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Research Paper

High Frequency Shoot Regeneration for Mass Multiplication of Desmodium Gangeticum (L.) DC—An Important Anticancer, Antidiabetic and Hepatoprotective Endangered Medicinal Plant

Biotechnology

KEYWORDS : Shalparni;Axillary shoot;Desmodium gangeticum; Node; Medicinal plant;Micropropagation

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ABSTRACT

Clonal multiplication protocol from in vitro culture of node and leaf explants from 4-week-old seedlings of Desmodium gangeticum (Fabaceae), a valuable, but endangered medicinal plant, was developed. An average of 24.1 shoots/nodal explant were obtained after 4 weeks of culture initiationon MS medium containing 4.40 µM of BA; addition of 1.13µM PVP or ascorbic acid had a small promotory effect, while presence of NAA at 1.07, 2.68 or 10.72 µM tended to suppress shoot regeneration, except in the case of 8.90 µM BA plus 2.68 µM NAA, whichyielded 29.1 shoots/explant from 100% of explants.

Multiple shoot (26 shoots/culture) regeneration was achieved from 84% of the cultures when callus derived from leaf explant was subcultured on medium supplemented with 28.90 µM BA and 2.68 µM NAA.The shoots were rooted by pulse treatment with 492.12 µM of indole-3-butyric acid for 30 min and then cultured on half-strength MS medium;~ 10 good quality roots/shoots were produced in 98% of shoots. Plantlets were successfully acclimatized and transferred to the field with survival rate of 92%, where they grew normally without any observable morphological variation. This protocol may help long-term conservation and mass propagation for commercial propagation and high biomass production of this valuable medicinal plant.

Introduction

Desmodium gangeticum (L.) DC (Fabaceae) is a small perennial shrub of tropical regions, which has been used in Indian system of medicine as a bitter tonic, febrifuge, digestive, anticatarrhal, antiemetic agent, and for treatment of various inflammatory conditions of chest and other organs due to vata disorder (Chopra et al., 1956; Bakshi et al., 2001) with [1-2]. *D. gangeticum* shoot extract is reported to contain alkaloids, pterocarpenoids, flavones and isoflavanoid glycosides [3]; and is expected to possess anti-cancer activities [4]. The aqueous extract of this species was reported to show severe antiwrithing activity, moderate central nervous system stimulant and antidepressant activity and antileishmanial activity [5-6]. Gangetin, a pterocarpenoid from *D. gangeticum*, has been sown to possess anti-inflammatory and analgesic activities [7]. The roots of *D. gangeticum* form one of the ingredients of a famous ayurvedic preparation, Dasmoola Kwatha, which is considered to be antipyretic alternative and bitter tonic. The aqueous extract of root exhibits anti-inflammatory, antibacterial and antifungal activities [8]; it also acts as a potent antiulcer agent that is effective in all models; its antiulcerogenic activity seems to be mainly due to its more cryoprotective effect in comparison to anti-secretory effects [9].

This plant has high commercial importance and its domestic demand is estimated as about 678.4 tones/year [10]. Department of Indian Systems of Medicine and Homeopathy, Ministry of Health and Family Welfare, Government of India, has formulated a Central Scheme for Cultivation and Development of Medicinal Plants. This plant is one of the species identified for promoting cultivation in order to reduce pressure on its natural habitat and to meet the shortage in biomass availability [11].

Efforts on propagation of this plant by seed and stem cuttings have been successful [12-13]. Vishwakarma et al. (2009) [14] reported *in vitro* multiplication protocol using *D. gangeticum* cotyledonary node explants; they obtained 9.2 shoots/explant on BA (8.8 µM) and NAA (21.2 µM) supplemented MS medium. A high rate of shoot multiplication from *D. gangeticum* nodal explants was obtained when these explants obtained from one-year old *D. gangeticum* plants were cultured on 4.40 µM BA + 1.14 µM IAA supplemented MS medium [15]. There are few reports of regeneration from other *Desmodium* species such as *D. heterocarpon* ssp. *angustifolium* and *D. ovalifolium*. Wofford et al. (1992) [16] attempted regeneration in six genotypes of two *Desmodium* species, *D. heterocarpon* ssp. *angustifolium* and *D. ovalifolium*; they observed organogenesis in only two genotypes on L2 basal salts + BA and Picloram, but no shoot regeneration in any genotype on MS basal salts + different concentrations of growth regulators (2,4-D, IBA, BA). Krottje et al. (1996) [17] estimated heritability for callus growth and regeneration by evaluating F2 and F3 generations from two crosses, viz., *D. heterocarpon* ssp. *angustifolium* × *D. ovalifolium* and *D. heterocarpon* ssp. *angustifolium* × *Desmodium* sp. by parent-offspring regression analysis. The heritability estimates ranged from 0.65 to 0.77 for callus growth and from 0.19 to 0.46 for regeneration suggesting a much greater environmental influence on shoot regeneration than on callus growth. It is important that some segregantes from these crosses showed more vigorous and prolific regeneration than the parental genotypes, indicating possibility of improving regeneration ability by a suitable breeding strategy. Devi and Narmathabai (2011) [18] obtained somatic embryogenesis in *D. motorium*. Embryogenic calli were obtained by culturing cotyledon segments lacking embryo axis on MS medium supplemented with IAA in combination with BA, while differentiation of globular and heart-shaped somatic embryos was achieved by transferring the embryogenic calli to growth regulator-free MS medium. Regenerated plantlets were transferred to pots with survival rate of 50%. Rey and Mroginski (1997) [19] reported *in vitro* regeneration from leaf callus of *D. affine* and *D. uncinatum*.

This study was undertaken to optimize protocols for axillary shoot proliferation from seedling derived node explants and for shoot regeneration from calli derived from leaf explants of *D. gangeticum*, induction of rooting of the shoots, and for establishing the plantlets in the field. The seedling derived explants were used during this study because, it showed better shoot regeneration than field explants as reported elsewhere [20-21]. So far, there has been no report on *in vitro* plant regeneration and

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organogenesis of *D. gangeticum* using leaf segment. This protocol may be used for the production of huge biomass of *D. gangeticum* as raw materials for manufacture of herbal formulations and extraction of crude drugs.

Materials and methods

Plant material, surface sterilization and explant selection

Seeds of *D. gangeticum* were collected from Ayurvedic garden of the Banaras Hindu University, Varanasi and washed under running tap water for 10 min. These seeds were kept in freshly prepared 1% cetrimide solution and agitated for 10 min, then treated with 0.1% (w/v) $HgCl_2$ (Hi media, India) solution for 10 min, followed by five washings with sterilized double distilled water. Finally, the seeds were soaked in sterilized double distilled water for 2 h. Further their seed coats were removed and the naked seeds (without seed coat) were cultured on growth regulator (GR)-free MS [22] medium supplemented with 30 g/l sucrose and 8 g/l tissue culture grade agar (Sigma, St. Louis, MO); the pH of medium was adjusted to 5.8 prior to addition of agar, and the medium was autoclaved at 121^{0}C for 20 min. Seeds were placed on this medium for germination and kept in culture room at 25^{0}C and illuminated at 20μmolm^{-2} s^{-1} from cool-white fluorescent tubes (16:8 h photoperiod). Node and leaf explants, excised from one-month-old seedlings grown *in vitro*, were cultured on media having different concentrations of growth regulators to achieve callus formation or shoot regeneration.

Bud break, shoot multiplication and its elongation

The mother explants along with regenerated shoots were repeatedly transferred on to fresh medium for 4 cycles and nodal explants were cultured in the presence of 2.22, 4.40 and 6.65 μM BA or 6.97, 9.30 and 23.2 μM KIN with and without additives (1.13 μM polyvinyl pyrollidine, PVP, or ascorbic acid). Since the two additives were comparable in their effects, data from PVP only are presented (Table 1). Nodal explants of *D. gangeticum* seedlings were cultured on MS medium supplemented with different (2.22, 4.40, 8.90 and 13.3 μM) concentrations of BA alone and in combination with NAA (1.07, 2.68, 5.36 μM) (Tables 2). The percent of explants showing shoot development, average number of shoots/explant and average shoot length (cm) were recorded after four weeks of culture. The excised shoots of 4-5 cm were used for rooting, while smaller shoots were kept on elongation medium (0.58 μM GA_3) as reported elsewhere [39]. Subculturing of mother nodal explants was continued for four passages on the most supportive medium (Table 3).

Callus initiation and plant regeneration

Leaf explants were inoculated horizontally on MS medium supplemented with additives (1.13 μM ascorbic acid or PVP) and auxin 2, 4-D (0, 1.13–9.05 μM) or NAA (0, 0.54– 2.68 μM). The callus was subcultured on the callus initiation medium (MS + 4.44 μM BA + 2.68 μM NAA) for 2–3 cycles before being evaluated for regeneration (data not shown). About 100 mg of callus was then subcultured onto MS medium having BA (2.22, 4.40, 8.90, 13.3 μM) + NAA (1.07, 2.68, 5.36 μM) (Table 4).

Rooting

Individual shoots of 4-5 cm were transferred to MS medium supplemented with various concentrations of IBA or NAA (0.54-1.07 μM) for rooting. Rooting frequency was low (< 10%) and in most of the shoots basal callus formed instead of rooting. Therefore, elongated shoots were pulse treated with an IBA or NAA solution (246.06-2460.6 μM) for 20 or 30 min duration and then cultured on half- strength MS medium (Table 5) since it was found to be more supportive for rooting than full strength MS medium [39].

Acclimatization and field transfer

Rooted plantlets were gently removed from the tissue culture vessels and washed thoroughly in tap water to remove all traces of medium from their roots. They were then transferred to small plastic pots containing a mixture of soilrite and soil (2:1 v/v), and kept in the culture room for acclimatization. The pots were covered with polyethylene bags to maintain high humidity, and irrigated with tap water and 1/8 strength of vitamin solutions on alternate days. The hardened plantlets were transplanted into 30 cm x 30 cm pits spaced at 80 cm x 80 cm and filled with vermiculite, and immediately irrigated; then irrigation was done on 4th and then on 8th day. Subsequently, plants were irrigated once every week during summer and once during two weeks during winter. Data were on stem length and number of branches per plant after 90 days from field transplantation.

Statistical analysis

The experiments were laid out according to completely randomized block design; each experiment had three replicates, and each replicate consisted of 50 culture, each tube containing a single explants/shoot. Analysis of variance (ANOVA) was carried out to detect the significance of differences among treatment means, and the means were compared using Duncan's new multiple range test (DMRT) at $P < 0.05$ [23]. Both ANOVA and DMRT analyses were carried out using the software SPSS ver. 16.0 (Statistical Package for the Social Sciences, SPSS).

Results

Axillary shoot multiplication from nodal explants

The *in vitro* seedling derived nodal explants did not show bud break even after 30 days when cultured on GR-free MS medium. However, in the presence of a cytokinin (BA or KIN) bud break and axillary shoot development was observable in about 5-7 days. Analysis of variance revealed that frequency of responding explants, mean shoot number per explant and mean shoot length (cm) were significantly affected by cytokinin concentration and the presence of additive (PVP or ascorbic acid). The optimum concentration of BA was found to be 4.40 μM BA; it yielded 25.2 and 24.1 shoots/explant from 100% of the explants, with and without the additive kinetin was found to be relatively less effective as compared to BA; the highest response was produced by 23.2 μM kinetin, which yielded 12.4 and 10.2 shoots/explant from 98% and 88% explants in the presence and absence of the additives, respectively. However, the average length of shoots was higher on media having KIN as compared to those having BA (Table 1). The additives PVP and ascorbic acid produced comparable effects; therefore, data for PVP only are presented here (Table 1). Presence of additives resulted in a small, but consistent and significant improvement in shoot proliferation and, particularly, shoot quality; the shoots regenerated on media supplemented with additives were stouter and looker healthier.

When nodal explants were cultured on MS medium supplemented with BA and NAA combinations, the maximum number of shoot buds developed on MS medium supplemented with 4.40 μM BA and 2.68 μM NAA, followed by that having 4.40 μM BA plus 5.36 μM NAA or 4.40 μM BA alone. In almost all the cultures maintained on media having NAA there was callus development, from which several shoot buds regenerated. The frequency of responding explants was the highest (100%) on media having 4.40 μM BA or 8.90 μM BA in combination with 2.68 μM NAA. Similarly, average shoot length was the highest on media containing 4.40 μM BA or 8.90 μM BA and 2.68 μM NAA (Table 2, Fig 1. A). Thus addition of NAA in the presence of BA had some beneficial effect on frequency of responding explants and average number of shoots per explant in some combinations, but in general, it had a depressing effect, which increased with NAA concentration.

Effect of successive transfer of mother nodal explants on shoot multiplication

Subculturing of mother nodal explants was continued for four passages on the most supportive medium (8.90 μM BA + 2.68 μM

NAA) for continuous production of healthy shoots; care was taken to remove any callus formed at the explant base. The number of shoots per explant as ranged from 31.2 to 34.6, which did not differ significantly from each other. Similarly average shoot length ranged from 2.5 cm to 2.91 cm, which were comparable with each other (Table 3). By adopting the procedure of shoot excision and re-culturing of the mother explants, a significant number of additional shoots (about 31–35 shoots) could be obtained from a single nodal explant in a period of four more weeks.

***In vitro* shoot regeneration from leaf calli**

When leaf explants were cultured on the callusing medium (MS + 2.26-22.62 µM 2,4-D or 5.36-16.1 µM NAA), up to 92% of them showed callus formation; the frequency of responding explants and the callus formed was the highest on the medium supplemented with 9.05 µM 2,4-D. These calli were transferred on regeneration media (MS + BA and NAA) for shoot regeneration (Fig 1. B). Calli kept on GR-free media did not show shoot regeneration, while those cultured on media containing BA with or without NAA showed shoot regeneration after about 15-20 days. The frequency of responding cultures increased with BA concentration up to 8.90 µM (80% response frequency), beyond which there was significant decline. Addition of NAA had some promontory effect on the frequency of responding cultures and the highest (92%) response was obtained on MS medium supplemented with 8.90 µM BA and 2.68 µM NAA. The average number of shoots per explant was promoted by NAA in combination with BA and the highest number of shoots per culture (26 shoots/culture) was obtained on medium having 8.90 µM BA and 2.68 µM NAA; this medium also produced the highest average shoot length (3.8 cm) (Table 4, Fig 1. C).

Rooting and transplantation

Shoots of 4-5 cm were cultured on half strength MS medium supplemented with either 0.54-10.72 µM NAA or 0.49-4.90 µM IBA. Only up to 10% of shoots showed regeneration of 1-2 roots per shoot primarily from callus formed at the cut ends of the shoots. Since satisfactory rooting could not be achieved by culturing the shoots on auxin containing media, pulse treatment with IBA and NAA was evaluated. The cut ends of shoots (4–5 cm) were dipped in different concentrations of IBA (0-2460.6 µM) and NAA (0–2678.6 µM) for 20-30 min, blotted dry with filter paper, and then cultured individually on half-strength MS medium, since it was earlier found to be superior to full-strength MS medium [38]. A 30 min treatment with 492.12 µM IBA seems to be the most suitable for rooting, while pulse treatment with NAA was relatively less effective as compared to that with IBA (Table 5, Fig 1. D and E).

The rooted plantlets were successfully transferred from culture tubes into plastic cups (Fig.1. F) containing soilrite and kept for acclimatization. In *D. gangeticum*, pulse treatment with IBA supported root induction, while addition of IBA into the medium itself was almost ineffective.

Finally, the plantlets after acclimatization were transferred into the field with 92% survival frequency (Fig.1G). There were no detectable variations among field transferred plants with respect to morphology and growth characteristics.

Growth and survival potential of field transferred plants

The performance of *D. gangeticum* plants transplanted in the field was evaluated after 90 days in terms parameters such as, stem length, which ranged from about 1.0-1.9 m, and number of branches per plant which ranged from 5 to 9 per plant.

Discussion

This is the first report on efficient shoot regeneration seedling from leaf callus cultures of *D. gangeticum*. Leaf calluses formed on MS medium containing 22.62 µM 2,4-D or 16.1 µM NAA were capable of regenerating large numbers (upto 26 shoots/culture) of shoots when subcultured on MS medium supplemented with BA with or without NAA, BA at 8.90 µM with 2.68 µM NAA being the optimum combination. The synergistic effect of BA and an auxin on shoot regeneration has been observed in other medicinal plants, such as *Rotula aquatica* [24] *Saussurea obvallata* [25], *Clitoria ternatea* L. [26], *Mucuna pruriens* [27], *Holarrhena antidysenterica* [28] and *Leucaena leucocephala* [29].

Nodal explants obtained from 4 week-old *in vitro* grown seedlings, and cultured on medium having a cytokinin (BA or KIN) with or without an additive (PVP or ascorbic acid) showed multiple axillary shoot proliferation, and 4.40 µM BA with 1.13 µM PVP was found to be optimum for shoot regeneration, and the additives had a small but consistent beneficial effect. Additives like PVP (0.1%) or activated charcoal (0.05%) are reported to enhance the shoot regeneration as well as average shoot length in aseptic seed cultures of *Sterculia urens*, but ascorbic acid (0.1%) was found to be most effective [30]. Kannaa and Jayabalan (2010) [31] reported that addition of BA with PVP in medium enhanced shoot number per cotyledonary node explant of *Solanum melongena* whereas shoot length and percent response were lower than those in case of BA alone.

The highest number of shoots/nodal explant was obtained on MS medium having BA at 8.90 µM and 2.68 µM NAA. Similar results were reported in *Sophora flavescens* [32], Oregano (*Origanum vulgare* × Appl II) [33], *Mucuna pruriens* [27], *Nyctanthes arbor-tristis* [34] and *Balanites aegyptiaca* [35] where a combination of BA and NAA was the most effective for shoot regeneration from nodal explants. Subculturing of mother nodal explants was continued for four passages and similar approach of subculturing of the mother explants for several passages was adopted for high-rate multiplication of other plant species [36-38].

After 4 weeks of culture, regenerated shoots were subcultured on the elongation medium as reported earlier [39] and elongated shoots (4-5 cm) were used for rooting. A 30 min pulse treatment with 492.12 µM IBA and culture on half-strength MS medium was optimum for rooting of the shoots. Similarly pulse treatment with IBA supported rooting of microshoots while addition of IBA into the medium was either ineffective or much less effective in some species like *Achras sapota* [40], *Celastrus paniculatus* [41] and *Santalum album* [42]. In contrast, IBA was found to be effective for rooting in several species like *Mucuna puriens* [27], *Withania somnifera* [43], *Acacia senegal* [44] and *Prosopis ceneria* [45] when added into culture medium.

The rooted plantlets were successfully transferred from culture tubes into plastic cups (Fig 1h) containing soilrite, acclimatized and then transferred into the field. There was no detectable variation among the field transferred plants with respect to morphology and growth characteristics. The appearance and size of the leaves were uniform.

The protocols reported here supports a high frequency multiple shoot regeneration from both leaf calli and seedling-derived nodal explants. Fully acclimatized plantlets ready for field transplantation were obtained after 4 months of nodal culture initiation, and a very high (92%) frequency of plantlets survived field transfer and this protocol may aid propagation and germplasm conservation of this endangered medicinal plant species.

Conclusion

This study reports an efficient and high multiplication from nodal explants and leaf derived calli of *D. gangeticum*. To our knowledge this is the first report on micropropagation and organogenesis from leaf derived calli of *D. gangeticum*. This may help rapid mul-

tiplication, large-scale production and conservation of this endangered valuable medicinal plant. *In vitro* regeneration from leaf derived calli may introduce somaclonal variations which can act as novel source of variation. This may compensate for the lack of natural variation in the surviving populations of endangered species with genetic bottlenecks, as suggested by Fay (1992) [46].

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Table 1: Effect of cytokinin and additive (1.13 µM PVP) on bud break and axillary shoot development from seedling derived nodal explants of *D. gangeticum*. Data recorded after 30 days of culture initiation, each mean is based on three replicates, each of which consisted of 50 culture tubes.

Plant Growth Regulator/ Concentration (µM)	Without PVP			With 1.13 µM PVP		
	Explants showing bud break* (%)	Average number of shoots/ explant*	Average shoot length* (cm)	Explants showing bud break* (%)	Average number of shoots/ explant*	Average shoot length* (cm)
BA						
2.22	94^{cd*}	21.6^{b}	1.9^{b}	96^{c}	22.4 ^{b}	2.0^{b}
4.40	100^{a}	24.1^{a}	2.1^{a}	100^{a}	25.2^{a}	2.3^{a}
6.65	96^{bc}	18.2^{c}	1.7^{bc}	98^{b}	18. 7^{c}	1.84^{c}
KIN						
6.97	80^{bc}	6.2^{c}	2.12^{abc}	86^{c}	7.1^{c}	2.5^{b}
9.30	82^{b}	7.6^{b}	2.36^{ab}	92^{b}	8.2^{b}	2.4^{b}
23.2	88^{a}	10.2^{a}	2.6^{a}	98^{a}	12.4^{a}	3.4^{a}

*Means having different letters in their superscript are significantly different from each other (P=0.05); comparison by DMRT within columns within cytokinin only.

Table 2: Effect NAA in combination with BA on per cent shoot formation, number of shoots/culture and average shoot length from nodal explant of *in vitro* germinated seedlings of *D. gangeticum*; data were recorded after 4 weeks of culture.

Treatment (µM)		Frequency of explants showing multiple shoots* (%)	Number of shoots/ explant*	Average shoot length* (cm)
BA	NAA			
0.0**	0.0**	0	0	0
2.22	0	94^{ab*}	21.6^{cd}	1.9^{b}
2.22	1.07	74^{ef}	11.7^{h}	1.4^{ef}
2.22	2.68	80de	15.8^{fg}	1.7^{cd}
2.22	5.36	84^{cd}	16.3^{f}	1.8^{bc}
4.44	0	100^{a}	24.1^{b}	2.1^{a}
4.44	1.07	88^{bc}	18.7^{e}	1.2^{g}
4.44	2.68	90^{bc}	19.2^{e}	1.54^{de}
4.44	5.36	94^{ab}	20.1^{de}	1.4^{ef}
8.90	0	96^{ab}	18.2^{e}	1.7^{cd}
8.90	1.07	92^{abc}	16.1^{f}	1.38^{ef}
8.90	2.68	100^{a}	29.1^{a}	2.2^{a}
8.90	5.36	94^{ab}	22.3^{bc}	1.9^{b}
13.3	0	92^{abc}	14.0^{g}	1.32^{fg}
13.3	1.07	70^{f}	10.2^{h}	1.6^{d}
13.3	2.68	78de	14^{g}	1.2^{g}
13.3	5.36	74^{ef}	12^{h}	1.2^{g}

Each mean is based on three replicates, each of which consisted of 50 culture tubes.

*Means having different letters in their superscript are significantly different from each other (P=0.05); comparison by DMRT within column only.

**GR-free MS medium.

Table 3. Effect of sub-culture cycles of mother stock of nodal explants cultured on MS medium supplemented with 8.90 µM BA + 2.68 µM NAA on shoot multiplication.

Subculture	Number of shoots per explant	Average shoot length (cm)
1st	31.2^{a}	2.5^{a}
2nd	33^{a}	2.72^{a}
3rd	34.6^{a}	2.91^{a}
4th	32^{a}	2.6^{a}

Each mean is based on three replicates, each of which consisted of 20 culture tubes. The values marked with different letters are significantly different from each-other at P < 0.05.

Table 4: Effect of different concentrations of BA and NAA on per cent shoot formation, average number of shoots/culture and average length of shoots from leaf derived calli of *D. gangeticum*; data were recorded after 4 weeks of culture.

Concentration (µM)		Frequency of explants with multiple shoots* (%)	Number of shoots/ culture	Average shoot length* (cm)
BA	NAA			
0	0	0	0	0
4.40	0	66^{gh*}	10^{i}	1.41^{g}
8.90	0	80^{bc}	14.1^{g}	2.2^{d}
13.3	0	70^{defg}	12^{h}	1.6^{fg}
4.40	1.07	70^{defg}	12.2^{h}	0.97^{h}
4.40	2.68	72^{cdef}	14.4^{fg}	1.04^{h}
4.40	5.36	76^{cde}	17de	1.1^{h}
8.90	1.07	78^{bcd}	19^{c}	2.6^{c}
8.90	2.68	92^{a}	26^{a}	3.8^{a}
8.90	5.36	84^{b}	22^{b}	3.1^{b}
13.3	1.07	64^{h}	18^{cd}	2.4^{cd}
13.3	2.68	72^{cdef}	21^{b}	1.89^{e}
13.3	5.36	68^{fgh}	15.9^{ef}	1.7^{ef}

Each mean is based on three replicates, each of which consisted of 50 culture tubes.

*Means having different letters in their superscript are significantly different from each other (P=0.05); comparison by DMRT within columns only.

Table 5: Effect of IBA and NAA pulse treatment on rooting of excised shoots of *D. gangeticum* cultured on half-strength MS medium

Pulse treatment		Frequency of root induction* (%)	Average number of roots per shoot*	Average root length* (cm)
Concentration (µM)	Treatment duration (min)			
0.0**	0	0.0	0.0	0.0
IBA				
246.06	20	64^{b*}	4.2^{b}	2.6^{b}
	30	68^{a}	5.0^{a}	3.2^{a}
492.12	20	92^{b}	6.2 ^{b}	4.4^{b}
	30	98^{a}	10^{a}	5.8^{a}
984.25	20	82^{b}	5.8^{b}	2.9^{b}
	30	88^{a}	7.0^{a}	3.3^{a}
2460.6	20	72^{a}	5.2 ^{a}	2.8^{a}
	30	66^{b}	4.8 ^{b}	2.3^{b}
NAA				
267.9	20	48^{b}	1.5^{d}	1.8^{b}
	30	58^{a}	3.0^{c}	2.1^{a}
535.9	20	70^{b}	3.5 ^{d}	3.1^{b}
	30	86^{a}	5.5 ^{c}	3.8^{a}
1071.8	20	50^{b}	2.1^{d}	1.7^{b}
	30	70^{a}	4.0^{a}	2.4^{a}
2678.6	20	46^{ab}	1.0^{b}	1.1^{b}
	30	52^{a}	4.0^{a}	1.9^{a}

Each mean is based on three replicates, each consisting of 50 culture tubes.

*Means having different letters in their superscript are significantly different from each other (P=0.05); comparison by DMRT within column within auxin only.
**Half-strength GR-free MS without pulse treatment.

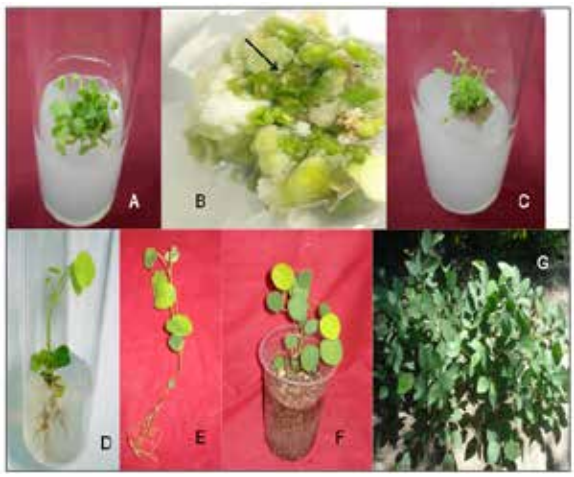

Fig 1.

Fig. 1 (A) Multiple shoot proliferation from nodal explants
(B) Leaf callus with shoot buds
(C) Multiple shoot regeneration from leaf callus
(D and E) Root induction on microshoots
(F) Hardened plantlets ready for transfer to the field
(G) Plants transferring to and growing in the field

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