Research in Biotechnology, 4(4): 25-29, 2013 ISSN: 2229-791X
www.researchinbiotechnology.com

Regular Article

Protoplast fusion studies in *Ocimum* species

Nitish Kholgade, Vijay Mohta, Rebecca Mathew and P. Deepa Sankar*

Plant Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore
*Corresponding author Email: pdeepasankar@vit.ac.in

Protoplasts of three *Ocimum* species, *viz.*, *O. basilicum*, *O. sanctum* and *O. gratissimum* derived from leaf samples were used for studying fusion. The isolation of protoplasts were carried out using 1 % cellulase and 0.5 % pectinase in combination with Cocking-Peberdy-White (CPW)–13% mannitol solution as it gave the best results. The protoplasts obtained by enzymatic digestion were purified by centrifugation. The yield of protoplasts was found to be highest when centrifuged at 800 rpm for 15 minutes at 4°C. Among the fusogen combinations tried to obtain homokaryons, 40 % Polyethylene glycol (PEG)-6000 fusogen solution gave better results when compared to the other combinations. The Ca^{2+} concentration and pH level (5-9) were altered and studied but major changes in time taken for fusion were not observed. The mean count of protoplasts per ml was found to be 209,200 for *O. basilicum*, 317,500 for *O. gratissimum* and 502,500 for *O. sanctum*. The Evans blue staining test showed that the average percentage of viable cells was 52.8 % for *O. basilicum*, 52.54 % for *O. sanctum* and 47.15 % for *O. gratissimum*.

Keywords – Ocimum, protoplast, fusion, PEG

Ocimum, commonly called as basil, a genus of about 35 species of aromatic annual and perennial herbs and shrubs belongs to the family Lamiaceae, and is mostly native to the tropical and warm temperate regions of the world. Some of the common plants of this genus are *Ocimum sanctum* (tulsi), *Ocimum basilicum* (sweet basil) and *Ocimum gratissimum* (African basil). The genus has been reported to possess anti-microbial, antioxidant and anti-cancer properties and have been used in Ayurvedic medicine. The basil extracts are endowed with various secondary metabolites including the phenols, flavonoids, alkaloids and terpenoids and active compounds like eugenol, ursolic acid, rosmarinic acid and nutritional components such as vitamin A and C, minerals like calcium, iron, zinc etc.,

Protoplasts derived from plant cells are the source for protoplast culture, gene transformation, development of transgenics, cytogenetic studies, and to derive homokaryons and heterokaryons for development of polyploids. Protoplasts differ from the normal plant cells in that the plasma membrane is the only barrier between the cytoplasm and its immediate external environment. The totipotency of the plant cell protoplasts can be expressed by providing optimum conditions for culturing the protoplasts. Protoplasts are particularly useful for genetic manipulation of plant genomes. Experiments have also been carried out on the uptake of naked DNA by the plant protoplasts for nuclear transformation. In addition, protoplasts have been used to study the internal cell structure

(Michael Davey *et al*., 2005). Protoplast studies form the basis for devising ways to enhance production of secondary metabolites possessing medicinal, aromatic and culinary properties through cell suspension cultures. Protoplast isolation and fusion has not been much studied in *Ocimum* species. Keeping this in mind, we tried to standardize a protocol for isolation, purification and fusion of *Ocimum* protoplasts.

Materials and Methods

Seeds of three *Ocimum* species, *viz*., *O. basilicum*, *O. sanctum* and *O. gratissimum*, were received from UAS, Bangalore and maintained at VIT University as pot cultures. Protoplast isolation, fusion and purification studies were carried out with the help of CPW-13% mannitol (CPW-13M) solution, 1% Onozuka Cellulase R-10, 0.5% Pectinase, CPW-21% sucrose (CPW-21S) solution, CPW-10% mannitol (CPW-10M) solution, PEG – 6000 fusogen solution and 0.5% Evans blue dye.

In 1960, Cocking devised a mechanism of isolating protoplasts from the leaves of plants by the application of enzymes. Leaf tissue is preferred for this purpose as large number of uniform cells is obtained without killing the plant. The primary and secondary structure of the cell wall is composed of cellulose and hemicellulose, while the middle lamella contains pectin. Cellulase enzyme is used to digest the cellulose and hemicelluloses in the cell wall, while pectinase is used to degrade pectin contained in the middle lamella. Once the protoplasts are released, there is a need to maintain the wall pressure with an equivalent osmotic pressure in a solution containing ionic or non-ionic solutes such as CPW solution. This solution is also used to treat the leaf tissue prior to enzymatic treatment for the purpose of pre-plasmolysis. Pre-plasmolysis is essential to minimize the accumulation of toxic contaminants that would be produced by direct enzymatic isolation. This is also used to enhance the yield and viability of the protoplasts. Enzyme digestion to obtain protoplasts in plants has been reported by Henn *et al.* (1998) and Liqing *et al.* (2005).

10-12 discs were placed in CPW-13M for 45 minutes. CPW-13M solution was sucked using a Pasteur pipette and replaced with 1 ml enzyme mixture and CPW-13M solution in a 1:1 ratio. Samples were incubated overnight at room temperature in dark. Leaf discs were agitated gently in order to release the protoplasts. Samples were centrifuged at 800 rpm for 15 minutes at 4°C. Supernatant was discarded, pellet resuspended in 400 µl of CPW-21S solution and centrifuged at 800 rpm for 10 minutes. Supernatant was collected and double the amount of CPW-10M washing solution was added. Centrifugation was carried out at 800 rpm for 5 minutes. Process was repeated till no pellet was observed. A drop of protoplast solution was placed on a glass slide. A drop of 0.5 % Evans blue dye solution was added and a cover slip was placed. Protoplasts were observed though a binocular compound microscope to differentiate between stained dead and unstained live cells. The percentage of viability was determined. Cells were counted using a hemocytometer.

PEG, which is slightly negative in polarity can form hydrogen bonds with water, protein, carbohydrate, etc., that possess positively polarized groups. PEG contributes to the closing of interbilayer gaps by removing most water molecules between two adjacent bilayers, owing to the polymer's high affinity for water. When the PEG chain is large enough, it acts as a molecular bridge between the surface of adjacent protoplasts and adhesion occurs resulting in the formation of tetraploid homokaryons (Razdan *et al*., 2010). PEG fusogen solution (sucrose 1.8g + $CaCl_2.2H_2O$ 154mg + KH_2PO_4 9.52 mg /100 mL) of different concentration *viz*., 20%, 25%, 30%, 35% and 40% was used for fusing the protoplasts. The

time taken for protoplasts to fuse and form binaries was recorded for the different PEG concentrations. The data was analyzed by one-way Analysis of Variance (ANOVA) using the software, SPSS 16.0.

Results and Discussion

Protoplast isolation

The protoplast isolation in the three species were tried with three different combinations of CPW-13% mannitol solution and enzyme mixture (1% cellulase and 0.5% pectinase) viz. 1:1, 1:2 and 2:1. Out of three, it was deduced that the highest protoplast yields were obtained with the 1:1 combination of the two solutions. Sigareva *et al*. (1999) isolated protoplasts for the purpose of regeneration of *Capsella bursapastoris* and somatic hybridization with *Brassica oleracea*. They used an enzyme mixture containing 0.2 % cellulase Onozuka RS, 0.1 % cellulysin cellulase and 0.2 % macerozyme R-10 in W5 inorganic salt solution.

Protoplast purification

To standardize the purification of isolated protoplasts, the following parameters were varied (Table 1.) to determine the optimum.

Table 1. Standardisation of procedure for purification of protoplasts

S.No	Parameter	Range	Optimum
1	Centrifugation Speed	600-1500 rpm	800 rpm
2	Time of centrifugation	5-20 minutes	15 minutes
3	Concentration of sucrose in CPW-sucrose solution	10-50 %	20 %
4	Concentration of mannitol in CPW-mannitol solution	10-20 %	10 %

Cell count and viability

The number of protoplasts per ml was calculated as 209,200 in *O. basilicum*, 317,500 in *O. gratissimum* and 502,500 in *O. sanctum* per ml of liquid sample using hemocytometer as an average of 5 microscopic fields. Out of the three species, the protoplast density was found to be maximum in *O. sanctum* and minimum in *O. basilicum*. The mean yield of protoplasts isolated from *in vitro* leaves of *Ulmus minor* Mill. by Paula Conde and Conceicao Santos (2006) was found to be 3.96 x 10^7 protoplasts per gram of fresh weight.

The average percentage of viable cells was found out to be 52.8 % in *O. basilicum,* 52.54 % in *O. sanctum and* 47.15 % in *O. gratissimum.* According to results obtained by Rafael Fernandez-Da Silva and Andrea Menéndez-Yuffá (2006) while investigating the viability of protoplasts isolated from *Coffea arabica*, Evans blue staining revealed 93.8 % viable protoplasts.

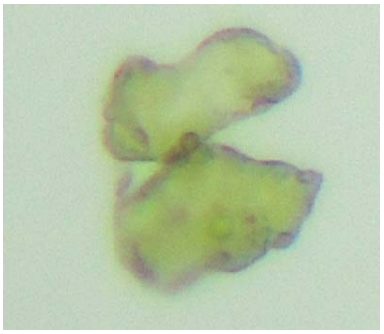

Fig.1. Adjacent protoplasts

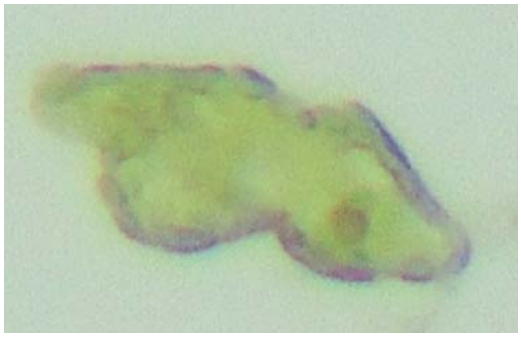

Fig.2. Fusion of protoplasts

Protoplast Fusion

Protoplast fusion was carried out by varying the PEG concentration and the time taken for fusion was observed (Fig. 1 and 2) and recorded (Table 2, 3, and 4) in all the three species under study. The experiment was analyzed using one-way analysis of variance. Treatments were found to be significantly different from each other.

Table 2. Standardization of Protoplast Fusion in *O. basilicum*

PEG Conc. (%)	Time taken for formation of binaries (min.)					
	R_1	R_2	R_3	μ	Standard deviation	Standard error
20	26	23	24	24.33	1.53	0.19
25	19	22	21	20.66	1.53	0.19
30	18	17	17	17.33	0.58	0.33
35	13	14	14	13.66	0.58	0.33
40	12	13	13	12.66	0.58	0.33

Table 3. Standardization of Protoplast Fusion in *O. gratissimum*

PEG Conc. (%)	Time taken for formation of binaries (min.)					
	R_1	R_2	R_3	μ	Standard deviation	Standard error
20	22	24	23	23.00	1.00	0.58
25	19	18	18	18.33	0.58	0.33
30	17	15	14	15.33	1.52	0.88
35	13	13	12	12.66	0.58	0.33
40	11	11	12	11.33	0.58	0.33

Table 4. Standardization of Protoplast Fusion in *O. sanctum*

PEG Conc. (%)	Time taken for formation of binaries (min.)					
	R_1	R_2	R_3	μ	Standard deviation	Standard error
20	25	25	27	25.66	1.15	0.66
25	21	21	19	20.33	1.15	0.66
30	16	15	14	15.00	1.00	0.58
35	12	13	11	12.00	1.00	0.58
40	10	12	11	11.00	1.00	0.58

40% PEG resulted in the formation of binaries by fusion in the least amount of time in all three species. Awatef Badr-Elden *et al*. (2010) used five different concentrations of PEG-6000 (15-35%) to fuse protoplasts isolated from *Beta vulgaris* L. to create heterokaryocytes from cultivars *viz*., Francesca and Meghribel by incubating them in PEG fusogen solution for 15-30 minutes. Qiuzhu Guan *et al.* (2010) investigated the process of regenerating somatic hybrids in ginger. They carried out somatic hybridization by inducing protoplast fusion through PEG-6000 at 30% concentration and found the fusion rate to be 13.5%.

References

Awatef M. Badr-Elden, Ahmed A. Nower, Nasr, M.I., and Ibrahim, A.I. 2010. Isolation and fusion of protoplasts in sugar beet (*Beta vulgaris* L). Sugar Tech. 12(1): 53-58.

Cocking, E.C. 1960. A method for the isolation of plant protoplasts and vacuoles; Nature. 187: 927-929.

Henn, H.J., Wigender, R. and Schnabl, H. 1998. Regeneration of fertile interspecific hybrids from protoplast fusions between *Helianthus annus* L. and wild *Helianthus* species. Plant Cell Rep 18, 220-224.

Liqing, Z, Bochu, W, Jing, Z, Lingxi, C, Chuanyun, D and Chuanren, D. 2005. Protoplast isolation of callus in *Echinacea augustifolia*. Colloids Surf B Biointerfaces. 44(1): 1-5.

Michael R. Davey, Paul Anthony, Brian Power, J., and Kenneth C. Lowe. 2005. Plant protoplasts: status and biotechnological perspectives. Biotechnology Advances 23: 131–171.

Paula Conde and Conceicao Santos. 2006. An efficient protocol for *Ulmus minor* Mill. Protoplast isolation and culture in agarose droplets. Plant Cell Tiss Organ Cult. 86: 359-366.

Qiuzhu Guan, Yinghua Guo, Yuxia Wei, Fanzhen Meng and Zhenxian Zhang. 2010. Regeneration of somatic hybrids of ginger *via* chemical protoplast fusion. Plant Cell Tiss Organ Cult. 102: 279-284.

Rafael Fernandez-Da Silva and Andrea Menéndez-Yuffá. 2006. Viability in protoplasts and cell suspensions of *Coffea arabica* cv. Catimor. Electron. J. Biotechnol. 9(5).

Razdan, M.K. 2010. *Introduction to Plant Tissue Culture*, New Delhi: Oxford & IBH Publishing Company Private Limited.

Sigareva, M.A., and Earle, E.D. 1999. Regeneration of plants from protoplasts of *Capsella* bursa-pastoris and somatic hybridization with rapid cycling *Brassica oleracea.* Plant Cell Rep. 18: 412-417.