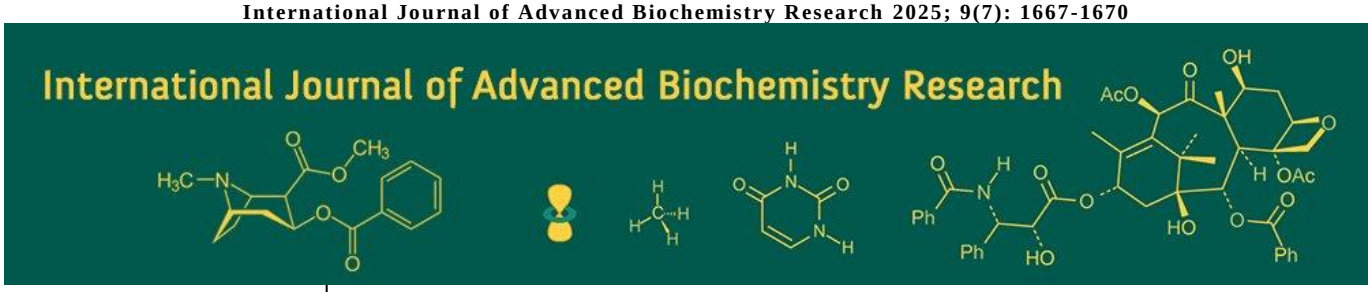

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Studies on protoplast isolation in soybean [(*Glycine max* (L.) Merr.]

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Abstract
This study establishes a reliable and efficient protocol for protoplast isolation from leaf explants of soybean (*Glycine max* L.) cv. MAUS 158, a stem fly-tolerant and high-yielding genotype. leaf tissue was utilized to evaluate explant-specific enzymatic digestion conditions. Preplasmolysis was performed using 13% mannitol, followed by enzymatic digestion with a combination of 2% cellulase and 1% macerozyme. Optimal incubation times 18 was determined for leaf tissue. The protoplast was purified using a 21% sucrose gradient and assessed for yield and viability. Leaf tissue showed the highest protoplast yield (3.60×10^4 cells/g), highest viability rates (>83%). The study confirms that the selected enzyme combination, in conjunction with explant-specific digestion times, supports the successful isolation of viable protoplast from soybean leaf tissues. This optimized protocol is applicable for advanced studies in plant regeneration, somatic hybridization, and genetic transformation.

Keywords: Protoplast isolation, preplasmolysis, enzymatic digestion, protoplast viability

1. Introduction
The soybean, (*Glycine max* L.) Merrill, is a legume crop originating in Eastern Asia, particularly China. Global soybean production in 2024-25 reached 420.6 million metric tons, with India ranking 5th globally (Food and Agriculture Organization of the United Nations. 2024). Madhya Pradesh and Maharashtra are the main soybean-growing states in India. Soybeans are valued for their oil content and protein-rich seeds, providing health benefits and serving as a vital protein source for humans and animals. However, soybean cultivation faces challenges from diseases, pests, and abiotic stresses like drought and floods. Plant tissue culture methods, including protoplast technology, offer opportunities for crop improvement through genetic modification. Protoplasts are plant cells without a cell wall, isolated from surrounding cells and consisting of the inner part of a plant cell surrounded by a protective plasma membrane. Protoplasts can regenerate into a complete plant by re-forming a cell wall. Protoplasts are single cells capable of producing identical clones (Ling *et al.*, 2010) [4]. A significant advancement in plant cell research was the enzymatic extraction of protoplasts from somatic tissues (Cocking, 1960) [1], enabling the isolation of a large number of protoplasts and expanding possibilities in protoplast culture. Protoplasts can form a new cell wall, divide to form cell colonies, and eventually develop callus tissue. Under suitable conditions, callus tissue can be induced to produce complete plants. This unique ability has facilitated the production of whole plants from cultured protoplasts of various plant species (Patil *et al.*, 2008) [7]. However, achieving this in many economically important crop plants has been challenging, primarily due to technical constraints or limitations in the methods used rather than any inherent inability of protoplasts primarily due to technical constraints or limitations in the methods used rather than any inherent inability of protoplasts. The successful isolation of viable protoplasts relies on specific conditions such as enzyme type, enzyme concentrations, temperature, osmotic stabilizers, and incubation periods. Protoplasts have intriguing characteristics, including the ability to merge through somatic cell hybridization, incorporate external genetic material (genetic transformation), and assimilate intact organelles like chloroplasts and mitochondria. These capabilities offer the potential to introduce beneficial genetic diversity from previously untapped sources into crop species. The advancement of protoplast systems has become essential in genetic engineering and crop breeding, enhancing plant adaptability (Maheshwari *et al.*, 1986) [5].

2. Materials and Methods

2.1 Plant Material

The plant material used in this study was soybean **(***Glycine max* L.) cultivar MAUS 158, seeds of the same were collected from soybean research station**,** VNMKV, Parbhani. Leaf explant was used for protoplast isolation. Soybean seeds were first washed thoroughly under running tap water and then subjected to surface sterilization under aseptic conditions, including: (i) soaking in 70% (v/v) ethanol for 30 seconds, (ii) triple rinsing with sterile distilled water, (iii) treatment with 0.1% (w/v) Bavistin solution for 3 minutes, followed by another triple rinse (iv) exposure to 0.1% (w/v) sodium hypochlorite ($HgCl_2$) for 3 minutes, followed by a final triple rinse with sterile distilled water. The sterilized seeds were germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) [6] supplemented with 1.5% (w/v) sucrose and solidified with 0.8% agar. The medium pH was adjusted to 5.8 before autoclaving. The Cultures were incubated in a growth chamber maintained at 25±1 °C, with a 16/8-hour light/dark photoperiod and a light intensity of 25 µmol m^{-2} s^{-1} using cool white fluorescent lamps**.** Leaf explant was excised from 7-day-old *in vitro* grown seedlings used for protoplast isolation experiments.

2.2 Preparation of Explants for Protoplast Isolation

Protoplast isolation involved preparing leaf tissue of soybean, using standardized procedures. Leaf tissue from 7-day old *in vitro* seedlings was cut into 1-2 mm pieces and incubated in 10 ml of CPW solution with 13% mannitol for 60 minutes for Preplasmolysis., following the method of Wu and Hanzawa (2018) [12]

2.3 Enzyme Incubation

Pre-plasmolysis was induced by treating plant explant with CPW salt solution containing 13% mannitol for 60 minutes, promoting the detachment of the protoplasm from the cell wall and enabling easier release during digestion. Following this, the CPW solution was replaced with an enzyme mixture, and each plasmolyzed sample was incubated with 10 ml of the enzyme solution at 27° C and shaken at 60-70 rpm for 18 hours to digest the cell walls and liberate the protoplasts. These enzyme mixtures (pH 5.8) was tested on leaf explant to find the most effective combination. The enzymes used included Cellulase 2% (w/v) and Macerozyme 1% (w/v). Mannitol (0.75 M) was added to the mixture to help maintain the osmotic balance and protect the protoplasts. The mixture also contained important salts like KCl (0.5 mM) and

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Table 1: Composition of Solutions Used for Protoplast Isolation and Purification

Sr. No.	Component	CPW Solution (mg/L)	W5 Solution (M)	WI Soluti-on (M)	Flotation Solution (%)
1	Potassium dihydrogen phosphate (KH_2PO_4)	27.2	-	-	-
2	Potassium nitrate (KNO_3)	101	-	-	-
3	Calcium sulphate ($CaCl_2.2H_2O$)	1480	125 mM	-	-
4	Magnesium sulphate ($MgSO_4.7H_2O$)	246	-	-	-
5	Potassium iodide (KI)	0.16	-	-	-
6	Copper chloride ($CuSO_4.5H_2O$)	0.025	-	-	-
7	Mannitol ($C_6H_{14}O_6$)	130,000	-	0.5 M	-
8	Potassium chloride (KCl)	-	5 mM	20 mM	-
9	Sodium chloride (NaCl)	-	154 mM	-	-
10	2-N-morpholino ethanesulfonic acid(MES) ($C_6H_{13}NO_4S$)	-	2 mM	4 mM	-
11	Sucrose ($C_6H_{22}O_{11}$)	-	-	-	21%

2.4 Protoplast Isolation and Purification

The resulting digested solution was filtered through a 150 µm nylon mesh using W5 solution to remove undigested tissue and debris. The filtrate was then centrifuged at 100 × g for 10 minutes, and the protoplast pellet was carefully re-suspended in a washing and incubation solution. To purify viable protoplasts, a 21% (w/v) sucrose solution was layered beneath the protoplast suspension to create a density gradient, followed by centrifugation at 100 × g for 5 minutes. The viable protoplasts, collected at the sucrose interface, were transferred to a clean tube, re-centrifuged to remove residual sucrose, and finally re-suspended in WI solution or an appropriate culture medium for further experimentation.

2.5 Counting of Protoplasts

The initial protoplast yield must be calculated since it will be used for all future operations, including fusion transformation and plant regeneration. Protoplast culture density needs to be established for ideal culture conditions and subsequent divisions. We used a Fuchs Rosenthal marking haemocytometer to count the protoplasts.(Lei *et al.*, 2015) [3]

The yield was determined using,

$$Y = n \times D.F. \times 10^4$$

Where,
Y = Yield (Total),

n = Average no. of protoplasts/square,
D.F.= (Dilution Factor),
10^4 = Conversion of volume into ml.

2.6 Protoplast Viability
Protoplast viability was evaluated by staining with trypan blue dye (0.4%). The protoplast was mixed with trypan blue solution in equal parts and incubated at room temperature for 3-5 minutes. The mixture was then placed on a Neuberger chamber with a cover slip. Healthy and damaged protoplast was counted using a 100x objective lens, focusing on the four corner squares and the central square of the chamber. This staining technique allowed for a quick and effective assessment of protoplast viability (Wang *et al.*, 2022) [11].

3. Results and Discussion
3.1 Effect of Explant and Incubation Time on Protoplast Yield and Viability
Protoplast was successfully isolated from leaf explants of soybean (*Glycine max* L.) cv. MAUS 158 using a combination of 2% cellulase and 1% macerozyme. The enzymatic digestion was performed at 27°C in the dark, with gentle agitation (70 rpm), and the incubation period was 18 hours. Leaf explants incubated for 18 hours yielded mesophyll derived protoplasts with high viability (84%) and highest protoplast density (3.60 x 10^4 cells/g). The prolonged digestion was essential to soften the rigid leaf tissues and release viable protoplasts. The denser tissue structure required prolonged enzyme exposure to achieve cell wall degradation. These results emphasize the importance of explant-specific incubation durations to maximize both protoplast yield and viability. Protoplasts have been successfully isolated from various plant tissues, such as tomato seedling root tips (Cocking, 1960) [1], leaves from *in vitro*-cultured *Lupinus angustifolius* and *L. subcarnosus* (Sonntag *et al.*, 2008) [10], and different organs like leaf mesophyll, petals, hypocotyls, roots, and nodules in *Phaseolus vulgaris* (Nanjareddy *et al.*, 2016). Additionally, callus tissues from cotton (Wang *et al.*, 2022) [12], rice (Poddar *et al.*, 2020) [8], and legume roots (Jia *et al.*, 2018) [2] have also been recognized as reliable sources for protoplast isolation. In leguminous crops such as chickpea and soybean, fully expanded leaves are typically considered the most suitable tissue for effective protoplast recovery (Wang *et al.*, 2020; Wu *et al.*, 2018) [11, 12]

3.2 Role of Enzymatic Composition and Osmotic Conditions in Protoplast Recovery
The combination of 2% cellulase and 1% macerozyme was crucial for efficient cell wall degradation across all explants. Cellulase helped dissolve cellulose micro fibrils, while macerozyme broke down the pectic substances of the middle lamella, enabling effective separation of plant cells. The use of 0.75 M mannitol in both preplasmolysis and enzymatic solutions played a key role in maintaining osmotic balance, thereby protecting the protoplasts from bursting or collapsing. The pH of 5.8, maintained using MES buffer, and the addition of calcium chloride and bovine serum albumin (BSA) helped stabilize the plasma membrane and preserve protoplast viability during isolation. These carefully controlled enzymatic and osmotic conditions ensured high-quality protoplast recovery. The findings align with previous studies (Wu & Hanzawa, 2018; Wang *et al.*, 2022) [12, 11], supporting the necessity for a tailored, tissue-specific approach in protoplast isolation protocols. This study demonstrates that the standardized enzyme concentration, when paired with explant-specific incubation times, results in efficient, high-quality protoplast recovery from leaf tissues of soybean.

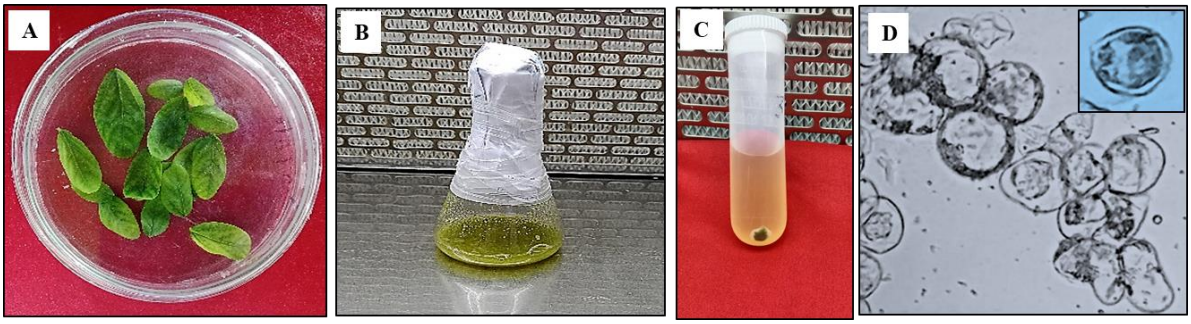

Fig 1: A) Excised Soybean Leaves Used for Protoplast Isolation. B) Enzymatic Digestion of Leaf Tissue in Cell Wall Degrading Solution. C) Filtration and Collection of Released Protoplasts D) Microscopic View of Isolated Leaf Protoplasts (*Inset: Single Intact Protoplast*)

4. Conclusion
This study successfully developed and optimized a reliable method for the efficient isolation of viable protoplasts from leaf explants of soybean (*Glycine max* L.) cv. MAUS 158. By employing a standardized enzyme mixture of 2% cellulase and 1% macerozyme and adjusting incubation durations based on explant type, the study achieved high protoplast yield and viability. Leaf tissue emerged as a productive source for protoplasts with superior viability. The findings underscore the necessity of tailoring enzymatic digestion conditions to specific explant types. This optimized protocol holds significant potential for applications in soybean improvement programs, particularly in somatic hybridization, transient gene expression studies, and future genetic engineering strategies.

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