



Efficient Isolation of Protoplasts from Hypocotyl-Derived Callus of *Brassica juncea* Var. B9.

Pijush Mallick^{1*}, Aisha Rana¹, Raja Gorai¹, Chandra Sekhar Mitra²

¹Department of Biotechnology, Srinath University, Adityapur, Jamshedpur, Jharkhand 831013, India

²Srinath College of Pharmacy, Srinath University, Adityapur, Jamshedpur, Jharkhand 831013, India

*Corresponding Author's Email: biology.pijush@gmail.com

Abstract

Protoplasts were isolated from 14 days-old hypocotyls-derived callus of *Brassica juncea* var. B9 using enzymatic tissue digestion. Callus was grown in artificial callus-inducing media i.e., MS basal added with growth hormones 2,4-D, NAA, and BAP at 1mg/ml concentrations. Cell wall degrading enzymes, Cellulase R10, Macerozyme, and Driselese were mixed in three different combinations (Set-1,2&3) in 0.6 M mannitol solution, and pH adjusted to 5.8. Callus tissue was digested under three different periods in all the enzyme sets and there was inconsistency in the number of protoplast yields. The highest protoplast yield i.e., 14.26×10^5 / gm tissue was recorded in Set-1 with 14 hours of incubation, and the lowest i.e., 5.22×10^5 / gm tissue was recorded in Set-3 with 18 hours of incubation. The protoplast yield was found consistent in the number in Set-2, and inconsistent in Set-1, and Set-3 against varied periods of incubation. The viability of the purified protoplasts was recorded at about 95.35% with a density of 20.32×10^5 cells/ml/gm tissue. The hypocotyl-derived callus formation and protoplast isolation in *Brassica juncea* var. B9 was optimized in this study which may further be used in commercial crop breeding programs in the field of agricultural biotechnology.

Keywords: *Brassica*; *Callus Tissue*; *Enzymatic Digestion*; *Protoplast Isolation*; *Viability Test*

Introduction

Genus *Brassica* is the most economically important plant under the family Brassicaceae (syn cruciferae) and known as 'mustard greens', 'Indian mustard', and 'leaf mustard'. It is grown as an oilseed crop in India (brown or Indian mustard), and as a leaf vegetable in China where leaf mustards have their greatest differentiation. Qualitative and quantitative improvement of mustard has greater values in crop breeding. Conventional and modern technologies have already been applied for the genetic improvements of this crop in which protoplast technology is one of the popular methods in the modern category.

The protoplast is a naked plant cell and contains all the components of a plant cell excluding the cell wall. Protoplast is a unique system for direct gene transfer in plants and the preparation of somatic hybrids (nuclear recombinants) and cybrids (cytoplasmic recombinants) through protoplast fusion. This capacity of the protoplast to take up foreign particles provides us with a powerful tool for the isolation and transplantation of nuclei in protoplast (Saxena & King, 1990) and also a material of choice for the study of the structure and function of cell organelles. Protoplast isolation was first attempted in *Stratiolates aloides* (L) by a mechanical method using microsurgery of plasmolyzed cells (Von Klercker, 1892). Later, Cocking, 1960 had used enzymes for the first time to release quality protoplasts from tomato (*Lycopersicon esculentum*) using cellulase and pectinase enzymes.

Callus-derived protoplast has many applications in plant regeneration, genetic transformation, and other cellular studies for the improvement of agricultural crops. Isolation of protoplast from callus tissues is the most refinement of single-cell regeneration efforts which have resulted in obtaining variant protoclonal in agriculturally important crop species including *Brassica* (Glimelius, 1984; Kirti & Chopra,

1989; Narasimhulu *et al.*, 1992; Razdan, 2003). In the past, in-vitro callus induction had been observed in canola and mustard seedlings where hypocotyl explants were excised and cultured on an MS medium supplemented with 2,4-D (Hussain *et al.*, 2014; Lone *et al.*, 2017). Effects of different carbohydrates and 2,4-D on callus induction ability were studied by culturing seedling-derived hypocotyl of *B. juncea* as explants on MS medium supplemented with 1, 3, or 10% fructose, glucose, sucrose, and ribose. It observed that the callus induction was significantly lower with ribose sugar than with other sugar sources and with higher sugar concentrations (Bogunia and Przywara, 2000). The effect of NAA and BAP hormones on efficient callus induction in Brassica genotypes had also been studied and found optimum in a combination of 2 mg/l BAP, 0.1 mg/l NAA, and 2 mg/l with added AgNO₃ (Khan *et al.*, 2009).

However, various studies on callus formation from different species of Brassica hypocotyl have been carried out followed by protoplast isolation. It was found that the discrete combinations of growth hormones, culture medium, and enzymes play a significant role in overall development. In this study, we have chosen *Brassica juncea* variety B9 and reported the efficient protocol for hypocotyl-derived callus formation followed by optimum protoplast isolation.

Materials and Methods

Plant material and hypocotyl development

Healthy seeds of mustard plants (*Brassica juncea*) var. B9 was obtained and sterilized following a modified protocol, previously established in our lab. Firstly, seeds were thoroughly washed in 50% liquid bleach solution for 10 minutes followed by 70% ethanol wash twice for 5 minutes each. Finally, the seeds were washed thrice with sterile distilled water for 2 minutes each and kept ready for culturing (Fig. 1a). Inside the sterile laminar airflow chamber, a total of 20 seeds were placed in sterile culture jars containing MS (Murashige and Skoog) basal media with added salt calcium chloride (CaCl₂) (Fig. 1b). Seed germination was observed under in-vitro conditions supported with 2500 lux light, 16 (light) / 8(dark) hours photoperiod at 23±1°C temperature.

Formation of callus

The 14-day-old sterile hypocotyls were excised into uniform pieces of 1 cm and cultured in a petri dish on MS media supplemented with callus induction hormones. We used 50 µl of 2,4-D, 150 µl of NAA, and 50 µl of BAP at 1 mg/ml concentration of each. Samples were cultured under in-vitro conditions and subjected to a total light intensity of 1500 lux (fluorescent lamps 16/8 h photoperiod) throughout the growing period and temperature was maintained at 23±1 °C.

Enzyme preparation and tissue digestion

A combination of Cellulase R10, Macerozyme, and Driselase enzymes was dissolved in 0.6 M mannitol solution and used as an osmotic stabilizer with CaCl₂·2H₂O (0.2%) and pH adjusted to 5.8. Various percentages and combinations of those enzymes are used to obtain the optimum and healthy protoplasts from Brassica callus (Table 1).

Table 1: List of enzymes and their percentage used in optimum protoplast isolation from hypocotyl-derived callus of *Brassica juncea* var. B9.

Enzyme Set	Cellulase R10 (%)	Macerozyme (%)	Driselase (%)
Set-1	1.5	1.0	0.5
Set-2	1.0	1.0	0.25
Set-3	2.0	1.0	0.5

About 1 gm of freshly induced hypocotyl callus tissue was collected in a sterile petri plate containing 5 ml filter-sterilized enzyme solutions. Callus was chopped with the help of scalpel and forceps and transferred the whole content of the petri dish into a 150 ml conical flask containing 15 ml (5 ml + 15 ml = Total 20 ml) enzyme solutions. The three sets of enzyme solutions (Set-1, Set-2 & Set-3) were used here. The flask was incubated at 25±1 °C for three different periods, i.e., 10 hours, 14 hours, and 18 hours, with shaking at 60 rpm.

Protoplast purification

The enzymatic suspension was sieved in a 15 ml screw-capped tube using sterile steel mesh having a porosity of 50-100 µm and centrifuged at 100 x g for 3 min. The supernatant was removed and 10 ml

washing solution (20% sucrose) was added to dissolve the protoplast pellet. Centrifuged again at 100 x g for 10 minutes to form a ring on the upper phase of the sucrose solution which indicates a healthy and uniform size of protoplast population. The ring was collected with the help of a Pasteur pipette carefully and suspended in washing solution at a ratio of 1:9 and centrifuged again at 100 x g for 3 minutes. The supernatant was discarded and the pure protoplast pellet was completely resuspended in 10 ml washing solution. Centrifugation and resuspension of pure protoplast pellet in washing solution were repeated thrice to remove traces of enzyme and sucrose. Finally, the pellet was suspended in 1 ml washing solution for further use.

Viability test and protoplast density

The purified protoplasts were incubated in 0.01% FDA (fluorescein diacetate) staining solution for 5 minutes and viability was calculated using a hemocytometer under the UV microscope (Widholm, 1972). The viable protoplast showed yellow-green fluoresce after FDA staining and the remaining showed dark brown (dead cells).

The Percentage of viable protoplast was calculated by the following formula:

$$\frac{\text{The average number of viable protoplasts counted}}{\text{The average number of total protoplasts counted}} \times 100$$

Results

Seedlings and callus induction

Seed germination was observed in 48 hours of seed culture on MS medium (Fig. 1c&d). Hypocotyls were collected from 14 days old fully germinated and healthy seedlings for callus induction. Hypocotyl-callus were formed within 14 days (Fig. 1e) which were used for protoplast preparation.

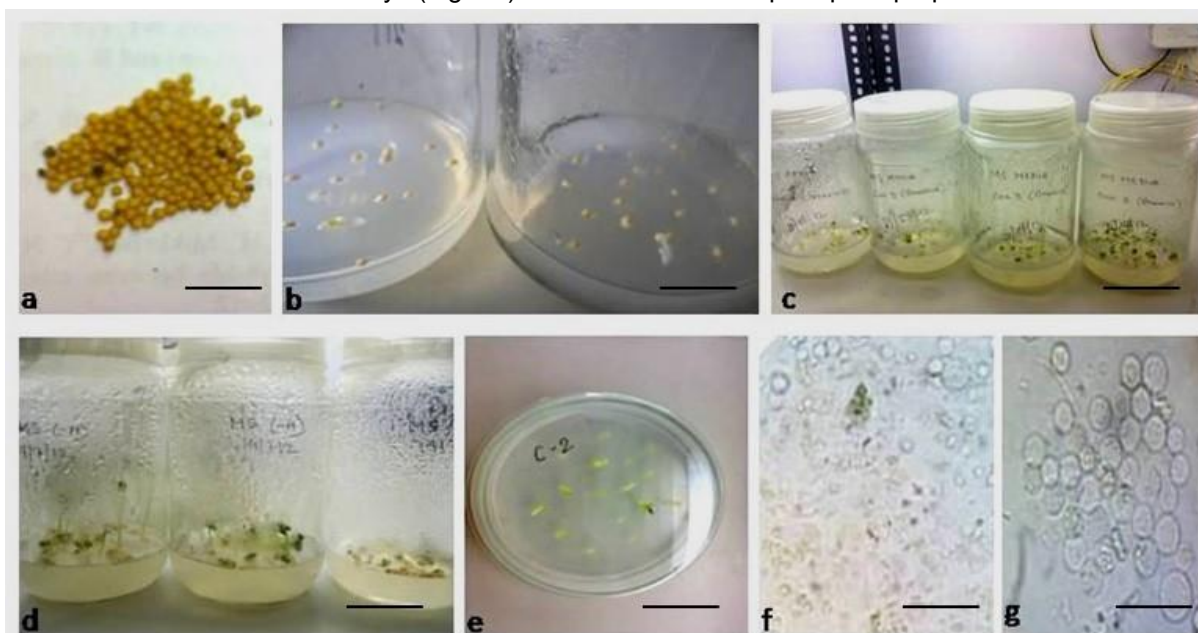


Figure 1: (a) Fresh surface sterilized seeds of *B. juncea*, var. B9. (b) seeds cultured on MS medium for germination, (c) germinated seedlings, (d) 7-days-old growing seedlings, (e) hypocotyl culture on callus induction medium, (f) isolated protoplasts, (g) purified protoplasts. Bar size (a) 1 cm, (b) 3 cm, (c) & (d) 70 cm, (e) 40 cm, (f) 200 μ m, (g) 100 μ m.

Optimization of enzymatic condition

Three sets of enzyme solutions were used which showed different protoplast yields (Table 2) and the microscopic views are shown in Fig. 1 f&g. The highest protoplast yield i.e., 14.26×10^5 / gm tissue was recorded in Set-1 with 14 hours of incubation, and the lowest i.e., 5.22×10^5 / gm tissue was recorded in Set-3 with 18 hours of incubation. Such results indicate that the combinations of various

cell wall degrading enzymes have a potential role in protoplast formation with treatment time. In all the sets, Macerozyme kept the same while Cellulase R10 and Driselese varied in percentage.

Table 2: Comparative study of protoplast yield under various conditions using 1 gm of callus tissue

Sl. No.	Protoplast yield after 10 hours	Protoplast yield after 14 hours	Protoplast yield after 18 hours
Set-1	9.44 x 10 ⁵	14.26 x 10 ⁵	7.92 x 10 ⁵
Set-2	6.12 x 10 ⁵	8.22 x 10 ⁵	9.66 x 10 ⁵
Set-3	11.36 x 10 ⁵	10.02 x 10 ⁵	5.22 x 10 ⁵

Viability and density of the purified protoplast

The viability of protoplasts was tested using the FDA staining method and 95.35% of the protoplasts were obtained as viable. This indicates that the maximum number of protoplasts are healthy enough and live cells which ultimately signifies the age of the tissue used. The density of the purified protoplast suspension of *Brassica juncea* var. B9 was measured with a hemocytometer and was recorded as 20.32 x10⁵ cells/ml/gm tissue.

Discussion

Protoplast isolation from hypocotyls callus of *Brassica juncea* var. B9 has been studied with changes in different enzyme combinations and incubation conditions. The cell-wall degrading enzymes of Cellulase R10, Macerozyme, and Driselese showed different results in various combinations under three different periods i.e., 10, 14, and 18 hours of incubation. Three sets of enzyme combinations were tested on hypocotyl-derived callus tissue of *Brassica juncea* var. B9 and found a discrete number in protoplast yield. In Set-2, where the enzyme Driselese is used in lower concentration, the protoplast yield was comparatively reduced than the other sets and gradually increased with higher time of incubation. But in Set-1, and Set-3, the protoplast yield was observed inconsistent with higher time of incubation. In Set-3, where 2% of Cellulase R10 was used, the protoplast yield was gradually decreased with higher time of incubation and was found to be the lowest after 18 hours of incubation. Only in Set-1, where 1.5% of Cellulase R10 was used, the protoplast yield increased from 10 hours state to 14 hours state but decreased after 18 hours of incubation. These results indicate that both the factors i.e., enzyme and time are found to be important to get optimum healthy protoplast yield. The viability% of the purified protoplasts was recorded above 95.35 which indicates that the maximum number of protoplasts were healthy enough and live and the similar findings had been reported in the past on different brassica species (Kaur et al., 2018; Stajic, 2023; Stelmach-Wityk et al., 2024). The density of the purified protoplasts was recorded as 20.32 x10⁵ cells/ml/gm tissue. These results occurred due to the use of callus tissue which is nothing but the mass of cells, unlike mesophyll tissue.

Conclusion

Protoplast technology in agricultural crop breeding becoming much more popular in the qualitative and quantitative development of crop varieties. It helps in somatic hybridization through the fusion of two parental protoplasts having sexual incompatibility. Gene transfer also can be continued using protoplast and their culture. However, the hypocotyl-derived callus formation and protoplast isolation in *Brassica juncea* var. B9 is optimized in this study which may further be used in commercial crop breeding programs in the area of agricultural biotechnology.

Acknowledgment

The authors are thankful to the Hon'ble Chancellor, Vice Chancellor, and Registrar of Srinath University, Jamshedpur for their kind support and encouragement.

Conflict of Interest

The authors declare no conflict of interest.

References

Bogunia, H., & Przywara, L. (2000). Effect of carbohydrates on callus induction and regeneration ability in *Brassica napus* L. *Acta Biologica Cracoviensia, Series Botanica*, 42(1), 79-86. Available at: <https://ruj.uj.edu.pl/server/api/core/bitstreams/e21c12e9-5586-423d-a1a0-7a29220a53b1/content>

- Cocking, E. C. (1960). A method for the isolation of plant protoplasts and vacuoles. *Nature*, 187(4741), 962-963. <https://doi.org/10.1038/187962a0>
- Glimelius, K. (1984). High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. *Physiologia plantarum*, 61(1), 38-44. <https://doi.org/10.1111/j.1399-3054.1984.tb06097.x>
- Hussain, S., Rasheed, A., Latif, M., Mahmood, T., & Naqvi, S. S. (2014). Canola (*Brassica Napus* L.) Regeneration and Transformation Via Hypocotyl and Hypocotyl Derived Calli, *Sarhad J. Agric*, 30(2), 165-172. Available at: <https://shorturl.at/3af42>
- Kaur, N., Vyvadilová, M., Klíma, M., Bechyně, M. (2018). A Simple Procedure for Mesophyll Protoplast Culture and Plant Regeneration in *Brassica oleracea* L. and *Brassica napus* L. *Czech J. Genet. Plant Breed*, 42, 103–110. <https://doi.org/10.17221/3649-CJGPB>
- Khan, M. M. A., Hassan, L., Ahmad, S. D., Shah, A. H., & Batool, F. (2009). In vitro regeneration potentiality of oil seed Brassica genotypes with differential BAP concentration. *Pakistan Journal of Botany*, 41(3), 1233-1239. Available at: <https://shorturl.at/cz8dc>
- Kirti, P. B., & Chopra, V. L. (1989). Plant regeneration from hypocotyl-derived protoplasts of *Brassica juncea* (L.) Czern and Coss. *Plant Cell Reports*, 7, 708-710. <https://doi.org/10.1007/bf00272067>
- Lone, J. A., Gupta, S. K., Wani, S. H., Sharma, M., Lone, R. A., & Shikari, A. B. (2017). Efficient callus induction and regeneration in *Brassica juncea* for environment friendly agriculture. *Indian Journal of Pure & Applied Biosciences*, 5(1), 135-141. <http://dx.doi.org/10.18782/2320-7051.2449>
- Narasimhulu, S. B., Kirti, P. B., Prakash, S., & Chopra, V. L. (1992). Rapid and efficient plant regeneration from hypocotyl protoplasts of *Brassica carinata*. *Plant Cell Reports*, 11, 159-162. <https://doi.org/10.1007/bf00232171>
- Razdan, M. K. (2003). *Introduction to plant tissue culture*. The Science Publishers, USA. Available at: <https://shorturl.at/7XFjP>
- Saxena, P. K., & King, J. (1990). Isolation and transplantation of nuclei into plant protoplasts. *Plant Cell and Tissue Culture*, 271-278. <https://doi.org/10.1385/0-89603-161-6:271>
- Stajič, E. (2023). Improvements in Protoplast Isolation Protocol and Regeneration of Different Cabbage (*Brassica oleracea* var. *capitata* L.) Cultivars. *Plants* (Basel, Switzerland), 12(17), 3074. <https://doi.org/10.3390/plants12173074>
- Stelmach-Wityk, K., Szymonik, K., Grzebelus, E. et al. (2024). Development of an optimized protocol for protoplast-to-plant regeneration of selected varieties of *Brassica oleracea* L.. *BMC Plant Biol*, 24, 1279. <https://doi.org/10.1186/s12870-024-06005-4>
- Von Klercker, J. (1892). A method for isolating living protoplasts. *Ofvers Vetensk. Akad. Forh. Stockholm*, 49.
- Widholm, J. M. (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology*, 47(4), 189-194. <https://doi.org/10.3109/10520297209116483>