Widholm 1972). In place of the usual method that uses calcofluor white (Galbraith 1981) to monitor the cell wall synthesis, a novel method of plasmolysis was utilized for the first time. The protoplasts were aliquoted for plasmolysis in 0.8 M mannitol solution and observed for wall formation. Following incubation for 7 days, 100 µl of CM I was added afresh at intervals of three days. The plating efficiency was determined at the end of three weeks. After 25 days, half the volume of spent medium was removed and an equal volume of CM II was added.

Table 1. Protoplast culture and regeneration of *C. annuum* cv. California Wonder—Media, culture conditions and responses.

Source tissues	Protoplasts			Cell colonies (microcalli)			Macrocalli			Organogenesis			Root induction		
	Culture medium	Period of culture (days)	Plating efficiency (%)	Culture medium	Period of culture (days)	Culture medium	Period of culture (days)	Culture medium	Period of culture (days)	Culture medium	Period of culture (days)	Culture response	Culture medium	Period of culture	
	CM I	30	—	—	—	—	—	—	—	—	—	—	—	—	
Mesophyll	CM I	25	20–25	CM II	20	CM III	20	CM IVa	25	Embryoids*	—	—	—	—	
								CM IVb	25	Calli	—	—	—	—	
								CM IVc	25	2-5 shoots	—	—	CM V	25	
								CM IVd	25	Calli	—	—	—	—	

CM I, Tomato medium (Shahin 1985) + 9% mannitol + 0.5% sucrose + 1.0 mg/L NAA + 1.0 mg/L 2,4-D + 0.5 mg/L BAP; CM II, Tomato medium + 6% mannitol + 0.5% sucrose + 2.0 mg/L NAA + 0.5 mg/L BAP; CM III, MS medium + 2.4% mannitol + 1.4% sucrose + 2.0 mg/L NAA + 0.5 mg/L BAP + 0.6% Agar; CM IVa, MS medium + 2.0% sucrose + 0.5 mg/L IAA + 2.0 mg/L Zea + 0.6% Agar; CM IVb, MS medium + 2.0% sucrose + 0.5 mg/L IAA + 1.0 mg/L BAP + 0.6% Agar; CM IVc, MS medium + 2.0% sucrose + 0.5 mg/L IAA + 2.0 mg/L GA + 10.0 mg/L BAP + 0.6% Agar; CM IVd, MS medium + 2.0% sucrose + 0.5 mg/L IAA + 2.0 mg/L GA + 10.0 mg/L Kin + 0.6% Agar; CM V, $\frac{1}{2}$ MS medium + 2.0% sucrose + 1.0 mg/L NAA + 0.5 mg/L BAP + 0.6% Agar.

*Embryoids that did not germinate into plants.



Figures 1-6. Plant regeneration from mesophyll protoplasts of *C. annuum*. (1) Freshly isolated protoplasts (bar = 10 μm). (2) Early divisions of a protoplast. (3) Microcalli on gelled medium. (4) Macrocalli. (5) Differentiation of shoots. (6) Regenerated plant.

The microcalli formed in CM II were transferred to CM III solid medium gelled with bacteriological grade agar (HI-Media, Bombay). After 65 days of incubation under $200\mu\text{Em}^{-2}\text{ s}^{-1}$ of light intensity for 16h photoperiod, the macrocalli formed were exposed to CM IV for shoot organogenesis. The microshoots formed were transferred to CM V for root induction.

3. Results and discussion

Protoplast yields varied with the source tissue used. Sufficient yields of protoplasts could not be obtained from hypocotyl explants, even after overnight incubation, while the cotyledon and leaf tissues yielded 1×10^5 and 8×10^8 protoplasts/gFW respectively. An overnight exposure to cell wall digesting medium resulted in enhanced yield and ensured complete digestion of cell walls. The yields were not affected by agitation during incubation for protoplast release. Therefore, protoplast isolation was carried out without agitation. The freshly isolated protoplasts (figure 1) maintained 70–75% viability. The plating density at 10^4 protoplasts m^{-1} was found to be ideal for culture. The cultivar California Wonder released large amounts of polyphenols into the medium which lead to browning of protoplasts. Similar observation was made earlier with other cultivars of *C. annuum* viz., Americano and Dulce Italiano (Diaz *et al* 1988). Antioxidant ascorbic acid and PVP were used to overcome this problem as was reported for tomato (Shahin 1985). We found that continuous presence of antioxidants and dark incubation for 25 days initially, controlled browning in our experiments.

Cell wall formation occurred two days after the protoplasts were plated in CM I. A novel method of subjecting protoplasts to plasmolysis was utilized to monitor cell wall synthesis. A circular ring observed around the plasmolyzed protoplast indicated the cell wall regeneration.

During the first few days of culture, the mesophyll protoplasts expanded as a prelude to cell division. The first division occurred 7–8 days after culture, second and subsequent divisions ensuing after 12 days. The plating efficiency was 20–25%, which is comparable to that obtained in *C. annuum* cv. Americano and Dulce Italiano by Diaz *et al* (1988).

The microcalli were formed by about 45 days in CM II (figure 2) and when transferred to CM III solid medium (figure 3) formed macrocalli by about 65 days (figure 4). Calli turned green and formed 2-5 microshoots per callus on CM IVc after 90 days of culture (figure 5). The microshoots excised and transferred to CM V for root induction, formed roots and developed into plantlets (figure 6). Experiments are underway to transfer the plantlets to green house.

Although the cotyledonary protoplasts formed cell wall and expanded into dumbbell-shaped cells, failed to divide and form cell colonies. The cells degenerated with time.

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