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Repetitive system of direct regeneration of soybean (*Glycine max* (L.) Merr) from hypocotyl explants

S. ARVINTH, S. ARUN AND R.K. SELVAKESAVAN
Division of Crop Improvement, Sugarcane Breeding Institute, Coimbatore - 641 007.

Abstract: *In vitro* culture establishment, shoot proliferation and root development responses of soybean (*Glycine max*) were examined using various combinations of growth regulators. Hypocotyl and cotyledon explants were taken of which hypocotyl explants initiated on Muragshige and Skoog (MS) solid medium supplemented with 2mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D) was the best for production of callus and with 2 mg L^{-1} of 6-benzylaminopurine (BAP) and 0.1 mg L^{-1} napthalene acetic acid (NAA) for shoot proliferation. Cotyledon explants inoculated remained dormant in the different combinations tried. Plants were rooted on half strength MS medium supplemented with 0.2 mg L^{-1} NAA. The regenerated plants grew normally in the greenhouse.

Key words : Glycine max, Hypocotyl, Direct Regeneration, Cotyledon.

Introduction

The *Glycine max* (L.) plant is a branched and non-frost tolerant (Johnson, 1987). It is an important source of high quality protein and oil and grown primarily for the production of seed. It has a multitude of uses in the food and industrial sectors, and represents one of the major sources of edible vegetable oil and of proteins for livestock feed use. Soybean provides about 64 per cent of the world's oilseed meal supply and is the major source of oil, accounting for about 28 per cent of total production (USDA, 2000). Soybean is the only common plant food that contains complete protein. Its protein provides all the essential amino acids in the amounts needed for human health. The amino acid profile of soy protein nearly equivalent in quality to meat, milk and egg protein.

Traditional plant breeding involves the movement of hundreds or thousands of genes and it is time consuming, whereas plant biotechnology allows for the transfer of only one or a few desirable genes with less labour and time. Most methods of plant transformation applied to genetically modified crops require a whole plant regeneration from isolated plant cells or tissue, which have been genetically transformed.

Soybean tissue culture is not only time consuming but also genotype dependent. This observation is true not only from *G. max* either regenerated through organogenesis (Barwale *et al*., 1986; Wright *et al*., 1986; Kim *et al*., 1990; Zhihui Shan *et al*., 2005) but also *via* embryogenesis (Ranch *et al*., 1985; Ghazi *et al*., 1986; Bailey *et al*., 1993; Rajasekaran and Pellow, 1997; Kim *et al*.,

Table 1. Effect of different hormonal compositions on the regeneration of soybean cotyledons and hypocotyl explants

Medium description	Constituents	Percentage Regeneration of cotyledons (%)	Percentage Regeneration hypocolys (%)	Average size of shoot (mm^2) per explant (6^{th} week)
MS1	0.5 mg L^{-1} BAP+0.1 mg L^{-1} NAA	0	0	0
MS2	1.0 mg L^{-1} BAP+0.1 mg L^{-1} NAA	0	0	0
MS3	2.0 mg L^{-1} BAP+0.1 mg L^{-1} NAA	0	12	26
MS4	1.0 mg L^{-1} 2,4 D	0	0	0
MS5	2.0 mg L^{-1} 2,4 D	0	0 (Callusing Observed)	0

2000). Embryogenesis has been achieved by culturing the cotyledonary explant of the seedling, while the organogenesis has been achieved by mainly using the primary leaf node explant of the seedling.

The process of production of plants *via* somatic embryogenesis is an efficient system but apart from the genotype specific, it is also accompanied by high levels of somaclonal variation in the regenerated plants (Finer and Nagasawa, 1988; Parrot *et al*., 1989; Finer and McMullen, 1991). But organogenesis is less genotype dependent and has become routine in several laboratories (Wright *et al*., 1986, 1987a, 1987b; Barwale *et al*., 1986; Dan and Reighceri, 1998).

Tissue culture is an essential step in transformation, except for *in planta* methods. Soybean tissue culture or transformation is relatively difficult compared to other plants (Hinchee *et al*. 1988). Plant genotype of *G. max* is one of the factors, which had influenced plant regeneration (Komatsuda *et al*., 1988; Bailey *et al*., 1993). It has been reported that *G. max* genotype contributed to variation in susceptibility to *Agrobacterium* and regenerability in tissue culture (Meurer *et al*., 1998; Donaldson and Simmonds, 2000).

Increased *G. max* transformation efficiency may be achieved by further optimizing culture conditions to promote regeneration and recovery of transformed plants. This report describes

Table 2. The effect of different media composition on the weight of calli of soybean hypcotyl explants

Medium	Average initial weight (mg)	Average weight after 6 weeks (mg)	Average weight increase (mg)
MS1	26	98	72
MS2	28	32	4
MS3	23	142	119
MS4	32	28	-4
MS5	26	187	161

the establishment of an efficient and repetitive system of direct regeneration of soybean plants.

Materials and Methods

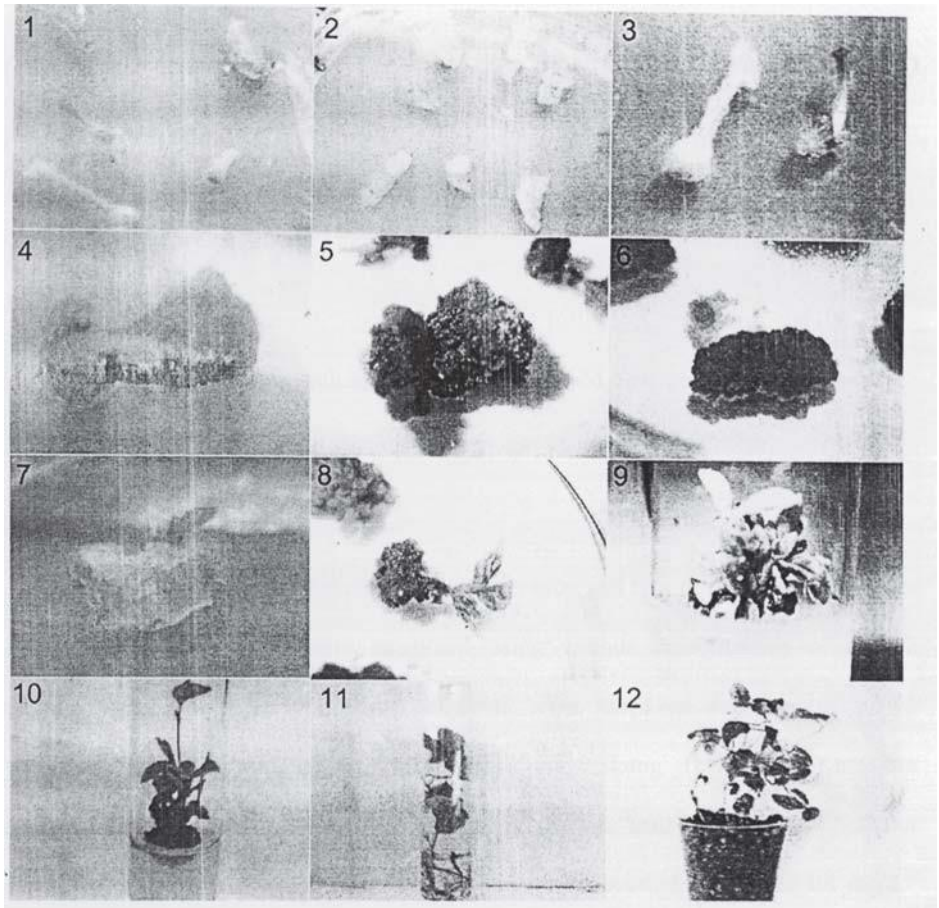
Aseptic seed germination, explant preparation and culture conditions

The seeds of CO 2 variety of soybean were obtained from Tamil Nadu Agricultural University, Coimbatore, India. These seeds were washed in running tap water for five minutes and then washed repeatedly in double distilled water. Now under aseptic conditions the seeds were surface sterilized with 70% ethanol for one minute followed by a twenty minute treatment with 2% sodium hypochloride and washed with sterilized triple distilled water five times followed by 0.1% mercuric chloride for ten minutes and then washed repeatedly in sterilized triple distilled water. The seeds were then plated on half strength MS basal medium (Muragshige and Skoog, 1962) solidified with 0.8% bacto agar for germination. This was incubated in dark at 26^{o}C till it germinated and then transferred to cool-white-fluorescent light room and incubated at 28^{o}C and allowed to grow.

The plant after reaching a height of 6 centimeters was taken in an aseptic condition and hypocotyledon and leaves were excised using a sterile scalpel and cut into 8-10 mm sections.

Plant regeneration

The excised tissues were then placed on MS medium supplemented with different combinations of hormones chosen after preliminary investigation in the laboratory (Table 1). A combination of 3% (w/v) sucrose and 0.7% (w/v) Bacto Agar was common in all the media. The pH of the media was adjusted to 5.8 with 0.1 M NaOH or with 0.1 M HCl before autoclaving. Ten explants were placed in each glass Petri dish (100 x 15 mm) containing 20 ml of MS medium augmented with plant growth regulators. Ten replicates were maintained under each hormonal combination. The cotyledons were deliberately wounded in a few areas and its abaxial side was placed in contact with the medium. All the cultures were incubated at 26-28^{o}C under a 16 hour light and 8 hour dark photo period provided by cool-fluorescent lights. Every two week the explants were transferred to fresh medium. The number of shoots produced was counted 6 weeks after culture. Isolated single shoots after reaching 5 centimeters in size were transferred to half strength MS medium supplemented with 0.2 mg/l NAA for rooting.



Figure

Direct Regeneration of soybean (*Glycine Max*). (1) Freshly inoculated hypocotyl explants (2) Leaf explants (3) Production of micro calli at the cut end of the hypocotyls explants (4) Spreading of callus from the cut ends of the hypocotyls (5 and 6) Characteristic variation in calli (7) Initiation of Shoots (8, 9 and 10) Elongation of shoots (11) Development of roots (12) Hardening of soybean plant in plastic containers.

Plantlets were transferred to the greenhouse for acclimatization and growth.

In a separate experiment the increase in fresh weight of the hypocotyl tissue was recorded for the same hormonal combination mentioned above. The experiment consisted of three replicates and per replicate 10 pieces of explant (25 mg per piece).

Results and Discussion

It was seen that medium MS 3 which was MS medium augmented with 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA and medium MS 5 which was MS medium augmented with 2.0 mg L^{-1} 2,4-D showed signs of regeneration when compared to the other media. Within the first week of inoculation the explants showed positive signs of regeneration.

The hypocotyl regions showed swelling in the cut ends (Fig. 1) but the leaves, which were inoculated, showed no signs of regeneration (Fig. 2). There was no callus proliferation until the second week when a majority of the incubated tissues exhibited signs of callusing. This activity started from the wounded ends (Fig. 3) and spread towards the middle region of the tissue segment (Fig. 4). The callus varied characteristically with the plant growth regulators and frequently sub culturing (Fig. 5). Medium MS5 produced callus, which enlarged due to multiplication of cells without any signs of differentiation (Fig. 6).

The tissues in medium MS 3 started showing small shoot like organogenic nodules by the fourth week (Fig. 7). The elongation of shoot buds into shoots is a critical step in legume regeneration. Few shoots elongated on the initial culture medium (Fig. 8), but after sub culturing the tissue to fresh medium there was elongation of many such organogenic nodules into shoots (Fig. 9). It was noticed that the detachment of the shoot bud clumps from the callus also affected shoot elongation but if it remained attached faster elongation of shoot buds into shoots was observed. These elongated shoots where allowed to grow for two weeks in the same culture medium (Fig. 10). After this the elongated shoots were efficiently rooted in half strength MS liquid medium containing 0.2 mg L^{-1} NAA, which produced roots in two weeks time (Fig. 11). The rooted plants were now transferred to a plastic containers containing sterilized coco peat and it was covered with transparent plastic bags. After two days, holes were punched on the plastic bags. After six days, the plastic bag was removed and the plastic containers were transferred to green house conditions where the plants grew autotrophically to maturity. These plants resembled the normal seed-derived plants, by flowering and producing viable seeds (Fig. 12) whereas plants did not produce seeds in previous studies (Zhihui Shan *et al*., 2005) were multiple bud tissue was used as explants.

Weight of the hypocotyl tissue taken after six weeks of inoculation showed clearly that explants inoculated on MS 3 and MS 5 medium gained the highest increase in weight around 119 mg from 23 mg original weight and 161 mg from 26 mg respectively (Table 2). While those explants in MS 1 showed a slight increase of 72 mg from its original weight of 26 mg. Explants on medium MS 2 and MS 4 did not show any increase in weight as they dried after a period of three weeks on the medium.

Soybean organogenesis was previously done by culturing the explants for two to four weeks on medium supplemented with cytokinin for the production of shoots and cultured on another medium for elongation or growth of shoots where meristems initiated and then developed into shoots. This sudden change in hormonal composition might delay the time required for the tissue to develop because it had to get adopted to the change in the hormonal composition and then start developing again. So the method described in this experiment used only one hormonal composition from the initiation of the meristematic tissue till it was transferred to the rooting media which had a very slight increase in the level of NAA.

Respective organogenesis in soybean was previously reported, by continuously culturing the explants on medium supplemented with BA (Wright *et al*., 1987a, 1987b; Shetty *et al*., 1992) and by culturing on TDZ and BA supplemented media in alternating cycles

(Barwale and Widholm, 1990). But the draw back in this method of repetitive organogenesis was though many shoots produced, only 2% developed into normal plants (Wright *et al*., 198a, 1987b). But the microcalli which were produced initially developed into shoots and 12 per cent (Table 2) of those shoots developed to produce normal rooted plants and organogenesis was less genotype dependent and had become routine in several laboratories (Wright *et al*., 1986, 1987a, 1987b; Barwale *et al*., 1986; Dan and Reigheeri, 1998). The regeneration depended on the proliferation of meristems in the cotyledonary node. Another draw back observed in previous studies were the recovery of transgenic plants capable of transmitting the target genes to R_1 was very low (Christou *et al*., 1990).

Soybean hypocotyls and epicotyls had previously been used for *in vitro* culture (Wright *et al*., 1987c; Shu *et al*., 1988; Kedlee *et al*., 1991; Cristia *et al*., 1992). The report proved that the process of development of the plant was very slow and highly recalcitrant (Hinchee *et al*., 1988) but the process of direct regeneration observed were repetitive and developed into normal plants.

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