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Short Communication

Polyethylene glycol mediated protoplast fusion of medicinally important *Canna*

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***Canna* is a spectacular ornamental plant which gains its importance in terms of nutritive and medicinal values. However, there are number of problems related to the production and breeding for improved varieties of *Canna*. Protoplast fusion technique was tried to generate genetically modified hybrid varieties of *Canna*. *In vitro* generated leaves and shoots of *Canna indica* and *Canna edulis* were used as the source for isolation of protoplasts. Viable protoplasts (viability range 60-75%) were generated in enzymatic combination of cellulase (1%) and pectinase (0.5%) with an incubation temperature of 24±3°C for 16-18 hrs in dark. In the present experiment, protoplast fusion in *Canna* was standardized using Polyethylene glycol (PEG). The fused protoplasts were cultured in a medium consisted of Banana Micropropagation medium, supplemented with nutrients and growth regulators for regeneration.**

Keywords: *Canna*; protoplast; Cellulase; Pectinase; PEG

Canna, the solitary genus of the family Cannaceae (Prince, 2010), is cultivated extensively around homes and public parks for its decorative and widely varying flower colors (Nakornthap, 1965). Besides, some of the *Canna* species/ cultivars are also treasured as medicine specially *Canna indica* Linn. and *Canna edulis* Ker Gawler (Choudhury *et al*., 2010; Joshi *et al*., 2009; Mishra *et al*., 2011; 2012). However, there are number of problems related to the production of *Canna* and breeding of *Canna* for the creation of improved varieties. Germination of seed in *Canna* is very difficult and not practiced very often. Therefore, production of new variety with mating and cross pollination is limited. We also found that standardization of tissue culture technique in *Canna*, particularly; varieties found in India have not been attempted. Besides, asexual reproduction, conventional breeding is not very popular in *Canna*, because it occurs through the multiplication of rhizome, which is a very slow process. Alternative methods like protoplast fusion for production of hybrid varieties has never been tried perhaps anywhere in the world. Looking all these into account, in the present study an efficient protoplast isolation and fusion technique was tried to generate genetically hybrid varieties.

Materials and methods

Young leaves of *Canna edulis* and immature healthy fruits of *Canna indica* were collected from our laboratory greenhouse for aseptic culture (accession no. 9588 and 9590 respectively and deposited in the "NBU Herbarium", Department of Botany, University of North Bengal). The explants were surface sterilized using the standard protocol of Goyal *et al*. (2014).

Surface sterilized fruits of *C. indica* were blot dried, and seeds were excavated. For germination, single cotyledonous seeds were cut to pieces and inoculated in MS medium supplemented with 3% sucrose, 0.42% Calcium chloride and 0.7% agar without any growth regulators.

Leaves of *C. edulis* were excised and aseptically cultured in Banana micro-propagation medium (BM) containing 1% agar, 30g/L sucrose, 0.17% NH_4NO_3, BAP (2 mg/L) and NAA (0.5 mg/L) and found to be effective in inducing callus. The same medium with BAP (2 mg/L) was found ideal for plantlet regeneration.

All the cultures were incubated at 25°C±2°C with 12 hours light exposure. The *in vitro* generated leaves of both *C. indica* and *C. edulis* were maintained for further study.

Isolation and purification of protoplasts were standardized as per Assani *et al*. (2002); De (1997) with some modifications. *In vitro* cultured leaves (4 to 5 weeks old) of *C. indica* and *C. edulis* were considered for isolation of protoplast. One gram leaves of each species were peeled and cut into pieces of 1 × 0.5 mm size separately. Cut pieces of leaves were placed with lower surface down in sterile petriplates in cell and protoplast washing medium-1 (CPW-1) (De, 1997). After 5 minutes, CPW-1 was replaced by the enzymatic solution of cellulase and pectinase. Different combinations of both cellulase (0.5%, 1%, 1.5%, 2%) and pectinase (0.15%, 0.3%, 0.5%, 0.75%) were tried to observe the best result. Peeled leaves were incubated at 24±3°C for 16-18 hrs in dark. After the incubation for enzymatic digestion, enzymatic solution was gently replaced by CPW-1 without disturbing the leaf pieces. The digested leaf pieces were gently agitated and squeezed with a pair of fine sterile forceps to facilitate the release of protoplasts. Purification of isolated protoplast was done by passing the digestion mixture through 100µm sterile metallic mesh to remove the debries and large cell colonies. The filtrate was transferred to centrifuge tube and spinned at 900 rpm (100Xg) for 5 minutes at room temperature (RT). The protoplasts were settled as pellet which was resuspended in second cell and protoplast washing medium-2 (CPW-2) (De, 1997) and centrifuged at 1,300 rpm (200Xg) for 7 minutes. The viable protoplasts forming dark green band (De, 1997) were collected from the surface of the mixture of CPW-2. Protoplasts were washed by suspending in CPW-1 and centrifuged at 900 rpm (100Xg) for 5 minutes for 2-3 times to remove the sucrose. Protoplast viability was checked by using phenosafranin according to the method described by De, 1997. Phenosafranin (0.1%) was mixed with protoplast preparation to view the staining.

Fusion of protoplast was achieved by using the protocol of Assani *et al*. (2005) with desired modification. Viable protoplasts of both the species were mixed in equal proportion in a fusion solution containing 0.5 M mannitol and 0.5 mM $CaCl_2$. Protoplast mixture was slowly dropped down to the sterile petriplate followed by the addition of Polyethylene glycol (PEG) solution (50% PEG, 0.5 M mannitol and 0.5 mM $CaCl_2$). After 30 minutes of incubation in RT, the PEG solution was replaced by liquid culture media. Further the protoplasts were studied under microscope for possible fusion.

Medium was prepared couple of days before the fusion of protoplasts. The fused protoplasts were sieved through 250 µm sterile metallic mesh to select the small cell aggregates for their culture. The culture medium consisted of BM media, 2 mg/L

BAP, 0.5 mg/L NAA, 2.8 mM glucose, 278 mM maltose, 116 mM saccharose, 2.5 mM myo inositol (pH 5.6±0.2) and 0.4% agar. The media was autoclaved for sterilization. The mixture was carefully poured into sterilized culture vessels. Then the sieved protoplasts (fused) were gently plated on the agar medium for the initiation of cell division and growth. The cultures were maintained at 25°C in the dark to observe further development of fused mesophyll cells.

Results and discussion

In vitro germinated leaves of *C. indica* and *in vitro* regenerated shoots of *C. edulis* were used as the source for isolation of protoplasts. It was observed that in both the plants, good quality of protoplasts was produced from *in vitro* leaf tissue (Fig 1). Of all the combinations of enzymes, 1% cellulase + 0.5% pectinase generated good quality protoplasts in both the plants. As per the literature, the viable protoplasts were viewed to be unstained with phenosafranin (De, 1997). The viability range of freshly isolated leaf protoplasts was 60-75%. Mesophyll protoplasts of *C. edulis* were found to be more uniform in size [20-30 micro meter (µm)] than that of *C. indica*.

The isolation of protoplasts in *Canna* depends significantly on both donor material and genotype used. It is essential to standardize the enzymatic mixture for the donor material to isolate high quality of protoplasts. This indicated that the digestion of cell wall components depends on enzymatic mixture. A number of studies have been performed to access the significance of the donor material in protoplast isolation. In banana and other monocotyledons such as rice, maize and wheat, lower yield and lower viability of protoplasts were observed when derived from mesophyll tissue (Assani *et al*., 2002; Jain *et al*., 1995; Prioli and So¨ndahl 1989; Vasil *et al*., 1990). The less viable protoplasts of mesophyll tissue could be connected to the sensitivity of leaves to enzymatic stress, resulting in breakage and damage during enzymatic digestion. Similar type of observation was found in our study.

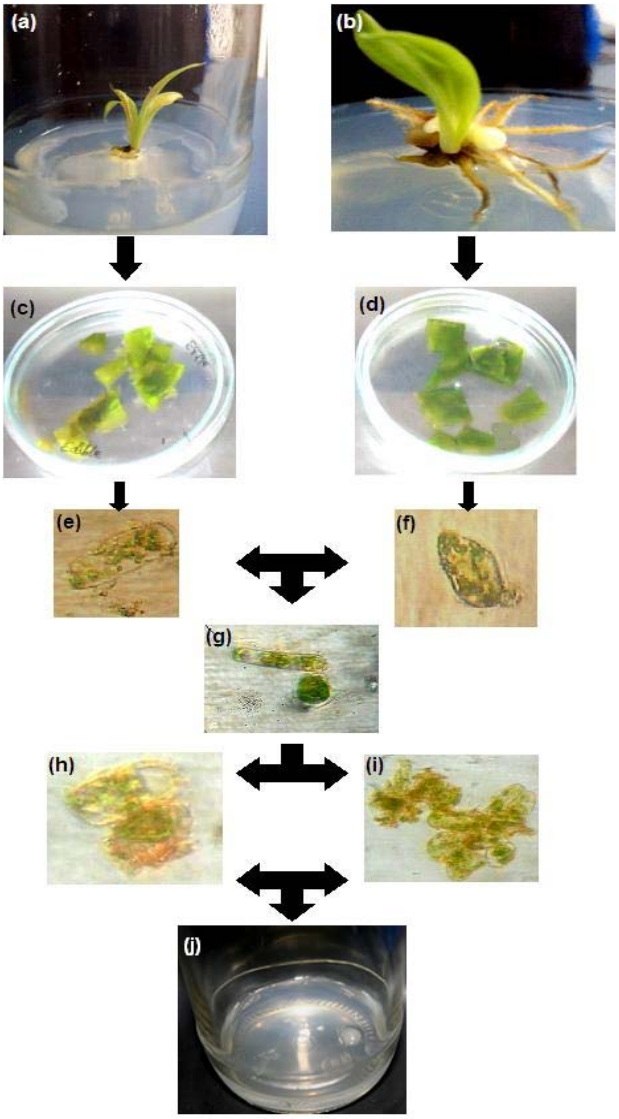

Figure 1- Protoplast isolation and fusion of *in vitro* regenerated *Canna*. (a) *In vitro* germinated *C. indica* (b) Callus regenerated *C. edulis* (c & d) Leaf pieces of *C. indica* and *C. edulis* for enzymatic digestion; (e & f) Freshly isolated mesophyll protoplasts (g) Protoplasts coming closer to each other after application of PEG (h) Fused protoplasts (i) Chain of Fused protoplasts (j) Initiation of callus.

In the present study, a standard protocol for isolation of protoplasts has been developed for the ornamental monocot like *Canna*. Since protoplast regeneration is a prerequisite for somatic hybridization, efficient isolation of protoplasts would be important for producing new cultivars of *Canna* through protoplast fusion.

Somatic hybridization could be an excellent tool for the breeding of ornamental and cultivated *Canna*. But till date there are very few reports of successful somatic hybridization in monocots. In the present experiment, protoplast fusion in *Canna* was standardized using PEG. Different steps of protoplast fusion are represented in figure 1.

A number of fused protoplasts were clearly seen under microscope. The rate of cell divisions of fused protoplasts may be affected by the toxic level of PEG, which hampers the viability of protoplasts. Less viable protoplasts do not participate in the fusion process and ultimately lowers down the mitotic activities (Mercer and Schlegel, 1979).

Callus like whitish mass of tissue was observed 9-10 days after the culture of fused Protoplasts. The frequency of formation of the above whitish tissue was very low i.e. 25-30% only. The callus like tissue was able to divide and increase in their size, although they did not differentiate further and died thereafter. This showed that the calli produced from the fused protoplast did not regenerate; however, they were alive for about 15 days while on the feeder layer. The whitish tissue may represent a type of wound tissue, which stimulates callus formation but was incapable of further differentiation and formation of somatic embryo. Lower regenerating capacity of fused plant protoplasts might be due to the suppression of totipotency. Reduced viability and cell division potential in fused protoplasts may be because of the presence of reactive oxygen species and reduced cellular antioxidant mechanism. Moreover, the loss of viability with due course of time might be due to different biotic and abiotic factors. The incapability of regeneration of fused plant protoplasts was described by many authors (Evans *et al*., 1984; Schieder and Kohn, 1986). This may be because of the effect of PEG, which ceases the development of fusion product and finally limiting the formation of callus and somatic embryos (Assani *et al*., 2005).

Conclusion

Thus, here we tried to develop some hybrid varieties of *Canna* through protoplast fusion. So far our knowledge goes this is the first attempt to fuse *Canna* protoplasts *in vitro*. Viable protoplasts were isolated as a result of enzymatic digestion of the *in vitro* leaves of *Canna*. Fusion of mesophyll protoplasts was standardized using Polyethylene glycol. However, detailed study has to be carried out to sort out the problems related to the regeneration of fused protoplasts.

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