Isolation and culture of protoplasts from leaf tissue of *Capsicum annnum* var. *accumnatum* Fingerh and *C. frutescens* L. [Syn. *C. minimum* Roxb.] (Bird chilli)

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Abstract

Isolation and culture of leaf protoplasts from two chilli cultivars (*Capsicum annuum* var. *accumnatum* and Bird chilli) were developed to enhance selection process in the somatic hybridization programmes. In order to isolate the protoplasts from leaves of these two chilli cultivars different incubation periods (3, 5 and 10 hours) were tested with combinations of enzyme mixtures containing cellulase and macerozyme. Leaves were incubated on three enzyme mixtures (2% cellulase + 0.4% macerozyme, 1% cellulase + 0.2% macerozyme and 0.5% cellulase + 0.1% macerozyme in 13% mannitol) at 251oC in the dark. Three hours of incubation using 2% cellulase and 0.4% macerozyme was the best for the protoplast isolation of both chilli cultivars tested. The yield was 5 x 108protoplasts/ml/g leaf tissue in both chilli varieties. It was found that in the mixed nurse method using Nagata and Takebe (NT) medium supplemented with 1.0mg/l 2,4-D, NAA and BAP with 0.5M mannitol and 1.2% Sea Plaque agarose is the best medium for protoplast culture. Protoplasts of *Capsicum annum* var. *accumnatum* were alive for 14 days forming cell walls and initiating cell division.

Key words: Protoplast culture. Chilli, sea plaque agarose

Introduction

Chilli (*Capsicum annuum* var. *accuminatum*Fingerh) is one of the important spice crops in Asia. Production has not been sufficient to meet the local demand due to number of reasons.

Disease susceptibility is one of the major problems in these cultivars. Production of disease resistant chilli varieties is possible through time consuming and laborious traditional breeding methods. However conventional crop improvement techniques should be supplemented with new biotechnological tools in order to expedite the selection process.

Among various strategies for genetic improvement, somatic hybridization appears to be a promising complement to classical breeding since protoplasts are amenable to plant regeneration (Assani *et* al, 2002). Protoplasts from number of dicotyledonous plants been regeneratedinto fertile plants. Solanaceae species pose the smallest problem in regeneration. The aggregation of two or more protoplasts is not enough to start a fusion. Protoplast surfaces bear strong negative charges due to phosphate groups. Intact protoplasts in suspension do thus repel each other. They can be linked and fused by the addition of calcium ions or PEG into the medium. Interspecific fusions can be generated rather easily. In order to further development of somatic hybridization within the genus, experiments were set up to develop a suitable protocol for protoplast isolation and culture.

Material and methods

Isolation of Protoplasts from Leaf Mesophyll Cells

a). Maintenance of plants:

Seeds of two chilli cultivars were sown aseptically on growth regulator free MS medium and incubated in dark for germination and then were kept at 271oC under cool-white fluorescent lights of 1000 lux illumination for 2 weeks for seedling establishment. After two weeks, seedlings were transplanted in propagator box and later transferred to pots and grown under green-house conditions. Plants were maintained under pathogen- free environment and sprayed with benlate twice a month.

b). Sample collection:

When eight-weeks old, the seedling was kept in dark for 24 hours and young fully expanded leaves were excised. 0.2g of the leaves was taken and surface sterilized with 3% NaOCl for 15 minutes and then with 70% ethanol for 1 minute. Each washing was followed by three successive washings with sterile water. They were then dried with sterile filter paper. The lower

epidermis was carefully removed completely with a sterile pair of forceps and cut into 2mm squares and placed lower surface down onto 10ml solution of mannitol (13%) and CPW solution. After 2 hour, mannitol and CPWsolution was removed using a pasture pipette and was replaced with filter sterilized enzyme mixture that contains cellulase and macerozyme at different concentrations (Table 1). Samples were incubated at 25oC for different time periods (3, 5 and 10 hours).

Table 1: Concentrations of enzymes (cellulase and macerozyme) tested on two varieties of Chilli

Treatment code	% Cellulase	% Macrozyme
T1	2.0	0.4
T2	1.0	0.2
T3	0.5	0.1

c). Purification of isolated protoplasts:

After incubation in cellulase and macerozyme for different time periods, leaves were agitated with sterile forceps. Larger pieces of leaf material was separated to one side of petridish by keeping at an angle of 15 degrees. The enzyme protoplast mixture was then transferred into a conical tip centrifuge tube and centrifuged at 50g for 10 minutes. The soft pellet in the bottom of tube was resuspended in a mixture of CPW and 20% sucrose. Mixture was re-centrifuged at 50g for 10 mins. This procedure was repeated two or three times till obtain a relatively clean protoplast preparation. Prepared protoplasts were later tested for the density and the viability.

d). Determination of viable protoplast plating density:

Before plating of protoplasts in culture medium, viable protoplast density of purified protoplast was determined. Purified protoplasts (one or two drops) were stained with 1% Evan's blue solution for 5 mins. The dead or dying protoplasts stained in blue, whilst those with an intact outer membrane will remain unstained. An aliquot of 2l was placed in 0.1mm

depth haemocytometer and number of viable (green) cells were counted in order to calculate the number of viable cells/ml (viable protoplast density).

e). Protoplast culture:

As a prelude to protoplast fusion, protoplast culture of two varieties was tested using different methods.

Method 1: Modified method for chilli varieties (Saxena *et al.*, 1981)

Protoplasts were cleaned by repeated centrifugation at 55g employing a culture medium which contained mineral salts of NT supplemented with 1.0mg/l 2,4-D, NAA (naphthalene acetic acid), BAP (benzyl amino purine), 2% sucrose and 0.5M mannitol. pH of the medium was adjusted to 5.7. The density of the suspension of protoplasts was adjusted 5x106/ml. 1ml of the protoplast suspension in theprotoplast medium (NT) was mixed with an equal volume of molten agarose medium (1.2% sea plaque agarose) in a plastic petridish. Then petridish was swirled gently to evently distribute protoplasts. Solidified agarose, containing the protoplasts was cut into 20mm x 20mm blocks and transferred into another plate containing 5ml of NT medium. Presence of nurse cells were absolutely required to induce high frequency division of protoplasts. Therefore, leaf mesophyll protoplasts from tomato, tobacco and potato were isolated and separately added to the protoplast cultures in the form of nurse cells (100mg/plate) to the liquid part of the culture (mixed nurse method). The cultures were sealed with parafilm and were incubated at 25 1oC in the dark for 10 days. Agarose blocks were transferred to new plates after ten days and nurse cells were completely removed by washing with the culture medium.

Method 2: Modified method for chilli varieties from tomato (*Lycopersicon esculenium* L.)

Same procedure described above was performed with slight modifications. B5medium was used as the protoplast culture medium (with nurse cells) and incubated in dark at 291oC for the first 21 days (Zapata *et al.*, 1977) or modified B5 medium supplemented with 1.0mg/l 2,4-D (2,4 dichloro phenoxy acetic acid), 0.5mg/l BA (benzyl adenine) and 0.5mg/l IAA (indole-3-acetic acid) was used as the protoplast culture medium (Morgan and Cooking, 1982).

Method 3: Protoplast culture employing liquid culture medium

Liquid MS salts and vitamin medium containing 1mg/l Kinetin and 1 mg/l NAA without nurse cells (Zapata and Sink, 1981) was used as the protoplast culture medium and incubated in dark at 291oC.

Method 4: Modified protoplast culture method for chilli varieties from *Solanum tuberosum L.* cv. May Queen. (Bajaj, 1986)

Protoplasts were washed in protoplast culture medium containing 0.4 M mannitol by centrifugation at 100g for 2mins. Then protoplasts were cultured at 5x106/ml density in plastic petridishes containing 4ml of liquid protoplast culture medium. Cultures were incubated at 251oC in dark. Cultures were replaced with fresh culture medium in every week.

Cells were viewed under the microscope and observations were made on cell wall formation after 7 to 10 days and after another additional 3 to 5 days for cell division and colony formation.

Results and discussion

Isolation of protoplasts:

The incubation period for 3 hours using 2% cellulase + 0.4% macerozyme was found to be the best for mesophyll protoplast isolation in both tested cultivars (Table 2). When the cultures were incubated for 5 hours, 6x106 protoplasts/ml were fully degraded, while incubation for 10 hours caused complete cell degradation in both tested chilli cultivars. The yield of viable protoplast in both chilli varieties in the best enzyme mixture was 5x108 protoplasts/ml.

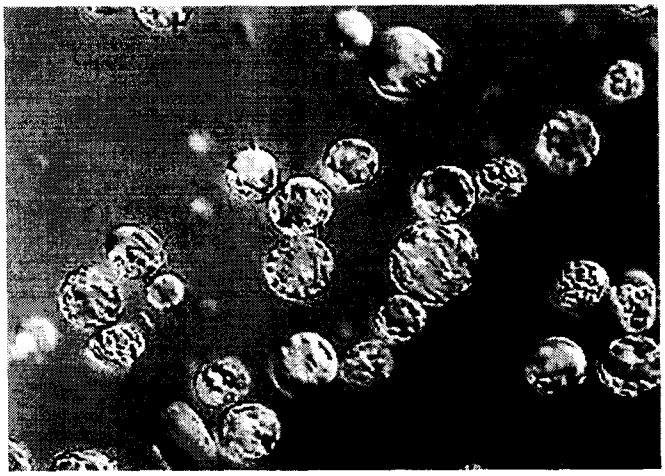

Figure 1: Isolated protoplasts from *Capsicum annuum* var. *accumnatum* after incubation in 2% cellulase + 0.4% macerozyme for 3 hours.

Protoplast culture:

In variety *Capsicum annuum* var. *accumnatum* protoplasts were alive after 14 days of incubation in NT supplemented with 1.0mg/l 2,4-D, NAA, BAP and 0.5M mannitol. Due to high osmotic pressure within the protoplasts, use of isotonic media which contains mannitol or/and sorbitol increase the viability of protoplasts. Growth regulators together with mannitol facilitates the maintenance of protoplasts capable of dividing for several days (Saxena *et al.*, 1981). Among the most striking properties of active protoplasts is the fast regeneration of a cell wall that does always precede protoplast division. It seems as if the development of a was a casual precondition of both cell division and polarity. Both are lost during protoplast regeneration and have to be develop if a new plant is going to regenerate. None of the other modified methods tested did not give positive results.

Table 2: Effect of different enzyme mixtures (T1 - T3) and different incubation periods o n cell wall digestion of leaf mesophyll cells. Cultures were incubated at 2510C in the dark

Leaf type	Enzyme mixture	Incubation period (hrs)	Partial cell wall digestion	Complete cell wall digestion	Complete cell degradation
		3	+	-	-
MI2	T1	5	+	-	-
		10	+	-	-
		3	+	-	-
Bird	T1	5	+	-	-
		10	+	-	-
		3	+	-	-
MI2	T2	5	+	-	-
		10	+	-	-
		3	+	-	-
Bird	T2	5	+	-	-
		10	+	-	-
		3	+	+	-
MI2	T3	5	-	+	+
		10	-	-	+
		3	+	+	-
Bird	T3	5	-	+	+
		10	-	-	+

+ Present - Absent

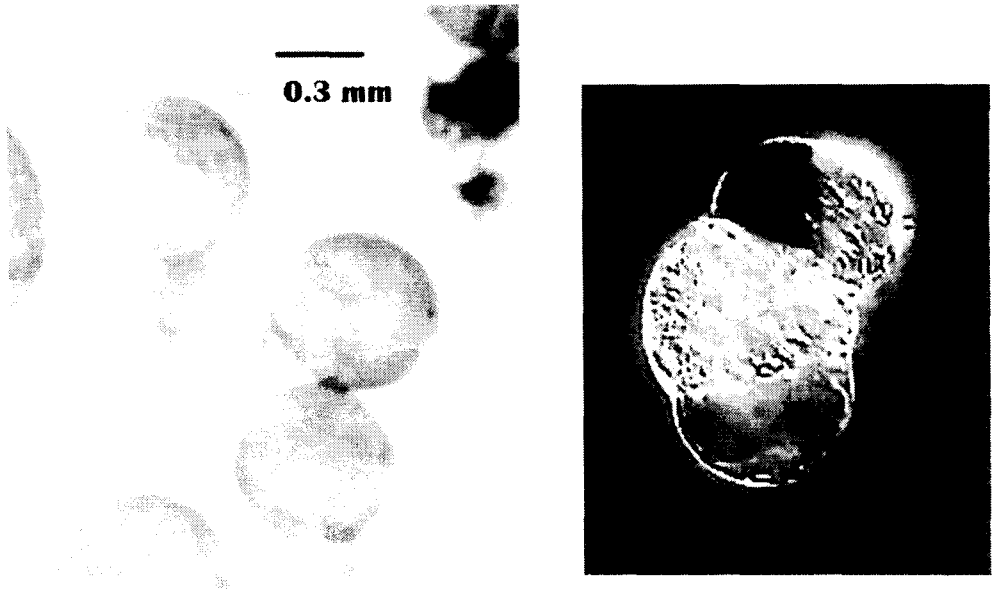

Figure 2: a) Live protoplasts of *Capsicum annuum* var. *accumnatum*in NT medium supplemented with 2,4-D, NAA, BAP and mannitol after 10 days. **b)** Cell division after 13 days.

Bacterial contamination was a problem in most of the cultures. About 30% of the total cultures get contaminated. Incorporation of antibiotics into the medium or following strict aseptic procedures have to be tested in order to eliminate the contamination problem associated with protoplast cultures of chilli varieties.

Conclusion

This study reveals that, three hours of incubation using 2% cellulase + 0.4% macerozyme was the best for the tested varieties of chilli, which yield viable protoplasts at the number of 5x108protoplast/ml/g leaf tissue. Among all the tested modified NT, MS, B5 and Potato protoplast culture media, it was found that mixed nurse method using NT medium supplemented with 1.0mg/l 2,4-D, NAA and BAP with 0.5M mannitol and sea plaque agarose is the best medium, where the protoplasts of cultivar *C. annum* var. *accumnatum*were alive for 14 days. But one of the major constraints was bacterial contamination (30% contamination) of the plates which was difficult to control. Incorporation of antibiotics into the medium would be one of the conclusions to control this problem. Further extensive research using other protoplast

culture media and advanced techniques like micro drop array technique are needed to achieve proliferation of protoplasts.

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