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Protoplast Isolation and Fusion in Amomum subulatum Roxb.

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Abstract

Amomum subulatum Roxb. is an important spice and cash crop of Himalayan region of Indian sub-continent. An attempt has been made to isolate and fuse protoplast from the *in-vitro* grown seedling of *Amomum subulatum* Roxb. Off the various enzyme combination tried, the one containing the enzyme combination of 2% cellulase "Ozonuka" RS (Yakult Pharmaceutical Ind. Co. Ltd, Japan) 1% Macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd, Japan) and 1 % Hemi-cellulase (Sigma-Aldrich, USA) prepared in 10.93% D- Mannitol (Himedia, India) and 0.055% calcium chloride yielded protoplast of 2 x 10^4/g of leaf tissue. Protoplast fusion was achieved using 20% Polyethylene Gylcol (PEG 4000) but in small quantity added to the mixed protoplast. To develop a successful somatic hybrid of commercial importance further research is required.

Key words: Protoplast, enzymes, cell wall digestion, protoplast fusion, somatic hybridization, Poly ethylene glycol

Introduction

Amomum subulatum Roxb., commonly known as Large Cardamom or Black Cardamom, is an important cash crop of Sikkim Himalayas. It belongs to the family Zingiberaceae under the order Scitaminae. The plant originated in trans-Himalayan region of present day Sikkim, India and Eastern Nepal in ancient times. Roxburgh was the first to describe the plant in his book "Plants of the coast of Coromandel" published in 1795. It is believed that The *Lepchas*, the aboriginal inhabitants of Sikkim were the first to collect wild Large Cardamom capsules from forest mainly for the purpose of medicine and as an edible aromatic wild fruit (Sharma et al, 2008). Now the crop is also cultivated in North-Eastern states of India and the hilly regions of Uttarakhand and Himachal Pradesh. India is the largest producer of Large Cardamom followed by Nepal and Bhutan. It is shade loving plants (Sciophyte) and grows well near the perennial source of water.

Currently, the crop is under severe crisis mainly due to the infestation by viral and newly emerged fungal diseases. The infestation by two viral diseases namely "Chirkey" and "Foorkey" is from the time immemorial. In the last 2 decades, a new fungal disease in Large Cardamom emerged which has devastated the crop to the large extent. The life expectancy of the crop has drastically reduced from 20-30 years to mere 5-6 years. The productivity and the production have declined and at the same time

the cost of production has gone up. It is primarily caused by *Colletotrichum gloeosporiodes*as blight disease followed by number of other opportunist fungus. Among the number of factors, the lack of variety resistant to these diseases is one of the main constrained to overcome the current problems. The new variety development through conventional breeding programme involves long and tedious process and may take 20-30 years in case of Large Cardamom due to longer gestation period of 2-3 years from seed germination to flowering. Somatic hybridization and marker assisted breeding is one of the option which can shorten the period of new variety development.

The possibility of introgression of desired traits from both distantly or closely related species through somatic hybridization was realized after the successful isolation of protoplast by Cocking (1960). The plant breeders are always in look-out to produce the hybrids which are high yielding, disease, herbicide or draught resistant. The incompatibility barrier is one of the main constraints to combine desired traits especially from the distantly related species. It has been largely found that the disease or draught resistant traits are found in the wild relatives or distantly related species. The combination of both complete nuclear and cytoplasmic genes simultaneously is possible through protoplast fusion which cannot be done by sexual hybridization or genetic engineering. The introgression of desired traits from wild relatives or distantly related species enriches the cultivated germplasm which are then widely used for crop improvement programme. The asymmetric somatic hybridization brings-in only the traits located in the extra-nuclear genome such as chloroplast. The nuclear genome of the donor plant is normally irradiated by gama or x-rays and destroyed to enable to combine only the extra-nuclear genome.

Few successful commercial varieties of potato and *Brassica* spp. have been developed. However, number somatically hybrids has been developed which are being used in breeding programme. There are number of limitations of somatic hybridization. The somatically hybridized plant does not always produce fertile and viable seeds due to genetic instability. It does not guarantee the successful expression of a specific trait. It is achievable to fuse the protoplast between different genus, species or varieties but it is not always possible to produce viable somatic hybrids (Bhatia, 2015). Development of protocol for somatic hybridization is a long and cumbersome process and regeneration of plantlets *in vitro* is poor in many species. However, it has great potential in transferring polygenic traits and it does not require regulatory approval as in transgenesis (Lidder, 2012). Since the transferring of single or few genes is not possible by protoplast fusion, the system can be efficiently utilized for producing new genetic variability by random combination of genes of the two parental genomes.

The initial process of protoplast isolation involves the use of hypertonic solution such a mannitol, sorbitol or sucrose to induce plasmolysis in cells which causes water to extrude from the vacuole causing a loss of turgor pressure. Different concentration of hypertonic solutions is maintained to induce appropriate plasmolysis in different plant cells during protoplast isolation. Mannitol is widely used as osmoticum. Mannitol at 11% concentration was used along with enzymes and other adjuvant to isolate the protoplast from embryonic cell suspensions of ginger (Guan, 2010). 8% (w/v) of mannitol was added in digesting solution to isolate the protoplast from the young leaves of rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.) during intergeneric somatic hybridization (Kisaka, 1997).

Cell wall digesting enzymes plays an important role in the isolation of protoplast from plant cells. Plant cell are bounded by various polysaccharides such as cellulose, hemicelluloses, pectin which gives structural support and protection. The enzyme cellulase, hemicellulase, pectolyase, macerozymes, drialeaseetc are used to macerate the cells, digest the cell wall of the plant cell at various concentrations

depending on the source of the plant material. Different workers have used various concentrations of these enzymes to isolate the protoplast from different plant species and sources.

An enzyme solution of 1% (w/v) cellulase (Ozonuka RS), 0.1% (w/v) pectinase dissolved in 0.6M mannitol was used to isolate protoplast from the suspension culture of *Taxus chinensis* and *Bupleurum schorzonerifolium* (Zhang, 2011). Geetha et al obtained protoplast yield of $3.5x10^5$/g of cardamom leaf tissue when incubated in an enzyme solution containing 0.5% macerozyme R10, 2% cellulose Ozonuka R10 and 9% mannitol for 18-12 h at 25°C in dark.

The protoplast from leaves of *in-vitro* grown seedlings of two varieties of sugar beet, Francesca and Meghribel, were successfully isolated in an enzyme solution containing 2% cellulose, 1% hemicellulase and 1% pectinase for 18 hr in dark at 25°C. The best protoplast yield of $4.55x10^5$ was obtained in Meghribel genotype (Elden, 2010). Guan et al followed the enzyme composition and conditions described by Guo et al (2007) for isolation of embryonic cell suspension of ginger.

The protoplast from the *in-vitro* grown potato leaf slices were released by gently swirling for 4-5 h at 40 rpm at 25-28oC in a enzyme solution containing Fish and Karp major salts, 0.15% macerozyme R-10 (Yakult, Tokyo, Japan), 0.7% cellulase R-10 (Yakult, Tokyo, Japan) and 7.5% mannitol, pH 5.6 and centrifugation at 700 rpm for 5 min (Ehsanpour, 2001). Before digestion of cell wall with enzymes, the leaves were preplasmolysed in wash solution containing mannitol at 7.5% pH 5.6 for 0.5-1h. Rose and Nolan used filter sterilized enzyme solution containing 0.5% cellulase RS (Yakult Honsha, Tokyo, Japan), 0.025% (w/v) pectolyase Y23 (Seishu Pharmaceutical Industry Co., Tokyo, Japan), 0.5% Macerozyme R-10(Yakult Honsha, Tokyo, Japan), 0.45 M mannitol, 7.0 mM $Cacl_2.2H_2O$, 0.5% (w/v) bovine serum albumin and 3.0 mM MES buffer pH 5.5 to isolate protoplast from *Medicago truncatula.* Protoplast of *N. sylvestris* and *A. belladonna* were isolated from leaves of young plants by incubation in an enzyme solution containing 0.4% cellulase Ozonuka R-10 (Serva),0.2% Driselase (Sigma), 0.5M sucrose and 5mM $CaCl_2$ for 14 h at 25°C in the dark (Yemets, 1999). Digesting solution containing CPW salts (Frearson et al., 1973), 1.5% cellulase R-10 (Yakult Honsha Co., Japan), 1.0% hemicellulase (Sigma), 1.5% Macerozyme (Yakult Honsha Co., Japan), 0.5M mannitol, pH 5.8 was used to isolate protoplast from the 2- week old flush leaves of mango (*Mangifera indica* L.). The incubation was carried out at 27°C for 16 h in darkness on gyratory shaker at 45 rpm (Rezazadeh, 2011). Keskitalo et al used 0.125% (w/v) Macerozyme R-10 (Yakult, Honsha, Japan), 0.25% (w/v) cellulase R-10, 0.25% (w/v) Cellulysin (Calbiochem),0.125% (w/v) Driselase and 0.02% (w/v) Pectolyase Y-23, 0.5M sucrose and 5.mM CaCl2.2H2O, pH 5.6-5.7 to isolate the protoplast from tansy and pyrethrum.

Kisaka et al added 0.1% $Cacl_2.2H_2O$ (pH 5.5) in the enzyme solution for stability of protoplast during protoplast isolation from the young leaves of rice and barley whereas Guo et al used 0.5% (w/v) $Cacl_2.2H_2O$ during isolation of protoplast from embryonic suspension of ginger. 0.1% (w/v) $Cacl_2.2H_2O$ was added during protoplast isolation from young leaves of *H. vulgare* and *D. carota* (Kisaka, 1996).

Washing solution is used to remove enzyme solution after enzymatic digestion of cell wall during protoplast purification process. Different workers used different composition of washing solution. Chaudhari et al diluted enzyme solution with equal volume of washing solution containing 12 um CaCl2, NaCl2, KCl before sieving protoplast during protoplast purification of sugarcane. Similarly, Akira et al used protoplast washing solution containing 0.8M D-Mannitol, 6mM CaCl2, 0.7mM NaH2PO4, 5mM Sodium Citrate (2H2O salt), 10mM KCl, 10mM MgCl2 to wash enzyme solution during protoplast purification. An attempt has been made to isolate and fuse protoplast derived from in-vitro grown seedling of *A. subulatum*.

Materials and methods

For isolation of protoplast and subsequent procedure, young leaves of in-vitro grown large cardamom seedlings were used. Various enzymes combination at different concentrations were tried to digest the cell wall of *in-vitro* grown seedling of large cardamom. These include Cellulase "Ozonuka" RS (Yakult Pharmaceutical Ind. Co. Ltd, Japan), Macerozyme R-10 ((Yakult Pharmaceutical Ind. Co. Ltd, Japan) and Hemi-cellulase (Sigma-Aldrich, USA). The cellulase enzymes was first dissolved by decreasing the pH to 4.00 and subsequently heating at 45oC for 15 minutes. The remaining enzymes were dissolved in this solution one by one. After complete dissolving the enzymes, the pH was raised to 5.8. To evaluate the suitable enzyme combination to digest the cell wall in large cardamom, the detail experimental set-up is tabulated in table 1.

Table 1: Study on the effect of various concentration of enzymes on protoplast isolation

Enzyme solution	Cellulase %	Macerozyme %	Hemi-cellulase %
ES-1	1	1	1
ES-2	1	2	1
ES-3	1	3	1
ES-4	1	1	2
ES-5	1	1	3
ES-6	1	2	2
ES-7	1	2	3
ES-8	1	3	2
ES-9	1	3	3
ES-10	2	1	1
ES-11	2	2	1
ES-12	2	3	1
ES-13	2	1	2
ES-14	2	1	3
ES-15	2	2	2
ES-16	2	2	3
ES-17	2	3	2
ES-18	2	3	3
ES-19	3	1	1
ES-20	3	2	1
ES-21	3	3	1
ES-22	3	1	2
ES-23	3	1	3
ES-24	3	2	2
ES-25	3	2	3
ES-26	3	3	2
ES-27	3	3	3

The young leaves of in-vitro grown seedlings were shredded into small pieces with the help of pointed forceps and surgical blade in small amount of enzyme solution on a petriplate. The shredded

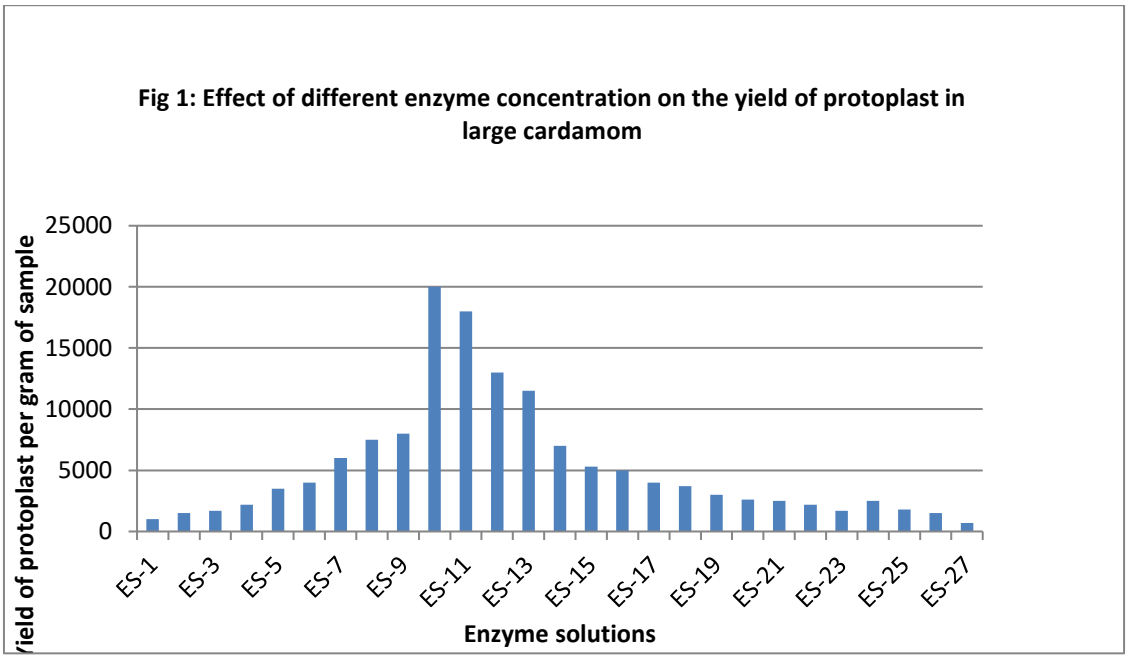
leaves were placed in the enzyme solution and incubated at 27ºC in water bath in static for 16 hrs. After 16 hrs the sample is incubated at 27ºC with shaking at 85 rpm.

The digested tissue was filtered through 8µ nylon filter mesh and collected on a falcon tube. The filtrate is then centrifuged at 1500 rpm for 5 minutes. The supernatant was removed with the help of Pasteur pipette. The pellet is diluted with CPW medium and again centrifuged at 1500 rpm for 5 minutes. This process was repeated two more times to ensure complete removal of enzymes. The pellet again dissolved in small amount of CPW medium and poured on top the 21% sucrose solution. It was then centrifuge at 500 rpm for 7 minute. The viable protoplast formed a ring in the intermediate region of the solution. It was carefully pipette out with the help of Pasteur pipette and collected in a tube. The high concentration of sucrose is diluted with CPW medium. The isolated protoplast was used for fusion.

Protoplast fusion was carried out with 20% Polyethylene Glycol (PEG 4000). The protoplasts isolated were mixed in equal proportion i.e. 1:1 ratio as drop in a sterile petriplates over the laminar air flow. The protoplast was allowed to settle for 15 minutes. The small amount of PEG solution was added from the side to the drop of mixed protoplast and allowed to fuse for 25 minutes. The fusion was observed under compound microscope for successful fusion.

Results and discussion

The isolation of protoplast is the pre-requisite for somatic hybridization. Off the various enzyme combination tried, the one containing the enzyme combination of 2% cellulase "Ozonuka" RS (Yakult Pharmaceutical Ind. Co. Ltd, Japan) 1% Macerozyme R-10 ((Yakult Pharmaceutical Ind. Co. Ltd, Japan) and 1 % Hemi-cellulase (Sigma-Aldrich, USA) and prepared in 10.93% D- Mannitol (Himedia, India) and 0.055% Calcium chloride yielded protoplast of 2 x 10^4/g of leaf tissue. The incubation temperature at 27ºC in static for 16 hrs was found to be suitable temperature for enzymatic digestion of cell wall and release of optimum level of protoplasts. It has been found that the cellulase enzyme dissolves completely at lower pH of around 4.0 and subsequent heating at 45ºC for 15 minutes in hot water bath. Properly dissolved enzymes works well than the partially dissolve one. Shaking additional 45 minutes in water bath shaker at 85 rpm releases more protoplasts.



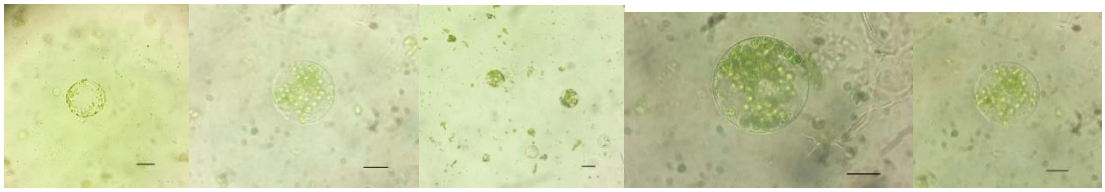
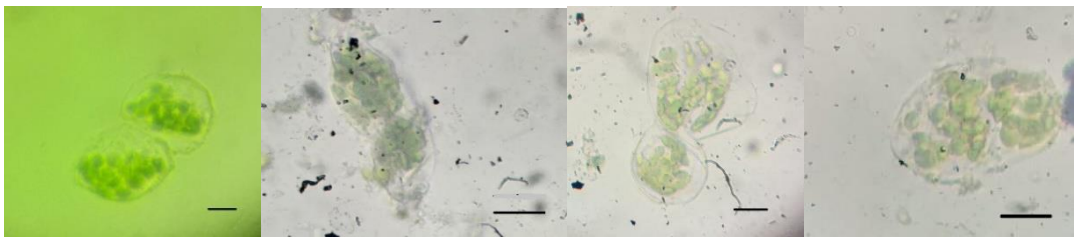


Fig. 2: Isolated protoplast after enzymatic digestion (Bar=20µm)



A B C D

Fig. 3: A-C: Fusing protoplast; D- fused protoplast (Bar=20 20µm)

Protoplast fusion was achieved using 20% Polyethylene Gylcol (PEG 4000) but in small quantity added to the mixed protoplast. Addition of high quantity of PEG led to fewer chances of two protoplasts to come close enough to get fused due to high viscosity. The fused products were cultured in protoplast culture medium. To develop into a true somatic hybrid of commercial importance requires further research which is beyond the scope of this research.

Conclusion

For gene introgression and development of new variety of large cardamom having commercial importance through somatic hybridization, further research is required to screen resistant gene in the genepool of large cardamom or its wild relative, raising of viable plants from fused products, molecular authentication of heterokaryon, testing of resistant traits and cleaning of unwanted traits.

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