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Protoplasts isolation of soy bean *Glycine max* (L.) Merrill from leaf explants

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Abstract
A simple protoplast isolation protocol that was designed to recover totipotent plant protoplasts with relative ease has been described. The key elements of the protocol are, tissue digestion at slightly elevated temperatures and use of protoplast releasing enzymes that are stable and efficient at higher temperatures· besides enzymes, the protoplast isolation cocktail consisted of an Osmoticum (Mannitol or $MgSO_4$), and a Protectant ($CaCl_2$ $2H_2O$), all dissolved in distilled water. The protocol has ensured reproducibility, higher yields and is gentle on protoplasts as the protoplasts obtained were amenable to cell wall regeneration and cell division· Plant regeneration was demonstrated for *Glycine maxo* L. from protoplasts isolated by this method· Wall regeneration and cell division were obtained in other species. The merits of the protocol are, simple and easy-to-handle procedure, non-requirement of preconditioning of donor plant and explants, incubation without agitation, satisfactory yields, cultivability of the protoplasts isolated and applicability of the protocol to a large number of species including mucilage-containing plants.

Keywords: Plant protoplasts; isolation; simple protocol; totipotency

Introduction
Legumes are a group of economically important plants valued for food, fodder, wood, ornamentals, and raw materials for industry and also for their role in biological nitrogen fixation (Duke 1981) [10]. Grain legumes are a major source of proteins for more than two billion people worldwide. Plant transformation technology has gradually become a useful tool for cultivar improvement as well as for studying gene function. A reliable regeneration system to be precise, is universally required in most currently available transformation protocol to recover pants. Plants are regenerated from cell or callus culture either through organogenesis or somatic embryogenesis (Hansen *et al.*, 1999) [17].

Soybean occupies top rank in the world as well as in India it has also secured first rank among oil seeds making its dent on agricultural scenario and farmers' economy. It provides the highest protein (approximately 40%) among the pulses, reasonably good amount of vegetable oil (approximately 20%) in seed, which is rare in other pulses. India ranks fifth inthe area and production in the world after USA, Brazil, Argentina and China. At present, it is cultivated in 108.834 lac hectares at an average total production of about 104.36 lakh million tonnes with average productivity 959 kg/ha in India (SOPA, 2014).

Soybean *G. max* (L.) Merr in one of the major grain legumes of tropical and subtropical regions and in grown for its high seed protein and oil contents. *In vitro* regeneration of plants via somatic embryogenesis has much potential for plant propagation and gene transfer (Sato *et al.*, 1993) [42]. In soybean somatic embryos have been obtained from cultured immature cotyledons (Lippman 1984) [28] leaf and stem (Ghazi 1986) [14], cotyledonary node (Kerns *et al.*, 1986) [20] and another (Santos 1997) [41] Some of the serious limitations of the somatic embryo genesis protocols are the low frequency, inconsistency, genotype specificity and occurrence of callus phase prior to embryogenesis. The author reports here a protocol for *in vitro* embryogenesis and plant regeneration of soybean from embryogenic callus line derived from the shoot tip explants.

Tissue culture and plant regeneration are required to generate of transgenic plants, and these techniques open new possibilities for improving soybean *Glycine max* (L.) Merril. The success of soybean transformation has been limited due to the low efficiency of transformation methods. The major critical prerequisite for all transformation procedures is the ability in establishing and maintaining a culture with highly responsive plant tissues. One of the most efficient methods for soybean regeneration is somatic embryogenesis, first described in 1983

(Christianson *et al*., 1983) [8]. After this, many reports of soybean somatic embryogenesis were published (Lippmann and Lippmann, 1984; Lazzeri *et al*., 1985; Ranch *et al*., 1986; Parrott *et al*., 1988, Tripathi and Tiwari 2003, Pathak 2009, Pathak *et al*., 2014) [3, 26, 28, 36, 37, 39, 47]. Nevertheless, most of the protocols were not successful for soybean transformation. In part, this limitation was due to the inability in producing a large number of embryos, which was overcome by applying high 2,4- Dichlorophenoxyacetic acid during induction and proliferation of the somatic embryos (Ranch *et al*., 1986) [39]. *In vitro* regeneration via somatic embryogenesis has drawn more attention than other methods because it can produce a large number of plants in a relatively short time (Wu *et al*., 2008) [51]. Somatic embryogenesis is defined as asexual reproduction in which a bipolar structure, resembling a zygotic embryo, is induced from a non-zygotic cell without vascular connection in the original tissue. In addition to a high number of regenerates, somatic embryogenesis is more attractive than organogenesis as a plant regeneration system due to the low frequency of chimeras and limited level of somaclonal variation (Ahloowalia 1991; Gaj 2001; Henry *et al*., 1998) [1, 13, 18]. Regeneration of soybean via somatic embryogenesis was first attempted by Beversdorf and Bingham (1977) [5], but somatic embryos have only been obtained sporadically since then. Christianson *et al*. (1983) [8] induced adventive somatic embryos from immature soybean cotyledons on medium containing moderately high auxin concentrations. Immature and meristematic soybean tissues are the most suitable explants for somatic embryogenesis. The selection of the explant is a critical factor that determines the success of most tissue culture experiments. Plant regeneration has been achieved via somatic embryogenesis from the immature cotyledons of developing seeds in soybean (Amberger *et al*. 1992; Bailey *et al*. 1993; Lazzeri *et al*., 1988; Liu *et al*., 1992; Walker and Parrott 2001) [2, 3, 25, 29, 50]. Thus, one of the most suitable targets for genetic manipulation in soybean is embryogenic tissue (Sato *et al*., 1993) [43].

Ugandhar *et al*., (2011) [48] has been also reported that high amount of cytokinin and lower amount of auxins is the best combination for somatic embryogenesis which is in accordance of our study. Positive effect of the cytokinins on somatic embryogenesis in soybean was also reported by Lazzeri *et al.* (1985) [26] and Tian *et al.* (1994 [46]). Between two explants, immature embryonic axes demonstrated better regeneration potential as compared to cotyledonary node for each of the treatment.

The development of protoplast systems has increased the versatility of plants for use in both biochemical and genetic research. They have become indispensable tools in genetic engineering and crop breeding of all the possible starting points for plant genetic manipulation, only protoplasts offer the opportunity to take advantage of all the technologies now available. Since the first successful isolation of protoplasts by Cocking (1960) [9], substantial progress has been made towards improving the technology. Attempts have also been made to isolate protoplasts from several crop species and protoplast-based plant regeneration systems are made available for a great number of species (Maheshwari *et al.*, 1986) [30]. The improvements that have occurred include modification of protoplast isolation procedures (Mei-Lei *et al.*, 1987) [31], media composition (Kao and Michayluk 1975) [19], preconditioning of protoplast donor tissues (Shahin 1985), utilization of conditioned media or feeder cells (Bellincampi and Morpugo 1987; Kyozuka *et al.*, 1987; Lee *et al.*, 1990) [4, 22, 27], and manipulation of culture environment (d'Utra Vaz *et al.*, 1992) [11].

The success of a protoplast culture system primarily lies with consistent yields of a large population of uniform and highly viable protoplasts. Several protoplast isolation and purification protocols have been published to optimize the yield and reproducibility. They are often procedures of elaborate nature, labor-intensive involving too many explant or protoplast handling steps, and require extended exposure of explant to digestion environment· Further, the efficacy of such protocols or that of enzyme combinations used therein could be limited to a few plant species· These restrictions must be overcome by improvement of the existing conditions and methods. A number of commercial cellulases and pectinases which allow protoplast release are available· by manipulating the source and concentrations of these, protoplasts may be released from most tissues; however, generalizations cannot be made.

The enzymes and techniques used for isolation of protoplasts have a bearing on their subsequent behaviour and development. Methods with too many steps involved often result in the introduction of cell contamination at some stage or the other. Here, we present a simple method in which slightly elevated temperatures and a set of new enzymes that are efficiency at higher temperatures have functioned synergistically to release protoplasts with relative ease in a number of plant species. The enzymes are hitherto not known as being used for protoplast isolation (Sankara Rao and Srikantha 1986) [40]. Consistently high yields of viable protoplasts from variety of explants of taxonomically widely separate plants were demonstrated. The cultivability of these protoplasts was examined. The overall efficiently and relative advantages of the method are discussed.

The protoplast, also known as naked plant cell refers to all the components of plant cell excluding the cell wall, Hanstein introduced the term protoplast in 1880 to designate the living matter enclosed by plant cell membrane. The isolation of protoplasts from plant cells was first achieved by micro surgery on plasmolyzed cells by mechanical method (Klercker, 1892) [21]. Protoplast can be isolated from plant tissues (or) cultured cells by enzymatic digestion to remove the cell wall. Besides been useful for cell fusion studies, higher plant protoplasts can also take up. Through the naked plasma membrane, foreign DNA cell organelles, bacteria or virus particles these unique properties of protoplasts, combined with totipotent nature of plant cells, have opened up an entirely area of fundamental and applied search in experimental biology and somatic cell genetic (Gleddie *et al.*, 1986).

Methods and procedure for protoplast isolation from plant tissues have long been known (Keller *et al.*, 1982) Recent advance in the isolation, culture and regeneration of plants from protoplasts of a wide diversity of species have been reported (Gleddie *et al.*, 1989) [16]. Essential in gradient of the technique of genetic modification of plant cells through the protoplast system are

1. Isolation of protoplast
2. Culture of protoplasts to raise whole plant
3. Cell fusion
4. Introduction of foreign genetic material cell organelles into the protoplasts.

A. Source of material

The most convenient and populous source of plant protoplasts is the leaf because it allows the isolation of large number of relatively uniform cells without the necessity of killing the plants. Moreover, the mesophyll cells are loosely arranged, the enzymes have an easy access to the cell wall. When protoplasts are prepared from leaves, the age of the plant and the conditions under which it has grown may be critical. To achieve maximum control on the growth conditions of source plants several workers have used *in vitro* grown shoots (Binding, 1975; Schieder, 1978a; Butenko and Kuchko, 1980) [6, 7, 43]. In some species where it is difficult to isolate culturable protoplasts from leaf cells alternative source material of cultured cells have been used. The yield of protoplasts from cultured cells depends on the growth rate and growth phase of cells. Frequently sub cultured suspension cultures, and cells taken from the early log phase are almost suitable (Vasil and Vasil, 1979) [49].

B. Enzyme treatment

The release of protoplasts is very much dependent on the nature and concentrations of enzymes used. The two enzymes regarded essential to isolate protoplasts from plant cells are cellulose & macerozyme. Driselase, having and number of zymolytic activities such as cellulose, pectinase laminarinase and zylanase has proved especially useful for isolating protoplasts from cultured cells. Increase in yield of mulberry proplasts by treatment with chemical substances has been reported earlier (Ohnishi and Kiyama, 1987) [33].

C. Osmticum

A fundamental property of isolated protoplasts is their osmotic fragility and hence the need for suitable osmotic stabilizer to the enzyme solution, the protoplast washing medium and protoplast culture medium is necessary. A variety of solutes, ionic and non-ionic, have been tested for adjusting the osmotic pressure of the various solutions used in protoplast isolation and culture, but the most widely used osmotica are sorbitol and mannitol. With advancement in plant tissue culture technology, plant regeneration systems from protoplasts have been developed in may mulberry species (Ohyama and Oka, 1975) [34]. Establishment of protoplast regeneration system using new technologies such as protoplast fusion and gene transfer would contribute to the improvement of mulberry varieties.

In the present study, attempts have seen made to study protoplast isolation and purification using leaf explants of *Glycine max*

2. Material and methods

A. Plant material

Seeds of *Glycine max* (L.) CV PK 472 obtained from ICRISAT, Hyderabad. The seeds were first washed with running tap water, then surface sterilized as follows: (i) Seeds were submerged in 70% (v/v) ethanol (EtOH) for 30 s, (ii) Rinsed with sterile distilled water (iii) Dipped in sodium hypochlorite (5% w/v) solution for 15 min, finally rinsed with sterile distilled water for three times. The seeds were then germinated on ½ strength Murashige and Skoog (1962) medium containing 1% (w/v) sucrose and 0.8% agar. For shoot regeneration, leaf (1 to 2 cm) excised from 4 week old sterile *in vitro* grown seedling were inoculated on to nutrient medium dispensed in 100 ml Erlenmeyer flasks.

B. Culture media and culture conditions

The pH of MS media used was adjusted to 5.8 using 1 N HCL or I N NaOH, before autoclaving at 120°C with 1.5 kgcm-2 pressure for 20 min. The cultures were incubated in a growth chamber at 25 ± 1°C under 16/8 (light/dark) photoperiod with 25 µmol m-2s-1 illumination from cool fluorescent tubes (Philips, India).

The *In vitro* leaves of 2-3 cm in length and 1- 1.5 cm in width were excised from 6 weeks old seedlings. The leaves were cut into pieces smaller than 1mm and incubated in filter sterilized enzyme solution. The enzyme solution consisted of 20% cellulose "onuzuka" R-10 and 1% macerozyme R 10 prepared in MS salts at pH 5.5 with 0.6 m mannitol and osmoticum the sliced leaf pieces of all the cultivars were incubated in 10 ml of enzyme solution at 27oC and shaken at 40- 50 rpm for 4 5 hrs in dark.

C. Isolation of protoplasts from callus cultures

Seeds of Seeds of *Glycine max* were soaked for 24 hrs in sterilized water and surface sterilized with 0.1% $HgCl_2$ for 3 to 5 minutes. Then these were washed 3 times with sterile distilled water for 5 minutes and germinated aseptically on MS basal medium. The cotyledon (0.5 – 0.8 cm2) from 4-weeks old axenic – seedlings were excised and inoculated to MS medium supplemented with 2.0 mg/L 2.4.D. alone and gelled with 0.8% agar cultures were maintained at 25 ± 2°C under a 16 hrs. Photoperiod was provided by cool white fluorescent lamps. 15 – 20 days old. One – gram friable callus derived from cotyledon explants was gently broken into small pieces of callus. Incubation was carried out in 10ml of digestion solution containing 1% cellulose and 0.5% macerozyme with 0.6 m mannitol as osmoticum. Flasks were incubated at 27° C and shaken at 50 rpm for 3-4 hours in dark.

D. Purification of mesophyll and callus derived protoplasts.

The protoplasts were then purified by 60 µ m steel mesh. The filtrate was collected in screw cap centrifuge tube and centrifuged at 50 g for 5 minutes. The supernatant was discarded and the pellet containing protoplasts was loaded on 20% sucrose solution for purification and centrifuged at 100g for 10 minutes to get a distinct protoplast band. The band was taken in a screw cap centrifuge tube and washed with 5ml of 0.6M mannitol by centrifuging at 50g for 5-7 minutes. The pellet was suspended in culture medium containing MS + 0.6 M mannitol + 2, 4-D and BAP at pH 5.7 and the protoplast yield was estimated using hemocytometer. Proto col is adopted for the purification of mesophyll derived protoplasts.

3. Results

A mixture of 2% cellulose and 1% macerozyme was suitable for isolation of viable protoplasts from mesophyll tissues of leaf cultures of *Glycine max* were investigated each enzyme was in effective by itself but when used in combination it resulted satisfactorily (Evans and Bravo 1983). For *in vitro* leaf explants above combination of macerozyme and cellulose gave an optimum yield.

In case of *Glycine max* cultivars, prolonged incubation period i.e 10-12hrs were observed to be unfavorable shrinkage of protoplasts in these cultivars. Cell digestion was taisly good when above mentioned conditions were applied for protoplast isolation. The number of protoplasts showed increase during shorter treatment time and reached a peak at 4-5 hours of

incubation in dark. Beyond 5 hours of incubation the protoplast yield gradually decreased and further resulted in complete shrinkage of protoplasts at 10-12 hours of incubation from these result it was estimate that the adequate time for enzyme treatment to isolate maximum number of protoplasts from mesophyll cells of *Glycine max* (Plate -I).

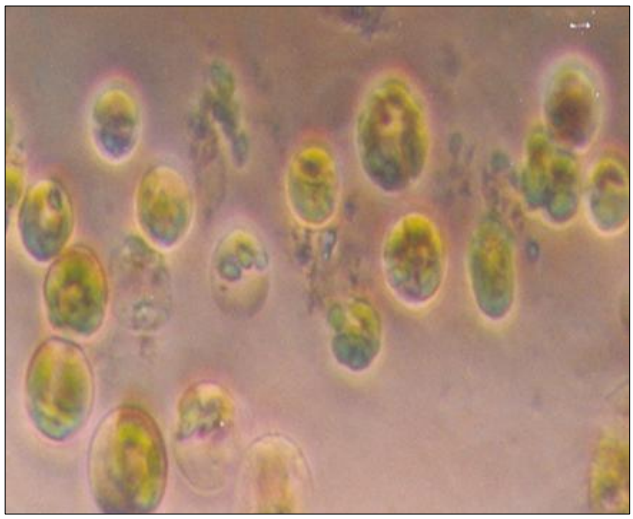

Fig 1: Protoplast Isolation in Leaf explants of *Glycine max:* Fresh and viable mesophyll derived Protoplast

4. Discussion

We were successful in protoplast isolation from callus culture and leaf mesophyll cells on the enzyme solution consisted of 2% cellulose "onuzuka" R- 10 and 1 % macerozyme R 10 prepared in MS salts at pH 5.5 with 0.6 M mannitol as osmoticum. Mesophyll cells and callus cells immersed in the enzyme mixture shaken at 40-50 rpm in dark for 10-12 hours resulted in better yield of protoplasts cell digestion was fairly good when above mentioned conditions were applied for protoplast isolation. Several authors have reported that cell digestion and protoplasts yield in case of mulberry was fairly good during 12-13 hours incubation in dark (Tewary and Lakshmisita 1992) [45].

However our findings agree with the results of who have examined a similar effect of enzyme treatment time for shorter duration (10-12 hours) on protoplast isolation from seedling cotyledons of *Glycine max.* While in the case of callus, cellulose 1% and macerozyme 0.5 % yielded maximum number of protoplast. The use of callus as source for protoplast isolation may overcome the difficulty encountered with the production of leaf material in some recalcitrant *Glycine max* cultivars. Callus developed on 2.0 mg/L 2.4-D was used for protoplast isolation. A part from sub cultured callus the use of cell suspension culture is also avoided here to isolate protoplasts; this is because the suspension culture may accumulate changes in ploidy and abbrerations due to soma clonal variations (Larkin and Scowcroft 1981) [24].

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