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RESEARCH ARTICLE

PROTOCOL FOR QUICK ISOLATION AND PURIFICATION OF *CAPSICUM* PROTOPLASTS USING LOWER CONCENTRATIONS OF MACEROZYME AND CELLULASE

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Abstract

Capsicum protoplast isolation protocols till to date is involved prolonged incubation with higher concentration of macerozyme and cellulase in the digestion solution. In this study we have developed a protocol for quick (4½ h) isolation of protoplasts using lower concentration of macerozyme and cellulase, further we developed a simple protocol for purification of protoplasts. We obtained 5.8 x 10^5 Protoplasts/ gram yield by using lower concentrations of macerozyme (0.2%) and cellulase (1%).

INTRODUCTION

Plant regeneration from chilli pepper cells is in its infancy. The importance of pepper protoplast cultures resides in the possibility of recovering either somaclonal variants or somatic hybrids in cases of sexual interspecific incompatibility. Somatic hybridization might provide an alternative method to conventional hybridization for improving *Capsicum*. Protoplast isolation, culture and regeneration of pepper plant (*C. annum* v. *California wonder*) was first reported by Saxena *et al*. (1981). Protoplasts were isolated from leaves of axenic shoot cultures (Saxena, 1981), and shoot cultures (Diaz *et al*., 1988). It has also shown that varieties of a given species differ markebly in their cultural requirements (Power *et al*., 1976; Bohorova *et al*., 1986). An improved regeneration of protoplast cultures of *C. annuum* var. California wonder reportd by Prakash *et al*. (1997).

Withers and Street (1977) reported on the capacity of *C. annuum* cell cultures to survive to freezing temperatures after pre treatment of somatic cells with mannitol. *Capsicum annuum* cell lines capable of growing in liquid media containing 1 or 2 % sodium chloride were isolated by Dix and Street (1975). Dix and Street (1976) also selected pepper cell lines with enhanced chilling resistance. Treatment with EMS promoted the isolation of stable resistant cell lines. Cell clones of chilli pepper with enhanced resistance to osmotic stress were isolated by Santos-Diaz and Ochoa-Alezo(1994).

Capsicum protoplast isolation protocols till to date is involved in prolonged incubation with higher concentration of macerozyme and cellulase in the digestion solution.Quick isolation of viable protoplasts is an essential requirement for the crop improvement through protoclonal variation. In this study we have developed a protocol for quick (4½ h) isolation of protoplasts using lower concentration of macerozyme and cellulase, further we developed a simple protocol for purification of protoplasts.

Materials and method:

The seeds of *Capsicum annuum* L. Cv G_4 were obtained from Lam-Farm, Chillies Research station, Guntur, Andhrapradesh. The seeds were first imbibed for 4-6 hrs in sterile distilled water. Then seeds were surface sterilized with 0.1 % Mercuric chloride (Hg cl_2) for 4 min. after appropriate sterilization, the sterilizing agent is decanted and the seeds are thoroughly washed three times with sterile distill water and dried on a sterile filter paper. Dried seeds were allowed to germinate in glass bottles with screw caps containing 50 ml of MS basal medium. 3 week old seedling shoot tips along with cotyledons (2 cm length) will taken and planted in culture bottles containing rooting medium. Shoot tips have developed in to healthy plantlets on the above medium. Cotyledons and leaves from 3 week old rooted plants were used for the study.

Protoplast Culture Medium (PCM):

For culturing of protoplasts, prepared a medium having modified macro and micro nutrients of MS medium additionally supplemented with PC vitamins, indole acetic acid (0.5 mg/L) and BAP (3 mg/L), sucrose and glucose were added as osmoticum (Table-1). This PCM was filter sterilized and used in several steps.

Protoplast Isolation Medium (PIM):

The digestion solutions (enzymes) were prepared in protoplast culture medium (PCM) additionally supplemented with 0.4 M sucrose as osmoticum and pH was adjusted to 5.8 with NaOH. The PIM was filtere sterilized and used in the incubation process.

Alginate Solution:

Morpholino Ethane Sulphonic acid (MES 7mM); $MgSO_4$ (10 mM); MgCl2 (10 mM); Alginate (2.4%) were dissolved in 100 ml of water and was filter sterilized. Mannitol was used as osmoticum.

Ca^+ Agar Stock:

Calcium Chloride (20 mM), MES (10 mM) were dissolved in 100 ml of water and Mannitol was used as osmoticum. This stock was filter sterilized and used in the experiment.

Methodology:

Leaves from three week old plants grown aseptically were gently sliced into 1 mm strips in 2ml of PIM. Incubation was carried out in 10 ml of digestion solution containing different levels of PIM, macerozyme and cellulase (mixed from their respective stock solutions under sterile conditions) with 0.4 M sucrose as osmoticum. Flasks were incubated at 27^0 C and 50 rpm for about 3-9 hrs in dark. After incubation, protoplasts were passed through stainless steel sieve of 60 µ. Filtrate was collected in a screw cap centrifuge tube and over layered with 2 ml of filter sterilized PCM containing PIM + 1.0 mg/L BAP + 0.1 mg/L NAA + sucrose 20 g/l + Glucose 65 g/L adjusted to a pH 5.8 and centrifuged for 10 min at 70 rpm(Step I = sedimentation).. This resulted in the formation of two distinct bands. Both the bands and also the pellet were collected with Pasteur-pipettes in separate centrifuge tubes and the volume of each was adjusted to exactly 10 ml with PCM. Contents were carefully mixed once by inverting the tubes and then centrifuged for 10 min at 50 rpm (Step II = sedimentation). Pellets were resuspended in 3 ml of PCM and were used for microscopic investigations. The yield of protoplasts was determined with a hemocytometer. Isolated protoplasts were cultured with an equal volume of PCM.

Isolated protoplasts were gentle mixed with alginate solution in a ratio of 1:1. 625 µl of the above mixture is loaded on to Ca^{+2} agar plate and immediately plastic grid was placed on the mixture and allowed to stand for 30 min. to 1 hr for solidification. The solidified grid along with the protoplast embedded inside it was removed and cultured in 10 ml PCM taken in petridishes.

Results and Discussion:

The number of protoplasts isolated from mesophyll tissue of *Capsicum* after being treated with macerozyme and cellulase increased upto 4½ hr of incubation, beyond4½ hr. incubation, the protoplast yield gradually decreased and further resulted in complete shrinkage of protoplasts at 8-9hr of incubation. The same result is also find in increasing macerozyme and cellulase concentrations, when filtrate was centrifuged at 70 rpm for 10minutes.

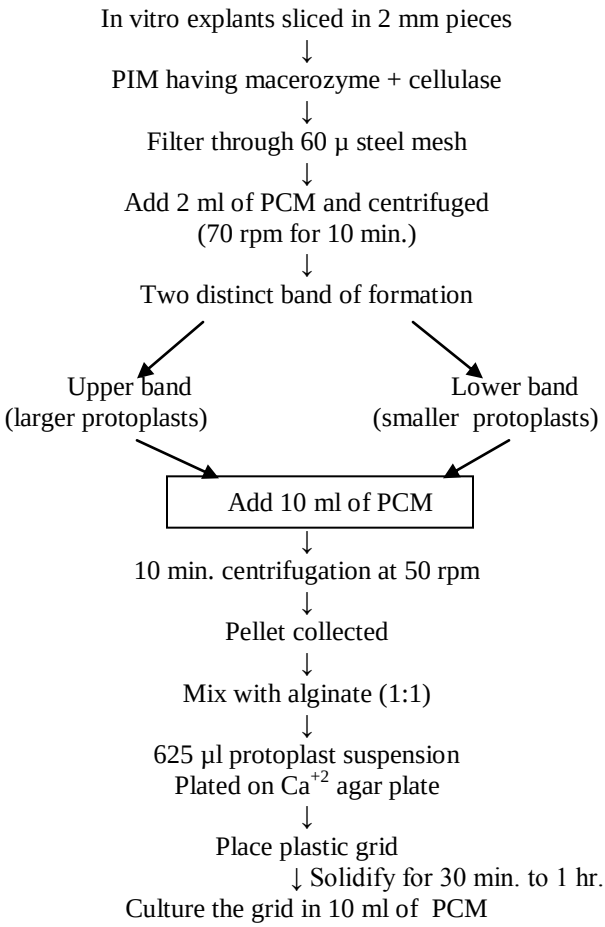
We obtained the best result of 5.8×10^5 protoplasts/ gram yield by using lower concentrations of macerozyme (0.2%) and cellulase (1%). Axenic shoot cultures were used for the isolation of mesophyll protoplasts. Leaves from *in vitro* grown plants were always found to be more promising for protoplast isolation than those of *in vivo* plants (Pavan *et al*., 2000). Saxena et al., (1981) reported the isolation of protoplasts from axenic shoot cultures of *Capsicum annuum* L. cv. California wonder by using 0.4% macerozyme and 2% cellulase. Diaz *et al*. (1988) isolated leaf protoplasts from shoot cultures established on MS medium without growth regulators of *Capsicum annuum*, shoot were plasmolysed and then treated with an enzyme mixture of 1% cellulase and 0.25%

macerozyme. Incubation was carried out for 14 h in dark. A protocol for protoplast isolation from leaf tissue of three cultivars of *Capsicum annuum* and two genotypes of *C. chinense* was developed (Murphy and Kyle, 1994).Plant regeneration from mesophyl protoplasts of chilli pepper, *Capsicum annuum* L. cv. California Wonder, was reported by Prakash et at. (1997).Both the larger and smaller protoplasts showed distinct division and differentiation in protoplast culture medium, whereas protplasts isolated from cotyledones failed to divide.

TABLE-1: **Composition of *Capsicum* protoplast culture medium(PCM)**

CONSTITUENTS	Amount in 1000ml Distilled water
Macro MS-modified	
KNO3(10mM)	1012mg
CaCl2.2H2O	440mg
MgSO4.7H2O	370mg
KH2PO4	170mg
NH4 Succinate (20mM)	10mg
Micro MS	10ml
PC-Vitamins	10ml
Sucrose	20g
Glucose	65g

Schematic presentation of isolation and purification method of capsicum protoplasts



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