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Full Length Research Paper

In vitro plant regeneration from epicotyl explant of *Withania somnifera* (L.) Dunal

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Studies were carried out to investigate the regeneration and rapid multiplication of *Withania somnifera* (L.) *in vitro*. Direct and indirect regeneration protocols for multiple shoots development from epicotyl explants of 50 to 60 days old seedlings were established. The shoots were initiated directly from epicotyl explant on 6-benzyl amino purine (BAP: 2.0 mg/L) along with indole-3-acetic acid (IAA: 0.2 mg/L), and the maximum of 15.5 ± 0.90 shoots/explant were achieved by subsequent subcultures at 4 weeks interval in the same medium. Calli (98.3%) were produced from epicotyl explant on 2,4-dichlorophenoxy acetic acid (2,4-D: 2.0 mg/L) along with kinetin (Kn: 0.6 mg/L), and shoots were initiated from calli on BAP (1.0 mg/L) along with adenine sulphate (AdS: 20.0 mg/L). Proliferation of shoots was achieved by subsequent subcultures at 4 weeks interval in the same medium. The maximum value of 25.3 ± 1.81 shoots/explant was achieved in the second subculture of indirect regeneration. Murashige and Skoog (MS) medium along with gibberellic acid (GA_3) at 1.0 mg/L produced maximum 73.3 and 95.5% of shoot elongation in direct and indirect regenerated shoots, respectively. On the other hand, MS medium with indole-3-butyric acid (IBA) at 0.8 mg/L induced maximum 86.7% and 90.0% of rooting from elongated shoots of direct and indirect regeneration, respectively. The rooted plants were transferred to small cups filled with sterilized mixture containing soil, sand and vermiculite (1:2:1, v/v/v) for hardening. About 90% of plants survived in the hardening process, and then the plants were established successfully in the experimental field. This protocol yielded a higher number of shoots within a short period. Consequently, the protocol developed in this study offers a simple and improved *in vitro* method to regenerate *W. somnifera*.

Key words: *Withania somnifera*, epicotyls, tissue culture, shoot elongation, root induction.

INTRODUCTION

Herbal medicines are still the mainstay of about 75-80% of the world population for primary health care because of the better acceptability with the human body and less side effects (Kamboj, 2000). *Withania somnifera* (L.) Dunal is a perennial plant belonging to the natural order of Solanaceae. It is a potential medicinal plant that has been used medicinally in the treatment of tuberculosis, rheumatism, inflammatory conditions, cardiac diseases, and it is used as a general tonic, anti-stress drug and as an antitumor, antibiotic, anticonvulsant and central nervous system (CNS)-depressant agent besides being an ingredient of several ayurvedic preparations (Uma Devi et al., 1993; Kandil et al., 1994; Tripathi et al., 1996; Mishra et al., 2000). It is also useful as abortificient, amoebicide, anodyne, bactericide, contraceptive and spasmolytic (Asthana and Raina, 1989). In previous studies, we observed the hypoglycaemic and hypo-lipidaemic effects of *W. somnifera* root and leaf extracts on alloxan-induced diabetic rats (Udayakumar et al., 2009) and suggested that the presence of phenolic

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compounds, including flavonoids in *W. somnifera* root and leaf extracts (Udayakumar et al., 2010a) and their antioxidant activity may play a vital role in reduction of blood glucose level in alloxan-induced diabetic rats (Udayakumar et al., 2010b). The pharmacological effect of *W. somnifera* may be attributed to withanolides, a group of steroidal lactones (Budhiraja and Sudhir, 1987). Among medicinal plants, the pharmacological and biochemical studies in *W. somnifera* are the most extensive (Deocaris et al., 2008).

With the increasing demand for the roots of *W. somnifera* in larger quantities, it is cultivated almost as a commercial crop in India. The annual requirement and production of the root of *W. somnifera* are about 7000 and 2000 tons, respectively. However, the demand for this medicinal herb has been increasing year after year. The production of *W. somnifera* roots through conventional methods of cultivation (seed) is less than the requirements due to several reasons, such as poor yield, poor viability of seeds, susceptibility of the seeds and seedlings to fungal infections like seedling mortality and blight, leaf blight, seed rotting etc (Misra et al., 1997). In addition, its propagation by conventional methods takes a long time. This medicinally important plant species has been depleted from its natural habitat and is now included in the list of threatened species by the International Union for Conservation of Nature and Natural Resources (Kavidra et al., 2000; Supe et al., 2006). The rapid multiplication of *W. somnifera* by tissue culture techniques can help to solve these problems and the benefits are extensive in the agricultural world. The plant tissue culture is desirable in order to satisfy production demands, which has been developed for the mass propagation of medicinal plants (Thomas and Philip, 2005; Thomas and Shankar, 2009), the conservation of particular and endangered species (Nagesh, 2008; Offord and Tyler, 2009), the bioenergy sources (Augustus et al., 2003: Kryzeviciene, 2006), and the bioactive compounds sources (Sudha and Seeni, 2001; Wadegaonkar et al., 2006). Now, it is an important technique for the production of economically valuable biochemicals, including enzymes, flavonoids, pigments, vitamins and cell biomass.

Regarding regeneration of *W. somnifera*, there are several reports describing the induction of shoot from germinating seeds and shoot tips (Sen and Sharma, 1991; Furmanowanet et al., 2001), axillary meristem explants (Roja et al., 1991), leaf explants (Abhyankar and Chinchanikar, 1996), and leaf callus (Pawar et al., 2001). Although efficient *in vitro* propagation protocols have been also developed using different explants like leaf, node, internode and hypocotyl of *W. somnifera* (Kulkarni et al., 1996, 2000), there is no *in vitro* regeneration report on epicotyl explant. Therefore, the present study was aimed at developing an efficient and rapid direct and indirect regeneration protocols from epicotyl explants of *W. somnifera*.

MATERIALS AND METHODS

Collection of seeds and sterilization of seeds

Seeds of *W. somnifera* were procured from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India, and maintained at room temperature since they have a long viability period of three years. The seeds were washed thoroughly in running water to remove the dust and other particulate matter adhering on the surface of the seeds. Then, they were washed with distilled water and soaked in 2.5% (v/v) commercial bleach “Teepol” (5.25% sodium hypochlorite; Reckitl and Benckiser, Ltd., Kolkatta, India) for 5 min. Teepol was poured off and the seeds were washed 3-4 times in distilled water. Subsequently, the washed seeds were transferred to an inoculation chamber and surface sterilized with 0.1% mercuric chloride (w/v) solution for 15 min. Then the solution was decanted and the seeds were washed thoroughly in sterilized distilled water to ensure that the last traces of mercuric chloride were removed. The sterilized seeds were used for inoculation.

Germination of seeds, seedling development and explant preparation

After sterilization, the seeds were inoculated in culture tubes (2.5 × 15 cm) containing sterile cotton moistened with sterile water. Following inoculation, the tubes were placed in darkness for 8 to 10 days to render uniform germination and then they were placed in light. The intensity of light was 2000 lux, with a photoperiod of 16 h light/ 8 h dark. The temperature was maintained for germination at 25 ± 2°C, and 60% humidity for the *in vitro* growth and development. After germination, the seedlings (20 to 30 days old) were transferred into 150 ml conical flask containing half strength MS medium (Murashige and Skoog, 1962) and maintained for another 30 days. The pH of the half strength MS medium was adjusted to 5.7 before autoclaving, and then autoclaved at 1.06 kg/cm^2 and at 121°C for 20 min. Epicotyl (0.5 cm) explants were excised from 50 to 60 days old *in vitro* raised seedlings with sterile scalpel and used as explants for both direct and indirect regeneration studies.

Tissue culture medium and plant growth regulators

To identify the most suitable medium for direct and indirect regeneration, MS medium containing 0.8% agar (w/v) and 3% sucrose (w/v) with different hormones (Moore, 1989) in various concentrations were tested. Auxins (2,4-D, IAA, NAA and IBA), cytokinins (BAP and Kn), GA_3 and AdS were used individually or in combination at different concentrations.

Media preparation

The stock solutions, including macro and micronutrients, iron chelates and vitamins were prepared separately and stored in amber colored reagent bottles. The growth regulators were dissolved separately in respective solvents and made up with double distilled water. All stock solutions were stored in the refrigerator. The stock solutions were pipetted out and final volume of the medium was made up with double distilled water with or without the addition of carbohydrate sources and growth regulators/organic additives depending on the nature of the experiments. About 15 and 20 ml of the medium were dispensed into the culture tubes and conical flasks respectively. The culture tubes and conical flasks were plugged tightly with non-absorbent cotton plug. The medium was gelled with 0.8% agar (w/v) in the case of solid medium. Prior to autoclaving, the pH of the medium

was adjusted to 5.6 to 5.8 using 1N NaOH or 1N HCl solutions, and then autoclaved at 1.06 kg/cm^2 (121°C) for 20 min. All the plant growth regulators used during the course of the present work were added before autoclaving the medium. Media without growth regulators served as a control.

Direct regeneration-media and growth regulators for shoot induction and subculture

The epicotyl explants from *in vitro* seedlings were individually inoculated in MS medium supplemented with various concentrations of BAP alone and in combination with IAA, 3% sucrose (w/v) and 0.8% agar (w/v) (shoot induction medium). The effects of different concentrations of BAP alone and in combination with IAA were studied to find the optimum concentration for multiple shoot induction. Epicotyl explants with emerging shoots were subcultured in MS medium supplemented with BAP (0.5 to 2.5 mg/L) alone and BAP (2.0 mg/L) in combination with IAA (0.1 to 0.5 mg/L). The explants were transferred to fresh medium with the same composition at 4-week intervals during the subculture.

Indirect regeneration-media and growth regulators for callus and shoot induction and subculture

The epicotyl explants from *in vitro* seedlings were individually inoculated in MS medium supplemented with various concentrations of 2,4-D either alone or in combination with Kn, 3% sucrose (w/v) and 0.8% agar (w/v) (callus induction medium). The effect of different concentrations of 2,4-D alone and in combination with Kn were studied to find the optimum concentrations for callus induction. Then the callus was transferred into shoot induction medium containing different concentrations of BAP alone or in combination with AdS to find the optimum concentration for multiple shoot development. Epicotyl explants with emerging shoots were subcultured in MS medium supplemented with BAP (0.5 - 2.0 mg/L) alone and BAP (1.0 mg/L) and in combination with AdS (10.0 - 40.0 mg/L). The explants were transferred to fresh medium with the same composition at 4-week intervals during the subculture.

Shoot elongation and root induction

In vitro regenerated shoots with a height of 1.0 cm and above were excised from the explants of both direct and indirect regeneration experiments and transferred to MS medium containing 3% sucrose and 0.8% agar fortified with different concentrations of GA_3 (0.5 to 2.0 mg/L) for shoot elongation. MS medium without growth regulator served as a control for all experiments. The elongated shoots (2 to 8 cm length) were transferred to MS medium containing 3% sucrose and 0.8% agar supplemented with NAA, IAA and IBA individually at various concentrations for root induction. MS medium without growth regulator served as a control for all experiments. Cultures were maintained under white fluorescent light at a photon flux of 30 µmol/m^2/s^1 for a 16 h light/8 h dark photoperiod at 25 ± 2°C.

Hardening (acclimatization) of regenerated plants

The rooted plants were removed from culture vessels and washed in running tap water to remove agar. The number of roots that developed was counted and the plants were transferred to plastic pots containing sterile soil: sand: vermiculite (1:2:1, v/v/v). The pots were covered with transparent plastic bags to retain 80% relative humidity and were maintained under a 16 h light/8h dark photoperiod at 25 ± 2°C. After 3 to 4 weeks, well grown plants were transferred to the field.

Statistical analysis

Each treatment consisted of at least 10 explants and each experiment was repeated six times. A complete randomized design was used in all experiments and a one-way analysis of variance (ANOVA) and comparisons between the mean values of treatments were carried out using Duncan's multiple range test (DMRT) with significance determined at 5% level.

RESULTS

In the direct regeneration experiments, the epicotyl explants were inoculated on MS medium supplemented with only BAP (0.5 to 2.5 mg/L) or BAP (2.0 mg/L) in combination with IAA (0.1 to 0.5 mg/L), and shoot bud formation was observed (Table 1). In the first set of experiments, BAP concentration was varied from 0.5 to 2.5 mg/L for evaluating the percentage response of explants in shoot bud induction and elongation. The frequencies of explants forming shoot buds increased with increase in BAP concentration. The maximum 3.2 ± 0.30 shoots per epicotyl explant was optimum at 2.0 mg/L BAP in the initial culture (Table 1). Four weeks after every subculture, 3.2 ± 0.30 shoots could be recovered up to the second subculture, resulting in 5.9 ± 0.32 shoots from a single epicotyl explant in a 12 week period. However, a combination of BAP (2.0 mg/L) and IAA (0.1 - 0.5 mg/L) showed increase in number of shoot bud formation when compared with medium containing BAP (0.5 to 2.5 mg/L) alone. The optimum number of 6.6 ± 0.52 shoots per epicotyl explant was induced on 2.0 mg/L BAP with 0.2 mg/L IAA in the initial 4 week culture period (Table 1, Figure 1a and b). During a 12 week period, the number of shoots of 6.6 ± 0.52 could be recovered up to the second subculture, resulting in 15.5 ± 0.90 shoots from a single epicotyl explant (Figure 1c and d).

In the indirect regeneration experiments, the epicotyl explants showed callus initiation within 20-25 days after inoculation. Tables 2 and 3 shows callus induction and shoot regeneration, respectively from epicotyl explants. Different concentrations of auxin-2,4-D (0.5 to 3.0 mg/L) alone and 2,4-D (2.0 mg/L) in combination with various concentrations of cytokinin- kinetin (0.2 to 1.0 mg/L) were tested for callus induction. Although the treatment of 2,4-D alone at the concentration of 2.0 mg/L induced more callusing (63.3%) (brown friable) than other concentrations, the most callusing (98.3%) was observed on MS medium supplemented with 2.0 mg/L 2,4-D and in combination with 0.6 mg/L kinetin (yellow greenish compact; Figure 2a). No callusing response was observed on MS medium without plant growth regulators. The calli were transferred to the MS medium containing BAP (0.5 to 2.0 mg/L) alone or in combination with AdS (10.0 to 40.0 mg/L) to induce shoot differentiation. BAP (1.0 mg/L) produced 5.2 ± 0.41 shoots per callus, whereas BAP

Table 1. Effect of different concentrations of BAP alone and in combination with IAA on multiple shoot formation from 50-60 days old epicotyl explants of *W. somnifera* on MS medium.

Plant growth regulators (mg/L)		Number of cultures developing shoots*	Explants developing shoots (%)	Initial culture		First subculture		Second subculture	
BAP	IAA			Number of shoots/ explant	Height of shoots (cm)	Number of shoots/ culture	Height of shoots (cm)	Number of shoots/ culture	Height of shoots (cm)
0	-	7	11.6	1.1 ± 0.20^{a}	0.9 ± 0.08^{a}	1.3 ± 0.52^{a}	1.1 ± 0.12^{a}	1.6 ± 0.49^{a}	1.0 ± 0.09^{a}
0.5	-	11	18.3	1.9 ± 0.15^{b}	1.2 ± 0.13^{b}	2.2 ± 0.21^{b}	1.3 ± 0.12^{ab}	2.7 ± 0.31^{b}	1.4 ± 0.13^{b}
1.0	-	17	28.3	2.2 ± 0.16^{b}	1.2 ± 0.09^{b}	2.5 ± 0.23^{b}	1.4 ±0.14^{bc}	3.6 ± 0.20^{c}	1.4 ± 0.18^{b}
1.5	-	21	35.0	2.8 ± 0.25^{c}	1.5 ± 0.18^{c}	3.5 ± 0.35^{c}	1.6 ± 0.18^{cd}	4.3 ± 0.52^{d}	1.6 ± 0.16^{bc}
2.0	-	28	46.7	3.2 ± 0.30^{d}	1.6 ± 0.18^{c}	4.2 ± 0.42^{d}	1.8 ± 0.18^{d}	5.9 ± 0.32^{ef}	2.0 ± 0.18^{d}
2.5	-	14	23.3	2.1 ± 0.15^{b}	1.3 ± 0.09^{b}	2.2 ± 0.26^{b}	1.6 ± 0.16^{cd}	3.0 ± 0.29^{bc}	1.8 ± 0.12^{cd}
2.0	0.1	25	41.7	2.0 ± 0.20^{b}	1.5 ± 0.13^{c}	4.2 ± 41^{d}	1.6 ± 0.18^{cd}	5.6 ± 0.65^{e}	1.8 ± 0.18^{cd}
2.0	0.2	51	85.0	6.6 ± 0.52^{g}	2.0 ± 0.18^{e}	10.2 ± 0.60^{h}	2.2 ± 0.27^{f}	15.5 ± 0.90^{i}	2.4 ± 0.27^{e}
2.0	0.3	46	76.7	4.8 ± 0.26^{f}	1.8 ± 0.17^{d}	8.8 ± 0.61^{g}	2.0 ± 0.18^{ef}	11.6 ± 0.71^{h}	2.2 ± 0.23^{e}
2.0	0.4	41	68.3	3.6 ± 0.32^{e}	1.6 ± 0.13^{c}	6.6 ± 0.71^{f}	1.8 ± 0.18^{de}	9.3 ± 0.69^{g}	1.9 ± 0.18^{d}
2.0	0.5	33	55.0	2.6 ± 0.27^{c}	1.6 ± 0.18^{c}	5.1 ± 0.45^{e}	1.7 ± 0.12^{d}	6.5 ± 0.45^{f}	1.8 ± 0.12^{cd}

Data shown are mean ± SD of six experiments each experiment consisted of 10 replicates. Values with the same letter within columns are not significantly different using Duncan's multiple range test (DMRT) at 5% level ($p \leq 0.05$). *Out of 60 explants inoculated.

(1.0 mg/L) and in combination with AdS (20.0 mg/L) produced 8.2 ± 0.45 shoots per callus in the initial 4-week culture period (Figure 2b). With repeated subcultures on the same medium at 4-week intervals, each epicotyl explant produced 14.6 ± 0.50 shoots per callus on medium containing BAP (1.0 mg/L) alone and 25.3 ± 1.81 shoots per callus on medium containing BAP (1.0 mg/L) and AdS (20.0 mg/L) in the second subculture (Figure 2c and d).

Furthermore, when the regenerated shoots reached a height of 1.0 cm or above, they were separated from the explants of both direct and indirect regeneration experiments and then transferred to MS medium with different concentrations of GA_3 (0.5 to 2.0 mg/L) for elongation. The MS medium containing GA_3 at 1.0 mg/L showed maximum shoot elongation response of 73.3 and 95.0% with a mean shoot length of 4.6 ± 0.36 and 6.4 ± 0.72 cm in direct and indirect regeneration experiments, respectively (Table 4, Figures 1e and 2e). At higher GA_3 concentrations (>1.0 mg/L), basal callusing and subsequent inhibition of shoot elongation were observed. In this study, the elongated shoots were transferred to root induction medium containing different concentrations of NAA (0.2 to 1.0 mg/L), IAA (0.2 to 1.0 mg/L) and IBA (0.2 to 1.0 mg/L). The percentage of shoots that produced roots increased with increasing IBA concentrations (Table 5). Among the various concentrations, 86.7 and 90.0% of shoots produced roots (6.0 ± 0.63 and 6.6±0.54 roots/shoot) with a mean root length of 3.4 ± 0.36 and 3.8 ± 0.36 cm at 0.8 mg/L IBA in direct and indirect regenerated shoots, respectively (Figures 1f and 2f). Moreover, IAA at 0.8 mg/L resulted in the next higher percentage (direct - 65% and indirect - 80%) of rooting, followed by NAA at 0.8 mg/L (direct - 51.7% and indirect - 63.3%).

Finally, the well rooted plants (4.0 - 6.0 cm height) obtained from rooting medium were transferred to cups for hardening (Figures 1g and 2g). Afterward, individual cups with single plant were partially covered with polythene bag to maintain high humidity. When the plants had shown signs of new leaf growth, the polythene covers were removed. Overall, 90% of plants survived in the hardening process (data not shown) and these plants were established successfully in the experimental field.

DISCUSSION

Plants have been used from time immemorial as food and as curative agents against several diseases. In view of this, herbal business of plants has increased several folds in recent years. To meet this increasing demand, ways and means have to be evolved to improve the biomass

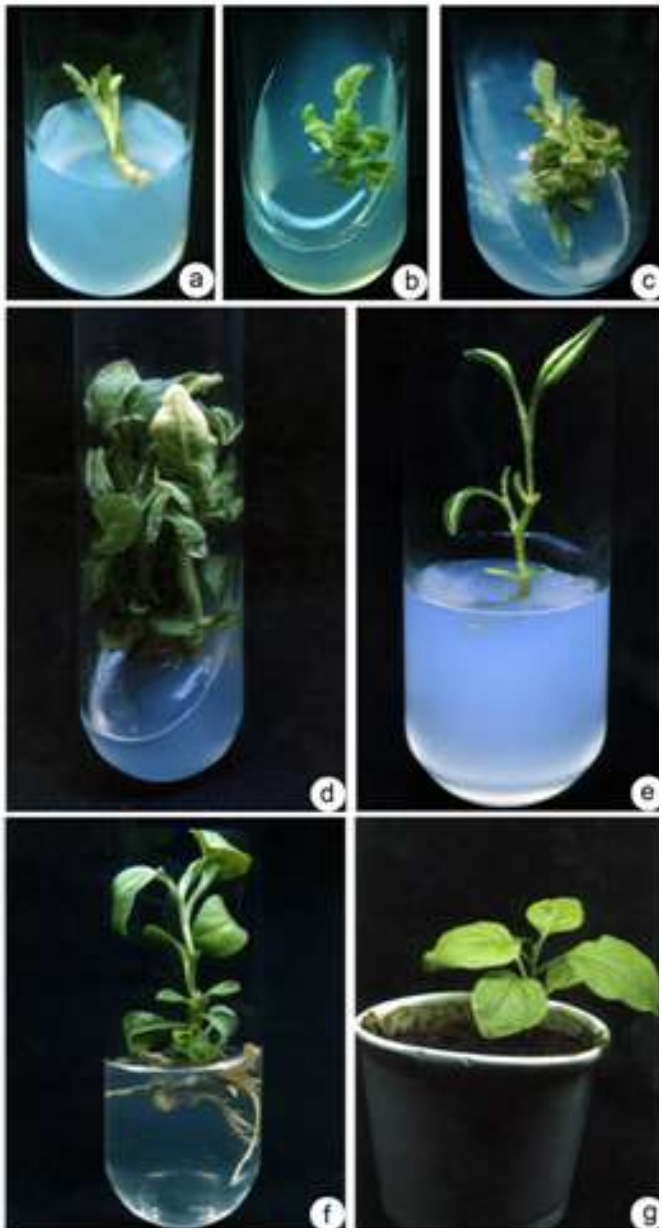

Figure 1. Direct regeneration of shoots from 50 to 60 days old epicotyl explants of *Withania somnifera* on MS medium. (a) Shoot bud initiation on BAP (2.0 mg/L) + IAA (0.2 mg/L); (b-d) multiple shoot production on BAP (2.0 mg/L) + IAA (0.2 mg/L) in 4, 8 and 12 weeks of culture; (e) shoot elongation on GA_3 (1.0 mg/L); (f) rooting of shoot on IBA (0.8 mg/L); (g) acclimatized plant.

productivity. Biotechnology is a viable alternative for the mass production of plants and plant parts. *W. somnifera* is of economic interest due to its wide range of pharmacological activity. It is observed that when the epicotyl explants from earlier or after 50 to 60 days old seedlings were used as explants, callusing was observed in all the treatments and there was no shoot bud formation in any of the treatments. Therefore, epicotyl explants of *W. somnifera* to produce adventitious shoots were selected from 50 to 60 days old *in vitro* seedlings in this study.

Direct regeneration experiments showed that the epicotyl explants formed shoot buds *in vitro*, when inoculated on MS medium supplemented with various concentrations of BAP alone or in combination with IAA. The threshold concentrations of these growth regulators required for optimal organogenesis response differed with explant types. Thus, the number of shoot produced per explants could greatly be increased by manipulating the growth regulators in the MS medium (Thorpe, 1993). In this study, shoot bud formation was observed in all the concentrations of BAP and IAA tested, though the number of shoots produced per explants differed in each treatment. The maximum number of shoots formed per explants was at 2.0 mg/L BAP with 0.2 mg/L IAA. However, the frequency of explants forming shoots decreased at 2.0 mg/L BAP with 0.3 mg/L IAA. This may be due to high endogenous auxin level, which led to a decrease in the percentage response of explants in forming shoots with an increase in the auxin concentration beyond a threshold value. Researchers have reported that the auxin and cytokinin combinations are to be fruitful for the formation of shoots from the explants of certain plant species (Dhar and Joshi, 2005: Shrivastava and Banerjee, 2008). However, very young explants fail to support adventitious shoot formation because of higher endogenous auxin level (Moore, 1989). The importance of cytokinin in shoot bud multiplication has been reviewed extensively (Ibanez et al., 2003; George et al., 2008). Development of shoot buds from epicotyl explants in the presence of a cytokinin only without the need of an exogenous auxin is not often an encountered phenomenon since the epicotyls do not have preformed meristems. This result may be attributed to the high intrinsic auxin levels in the epicotyls of this plant. By supplementing with high concentrations of BAP, it is possible to balance both the growth regulators in such a way that the explants become competent for organogenesis (Christianson and Warnick, 1985).

Indirect regeneration experiment reveals that the epicotyl explants produced the most calli in MS medium supplemented with 2.0 mg/L of 2,4-D in combination with 0.6 mg/L of kinetin. Callus proliferation declined at lower (< 2.0 mg/L) concentrations of 2,4-D and only small amounts of hard calli were obtained at higher (> 2.0 mg/L) concentrations of 2,4-D and it may be due to cessation of growth. Similar findings had been observed by Sita et al. (1986) in rose wood. The importance of auxin and cytokinin for optimal development of callus has also been observed by George et al. (2008). Conversely, no callus formation was observed on MS medium without treatment of plant growth regulators. These results are in agreement with the earlier findings in other plant species of Solanaceae (Emke and Eilert,

Table 2. Callus induction from 50 to 60 days old epicotyl explants of *W. somnifera* grown on MS medium supplemented with various concentrations of 2.4 -D alone and in combination with Kn.

Plant growth regulators (mg/L)		Number of cultures forming callus*	Percent callusing (%)	Nature of callus
2,4-D	Kn			
0.5	-	23	38.3	Brown friable
1.0	-	27	45.0	Brown friable
1.5	-	34	56.7	Brown friable
2.0	-	38	63.3	Brown friable
2.5	-	36	60.0	Brown friable
3.0	-	32	53.3	Brown friable
2.0	0.2	47	78.3	Yellow greenish compact
2.0	0.4	55	91.1	Yellow greenish compact
2.0	0.6	59	98.3	Yellow greenish compact
2.0	0.8	42	70.0	Yellow greenish compact
2.0	1.0	35	58.3	Yellow greenish compact

*, Out of 60 explants inoculated.

Table 3. Effect of different concentrations of BAP alone and in combination with AdS on shoot regeneration from 50 to 60 days old epicotyl explants derived callus of *W. somnifera* on MS medium.

Plant growth regulators (mg/L)		Number of cultures forming multiple shoots*	Explants forming shoots (%)	Initial culture		First subculture		Second subculture	
BAP	AdS			Number of shoots/ explant	Height of shoots (cm)	Number of shoots/ explant	Height of shoots (cm)	Number of shoots/ explant	Height of shoots (cm)
0.5	-	27	45.0	4.1 ± 0.24^{a}	1.1 ± 0.05^{a}	6.2 ± 0.37^{a}	1.0 ± 0.05^{a}	9.4 ± 0.80^{a}	1.2 ± 0.09^{a}
1.0	-	38	63.3	5.2 ± 0.41^{b}	1.5 ± 0.14^{c}	9.7 ± 0.80^{c}	1.6 ± 0.09^{cd}	14.6 ± 0.50^{c}	2.0 ± 0.27^{cd}
1.5	-	34	56.7	4.9 ± 0.33^{b}	1.3 ± 0.09^{b}	8.2 ± 0.58^{b}	1.5 ± 0.14^{c}	12.8 ± 0.75^{b}	1.7 ± 0.14^{c}
2.0	-	29	48.3	4.2 ± 0.36^{a}	1.1 ± 0.09^{a}	7.4 ± 0.74^{b}	1.2 ± 0.09^{b}	10.8 ± 0.72^{a}	1.5 ± 014^{b}
1.0	10.0	30	50.0	5.8 ± 0.36^{c}	1.4 ± 0.09^{bc}	10.8 ± 0.89^{d}	1.5 ± 0.09^{c}	16.4 ± 1.25^{d}	1.8 ± 0.09^{c}
1.0	20.0	52	86.7	8.2 ± 0.45^{e}	1.8 ± 0.18^{c}	16.2 ± 0.92^{g}	2.0 ± 0.14^{e}	25.3 ± 1.81^{g}	2.2 ± 0.18^{e}
1.0	30.0	43	71.7	7.3 ± 0.58^{d}	1.8 ± 0.14^{d}	14.8 ± 1.34^{f}	1.9 ± 0.18^{e}	21.8 ± 1.43^{f}	2.1 ± 0.18^{de}
1.0	40.0	32	53.3	6.0 ± 0.49^{c}	1.5 ± 0.14^{d}	13.2 ± 0.89^{e}	1.7 ± 0.09^{d}	19.6 ± 1.80^{e}	1.9 ± 0.09^{c}

Data shown are mean ± SD of six experiments each experiment consisted of 10 replicates. Values with the same letter within columns are not significantly different using Duncan's multiple range test (DMRT) at 5% level ($p \leq 0.05$). *Out of 60 explants inoculated.

1986; Filippone and Lurquin, 1989; Shahzad et al., 1999). The callus growth induction, and subsequent differentiation through organogenesis are accomplished by the differential application of growth regulators and the control of culture conditions. With the stimulus of endogenous growth substances or by the addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation

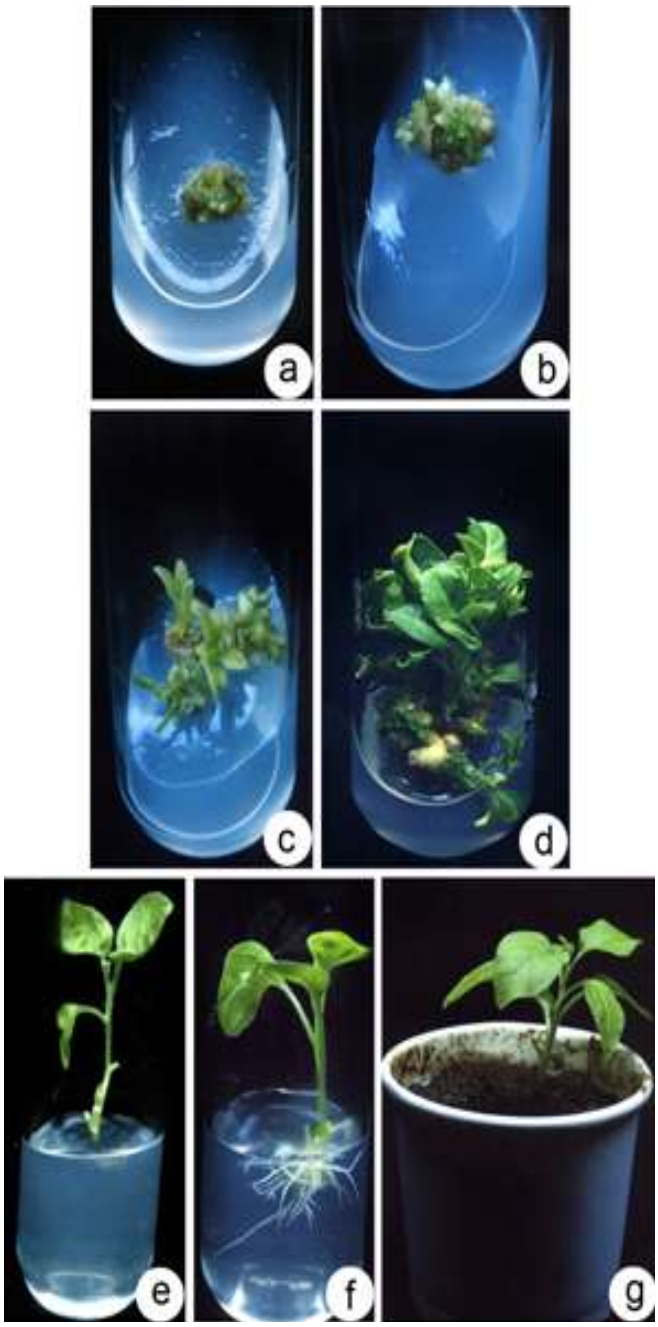

Figure 2. Regeneration of shoots from 50 to 60 day-old epicotyl derived callus of *Withania somnifera* on MS Medium. (a) Greenish compact nodular callus on 2,4-D (2.0 mg/L) + kinetin (0.6 mg/L); (b-d) initiation of adventitious shoots and proliferation from epicotyl derived callus on BAP (1.0 mg/L) + AdS (20.0 mg/L); (e) shoot elongation on GA_3 (1.0 mg/L); (f) rooting of shoot on IBA (0.8 mg/L); (g) acclimatized plant.

are induced. The regeneration of shoots from callus was enhanced usingappropriate concentrations of auxins and cytokinins. *In vitro* propagation was reported through embryo and nodal explants of miracle berry using different levels of combinations of auxins and cytokinins on MS medium (Ogunsola and Ilori, 2008). The formation of adventitious shoots either directly from explanted tissues or indirectly from callus is regulated by the interaction between auxins and cytokinins (George et al., 2008).

The maximum number of shoots produced per callus was observed on MS medium containing 1.0 mg/L of BAP and 20.0 mg/L of AdS. Cytokinins are very effective in promoting shoot initiation and multiplication from various explants of *W. somnifera* (Kulkarni et al., 2000).

The AdS showing a synergistic effect with cytokinins, which stimulates cell growth and gently enhances shoot formation (Raha and Roy, 2001). This suggested the influence of additives like AdS in shoot formation along with cytokinins, which was also reported in *Jatropha curcas* (Shrivastava and Banerjee, 2008). The maximum shoot elongation response in direct and in indirect regeneration experiments was observed on MS medium containing GA_3 at 1.0 mg/L. Similar response was reported in the shoot buds of *Cicer arietinum* that elongated on media containing GA_3 (Srivastava et al., 2001). Shoots treated with GA_3 to increase the length of shoots during multiplication or prior to rooting have been reviewed (George et al., 2008). Shoot elongation was observed with adventitious shoot buds obtained from young leaf explants of chile pepper on the MS medium fortified with BAP and GA_3 (Golegaonkar and Kantharajah, 2006).

In the present study, auxins were successfully used to produce roots from regenerated shoots. The number of roots produced per shoot was low in IAA and NAA containing MS medium. The roots were very thin and delicate in both IAA and NAA amended MS medium. However, more percentage of shoots produced roots was achieved in MS medium containing IBA. Similar results were observed in which maximum shoots rooted when cultured on MS medium containing optimal concentration of IBA (Manickam et al., 2000; Rani et al., 2003). Additionally, direct rooting from leaf explants of *W. somnifera* was achieved on half strength MS medium supplemented with sucrose and different concentrations of growth regulators like IAA and IBA achieved maximum number of roots (Wadegaonkar et al., 2006; Supe et al., 2006). This suggested that the addition of IBA to rooting medium improved rooting efficiency of shoots.

In conclusion, the protocol developed in this study offers a simple and improved *in vitro* method to regenerate *W. somnifera* from epicotyls explants and also yielded a higher number of shoots within a period of 80 - 100 days. This technique may be useful in the genetic transformation of *W. somnifera* as to it allows the production of multiple shoots in a shorter period.

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Table 4. Effect of GA_3 on shoot elongation from regenerated shoots of *W. somnifera.*

Plant growth regulator (mg/L) GA_3	Number of cultures responding elongation*		Shoot elongation response (%)		Mean shoot length (cm)		Mean number of nodes	
	Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
0	8	12	13.3	20.0	1.5 ± 0.09^{a}	2.4 ± 0.23^{a}	2.0 ± 0.23^{a}	3.2 ± 0.36^{a}
0.5	30	34	50.0	56.7	2.6 ± 0.27^{c}	3.6 ± 0.27^{b}	4.2 ± 0.45^{c}	5.4 ± 0.54^{b}
1.0	44	57	73.3	95.5	4.6 ± 0.36^{e}	6.4 ± 0.72^{d}	6.5 ± 0.54^{e}	9.8 ± 0.63^{d}
1.5	36	44	60.0	73.3	3.1 ± 0.27^{d}	4.8 ± 0.45^{c}	5.0 ± 0.27^{d}	7.2 ± 0.45^{c}
2.0	23	28	38.3	46.7	2.0 ± 0.18^{b}	3.4 ± 0.36^{b}	3.2 ± 0.22^{b}	5.0 ± 0.45^{b}

Data shown are mean ± SD of six experiments; each experiment consisted of 10 replicates. Values with the same letter within columns are not significantly different using Duncan's multiple range test (DMRT) at 5% level ($p \leq 0.05$). *Out of 60 explants inoculated.

Table 5. Effect of NAA, IAA and IBA on root induction from regenerated shoots of *W. somnifera.*

Plant growth regulators (mg/L)		Number of cultures producing roots		Cultures producing roots (%)		Mean number of roots / shoot		Mean root length (cm)	
		Direct	Indirect	Direct	Indirect	Direct	Indirect	Direct	Indirect
NAA	0.2	10	17	16.7	28.3	2.1 ± 0.18^{a}	2.2 ± 0.18^{a}	0.8 ± 0.09^{a}	1.5 ± 0.09^{a}
	0.4	15	22	25.0	36.7	2.4 ± 0.22^{ab}	3.0 ± 0.27^{b}	1.1 ± 0.09^{b}	1.8 ± 0.14^{bcd}
	0.6	23	32	38.3	53.3	2.8 ± 0.27^{bc}	3.9 ± 0.45^{cd}	1.5 ± 0.14^{c}	2.2 ± 0.18^{ef}
	0.8	31	38	51.7	63.3	3.8 ± 0.36^{dc}	4.4 ± 0.40^{ef}	2.0 ± 0.23^{d}	2.8 ± 0.18^{hi}
	1.0	18	36	30.0	43.3	2.4 ± 0.18^{ab}	3.5 ± 0.31^{c}	1.2 ± 0.09^{b}	2.1 ± 0.14^{de}
IAA	0.2	22	25	36.7	41.7	2.5 ± 0.23^{ab}	3.5 ± 0.31^{c}	0.8 ± 0.09^{a}	1.6 ± 0.14^{ab}
	0.4	26	32	43.3	53.3	3.2 ± 0.27^{c}	4.0 ± 0.36^{de}	1.2 ± 0.09^{b}	1.8 ± 0.18^{bc}
	0.6	34	38	56.7	63.3	4.1 ± 0.36^{e}	4.6 ± 0.45^{f}	2.1 ± 0.23^{d}	2.5 ± 0.27^{g}
	0.8	39	48	65.0	80.0	4.8 ± 0.45^{f}	5.1 ± 0.36^{g}	2.4 ± 0.27^{e}	3.2 ± 0.36^{j}
	1.0	29	35	48.3	58.3	3.6 ± 0.36^{d}	4.2 ± 0.40^{def}	1.6 ± 0.14^{c}	2.0 ± 0.09^{cde}
IBA	0.2	27	34	45.0	56.7	2.8 ± 0.31^{bc}	4.4 ± 0.27^{ef}	1.5 ± 0.14^{c}	1.8 ± 0.05^{abc}
	0.4	36	39	60.0	65.0	3.1 ± 0.31^{c}	5.1 ± 0.36^{g}	2.0 ± 0.18^{d}	2.3 ± 0.14^{fg}
	0.6	44	48	73.3	80.0	4.8 ± 0.45^{f}	5.8 ± 0.45^{h}	2.8 ± 0.27^{f}	3.0 ± 0.27^{ij}
	0.8	52	54	86.7	90.0	6.0 ± 0.63^{g}	6.6 ± 0.54^{i}	3.4 ± 0.36^{g}	3.8 ± 0.36^{k}
	1.0	39	42	65.0	70.0	4.0 ± 0.36^{de}	5.2 ± 0.45^{g}	2.1 ± 0.18^{d}	2.6 ± 0.22^{gh}

Data shown are mean ± SD of six experiments each experiment consisted of 10 replicates. Values with the same letter within columns are not significantly different using Duncan's multiple range test (DMRT) at 5% level ($p \leq 0.05$). *Out of 60 explants inoculated.

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