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Plant Regeneration in *Glycine clandestina* Wendl. from Explants of Cultured Cotyledons

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Abstract

The ability of nodal shoot segments regenerated *in vitro* to form plantlets under culture conditions has been investigated in *Glycine clandestina*, a wild relative of cultivated soybean (*Glycine max*). The nodal shoot segments (NSSs) were excised from *in vitro* regenerated shoots which differentiated from the cultured cotyledons on B5 medium supplemented with IBA (0.05 mg/l) and BAP (3.0 mg/l). Plant regeneration from NSSs occurred through direct multiple shoot as well as via callus formation on B5 medium with different levels of IBA (0.005 to 0.05 mg/l) and BAP (1 to 10 mg/l). Clumps of the regenerated shoot segments were further subcultured on B5 medium with IBA (0.005 mg/l) and BAP (1.0 mg/l) for continuous shoot proliferation. Plants can be obtained from shoot buds when transferred to low levels of IBA and BAP. It was found that on this medium new shoot buds were also differentiated while small shoot buds elongated into shoots without any loss of morphogenic potential for more than six years. The differentiated shoots were rooted on hormone-free B5 medium containing 6% sucrose. This approach of plant regeneration can be applied to improve the soybeans through transformation by *Agrobacterium* as well as particle bombardment.

Introduction

The genus *Glycine* contains 14 species out of which 12 are perennial wild, one is an annual wild and only one species, i.e., *G. max* (soybean) is cultivated (Hymowitz and Singh 1988). Wild species of soybean which are adapted to

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tropics and subtropics mostly found in Australia (Singh and Hymowitz 1985). They grow slowly, have a procumbent habit and produce a fewer pods (Hymowitz and Burridge 1989). These species provide considerable variability for breeding programmes but the seed availability is limited. *Glycine clandestina* possesses several agronomically valuable traits such as heat, drought and cold tolerance and resistance to rust, yellow mosaic virus and powdery mildew diseases (Marshall and Broue 1981) that would be desirable if transferred into *G. max* whose germplasm base is rather limited. Incorporation of these traits by conventional hybridization of soybean with wild species is difficult (Chung and Kim 1990) and attention has been focused on somatic hybridization (Davey et al. 1988) and genetic transformation (Rech et al. 1988) to improve the cultivated soybean. This approach needs the multiplication and conservation of rare genotypes of wild soybean through efficient and reproducible regeneration of plants from tissues and protoplasts. Plant regeneration has been reported from different seedling explants of *G. clandestina* such as cotyledons, leaves and petioles (Hammatt et al. 1986); leaf explants (Hymowitz et al. 1986; Kollipara and Hymowitz 1989) and various other seedling explants (Sharma and Kothari 1993); and protoplasts (Hammatt et al. 1987; Jones and Davey 1991). To circumvent the problem of limited availability of seed/seedling material, it was decided to micropropagate *G. clandestina* using *in vitro* differentiated nodal shoot segments (NSSs). As far as we know the NSSs taken from *in vitro* differentiated shoots were not used previously for plant regeneration in any species of the genus *Glycine* including *G. clandestina*.

Materials and Methods

Seeds of *G. clandestina* Wendl. (Accession G1826) were obtained from the CSIRO, Division of Plant Industry, Canberra, Australia. Seeds were germinated and cotyledons excised as described (Sharma and Kothari 1993). Cotyledon explants were cultured on B5 medium (Gamborg et al. 1968) supplemented with IBA (0.05 mg/l) and BAP (3.0 mg/l) for shoot bud differentiation. The differentiated shoot buds were elongated and maintainted on B5 medium with IBA (0.05 mg/l) and BAP (1.0 mg/l) for the source of NSSs (Fig.1). For this purpose, the differentiated shoots (3 - 6 cm) were aseptically cut into 1 - 2 cm segments having one to three nodes and cultured on B5 medium containing 3% sucrose and different concentrations of IBA (0.005, 0.05 or 0.5 mg/l) and BAP (1, 3, 5 or 10 mg/l) added in combination for plant regeneration. All media were solidified with 0.8% agar (w/v; Qualigens), pH adjusted to 5.8 and approximately 40 ml medium was poured in a flask (Borosil, 100 ml) and

20 ml in a culture tube (Borosil, 38 x 200 mm). Media were sterilized in an autoclave at 121°C and 1.06 kg/cm^2 pressure for 20 min. A total number of 20 - 25 NSSs (three - four explants per flask) were cultured for each treatment.

Subcultures were done on B5 medium supplemented with IBA (0.005 mg/l) and BAP (1.0 mg/l) and two methods were adopted for subculturing. In the first method, 4 - 6 cm long regenerated shoots were cut from the tip leaving 1 - 2 cm long basal part, callus removed from the base and the basal parts of the cut shoots (clumps, 1 - 2 cm) were used; and in the second method only the pieces of organogenic callus (about 0.5 gm) were used. Hormone-free B5 medium containing 6% sucrose instead of 3% sucrose was used for rooting of the differentiated shoots.

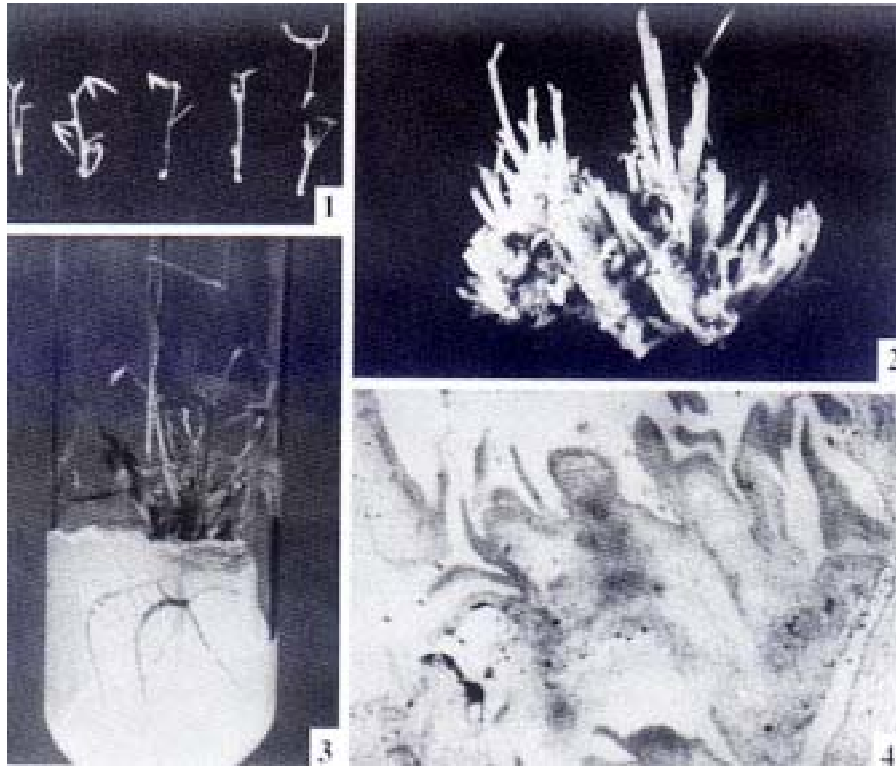
Morphogenic data were recorded after six weeks of initial culture period, and after four weeks of each subculturing passage. Cultures were incubated at 26 ± 1°C with light intensity of 1600 lux for a 16 hr photoperiod provided by fluorescent tubes (40 W) and incandescent bulbs (40 W) in the growth chamber. The entire protocol was repeated several times over a period of six years to test the efficiency and reproducibility of results.

For histological studies, pieces of the differentiated NSSs with organogenic callus were fixed in FAA, preserved in 70% alcohol, dehydrated in TBA-xylol series, embedded in paraffin wax and serial sections of the material were cut at a thickness of 10 µm (Johansen 1940).

Results and Discussion

Cultured cotyledon explants showed *de novo* shoot bud differentiation from the entire surface along with the dark green/brown, hard and compact callus after six weeks on B5 medium supplemented with IBA (0.05 mg/l) and BAP (3.0 mg/l) as reported previously (Sharma and Kothari 1993). The NSSs of these differentiated shoots were used as explants as described in the Methods. Initially, the proliferation of axillary shoots (two - four) was observed after two weeks and organogenic callus developed from the cut ends of the cultured NSSs in four weeks. After six weeks of incubation the number of early formed shoot buds increased in the same cultures (Fig. 2). It was observed that the shoots differentiated through multiple shoot as well as via callus formation. Direct shoot multiplication response was seen from the nodal parts of the NSSs on B5 medium with lower levels of IBA (0.005 and 0.05 mg/l) and BAP (1.0 mg/l). While IBA at 0.5 mg/l with higher levels of BAP (3 - 10 mg/l) in the medium evoked shoot bud differentiation along with callusing. Mostly the callus was green or brown to dark brown in color, compact, and nodular in

texture. The higher doses of BAP (5 and 10 mg/l) favoured more browning in the callus while the lower doses of BAP (1 and 3 mg/l) favoured green callus which also promote shoot proliferation. The amount of shoot organogenic callus along with shoots/shoot buds inhanced on increasing the levels of BAP in the medium (Table 1).



Figs. 1 - 4: Plantlet regeneration from different explants of *Glycine clandestina.* 1. Nodal shoot segments taken from *in vitro* differentiated shoots. 2. Shoot buds/shoots differentiated from nodal shoot segments. 3. Rooted shoot on hormone-free B5 medium containing 6% sucrose. 4. Section of a portion of shoot clump showing meristematic shoot buds.

Regenerating shoot clumps/calli were transferred to subculturing medium as mentioned in the Methods. It was observed that new shoot buds were differentiated on this medium and early formed buds elongated to develop into shoots in each passage. The number of regenerated shoots from one clump ranged from 7.0 to 10.5 (Table 2). A continuous line of shoot production could be achieved without losing the shoot regenerating potential in the passages of subculturing. Shoots (4 - 6 cm) were removed from parental cultures and rooted to form plantlets. The well developed root and shoot systems were observed

Table 1. Shoot buds/shoots developed from nodal shoot segments of *Glycine clandestina* **on B5 medium with different levels of IBA + BAP.**

IBA + BAP (mg/l)	Number of shoot segments cultured	Number of regenerating shoot segments	Number of shoots produced per segment (Mean ± S.E.)
0.005 + 1.0	22	10	16.5 ± 2.5
0.005 + 3.0	24	20	22.5 ± 2.5
0.005 + 5.0	25	25	29.4 ± 0.8
0.005 + 10.0	23	23	31.2 ± 1.1
0.05 + 1.0	20	15	12.6 ± 0.8
0.05 + 3.0	22	20	17.0 ± 3.0
0.05 + 5.0	24	21	25.0 ± 5.0
0.05 + 10.0	25	24	26.8 ± 2.6
0.5 + 1.0	23	15	10.0 ± 3.0
0.5 + 3.0	22	15	12.5 ± 2.5
0.5 + 5.0	20	20	20.0 ± 1.6
0.5 + 10.0	25	24	22.5 ± 2.5

S.E. = Standard error.

Table 2. Morphogenic potential of regenerating shoot clumps of *Glycine clandestina* **on B5 medium with IBA (0.005 mg/l) and BAP (1.0 mg/l) in succeesive passages of subculturing over a period of six years.**

No. of passages*	Clumps plated (No.)	Clumps producing shoot buds/ shoot (No.)	Total shoot buds/ shoots produced (No.)	No. of shoot buds/shoots produced per clump (Mean ± S.E.)
1	22	16	142	8.8 ± 4.1
2	25	20	175	8.7 ± 1.3
3	20	20	168	8.5 ± 3.2
4	25	25	196	7.8 ± 2.2
5	25	21	172	8.1 ± 1.1
6	22	20	160	7.9 ± 3.6
7	24	14	134	9.5 ± 0.8
8	20	15	158	10.5 ± 1.3
9	20	13	127	9.7 ± 2.9
10	20	17	148	8.7 ± 2.0
11	18	13	93	7.0 ± 0.6
12	23	15	141	9.2 ± 1.8
24	22	15	146	8.2 ± 3.8
36	24	20	171	10.8 ± 1.1
48	18	18	182	9.3 ± 2.7
60	24	21	153	8.5 ± 1.9
72	24	23	166	9.5 ± 2.1

S.E. = Standard error. *Twelve passages count for one year.

observed on hormone-free B5 medium containing 6% sucrose after four weeks (Fig. 3). Histological studies of the organogenic callus revealed the formation of several shoot buds each with a central dome shaped shoot apex and leaf primordia (Fig. 4).

Rapid and reproducible plant regeneration system in grain legumes is difficult in tissue cultures (Nagl et al. 1997; Das et al. 2002) including soybean (Yuan et al. 2001), although plant regeneration has been reproted in soybean from various explants (Dan and Reichert 1998, Meurer et al. 2001, Tripathi and Tiwari 2003). Axenic NSSs taken from *in vitro* differentiated shoots can also be used as a source of explant for an efficient plant regeneration in *G. clandestina* as described in this report. This system might be useful for *Agrobacterium* as well as particle bombardment mediated genetic transformation to improve the soybeans.

References

Chung GH and **Kim JH** (1990) Production of interspecific hybrids between *Glycine max* and *Glycine tomentella* through embryo culture. Euphytica **48** : 97-101.

Dan Y and **Reichert NA** (1998) Organogenic regeneration of soybean from hypocotyl explants. In Vitro Cell. Dev. Biol.-Plant. **34** : 14-21.

Das DK, Bhomkar P, Prakash NS and **Bhalla-Sarin N** (2002) Improved method of regeneration of black gram (*Vigna mungo* L.) through liquid culture. In Vitro Cell. Dev. Biol.-Plant **38** : 456-459.

Davey MR, Hammatt N and **Jones B** (1988) Somatic cell techniques in soybean improvements: Problems, progress and potential. Soybean Genet. Newslett. **15** : 41-47.

Gamborg OL, Miller RA and **Ojima K** (1968) Nutrient requirement of suspension cultures of soybean roots. Exp. Cell Res. **50** : 151-158.

Hammatt N, Davey MR and **Nelson RS** (1986) Plant regeneration from seedling cotyledons, leaves and petioles of *Glycine clandestina*. Physiol. Plant. **68** : 125-128.

Hammatt N, Kim H, Davey MR, Nelson RS and **Cocking EC** (1987) Plant regeneration from cotyledon protoplasts of *Glycine canescens* and *Glycine clandestina*. Plant Sci. **48** : 129-135.

Hymowitz T and **Burridge JA** (1989) Management of the USDA wild perennial *Glycine* collection. Soybean Genet. Newslett. **16** : 89-92.

Hymowitz T and **Singh RJ** (1988) Updated information on the biosystematics of the genus *Glycine*. Soybean Genet. Newslett. **15** : 112-113.

Hymowitz T, Chalmers NL, Constanza SH and **Saam MM** (1986) Plant regeneration from leaf explants of *Glycine clandestina.* Plant Cell Rep. **3** : 192-194.

Johansen DA (1940) Plant Microtechnique. McGraw Hill Co., New York.

Jones B and **Davey MR** (1991) Regeneration of plants from protoplasts of *Glycine clandestina* Wendl. Accession G1826. Soybean Genet. Newslett. **18** : 174-176.

Kollipara KP and **Hymowitz T** (1989) Screening the *Glycine clandestina* Wendl. collection for shoot regeneration from leaf explants. Soybean Genet. Newslett. **16** : 83-85.

Marshall DR and **Broue P** (1981) The wild relatives of crop plants indigenous to Australia and their use in plant breeding. J. Aust. Inst. Agric. Sci. **47** : 149-154.

Meurer CA, Dinkins RD, Redmond CT, McAllister KP, Tucker DT, Walker DR, Parrot WA, Trick HN, Essig HM, Finer JJ and **Collins GB** (2001) Embryogenic response of multiple soybean [*Glycine max* (L.) Merrill.] cultivars across three locations. In Vitro Cell. Dev. Biol.-Plant. **37** : 62-67

Nagl W, Ignacimuthu S and **Becker J** (1997) Genetic engineering and regeneration of *Phaseolus* and *Vigna*. State of the art and new attempts. J. Plant Physiol. **150** : 625-644.

Rech EL, Golds TJ, Hammatt N, Mulligen BJ and **Davey MR** (1988) *Agrobacterium rhizogenes* mediated transformation of the wild soybeans *Glycine canescens* and *Glycine clandestina*: Production of transgenic plants of *Glycine canescens*. J. Exp. Bot. **39** : 1275-1285.

Sharma VK and **Kothari SL** (1993) High frequency plant regeneration in tissue cultures of *Glycine clandestina*-A wild relative of soybean. Phytomorphology **43** : 29-33.

Singh RJ and **Hymowitz T** (1985) The genomic relationships among six perennial species of the genus *Glycine* subgenus *Glycine* Willd. Theor. Appl. Genet. **71** : 221-230.

Tripathi M and **Tiwari S** (2003) Epigenesis and high frequency plant regeneration from soybean (*Glycine max* (L.) Merr.) hypocotyl. Plant Tissue Cult. **13** : 61-73.

Yuan Y, Liu DP, Zheng PH, Xu WJ and **Li HL** (2001) Study on plant regeneration from soybean cultures. Soybean Sci. **20** : 9-13.