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Efficient 2.4-D and TDZ-assisted somatic embryogenesis and plant let regeneration in Indian soybean (*Glycine max* L.) from cotyledonary explants

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Abstract
An efficient system for somatic embryogenesis was standardized. Immature cotyledonary explants of *in vitro* grown plants of Indian soybean cultivar (Pk 416). Somatic embryos were induced directly from cotyledon explants on Murashige and Skoog's (MS) medium fortified with different concentrations of 2,4- dichlorophenoxyacetic acid, (1.0 mg/L to 5.0mg/L), fast growing, greenish nodular callus lines containing somatic embryos were established on initiation medium supplemented with (3.0 mg/L 2.4-D respectively. On subculture of Embryo development and well maturation was achieved on MS medium supplemented with (3.0mg/L 2, 4-D + 1.0mg/L TDZ). The well formed embryos germinated into complete plantlets on MS medium containing (3.0mg/L 2, 4-D+1.0mg/L) TDZ. Individual shoots were aseptically excised and sub cultured in the same media for shoot elongation. The elongated shoots were transferred to Indole Butyric Acid (IBA) (1.0mg/L–5.0mg/L) for root induction. Rooting was observed within two weeks of culture. Rooted plantlets were successfully hardened under culture conditions and subsequently established in the field conditions. The recorded survival rate of the plants was 86%. Plants looked healthy with no visually detectable phenotypic variations.

Keywords: *Glycine maxo*, somatic embryogenesis, plantlet regeneration, *In Vitro* rooting

Introduction
Legumes are members of a family of flowering plants known as Leguminaceae. It is one of the three largest families of flowering plants, with approximately 690 genera and about 18,000 species. Soyabean has been cultivated in china from prehistoric times, It was an important food plant in the orient (China, Manchuria, Korea and Japan) since the earliest times and was carried to Europe by French missionaries in 1740 and to the Royal Botanic Garden Kew in 1790. Soybeans were first brought to the United States in 1804 but failed to receive any recognition. At present it ranks high among the leguminous crops in its nutritional value owing to a high protein content (as high as 43 percent). Also it has about 20 percent oil. Soya bean is an excellent protein supplement for enriching our cereal diet. It has earned a special place in the nutrition programme. So numerous are the modern uses of the Soya bean that it in called the wonder bean Soya bean was introduced to India in about 1880 and is now being cultivated in the northern region, particularly in the hills of Assam, Uttar Pradesh, Himachal Pradesh, Manipur, Nagaland, Madhya Pradesh and the adjoining areas of Maharashtra and Gujarat. Soybeans is an erect much branched, pubescent annual 0.6-1.8m high, depending on cultivar, although in some varieties it denotes to be creeping or twining. Black seeded varieties are richest in protein and have low percentage of oil. Yellow – seeded forms, on the other hand, have higher oil content but are low in protein. To fatty acid constituents of soybeans oil are olcic acid, h 23-34 percent Linoleic acid. 52-6o percent, Palmitic acid 7-14 percent, Stearic acid, 2-6 percent Linolenic acid 3.0 percent and higher saturated acids up to 2.0 percent . Soyabean contains the glycosides genistin and diadzin (daidzin) and four Saponins. It has a higher perecentage of proteins than many other food stuffs. The proteins being of a higher biological value.

Soybean [*Glycine max* (L) Merr.] is a major protein source for humans and other animals. About 90% of soluble proteins in soybean seeds are globulins and more than 70% of globulins are glycinin (G) (11S globulin) and β-conglycinin (βc) (7S globulin). G is relatively rich in scontaining amino acids (methionine and cysteine) (3%- 4.5%) and is stored primarily in cotyledons of seeds where it is deposited in protein bodies

Soybean [*Glycine max* (L.) Merrill] is the world's most important oil and protein crop, and

efforts have been made to develop efficient techniques for its *in vitro* culture and genetic engineering. Such cultures can be a convenient system for genetic transformation of soybean via particle bombardment and recovery of transgenic plants (Finer and McMullen, 1991; Sato *et al.*, 1993; Parrott *et al.*, 1994; Stewart *et al.*, 1996) [6, 25, 21, 28].

Although maintenance of soybean embryogenic cultures in liquid medium was facilitated by the development of FN medium by Finer and Nagasawa (1988) [7] the efficiency of soybean tissue culture manipulations *in vitro* still remains low relative to that of other crops. In recent years there have been numerous reports of regenerating plants from tissue explants, cultured cells and protoplasts of many species however, the soybean (*Glycine max* L.) has not proven generally responsive to procedures that have been successfully applied to other species. Soybean has been used extensively in tissue since 1960,s but regeneration of plants from undifferentiated tissue was reported by Christianson *et al.*, (1983) [4]. Growth centers, clusters of meristamatic cells, embryo like structure and embryos have been produced in cultures using different genotypes (Phillips and Collins, 1981) [22] but these did not develop into whole plants. Several protocols for regeneration of soybean plants via somatic embryogenesis have been documented (Komatsuda *et al.*, 1988) [11, 12]. Several factors that have influence on plant recovery include the length of exposure of explants to auxins plants genotype (Komatsuda *et al.*, 1988; Bailey *et al.*, 1993) [11, 12]; Somatic embryo morphology (Lazzeri *et al.*, 1985) [14]; extentent of hypocotyls elongation (Komatsuda *et. al.*, 1992) [11, 12]; extent of cotyledon development and a partial desiccation treatment prior to germination. Hence, the availability of a suitable tissue culture technology for soybean embryo proliferation and regeneration maybe a limiting step for efficient soybean genetic transformation.

Therefore, the objective of this study was to identify the roles that individual medium components have on somatic embryogenesis in order to improve proliferation of soybean suspension cultures in liquid medium. Towards that end, the effect of a number of factors, such as carbohydrate type and concentration, total nitrogen, ammonium, nitrate, and other macronutrients on proliferation of suspension embryogenic cultures were evaluated, resulting in the development of an optimized medium we refer to as FN Lite.

In this communication, we reported a plant let regeneration system in *Glycine max* (L) through direct somatic embryogenesis from Cotyledonary explants using TDZ, without involving callus phase.

Material Methods

Seeds of *Glycine max* CV PK- 416 are used in our study the seeds were obtained from ICRISAT Hyderabad. Such seeds were not damaged and uniform in size were used. These selected seeds were rinsed in 70% alcohol for 1 min. then sterilized with 0.2% Aqueous Mercuric chloride ($HgCl_2$) for 3 min and subsequently washed five times with sterilized distilled water. The sterilized seeds were germinated aseptically on MS (Murashige and Skoog 1962) Basal medium.

Culture media and culture conditions

Cotyledonary explants (4 week old) of different sizes (0.5 - 10 mm) were cultured with the abaxial surface in contact with induction MS medium consisting (1.0-5.0 mg/L) 2.4-D and (0.5-1.0 mg/L) TDZ for maturation and plantlet regeneration in the second culture phase, these somatic embryos developed from Cotyledonary explants were transferred to MS medium fortified with (2.0 - 4.0 mg/L) 2, 4-D + (0.5-1.0mg/L) TDZ respectively. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 ^{0}C for 15-20 min. All the cultures were incubated under 16/8hr light/dark photoperiod at 25 ± 2 ^{0}C. A light intensity of 40 mol m-2s-1 was provided by cool white fluorescent tubes. The cultures were transferred to fresh medium after an interval of 4 weeks. For germination and plantlet formation somatic embryos were transferred to MS medium supplemented with (3.0mg/L) 24-D + (1.0mg/L) TDZ and incubated under the same culture conditions. (Table-2).

Results and Discussion

Results on somatic embryogenesis in *Glycine max* CV PK-416 are presented in (Table 1). Cotyledonary explants cultured on various concentrations of 2, 4-D (1.0- 5.0 mg/L) become Swollen and generally dedifferentiated and developed friable callus after 8-10 days of culture. Maximum number of somatic embryos / explant and higher percentage of response for somatic embryos formation have been found at (3.0 mg/L) 2, 4-D in cotyledonary explants (Plate I fig a) with the increase of 2, 4-D concentration up to (2.0 mg/L) within 25-30 days of culture, globular, cotyledonary, and heart shaped embryos have formed directly on the surface of callus (Plate I fig b). But when the concentration of 2, 4-D was increased above (3.0 mg/L) percentage of response and somatic embryo induction were decreased it was found that at higher concentration of 2, 4-D (5.0 mg/L). When the explants of primary somatic embryos were cut in to fragments and cultured on the same induction medium secondary somatic embryos were induced within two weeks. Thus proliferation of somatic embryos occurred in two ways:

Table 1: Effect of different concentration of 2, 4-D on induction of Somatic embryogenesis from Cotyledonary explants culture of *Glycine max* cv PK-472

Growth regulators mg/ MS+2,4-D	% of cultures responding	Mean no of embryos per explants
1.0	40	25.0 ± 0.12
1.5	43	30.0 ± 0.12
2.0	56	33.0 ± 0.13
2.5	60	36.0 ± 0.13
3.0	72	43.0 ± 0.13
3.5	56	38.0 ± 0.15
4.0	50	32.0 ± 0.15
4.5	42	26.0 ± 0.05
5.0	38	20.0 ± 0.05

1. Multiplications of somatic embryos from the explants through primary Somatic embryogenesis and
2. Proliferation of secondary somatic embryos from already formed. The percentage of explants responding was evaluated after 4 weeks of culture.

Responses scored were the percentage of explants with evidence of globular stage embryos. All the cultures were incubated under 16/8 h. light/ dark photoperiod at 25 ± 2^{O}C a light intensity of 40 µ mol m-2 $^{s-1}$ was provided by cool-white florescent tubes. The cultures were transferred to fresh medium after an interval of 4 weeks. For germination and plant let formation somatic embryos were transferred to MS medium supplemented with (2.0-4.0 mg/L) 2.4-D + (0.5-1.0 mg/L) TDZ and incubated under the same culture conditions.

Table 2: Effect of different concentration of 2.4-D in combination with (0.5mg-1.0/L TDZ) on germination of somatic embryos derived from Cotyledonary explants of *Glycine max* cv PK-472

Growth regulators mg/L MS+2,4-D +TDZ	% of cultures responding	Average No of Embryos /explants (S.E)**
2.0+0.5	46	12.0 ± 0.12
2.5+0.5	52	18.0 ± 0.12
3.0+0.5	60	20.0 ± 0.13
3.5+0.5	56	13.0 ± 0.13
4.0+0.5	42	10.0 ± 0.13
2.0+1.0	48	8.0 ± 0.15
2.5+1.0	56	16.0 ± 0.15
3.0+1.0	76	18.0 ± 0.05
3.5+1.0	62	16.0 ± 0.05
4.0+1.0	50	10.0 ± 0.05

Maximum number of somatic embryos /explants and higher percentage of response for somatic embryo formation have been found at (3.0 mg/L) 2, 4-D in Cotyledonary explants of *Glycin max* cv PK-416. The calli developed form Cotyledonary explants containing globular embryo were transferred to maturation medium containing MS medium supplemented with (2.0-4.0 mg/L) 2,4-D + (0.5-1.0 mg/L) TDZ respectively. Hence the somatic embryos with various developmental stages (heart and Globular) were further sub cultured on fresh. MS medium containing various concentration of TDZ (0.5-1.0 mg/L) in combination with (3.0 mg/L) 2.4-D for germination of somatic embryos induced form cotyledonary explants of these media tested MS + (3.0 mg/L) 2,4-D + (1.0 mg/L) TDZ proved to be the best for somatic embryos germination and plantlet formation after 4 weeks of culture. The embryos turned green with folded cotyledons, which subsequently developed into whole plantlets. Only fully matured embryos when transferred to MS basal medium without growth regulators, produced good shoot and root systems with 20% frequency, but not the heart-shaped ones. This implies that embryos need to mature enough for germination on basal medium.

A. **B.** **C.** **D**

Plate 1: Somatic embryogenesis and plant regeneration in Cotyledonary explants cultures of *Glycine max* L.(Merr) a) Embryogenic callus induction on MS+ 2.4-5 D 3.0mg/L after four weeks b) callus showing various stage embryos of i.e. Heart, Torpedo and Globular shaped embryos on MS+ 2.4-5 D 3.0mg/L after 6 weeks c) Germination of somatic embryos on 3.0mg/L 2.4-D + 0.5mg/L TDZ e) plant with normal shoot and root system developed from somatic embryos on 3.0mg/L 2.4-D + 0.5mg/L TDZ after six weeks.

In the present investigation, the results on somatic embryo genesis have shown that auxin such as 2,4-D along with cytokinin TDZ are essential for inducing the somatic embryogenesis from Cotyledonary explants of *Glycine max* cv PK- 416 A major factor for somatic embryo genesis in the nature of growth regulators used in the induction medium. The type of auxin or auxin in combination with cytokinin used in the medium can greatly influenced somatic embryo frequency. Proliferated embryo genetic suspension cultures were established for tested in PK- 416 Genotype. This regeneration system may be widely applicable.

For germination, globular, heart and torpedo shaped embryos were transferred to MS medium supplemented with different concentration of auxin such as 2.4-D (3.0mg/L) in combination with TDZ (0.5 mg/L). Highest (60%) frequency of embryo germination was noticed on a medium containing (0.5mg/L) TDZ in combination with (2.0mg/L) 2.4-D .While TDZ and IAA was better for germination of somatic embryos in the cultivar *Glycine max* cv PK- 416. When the concentration of TDZ was increased above (3.0 mg/L) percentage of response and somatic embryo germination frequencies were decreased it was found that at higher concentration of TDZ (5.0 mg/L) in combination with (0.5 mg/L) IAA. Maximum number of somatic embryos germination and higher percentage of response for somatic embryos germination have been found at 3.0 mg/L TDZ + 0.5 mg/L IAA in leaf explants derived Somatic embryogenesis of *Glycine max* cv PK- 416 (Table 2).

However the magnitude of several tissues (Induction, growth, embryo yield, germination and conversion) essential for efficient plant recovery was different among the PK- 416 Genotype. Various studies have documented genotype effects on induction of somatic embryo genesis from immature cotyledon explants of soybean using a variety of protocols (Komatsuda and Ohyama, 1988; Komatsuda *et al.*, 1991; Parrott *et al.*, 1989; Ranch *et.al.*, 1985; Shoemaker *et.al.*, 1991) [11-13, 14, 27]. The development stage of cotyledon is known to be critical for induction of somatic embryo genesis (Lippmann and Lippmann, 1984; Lazzeri *et al.*, 1985; Ranch *et al.*, 1985) [14, 23] and for this reason explants were prepared from immature seeds of 3-5 mm in length. However, selection of explants from uniform seeds many not have ensured uniform developmental status among genotypes differing in mature seed size. These genotypic differences for induction capacity might be altered by selection of explants based on criteria other than equal seed length. Komatsuda *et al.*, (1992) [10] selected immature embryos for culture which were one – half of the length of mature seeds. While Reddy and Reddy (1993) have reported the improved response of auxin 2,4-D alone for induction of somatic embryo genesis compared to

2,4-D and cytokines combination in *Arachis hypogea*.

Somatic embryo genesis is generally believed to be triggered by an auxin and for many plants, 2,4-D has been widely regarded to be effective for somatic embryo genesis (Litz *et al* 1992: Ammirato 1983b, Finner, 1988) [16, 1, 7, 8]. Whereas Binzal *et al.*, (1996) [13] reported that the entire process of induction and maturation of the embryos was completed on the same MS medium containing auxin and cytokinins (2,4-D + TDZ) in *Glycine max* cv PK- 416 as it was observed the requirement of both the hormones in the investigations, similarly somatic embryos maturation on MS medium containing the combination of auxins (2,4-D) and cytokinins (BAP) was observed in *Cajanus cajan* (Mallikarjuna *et.al.*, 1996). Thus somatic embryo genesis always appeared to be depended on the type of auxin / cytokinin /auxin + cytokinin and their concentration in the medium. The type of phytohormone and its concentration of auxin in combination with less concentration of cytokinin induced the somatic embryo genesis and maturation of somatic embryos in *Glycine max* cv PK- 416.

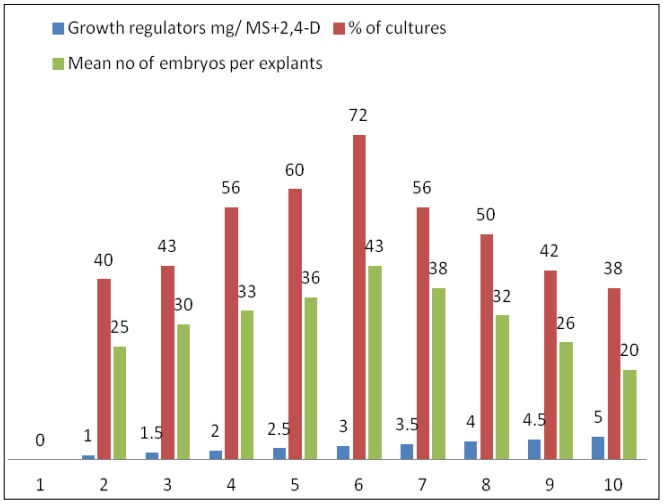

Fig 1: Effect of Different concentration of 2, 4-D on induction of Somatic embryo genesis from Leaf explants of *Glycine max cv* PK-472

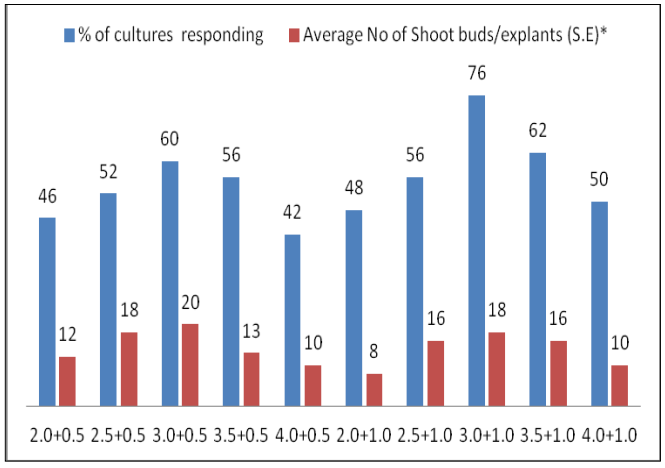

Fig 2: Effect of different concentration of 2.4-D in combination with (0.5mg-1.0/L TDZ) on germination of somatic embryos derived from leaf explants of *Glycine max cv* PK-472

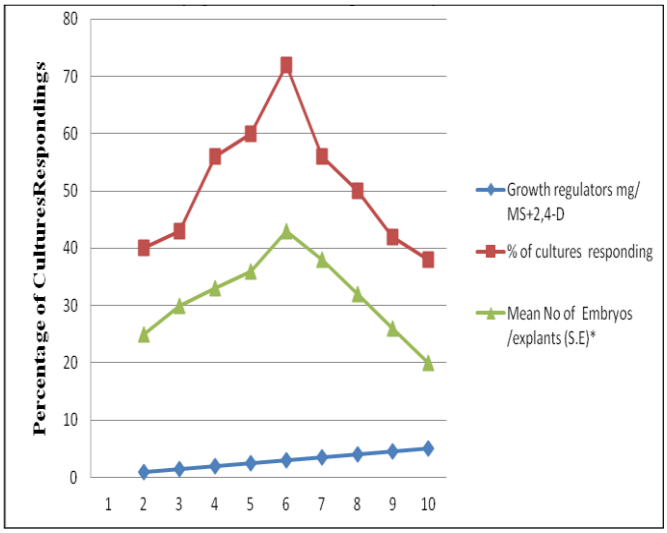

Fig 3: Effect of Different concentration of 2,4-D on induction of Somatic embryo genesis from Leaf explants of *Glycine max cv* PK-472 80

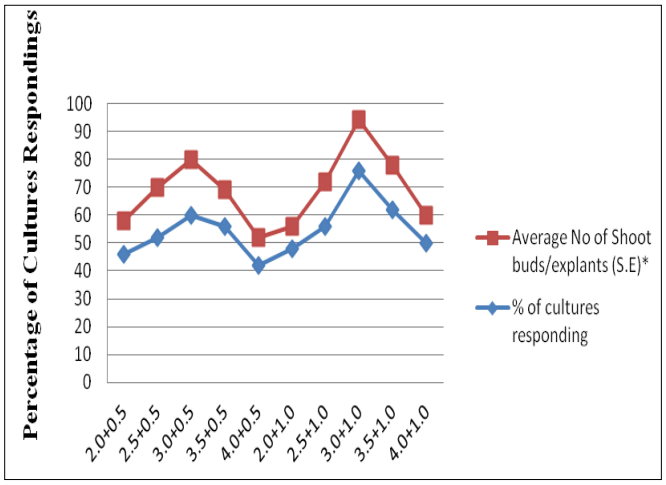

Fig 4: Effect of different concentration of 2.4-D in combination with (0.5mg-1.0/L TDZ) on germination of somatic embryos derived from leaf explants of *Glycine max cv* PK-472

However for germination of somatic embryos low level of auxin and high concentration of cytokinin combination is required. Somatic embryo genesis is also preferred because it allows production of plants without somaclonal variation and in efficient cloning and genetic transformation (Sharp, *et.al.*, 1980).

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