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Epigenesis and High Frequency Plant Regeneration from Soybean (*Glycine max* (L.) Merr.) Hypocotyls

Manoj Tripathi and Sharad Tiwari

Plant Biotechnology Laboratory, Department of Plant Breeding and Genetics, Jawaharlal Nehru Agricultural University, Jabalpur 482004 MP, India

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Abstract

High frequency somatic embryo induction was achieved in *Glycine* hypocotyls depending upon genotype and the explant inoculation medium. Plant regeneration efficiency was in excess of 85% following plural shoot initiation in genotype JS 90-41 when the *in vitro* inoculation background was a combination of 0.5 mg/l NAA and 3.0 mg/l BAP and contained MS macro- and micro-nutrients, vitamins and agar. Phenotypically normal plants were regenerated from the hypocotyl explants.

Introduction

Soybean (*Glycine max* (L.) Merr.) is an important source of high quality protein (~ 40%) and oil (~ 20%) for vegetarians, particularly in the third world. The genotypes currently under cultivation in India are pure inbred lines. Hybrid *Glycine* has not been successful in view of the high production costs and lack of a clear heterotic response. The conventional breeding of this crop has made some advances but *Glycine* needs to be improved further to enhance not only yield but also the oil and protein contents and their qualities. Futhermore, resistance to diseases and pests and tolerance to soil salinity, acidity and drought are also desirable. Despite the fact that biotechnology offers the best option for genetic enhancement of crop plants, very little *in vitro* work has been done on Indian *Glycine* genotypes.

Hypocotyl is one of the tissues of choice for plant genetic manipulations (Liu et al. 1996) in view of the fact this tissue is amenable to *in vitro* techniques in addition to contributing substantially to the plantlet biomass. Hypocotyl is quick and convenient to obtain from the seed and it is possible to homogenize the physiological state of the tissue in terms of its age, i.e. days after seed germination. Glycine hypocotyls and epicotyls have previously been used for *in*

vitro culture (Wright et al. 1987; Shu and Yeh 1988; Li and Keller 1989; Kadlec et al. 1991; Cristea and Cachita-Cosima 1992) including somaclone studies (Freytag et al. 1989) and the production of transgenics. Hypocotyl protoplasts (Newell and Luu 1985; Hammatt and Davey 1988) and cell suspension cultures (Philips and Collins 1981; Shao et al. 1985) have also been used for regenera-tion. Nonetheless, inter- and intrespecific variations for micropropagation have been observed in several studies (Komatsuda and Ohyama 1988; Ferreira et al. 1990; Komatsuda 1990; Komatsuda et al. 1991; Bialey et al. 1993a,b; Bodanse-Zanettini et al. 1993; Thome et al. 1995; Nawracala et al. 1996).

Prior to the transformation of *Glycine* to cater to the regional needs and to address the acclimatization issue, responding genotypes need to be identified. This paper reports on the induction of embryogenesis and plural shoots to screen *in vitro* responding genotypes and describes a suitable nutrient medium.

Materials and Methods

Seeds of 11 genotypes of *Glycine* (Table 1) were obtained from All India Coordinated Research Project on Soybean, Jawaharlal Nehru Agricultural University, Jabalpur. These were established commercial genotypes with the exception of JS 90-41, which was an advanced breeding line. For all *in vitro* work, tissue culture grade water was used. Furthermore, each of the treatments was replicated twice. A replication consisted of 70 - 130 hypocotyl sections harvested from different seedlings of a single genotype, randomized and cultured on a specific medium. Ready-made powdered MS basal medium and all other additions were procured from HiMedia, Mumbai, India.

Hypocotyl initiation and culture : The seeds were surface sterilized with 70% (v/v) ethanol for one min followed by a five min treatment with 1% (w/v) purified mercuric chloride and three subsequent rinses in sterile water under asceptic condition. These were then germinated under dim light in tubes containing 0.8% (w/v) bacto agar medium without any nutrients. The hypocotyls, obtained four days after the imbibition of seeds, were excised and cut transversely into 8 - 10 mm sections. Between 8 and 10 sections were placed on to the explant inoculation medium in 100 ∇ 15 mm glass Petri dish for incubation. The media short-listed on the basis of preliminary investigations in this laboratory were supplemented with (a) 3.0 mg/l BAP + 0.5 mg/l NAA, (b) 1.0 mg/l BAP + 1.0 mg/l NAA and (c) 0.5 mg/l BAP + 0.2 mg/l IAA, hereinafter referred to as *GHyl, GHy2* and *GHy3,* respectively. Unless specified otherwise, all the media contained MS macro- and micronutrients, vitamins, 30 g/l sucrose and 0.8% (w/v) bacto agar and were autoclaved at

121_C under 15 psi pressure for 45 min after adjusting the pH to 5.6 ± 0.1 with 1N KOH.

Callus induction : The cultured dishes were sealed with Parafilm and incubated under complete darkness at 25 ± 2_C for one week after which the incubates were subjected to a photoperiod regime of 30 μ mol $m^{-2}s^{-1}$ luminance provided by white fluorescent lamps for 12 h. The performance of each of the genotypes in terms of callus induction on explant inoculation media was recorded after five weeks in incubation. Based on the appearance, the callus types were classified into three categories, *viz.* (i) embryogenic - green and compact with bead like structures sometimes coexistent with white loose callus, (ii) organogenic - light green with dense and glossy appearance and (iii) undifferentiated - cream and friable. Callusing efficiency was defined as the percentage of explants that produced callus. Data on the first two categories were grouped together to constitute morphogenic callus for statistical analyses. Morphogenic efficiency was defined as the percentage of explants in which such callus proliferated.

Plantlet production : All calli types were transferred to a plantlet regeneration medium save the embryonic callus, which was cultured, on PGR free MS medium for the germination of somatic embryos. The MS for plantlet regeneration was modified with 0.4 mg/l BAP and 0.4 mg/l NAA and the sucrose concentration was alleviated to 20 g/l. Where necessary, the *in vitro* shoots were subsequently transferred to a rooting medium which was MS supplemented with 1 mg/l IBA and further reduced level of 15 g/l sucrose. The competence of genotypes for *in vitro* shoot proliferation in the background on the explant inoculation media was evaluated four weeks after the transfer of the respective calli to the plantlet regeneration medium. Each callus body was counted as one irrespective of the number of shoots initiated. PSI was not accounted for separately at this stage and shooting efficiency was defined as the percentage of explants from which shoots emerged.

Complete plant regeneration : A third and final set of data were recorded for complete plant regeneration when the plantlets were successfully transferred to the soil. The roots of plantlets were rinsed in sterile lukewarm water to wash the agar. The plantlets were potted in autoclaved 30 ∇ 30 mm white plastic containers containing a 3 : 1 mixture of soil and sand autoclaved individually prior to mixing. The plants were raised under glasshouse conditions. The number of complete plant(s) produced per 100 explants constituted plant regeneration efficiency.

Results and Discussion

In the first week of hypocotyl *in vitro*, the explants enlarged but no callus proliferation was evident until the second week when a majority of the incubated tissue exhibited signs of callusing (Plate 1A). This activity started from the wounded ends and spread towards the middle region of the tissue

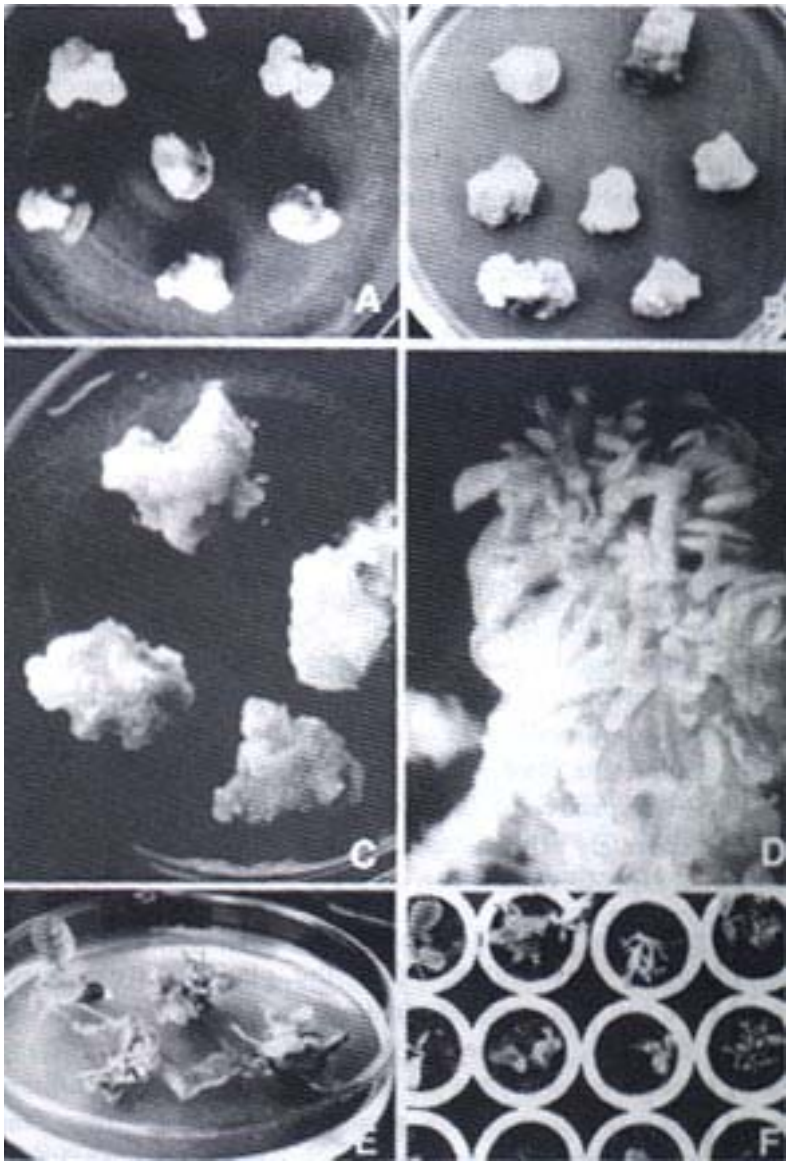

Plate 1A - F : A. Cultured hypocotyl after ten days in culture. B. After 20 days. C. Organogenic calli. D. Embryogenic calli with germinating plantlets. E. Regenerated plantlets. F. Transferred into pots for hardening.

segment (Plate 1B). The callus varied characteristically not only with the genotype but also with the PGR regime and their distinct phenotypes *viz.* wet, rough, hard, dense and glossy, reflected diversity in the developmental potentials. Visual selection and subcultures of these pheno-variants produced cultures where plantlet regeneration occurred routinely and efficiently.

The data of organogenic (Plate 1C) and embryogenic calli (Plate 1D) categories were clustered together to form the 'morphogenic callus' group. In the case of embryogenic calli embryo like structures (ELS) formation started in two weeks of inoculation on the explant inoculation medium although in some incubates this was delayed. The ELS were globular or beaklike, with irregular boundaries, usually appeared in clusters and did not form a bipolar structure. In a few cases, both types of growth patterns (embryogenesis as well as organogenesis) were observed on the same callus body and thus PSI was frequently apparent.

The analysis of variance revealed that there were highly significant ($p < 0.01$) differences between the response of genotypes as well as nutrient media in terms of overall callogenesis, morphogenic callus and shoot induction. Furthermore, although the genotypes varied highly significantly ($p < 0.01$) and significantly ($p < 0.05$) for the induction of morphogenic callus and shoot respectively, in response to incubation on three different PGR regimes, there were non-significant interactions between genotypes and nutrients media for overall callus initiation.

The medium *GHy1* was consistently superior to *GHy2* and *GHy3* (Table 1) for overall callus initiation (87.43%), morphogenic callus (56.04%) and shoot induction (35.31%) in all the 11 genotypes. The superiority of *GHy1* over the other media was significant ($p < 0.01$) for overall callus and shoot initiation. Nonetheless, the difference between the induction of morphogenic calli on *GHy1* and *GHy2* was non-significant ($p < 0.05$) despite the fact that numerically higher number of morphogenic calli were observed in cultures on *GHy1* in comparison to *GHy2* medium. The medium *GHy3* proved to be significantly ($p < 0.05$) inferior to *GHy1* and *GHy2* for all the parameters recorded for evaluation.

In terms of the varietial response to *in vtiro* culture, three of the 11 genotypes studied were more responsive than the rest for the parameters investigated. These were JS 80-21, JS 335 and JS 90-41 (Table 1). When compared with the other genotypes, JS 80-21 produced the largest number of calli numerically (89.73%) but the difference between this genotype and MACS13 (88.86%), JS 90-41 (85.41%), JS 75-46 (84.42%) was non-significant ($p > 0.05$).

Table 1. ***In vitro*** **response from hypocotyl cultured on three different media.**

Genotypes/ culture media	Callus induction (%)				Morphogenic calli (%)				Shoot forming calli (%)			
	GHy1	*GHy2*	*GHy3*	Mean	*GHy1*	*GHy2*	*GHy3*	Mean	*GHy1*	*GHy2*	*GHy3*	Mean
Bragg	91.01	77.30	77.37	81.92	48.93	58.98	27.61	45.17	17.25	35.38	16.51	23.0
JS 72-280	83.28	79.38	69.04	77.23	49.00	39.75	25.66	38.13	33.33	32.46	14.33	26.70
JS 72-44	84.19	84.09	72.80	80.36	41.40	51.25	25.95	39.53	18.15	30.28	13.52	20.65
JS 75-46	93.06	91.18	69.04	84.42	68.96	31.46	29.80	43.41	35.85	22.63	13.96	24.14
JS 80-21	91.54	92.25	85.41	89.73	61.63	56.41	30.24	49.40	48.02	25.64	11.94	28.53
JS 90-41	93.55	85.14	77.77	85.49	81.03	50.23	30.83	54.03	62.65	28.18	14.14	34.99
JS 335	81.25	72.25	79.79	77.76	69.99	45.57	55.04	56.86	34.56	21.93	21.48	25.99
MACS 13	93.01	89.20	84.38	88.86	57.11	47.82	41.45	48.79	33.06	22.78	14.83	23.55'
NRC 2	85.61	74.26	79.50	79.79	65.20	42.56	32.04	46.60	53.23	26.32	18.86	32.80
Panjab 1	79.22	73.86	69.82	74.30	41.10	42.67	27.50	37.09	30.27	27.40	18.82	25.49
PK 472	86.00	87.93	7.12	84.35	32.15	62.27	28.11	40.84	22.12	28.38	14.55	21.68
Mean	87.43	82.43	76.73		56.04	48.08	32.20		35.31	27.40	15.72	
CD (0.05)												
Genotypes				4.52				5.45				3.43
Media				2.36				2.85				1.79
Genotype ∇ medium				7.84				9.45				5.95

Of this overall calli, the proportion of calli resulting in morphogenesis was hgihest for the genotypes JS 335 (56.86%) followed by JS 90-41 (54.03%), JS 80-21 (49.40%) and MACS13 (48.79%) but the differences between these were not significant at 5% probability.

The data presented in Table 1 suggest that the difference in the *in vitro* performance of JS 90-41 and NRC 2 for shoot initiation was non-significant ($p > 0.05$). Furthermore, the shoots induced in the genotype JS 90-41 regenerated at a high frequency into plantlets (Fig. 1, Plate 1E) that were also transferred successfully to the soil (Plate 1F). The histograms clearly illustrate that although whole plant regeneration was observed in all the genotypes, the overall response of JS 90-41 was superior to such other highly responsive genotypes as JS 80-21, NRC 2 and JS 335. The performance of genotype Pubjab 1 was inferior to the other genotypes in terms of callus and shoot proliferation (Table 1) but the production of total number of plants was at par with JS 335 (Fig. 1).

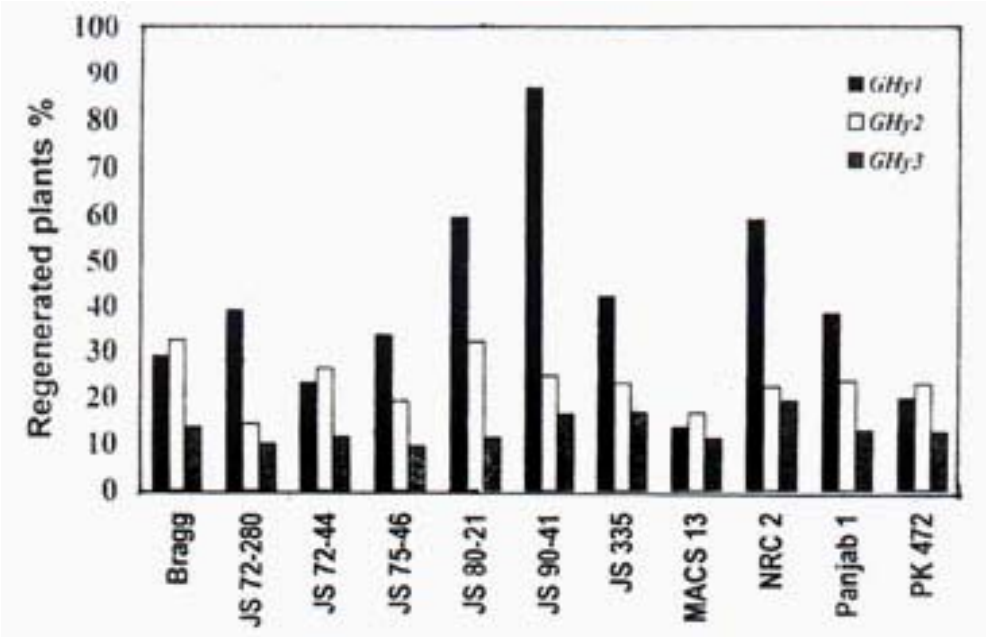

Fig. 1. Regeneration potential of cultured hypocotyls of 11 soybean genotypes on three different MS culture media.

As well as at the *in vitro* level, the interaction between the genotypes and the media was also evident from the media dependent response of the genotypes for plant regeneration (Fig. 1). Whilst the *GHy1* PGR regime promoted the highest rate of plant regeneration in genotype JS 90-41 followed by JS 80-21 and NRC 2, *GHy2* stimulated the regeneration of maximum number

of plants in the genotype JS 80-21 and Bragg. The PGRs incorporation in the *GHy3* combination were best suited for the genotype NRC 2 followed by JS 335 and JS 90-41, respectively in terms of the regeneration leading to entire plant. Fig. 1 indicates that, of the 11 genotypes, seven produced higher number of plants when incubated initially on the *GHy1* as opposed to *GHy2* or *GHy3* regimes. The remaining four responded better to the *GHy2* nutrient background in contrast to the other two.

The plants, after survival in the glasshouse conditions, were evaluated visually on the basis of appearance. Although the traits were not scored quantitatively, the plants obtained repeatably via micropropagation were phenotypically normal and true to the type.

The PGRs were compared with each other in combinations only and the effect of a cytokinin or an auxin *per se* was not investigated in the work being reported. This was so because during the course of preliminary investigation (data not presented), it was observed that both cytokinin as well as auxin was essential for increased morphogenesis but they were most effecitve when used in combination, and that the type and pace of callus growth was dependent on their ration. Similar response was also reported by Pavlina (1986).

Callus initiation was observed in all the treatments studied here but there were qualitative and quantitative differences. Of the three PGR combines, the *GHy1* regime with comparatively higher cytokinin and a relatively lower auxin concentration produced primarily embryogenic calli which is known to exhibit a high morphogenesis rate and regeneration potential. This observation suggests that a high auxin concentration may not be imperative for *in vitro* embryogenesis but is in contrast with the reports that favour a high auxin concentration in explant inoculation *milieu* for enhanced embryogenesis (Ancelet et al. 1988; Yeh 1989; Nawracala and Konieczny 1996). The inputs that elicit the stimulation of embryonic or organogenic pathway are not well understood but IEDCs are known to respond to a suitable PGR which might be a specific combination of auxin and cytokinin as in the present case.

Morphogenesis was primarily through embryogenesis but organogenesis was also observed at a lower frequency in this study. This followed the generation either of plantlets (shoot and root) or of shoots that were later rooted separately. Besides, rhizogenesis alone was also observed but never lead to viable plants. Visual observations revealed that the meristems located at periphery of the callus mass developed into shoots whereas those embedded deep into the bodies lead to root formation. Similar observations have been made in *Nicotiana* (Thorpe and Murashige 1970) and *Linum* (Sarathe and

Tiwari 1997) although the meristems arising from a group of cells called 'meristemoids" (Bunning 1952) develop into shoots and roots as observed occasionally during the present work.

The embryogenic and organogenic calli were found to be morphologically distinctively dissimilar at the time of data collection in this study. Despite this, a system of clubbing the data on the two together as 'morphogenic' calli was followed not only for the case of analysis and interpretation but also in view of the fact that anatomical and biological examinations were not carried out to confirm ELS as true embryoids. There have been suggestions that the ELS, speculated as embryoids, may in fact be shoot and root structures initiation from neighboring cells and resembling pseudo-embryos. (Swamy and Krishnamurthy 1981).

The observations made during the course of present study revealed that orgnogenesis and embryogenesis coincided spatially as well as temporally, though at varying degrees, between genotypes. Normally, only one event is reportedly observed at a time (Henshaw et al. 1982). Not much is known about the mechanism of epigenesis but it has been suggested that the time and venue of embryo development is possibly governed by a complex interaction of biochemical and physical environments with the genetic factors.

For complete plant regeneration from *Glycine* hypocotyl explants, genotype JS 90-41 facilitated the production of highest number of regenerants followed by JS 80-21 when compared to other genotypes. Within each medium treatment, the variation observed for the *in vitro* response resulted from genetic and physiological differences between genotypes as the culture condition were standardized.

Organogenesis *in vitro* depends on the application of exogenous phytohormones, in particular auxin and cytokinin, and also on the ability of the tissue to respond to these phytohormone changes during culture. Thus the manipulatable nature of tissue culture can be exploited for the physiological analysis of organogenesis *in vitro* (Christianson and Wamick 1983, 1985; Attfield and Evans 1991; Burritt and Leung 1996; Lakshmanan et al. 1977). In addition, a difference between the endogenous hormone levels (Norstog 1970) amongst the genotypes also contributes to variability. Genotypic variations have been widely recorded in *Glycine* tissue cultures (Hammatt and Davey 1987; Parrott et al. 1989; Kllipara and Hymowitz 1989; Li and Grabau 1996).

The present report clearly illustrates the intriguing relationship between initial and final results in micropropagation studies, not so much with the chemical parameters as with the biological ones that are less predictable. The

cultures demonstrating a high organogenesis rate produced plural shoots though this did not necessarily mean an inflated plant regeneration frequency. The translation and manifestation of PSI into complete plant regeneration varied from 0 (no plant) to 19 per callus mass suggesting that not all the shoot developed into complete plants. This variation may, at least in part, be attributed to the physiological state of the mother plant at the time of excision of the explant. On some instances, shoots ceased to develop after initiation, were deformed, or did not initiate roots even on rooting medium. The relationship between initial and end results is best explained by the performance of genotypes MACS 13 and NRC 2. The former exhibited high callus proliferation and morphogenesis but the number of complete plants produced was comparatively poor. On the contrary, genotype NRC 2 did not produce very many calli initially but the total number of complete plants regenerated was high.

Under reported condition, plant regeneration from *Glycine* hypocotyls took place after an intervening callus phase through embryogenesis as also via PSI. Direct somatic embryogenesis with singular shoot initiation was seldom observed. In the light of the above, genetic instability of the callus cells is expected and the possibilities of somaclonal variations cannot be ruled out as was observed by Freytag et al. (1989). PSI is ideal if somaclonal variations are to be screened and beneficial aberrants selected. Moreover, PSI is not undesirable in direct DNA uptake procedures involving transient plasma poration as well as in electro and chemo-amalgamation of the target protoplasts. That said PSI needs to be avoided in transgene expression studies employing *Agrobacterium*-mediated or ballistic approaches in view of the fact that the number of escapes may be higher. For such studies, shoot initiation only from the transgenic cell or tissue following somatic embryogenesis is preferable and has been reported to be amenable to *Agrobacterium*-mediated gene delivery (Trick and Finer 1997) in addition to the direct uptake protocols (Santrem and Finer 1999)

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