Plantlet regeneration from tissue cultures of *Sesbania grandiflora*

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A method has been established for the production of whole plantlets in *Sesbania grandiflora* tissue cultures. Hypocotyl and cotyledon explants obtained from 10–12-day-old seedlings were cultured on B_5 medium supplemented with BAP, BAP+NAA or BAP+NAA+GA_3 to induce callusing and organogenesis. Hypocotyl explants were more amenable than cotyledon explants in terms of callusing and differentiation of shoot buds. The number of shoot buds formed in a period of 4 weeks from the hypocotyl and cotyledon cultures ranged from 2 to 12 and from 1 to 4 respectively. Serial transfer of cultures (after every 4 weeks) on BAP, BAP+NAA, BAP+NAA+GA_3 and BAP+GA_3 enhanced the number of shoot buds to 15–25 in hypocotyl cultures and to 4–8 in cotyledon cultures in 12 weeks. Subsequently, however, the callus lost the ability to either proliferate or differentiate more shoot buds. Ninety to 160 shoot buds could be obtained from the explants of a single seedling in a period of three months. Whole plantlets were obtained when the elongated shoots were planted on B_5 medium supplemented with IBA (5×10^{-6} M). A histological study of the cultures showed the presence of 'meristemoids' and vascular 'nodules'. The former developed into shoot-bud meristems.

MUCH interest has been evinced in recent times in the propagation of leguminous trees by tissue culture. Micropropagation can speed up the production of planting stocks. This has also formed the basis of creating somaclonal variants and for producing a novel source of genetic variability with potential for improvement[1,2]. In leguminous trees, isolation of somaclonal variants with high nitrogen-fixing capabilities and high biomass production will be of immense value.

Regeneration of whole leguminous tree species from callus via shoot production has been reported in *Acacia*, *Albizia*, *Dalbergia*, *Leucaena*, *Prosopis* and *Robinia* (see the recent review by Bajaj[3]). The arborescent *Sesbania* spp. have been recommended in social forestry programmes in India because of their multiple uses.

We have published preliminary data on shoot bud differentiation in tissue cultures of *Sesbania sesban*[4] and *S. grandiflora*[5]. The other reports demonstrating morphogenetic potential in tissue cultures of *Sesbania* are those on *S. bispinosa*[6] and *S. rostrata* (considered to be one of the most powerful N_2-fixing systems (about 200 kg N_2/ha in 50 days)[7]) by Vlachova *et al.*[8] The latter authors have also attempted to induce *Agrobacterium*-mediated infection to develop a transformation system in *S. rostrata*. Mathews *et al.*[9] reported that regeneration of shoots from stem and leaf explants occurs at a low frequency in *S. rostrata*. However, in the crown gall tumours induced in this plant (with *Agrobacterium tumefaciens*), the frequency of regeneration of normal, non-transformed, nopaline-negative shoots was substantially increased.

In this paper we outline the procedure for whole plantlet regeneration and maintenance of cultures through serial transfers of *S. grandiflora*, a versatile tree legume. *S. grandiflora* grows amazingly quickly and provides forage, firewood, pulp and paper, food, and green manure. It is also used for landscaping. This species is ideally suited for afforestation of eroded and grassy wastelands throughout the tropics.

Methods

Seeds of *S. grandiflora* were purchased from the market in Tiruchirapalli, Tamil Nadu, India. They were surface-sterilized and implanted in tubes containing agar (0.8%)+sucrose (2%) (for details see Khattar and Mohan Ram[5]). In the initial experiments it was observed that 50–60% of the seeds germinated in 5–6 days. However, soaking them in dilute sulphuric acid (1:4 v/v) for 10 min before sterilization promoted a high percentage (90–100%) of germination within 2–3 days of planting.

Germination was hypogeal. Hypocotyl explants (1 cm long) were prepared from aseptically grown seedlings of the same length. Each cotyledon was cut transversely to obtain ~0.5-cm-long pieces. While preparing explants from the cotyledon, care was taken to remove the basal 2–3 mm as this could contain a pre-existing meristem. A comparison of the performance of explants obtained from seedlings that were 4, 6, 8, 10, 12 and 14 days old indicated that explants from 10- and 12-day-old seedlings were the most responsive.

B_5 medium[10] was used as the basal medium (BM). Explants were cultured on BM supplemented with 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA) and gibberellic acid (GA_3) individually and in several combinations at concentrations ranging from 10^{-7} to 10^{-4} M.

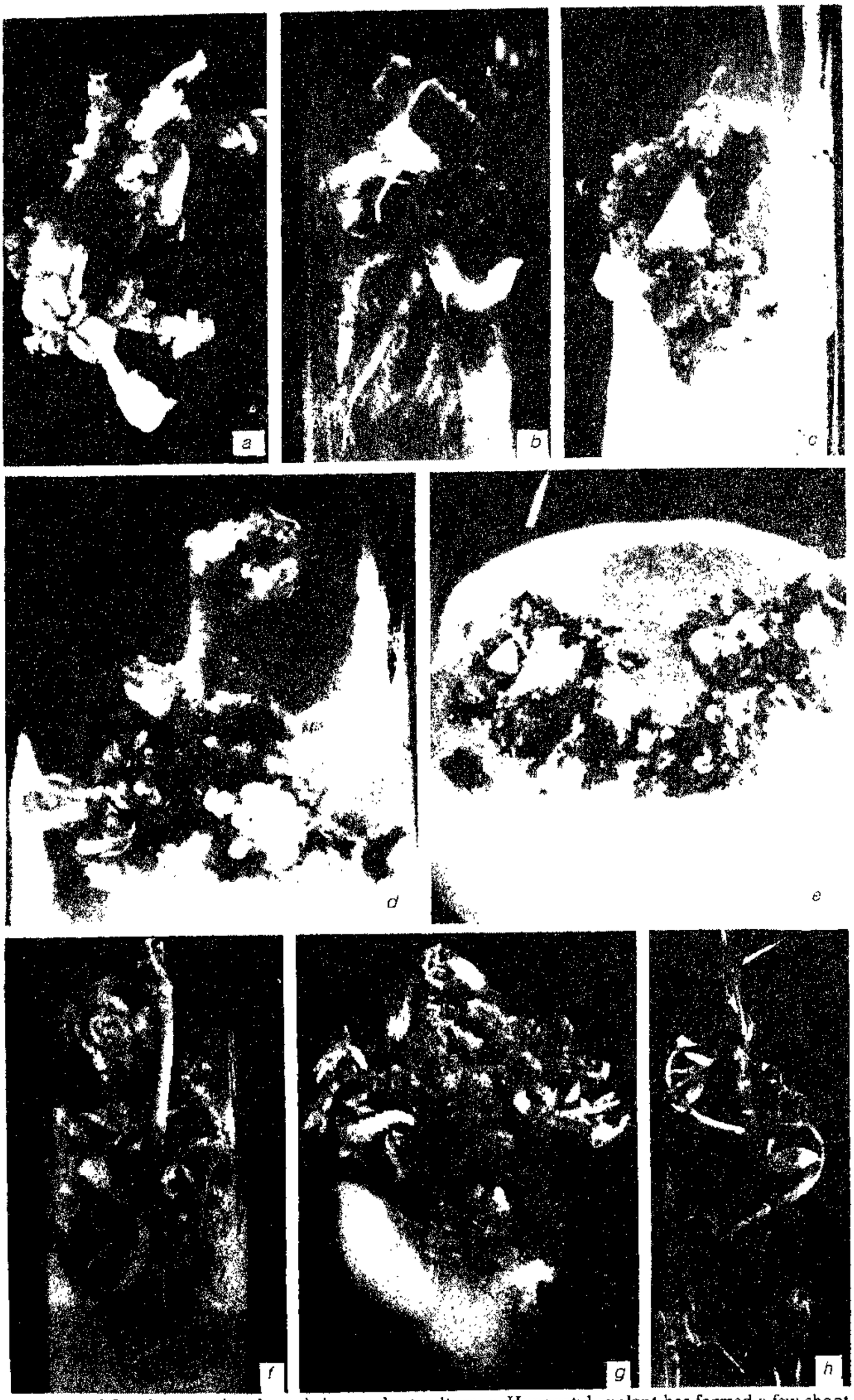

Figure 1. *Sesbania grandiflora* hypocotyl and cotyledon explant culture. *a*, Hypocotyl explant has formed a few shoot buds from the slightly callused cut ends on BAP (5×10^{-6} M) (4 weeks old). *b*, Numerous thick roots have arisen from cotyledon callus (in contact with the medium) on BAP (10^{-7} M) + NAA (10^{-5} M) (4 weeks old). *c*, Callus formed from hypocotyl explants differentiated numerous shoot buds on BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (4 weeks old). *d*, Induction of more shoot buds occurred when hypocotyl cultures initially raised on BAP (10^{-6} M) were transferred to BAP (10^{-6} M) twice (after every 4 weeks). The shoot buds, however, remained suppressed in their growth. *e*, Fate of hypocotyl explants grown through different passages: BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) → same medium → BAP (10^{-6} M) in long-term callus cultures. Numerous tiny shoot buds can be seen. *f*, Elongation of shoot buds occurred when the hypocotyl cultures were transferred to BM after raising them on BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (for 12 weeks). *g*, Cotyledon culture raised on BAP (5×10^{-6} M) for 12 weeks. Shoot buds have become slightly elongated. *h*, A cultured shoot after 6 weeks growth on BM + IBA (5×10^{-6} M). Note root development from the base.

The cultures were stored in a sterile room at 26±2°C under a 16/8 h light/dark cycle. Light supplied from fluorescent tubes had an intensity of 200 μE m^{-2} S^{-1}. For every treatment 48 explants (24 each for hypocotyl and cotyledon explants) were raised. The cultures were maintained through serial transfers at 4-week intervals. The parameters studied were extent of callusing, number of shoot buds formed, and length of shoot buds.

To prepare microtome sections the callus masses containing shoot buds were fixed in FAA (formalin/glacial acetic acid/70% ethanol) for 24 h and preserved in 70% ethanol. The material was dehydrated through an ethanol–xylene series and embedded in a mixture of paraffin and Histowax (3 : 1). Sections of 10 μm were cut using a rotary microtome, and stained with haematoxylin and counterstained with erythrosin.

Shoot and root induction

Explants from hypocotyl and cotyledon yielded axenic cultures. In tree species characterized by a shift from juvenile to adult phase, it has been reported by various workers that explants of mature (adult) parts either do not survive or perform poorly, whereas those from embryonal, seedling or juvenile tissue yield vigorously growing cultures that are potentially more regenerative[11–15].

The main objectives of the present investigation were to identify a suitable treatment to (a) promote and sustain callus growth from the hypocotyl and cotyledon explants and (b) induce high incidence of shoot-bud formation. Of all the treatments tested, only a few could induce callusing, and root and shoot formation.

Addition of BAP (10^{-7}–10^{-4} M) to BM caused slight callusing and shoot-bud (2–12) production from hypocotyl explants. Callusing and shoot-bud formation were more abundant at cut ends than on the surface (Figure 1*a*). At these concentrations cotyledon explants formed only 1–4 shoot buds. Roots (2–9) were formed from hypocotyl and cotyledon explants on a medium containing BAP (10^{-7} M) + NAA (10^{-5} M) and BAP (10^{-7} M) + NAA (10^{-5} M) + GA_3 (10^{-7} M) (Figure 1*b*). In the remaining BAP + NAA and BAP + NAA + GA_3 treatments, either only callusing or callusing with shoot buds (2–5) was noted. BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) proved the best among several combinations for callusing and shoot-bud (2–13) differentiation (Figure 1*c*) from hypocotyl explants. Cotyledon explants were less responsive and formed only 3 or 4 shoot buds in this treatment.

Serial transfers

Because of their better responsiveness hypocotyl explants were selected to assess response in subsequent transfers. With the objective of causing further proliferation of the callus and enhancing shoot-bud regeneration, hypocotyl cultures were transferred (as a whole) serially on various media. The study on serial transfer of cultures showed that (i) BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) promoted good callusing, (ii) BAP (10^{-6} M) (Figure 1*d*, *e*) and BAP (10^{-6} M) + GA_3 (10^{-7} M) stimulated production of more shoot buds, and (iii) BM (Figure 1*f*) and BAP (10^{-6} M) + GA_3 (10^{-7} M) caused elongation of shoot buds already present (Table 1).

The number of shoot buds from hypocotyl cultures increased to 15–25 till the third passage (Table 1). After the third transfer no appreciable change in either callus growth or number of shoot buds was observed in the cultures. During the same period, cotyledon cultures formed only 4–8 shoot buds on medium containing BAP (10^{-6} and 5×10^{-6} M) or BAP (10^{-6} M) + NAA

Table 1. *Sesbania grandiflora*: Growth of hypocotyl explants in serial transfers*

	Treatment and response				
Initial medium	BAP (10^{-6} M) (+, 7, 0.2–1)	BAP (10^{-6} M) (+, 7, 0.2–1)	BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (++, 8–10, 0.2–0.5)	BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (++, 8–10, 0.2–0.5)	BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (++, 8–10, 0.2–0.5)
⇩ First transfer	⇩ BAP (10^{-6} M) (+, 10, 0.2–1)	⇩ BAP (10^{-6} M) (+, 10, 0.2–1)	⇩ BM (++, 8–10, 0.5–2)	⇩ BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (+++, 8–10, 0.2–0.8)	⇩ BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (+++, 8–10, 0.2–0.8)
⇩ Second transfer	⇩ BAP (10^{-6} M) (+, 15–20, 0.2–1)	⇩ BAP (10^{-6} M) + GA_3 (10^{-7} M) (+, 12–15, 0.2–3)	⇩ BM (++, 8–10, 0.5–3)	⇩ BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (+++, 15–17, 0.2–1)	⇩ BAP (10^{-6} M) (+++, 15–25, 0.2–1.3)
⇩ Third transfer	⇩ BAP (10^{-6} M) (+, 18–25, 0.3–1.2)	⇩ BAP (10^{-6} M) + GA_3 (10^{-7} M) (+, 12–18, 0.4–3)	⇩ BM (++, 8–11, 0.5–3.5)	⇩ BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) (+++, 15–17, 0.2–1)	⇩ BAP (10^{-6} M) (+++, 15–25, 0.2–1.3)

Data based on 24 cultures.
Figures in parentheses are extent of callusing (denoted by + signs: + (slight), ++ (moderate), +++ (profuse); range of number of shoot buds; shoot length (in cm).
*Culture period for each treatment is 4 weeks.

Figure 2. Sections of *S. grandiflora* hypocotyl and cotyledon explant cultures. *a–e*, Portions enlarged from transections of hypocotyl (*a*, *b*) and cotyledon cultures (*c*) (raised on BM + BAP (10^{-6} M) + NAA (10^{-6} M) + GA_3 (10^{-6} M) to show meristemoids (arrows) in the callus). *d*, Development of young shoot-bud meristems in peripheral region of nodule. Differentiation of many zones consisting of elongated tracheid-like cells can be seen. *e*, Transverse section of part of hypocotyl culture showing several shoot-bud meristems (arrows) arising from meristemoids. *f*, A young developing shoot bud consisting of a meristem surrounded by a pair of leaf primordia in longitudinal section. *g*, An elongated bud in longitudinal section.

(10^{-6} M) + GA_3 (10^{-6} M), and transferred after every 4 weeks (figure 1*g*). Thus, using hypocotyl and cotyledon explants of a single seedling (4 cotyledon and 5 hypocotyl explants), it is possible to obtain 90–160 shoot buds in 3 months.

The cultures of *S. grandiflora* responded differently from those of *S. sesban*[4,16]. Whereas *S. sesban* cultures were harder, highly nodular, and secreted copious amount of dark (brown-black) leachates (phenolics) into the medium, the cultures of *S. grandiflora* were less nodular, light brown, and produced no leachates. The shoot-forming ability of cultures of *S. sesban* was retained till the 12th passage[16] (i.e. over a period of one year). In contrast, *S. grandiflora* cultures lost their ability to regenerate after 3 passages (3 months after culture).

In vitro-raised shoots measuring 3–4 cm were excised and rooted in a semisolid basal medium containing indole-3-butyric acid (IBA, 5×10^{-6} M). This treatment not only induced up to 10 roots per culture with laterals in 80% of cultures but also promoted shoot growth (length as well as number of axillary branches, Figure 1*h*). Semisolid medium facilitated the development of a strong root system in the plantlets. Attempts are being made to achieve successful transplantation of these plantlets to the field.

Histology of cultures

In sections the differentiating cultures showed the presence of meristemoids and vascular nodules. Whereas the nodules were accompanied by well-differentiated vasculature, the meristemoids lacked vascularization altogether (Figure 2*a–d*). Several shoot-bud meristems could be seen in sections of hypocotyl cultures (Figure 2*e,f*) suggesting that meristemoids eventually develop shoot-bud meristems. Figure 2*g* is an elongated bud in longitudinal section with the shoot meristem in the centre, surrounded by a pair of leaf primordia.

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