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# Isolation of Mesophyll Protoplasts from Leaves of *Dalbergia sissoo* Roxb

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**ABSTRACT:** *Dalbergia sissoo* is an important timber tree facing mysterious die back and wilting problem. In case of die back, *Dalbergia* is facing the threat of destruction in its natural habitats due to lack of potential pathogenicity test which is the major bottleneck in pathogen assessment and tree improvement programmes. Isolation of protoplasts was attempted to produce an effective source for the pathogenicity test. This study described a procedure for the rapid isolation, in high yield, of photosynthetically active mesophyll protoplasts from young leaves of *D. sissoo*. The present study reports the isolation of protoplasts from leaf mesophyll of *D. sissoo*. Leaf strips were suspended in the enzyme solution for the isolation of protoplast. Different concentrations of enzymes were used to optimize the suitable combination for the protoplast isolation. Enzyme solution turned green after a gentle swirling motion, which indicates the release of protoplasts. Release of protoplast was checked in the solution under the microscope. A combination of filtration, centrifugation and washing was used to purify the protoplasts. The optimum combination of enzymes for protoplast isolation was 1.5% cellulase R-10+ 0.5 % pectinase R-1 after incubation for 6 hr at 28° C. The isolated protoplasts were round and filled with chloroplasts. The size of protoplasts was 20~35 µm. The protoplast yield was $2 \times 10^5$ per g of leaf tissue. The protoplast viability as assessed by 0.01% Phenosaphranine staining was 77%. @JASEM

*Key words: Dalbergia sissoo*, Leaf mesophyll, Protoplasts, Phenosaphranine.

*Dalbergia sissoo* Roxb, a deciduous tree of family Papilionaceae, is an important multipurpose timber tree timber tree of great economic importance. *D. sissoo* has been inflicted with decline due to major diseases viz. wilt and dieback. It was in 1998 that decline was reported as an epidemic in central tract of Punjab Province (Naz 2002, Bajwa et al. 2003). It is observed that dieback is an old age tree disease and can not be induced in young plant for the establishment of causal pathogen. With the reference to dieback no satisfactory tool has been designed for pathogenicity test.

Protoplasts offer an attractive tool to study several aspects of plant cell biochemistry and physiology, such as photosynthesis (Devi et al. 1992, Yoo et al. 2007), intracellular distribution of metabolites (Robinson and Walker 1980), isolation of intact chloroplasts (Walker 1988) and transport/accumulation of organic/inorganic compounds. Protoplasts are also used in production of hybrids, cybrids, genetic transformation and to study several aspects of modern biotechnology, including the synthesis of pharmaceuticals (Sheen 2001, Yanagisawa et al. 2003, Davey et al. 2005). Plant protoplasts have also been used to determine the mechanism of fungal toxin action at the cell level (Earle et al. 1978, York et al. 1980) in bioassays of crude filtrates and partially or fully purified toxins (Nachmias et al. 1990, Wolf and Earle 1991, Gentile et al. 1992, Asai et al. 2000, Zhang et al. 2004, Daub et al. 2005, Iakimoval et al. 2007), differential responses between susceptible and resistant protoplasts has been noted. Protoplasts can be isolated from different plant organs. Mesophyll tissues of leaves are one of the convenient sources for a large number of uniform cells for protoplast isolation. Protoplasts isolated from plant tissues retain their cell identity and differentiated state. Therefore, protoplasts are regarded as totipotent cells. So, an ideal technique for protoplast preparation aims at achieving rapid isolation with maximum yield and high metabolic integrity of protoplasts. Therefore, the objective of this investigation was to establish a optimized system for the protoplasts isolation from *D. sissoo* leaves, in order to offer an important technique/tool for the research of plant response in the presences of its pathogen, forest engineering, plant cell mechanics.

## MATERIALS AND METHODS

*Plant material:* Fully expended young leaves age of 3~4 wk were collected from mature tree of *Dalbergia sissoo*. Detached leaves were rinsed with thoroughly with running water to remove dust and other entities form the leaf surface, blotted and dry.

*Enzyme solution*: One-step method of enzyme digestion was used to release the protoplasts, i.e. the tissue was digested with a mixture of macerozyme and cellulase. For enzyme solution cellulose R-10 (wt/vol), pectinase R-10(wt/vol) were dissolved in Cell Protoplast Washing (CPW) medium (Table 1) at 5.8 pH. Solution was warmed at 55° C for 10 min to inactivate DNAse and proteases and enhance enzyme solubility and was cooled at room temperature (25~30℃). Different concentrations of these 2 enzymes were used (Table 2) for the optimization. For osmotic strength of cytoplasm and the isolation

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medium, different concentrations of mannitol (7, 8, 9, and 10%) were also optimized. Final the prepared enzyme solution was clear light brown. Final enzyme solution was filtered through a 0.45-mm syringe filter device into a 50 mm Petri dish solution.

*Protoplast isolation*: Leaves were cut 0.5~1-mm leaf strips from the middle part of a leaf using a fresh sharp razor blade without tissue crushing at the cutting site to ensure proper enzymatic digestion, as it is difficult to peel-off the epidermis. Leaf strips were transferred quickly and gently into filter-sterilized enzyme solution (7~14 leaves in 5~10 ml) in 50-mm Petri dishes by dipping both sides of the strips using a pair of flat-tip forceps. The material was kept for different time interval at 28°C without shaking under continuous illumination up to 8 hr. Changes occurring during incubation were observed at 2 hr intervals. Enzyme solution turned green after a gentle swirling motion, which indicates the release of protoplasts. Release of protoplast was checked in the solution under the microscope. Enzyme/protoplast solution was diluted with an equal volume of CPW salt solution (Table 1) before filtration to remove undigested leaf tissues.

*Purification of protoplast:* A combination of filtration, centrifugation and washing was used to purify the protoplasts. Isolated protoplasts were filtered through a nylon mesh (60~72 µm) and transferred into 10 ml centrifuge tubes. CPW salt solution (Table 1) was added to the protoplast suspension and centrifuged at 100 g for 5 minutes. The supernatant was removed and the protoplast pellet was re-suspension in CPW solution by gentle swirling and centrifuged at the same parameters.

Calculation of protoplast was done by hemacytometer (Vancha et al. 2004) that has the chamber depth of 0.2 mm. The ruling consisted of 16 large square of 1 mm each. Each large square was divided onto 16 subsquare with a side of 0.25 mm and an area of 0.0625 mm. For counting the protoplast, a drop of protoplast suspension was gently transferred into the chamber. Chamber with covered with cover slip, protoplast s were counted using a light microscope with a 10 × 10 objective. Several minisquares were counted and average number per minisquare was calculated. Protoplast (pp) concentration was calculated as:

Number of pp × 16 × 10,000 = Number of protoplast/ml

Protoplasts were re-suspended in CPW solution after counting cells under the microscope using a hemacytometer. All yield assessments were repeated at least 3 times.

*Protoplast viability:* One to 2 drops of 0.1% phenosaphranine was added to 10 ml of CPW solution and mixed well. Equal volumes of a dense protoplast suspension and CPW+ dye solution were mixed. After 10 min of incubation viability was determined using microscope. Protoplasts exhibiting green color were regarded as being viable and red stained were considered dead. Results were expressed as the percentage of viable protoplasts per field with each count including at least 500 randomly-chosen protoplasts.

## RESULTS AND DISCUSSION

Isolation procedures that yield highly purified and functional protoplasts have been described for many species. The isolation of plant protoplasts was first reported more than 50 yr ago (Cocking 1960). Isolated protoplasts allow the study of various metabolic processes. Freshly isolated protoplasts have been proved to be physiological and versatile cell systems for studying a broad spectrum of plant physiology, plant cell biology, plant gene engineering, biomechanics, stress responses and cell death controls (Sheen 1999, Asai et al. 2000, Cocking 2000, Bethke and Jones 2001, Sheen 2001, Tena et al. 2001).

**Table 1.** Composition of Cell ProtoplastWashing (CPW) media used for protoplast isolation

| Components | CPW medium (mg /L) |
|---|---|
| $KNO_3$ | 101.0 |
| $CaCl_2.2H_2O$ | 1480.0 |
| $MgSO_4.7H_2O$ | 246.0 |
| $KH_2PO_4$ | 27.2 |
| KI | 0.16 |
| Mannitol* | 7% |
| BSA | 0.1% |
| pH | 5.8 |

* Was added in g $l^{-1}$.

Leaf mesophyll tissues of a wide range of plants, have been used as a protoplasts source with success. Novel observations on the intriguing interactions of chloroplasts with other organelles in a green cell are also reported through studies using mesophyll protoplasts (Krömer and Heldt 1991, Saradadevi and Raghavendra 1992, Igamberdiev et al. 1997).

In this study, concentration levels of enzymes, incubation time, and osmolarity were considered for optimize protoplast isolation form mesophyll tissues. Young expended *D. sissoo* leaves were used for the isolation of protoplasts. Since it was difficult to peal-off lower epidermis in *D. sissoo*, leaf tissue strips

were incubated in different concentrations of mannitol and combinations of enzyme solutions. The protoplast isolation solution containing 7% mannitol was found needed for releasing and maintaining viable protoplasts.

**Table 2.** Composition of Mannitol and Enzymes for mesophyll protoplast isolation

| Mannitol (%) | Pectinase (%) | Cellulose (%) |
|---|---|---|
| 10 | 0.5 | 0.1 |
| 9 | 0.5 | 0.1 |
| 8 | 0.5 | 0.1 |
| 7 | 0.5 | 0.1 |
| 6 | 0.5 | 0.1 |
| 5 | 0.5 | 0.1 |
| 10 | 0.5 | 0.2 |
| 9 | 0.5 | 0.2 |
| 8 | 0.5 | 0.2 |
| 7 | 0.5 | 0.2 |
| 6 | 0.5 | 0.2 |
| 5 | 0.5 | 0.2 |
| 10 | 0.1 | 0.3 |
| 9 | 0.1 | 0.3 |
| 8 | 0.1 | 0.3 |
| 7 | 0.1 | 0.3 |
| 6 | 0.1 | 0.3 |
| 5 | 0.1 | 0.3 |

* Enzyme solutions were prepared in CPW medium.

On the basis of periodical microscopic observations, the liberation of cell clusters and individual cells after 2 hr of incubation in enzyme solution were monitored (Fig. 1a, b).

For mesophyll tissues, 0.5% pectinase was adequate to digest the middle lamella and separate the cells. Among the tested enzyme solutions concentrations, only 0.5% of pectinase and 1.5% of cellulose was found sufficient to digest the cell wall and release protoplasts after incubation for 6 h. The results also showed that the yield of protoplasts was lower with lower cellulase concentration. When cellulose concentration was increased to 1.5% (w/v), yield of protoplasts release also increased, but when increased, result was reversed. Lower concentration of cellulase cannot liberate concentration protoplasts sufficient, but higher cellulase concentration affect on integrity of membrane and reduces their physical activities, even may cause over-digestion of plant materials (Kim et al. 2000, Koster et al. 2003, Monteiro et al. 2003). The isolated protoplasts were round and filled with chloroplasts (Fig. 1c). The size of protoplasts was 20~35 µm. The protoplast yield was $2 \times 10^5$ per g of leaf tissue. The protoplast viability was assessed 77% by Phenosaphranine staining.

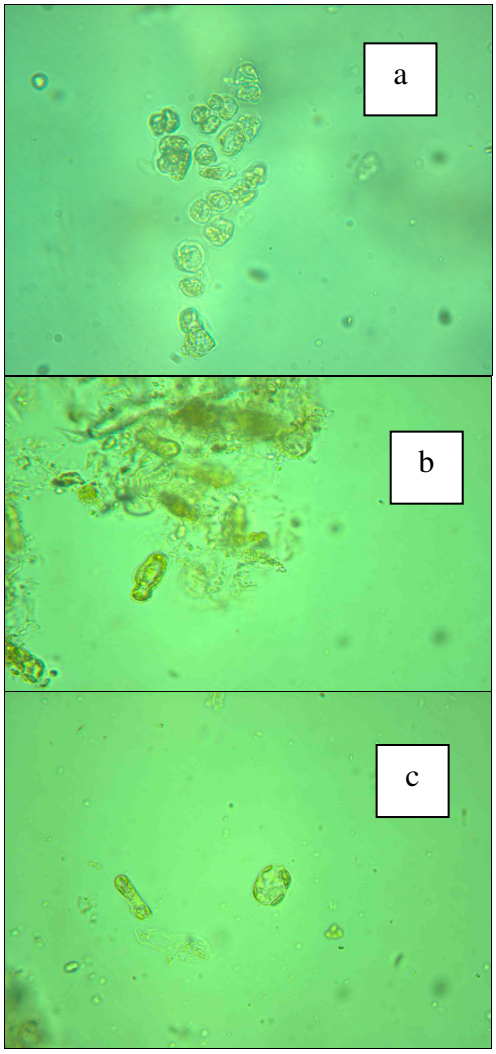


**Fig 1. a,** liberation of cell clusters; b, Liberation of protoplast; c, Round protoplast.

The enzymatic (cellulase) isolation of protoplasts was first reported in tomato from root tips by Cocking (Cocking 1960). The easy availability of commercial, purified enzymes such as cellulase, cellulysin, pectinase, macerozyme, driselase, rhozyme and hemicellulase has now increase in the yield and viability of protoplasts and their subsequent response in the culture medium. Commonly a combination of pectinase and cellulase is used to digest the cell walls and also liberate protoplasts in a single cell (Power and Cocking 1970). The concentration and combination of enzymes for the isolation depend upon age, genotype and stage of differentiation of the tissue from which the protoplasts are to be isolated. Though protoplasts can be isolated from a variety of tissues, young *in vitro*-grown plants (Bajaj 1972), tissues and explants such as root tips (Xu et al. 1982), hypocotyl, cotyledons (Hammatt et al. 1987) and shoots (Russell and McCown 1986) and leaves from

old or mature plants (Sheen 2001). Protoplasts were isolated from *D. sissoo* 20~35 µm in diameter. Chloroplasts were arranged around the periphery of cell but also observed in the in middle of some cells.

The present study indicated that low concentrations of pectinase and cellulase were sufficient for release of protoplasts in short incubation period. The visibly distinguishable nature of protoplasts can be exploited for host - pathogen relationship, genetic transformation by cell modification and somatic hybridization, which could have far-reaching implications in disease assessment and sustainable improvement programmes of this commercially important timber tree.

## REFERENCES

Asai T, et al. 2000. Fumonisin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell 12:1823-35.

Bajaj YPS. 1972. Protoplast culture and regeneration of haploid tobacco plants. Am J Bot 59:647.

Bajwa R, Arshad J, Saleh A. 2003. Extend of shisham (*Dalbergia sissoo* Roxb.) decline in Sialkot, Gujaranwala, Lahore and Sargodha districts. Mycopath 1:1-5.

Bethke PC, Jones RL. 2001. Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. Plant J 25:19-29.

Cocking EC. 1960. A method for the isolation of plant protoplasts and vacuoles. Nature 187:927-9.

Cocking EC. 2000. Turning point article plant protoplasts, In Vitro Cell. Dev Biol Plant 36:77-82.

Daub E, Herrero S, Chung K. 2005. MiniReview; Photoactivated perylenequinone toxins in fungal pathogenesis of plants. Margaret FEMS Microbiol Lett 252:197-206.

Davey MR, Anthony P, Power JB, Lowe CL. 2005. Plant protoplasts: status and biological perspectives. Biotechnol Adv 23:131-71.

Devi MT, Vani T, Reddy MM, Raghavendra AS. 1992. Rapid isolation of mesophyll and guard cell protoplasts from pea and maize leaves. Indian J Exp Biol 30:424-8.

Earle ED, Gracen VE, Yoder OC, Gemmill KP. 1978. Cytoplasmspecific effects of *Helminthosporium maydis* race T toxin on survival of corn mesophyll protoplasts. Plant Physiol 61:420-4.

Gentile A, Tribulato E, Continella G, Vardi A. 1992. Differential responses of citrus calli and protoplasts to culture filtrates and toxin of *Phoma tracheiphila*. Theor Appl Genet 83:759-64.

Hammatt N, Kim HI, Davey MR, Nelson RS, Cocking EC. 1987. Plant regeneration from cotyledon protoplasts of *Glycine canascens* and *G. glandestina*. Plant Sci 48:129-35.

Iakimoval ET, Weltering EJ, Yordanova ZP. 2007. Toxin- and cadmium-induced cell death events in tomato suspension cells resemble features of hypersensitive response. J Fruit Ornamental Plant Res15:5-19.

Igamberdiev AU, Zhou G, Malmberg G, Gardeström P. 1997. Respiration of barley protoplasts before and after illumination. Physiol Plant 99:15-22.

Kim BK, Kang JH, Jin M, Kim HW, Shim MJ, Choi EC. 2000. Mycelial protoplast isolation and regeneration of *Lentinus lepideus*. Life Sci 66:1359-67.

Koster KL, Reisdorph N, Ramsay JL. 2003. Changing desiccation tolerance of pea embryo protoplasts during germination. J Exp Bot 54:1607-14.

Krömer S, Heldt HW. 1991. On the role of mitochondrial oxidative phosphorylation in photosynthesis metabolism as studied by the effect of oligomycin on photosynthesis in protoplasts and leaves of barley (*Hordeum vulgare*). Plant Physiol 95:1270-6.

Monteiro M, Appezzato-da-Gl´oria FB, Valarini MJ, Oliveira I de CA, Carneiro ML, Vieira I. 2003. Plant regeneration from proroplasts of alfalfa (*Medicago sativa*) via somatic embryogenesis. Scientia Agricola 60:683-9.

Nachmias A, Orenstein J, Tal M, Goren M. 1990. Reactions to a *Verticillium dahliae* phytotoxin in tissue cultures derived from susceptible and tolerant potato. Plant Sci 68:123-30.

Naz SI. 2002. The vanishing shisham tree. The daily

Dawn, Jan. 4th 2002. Lahore, Pakistan.

Power JB, Cocking EC. 1970. Isolation of leaf protoplast: Macromolecule uptake and growth substance response. J Exp Bot 21:64-70.

Robinson SP, Walker DA. 1980. Distribution of metabolites between chloroplast and cytoplasm during the induction phase of photosynthesis in leaf protoplasts. Plant Physiol 65:902-5.

Russell JA, McCown BH. 1986. Culture and regeneration of Populus leaf protoplasts isolated from non-seedling tissue. Plant Sci 46:33-142.

Saradadevi K, Raghavendra AS. 1992. Dark respiration protects photosynthesis photoinhibition in mesophyll protoplasts of pea (*Pisum sativum*). Plant Physiol 99:1232-7.

Sheen J. 1999. C4 gene expression. Ann Rev Plant Physiol Plant Mol Biol 50:187-217.

Sheen J. 2001. Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127:1466-75.

Tena G, Asai T, Chiu WL, Sheen J. 2001. Plant MAP kinase signaling cascades. Curr Opin Plant Biol 4:392-400.

Vancha AR, Govindaraju S, Parsa KVL, Jasti M, Gonz´alez- Garc´ıa M, Ballestero RP. 2004. Use of polyethyleneimine polymer in cell culture as attachment factor and lipofection enhancer. BMC Biotechnol 4:23.

Walker D. 1988. The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. Sheffield, UK: Univ of Sheffield Press. p 188.

Wolf SJ, Earle ED. 1991. Effects of *Helminthosporium carbonum* race 1 toxin on host and non-host cereal protoplasts. Plant Sci 70:127-37.

Xu ZH, Davey MR, Cocking EC. 1982. Organogenesis from root protoplasts of the forage legumes *Medicago sativa* and *Trigonella foenum-graecum*. Z. Pflanzenphysiol 107:231-5.

Yanagisawa S, Yoo SD, Sheen J. 2003. Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425:521-5.

Yoo S, Cho Y, Sheen J. 2007. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protocols 2:1565-72.

York DW, Earle ED, Gracen VE. 1980. Ultrastructural effects of *Helminthosporium maydis* race T toxin on isolated corn mitochondria within corn protoplasts. Can J Bot 58:1562-70.

Zhang KH, Zhang X, Mao BZ, Qun L, Zu HH. 2004. Alpha-picolinic acid, a fungal toxin and mammal apoptosis-inducing agent, elicits hypersensitive-like response and enhances disease resistance in rice. Cell Res 14:27-33.