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Indirect Regeneration of *Withania somnifera* from Nodal Explants

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Authors' contributions

This work was carried out in international collaboration between the four authors. Author RU performed experiments, statistical analysis and wrote the initial draft of the manuscript. Author AG as a co-corresponding author designed the study and lead this project. Author SCK guided the first author's experiment and analysed the data. Author CWC as a co-corresponding author funded a grant and made the final draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Withania somnifera is an important medicinal plant and used to cure many diseases. Indirect regeneration protocol for multiple shoots development was established using nodal explants of *W. somnifera* from 50-60 days old seedlings. The callus induction was observed from nodal explants, grown on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin (Kn). Maximum level of callusing response (80.0%) was recorded on MS medium supplemented with a combinations of 2.0 mg/l 2,4-D and 0.2 mg/l Kn. The callus (greenish compact) was transferred into MS medium containing various concentrations (0.5–2.0mg/l) of 6-benzyl amino purine (BAP) alone and in combination (0.1–0.4mg/l) with indole-3-acetic acid (IAA) for shoot initiation and

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proliferation. The maximum number of shoots was initiated from callus on 1.0mg/l BAP along with 0.2 mg/l IAA and proliferation of shoots achieved by subsequent subcultures at 4 weeks interval in the same medium. The maximum of 31.4 shoots/explant were achieved in the second subculture. MS medium along with 1.0 mg/l gibberellic acid (GA_3) induced maximum elongation (96.7%) of regenerated shoots and MS medium supplemented with 0.8 mg/l indole-3-butyric acid (IBA) induced maximum rooting (96.7%) from elongated shoots. After a hardening period, the plantlets were transferred to the field with 98% of survival.

Keywords: Callus; indirect regeneration; nodal explant; root induction; shoot elongation; Withania somnifera.

1. INTRODUCTION

Herbal medicines are still the mainstay of about 75-80% of the world population, for primary health care because of better acceptability with the human body and less side effects [1]. Over exploitation of these plant species leads to the extinction in natural habitat. To meet this increasing demand, ways and means have to be evolved to improve the biomass productivity. Plant tissue and cell culture technologies in this context are seen as a savior in channelizing the resources of nature for the benefit of mankind by conservation of elite, endangered plants and eco-friendly production of drugs and drug intermediates. So, the plant tissue culture system is actively involved to produce multiple copies of a plant, which is also known as micro propagation and it is used in plant sciences, forestry and horticulture. Some of the plant breeders and nurseries have their own labs for propagating plants by micro propagation, which can also be used to conserve rare or endangered plant species. To date, plant tissue culture techniques are extensively used for mass production of elite plants.

Withania somnifera is a perennial plant belonging to the natural order of Solanaceae. A number of steroidal lactones of withanolides have been isolated from the leaves of *W. somnifera* [2]. Alkaloids constitute another major group of components which have been isolated from *W. somnifera* [3]. The leaves contain a number of withanolides like withaferin-A and exhibit antibacterial, antifungal and antitumor properties [4]. The entire plant of *W. somnifera* is used in the treatment of tuberculosis, rheumatism, inflammation and cardiac diseases and as a general tonic, antistress drug and as an antitumor, antibiotic, anticonvulsant and CNS-depressant agent [5]. In our previous studies, we observed the hypoglycaemic and hypolipidaemic effects of *W. somnifera* root and leaf extracts on alloxan induced diabetic rats [6], and suggested that the phenolic compounds including flavonoids in *W. somnifera* root and leaf extracts [7] and their antioxidant activity may be responsible for the reduction of blood glucose level in alloxan induced diabetic rats [8]. *W. somnifera* is of economic interest for its wide ranging pharmacological activity and one of the major constraints in utilizing natural populations of the existence plant. 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. *W. somnifera* is also being exploited on a large scale commercial basis for its medicinal value. Due to this over exploitation, it is becoming a member in the endangered plant species. Therefore, the tissue culture technology is a viable alternative for the mass production of medicinal plants. Indirect *In vitro* regeneration is an important efficient method for regenerating a number of plants from an intervening callus

stage and the regenerated shoots showed somaclonal variation [9,10]. Somaclonal variation is quite often associated with indirect *In vitro* regeneration involving an interviewing callus stage. Bouman and De Klerk [11] showed that the rate of somaclonal variation among regenerated shoots derived from the callus. During the callus period, differentiated cells undergo dedifferentiation, induction and redifferentiation [12].

Sen and Sharma [13] reported regeneration from germinating seeds and shoot tips of *W. somnifera*. Many reports are available on *in vitro* regeneration of *W. somnifera* species using leaf, stem, hypocotyl, node, internode, embryo, axillary shoots and root explants [14,15,16,17,18,19]. Micropropagation of *W. somnifera* using various explants like shoot-tip and nodal explants [20], apical bud [21] and cotyledonary nodes [22] were reported. Recently, *in vitro* plant regeneration via direct adventitious shoot proliferation from leaf explants [23] and from mature cotyledon, embryo and hypocotyl explants [24] of *W. somnifera were* also developed. But there are only a very few reports [25,26] are available on regeneration and shoot multiplication using nodal explants of *W. somnifera*. In the present study it was aimed to develop an efficient and rapid indirect regeneration protocol from nodal explants of *W. somnifera* due to over exploitation of it.

2. MATERIALS AND METHODS

2.1 Chemicals

The chemicals used during the course of the study were of analytical grade. Inorganic salts, agar agar and sucrose were obtained from Qualigens, SRL Chemicals and Himedia Chemicals, Mumbai, India. All vitamins, plant growth regulators and phytagel were purchased from Sigma Chemicals Company, St. Louis, MO, USA.

2.2 Collection and Sterilization of Seeds

Seeds of *W. somnifera* were procured from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India. 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, (India) Ltd., Kolkatta, India) for 5 min. Teepol was poured off and the seeds were washed 3-4 times in distilled water. The washed seeds were transferred to an inoculation chamber and surface sterilized with 0.1% mercuric chloride (w/v) (Qualigens, Mumbai, India) 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.

2.3 Germination of Seeds

After sterilization, the seeds were inoculated in culture tubes (2.5x15cm, Borosil, India) containing sterile cotton moistened with sterile water. After inoculation, the tubes were placed in darkness for 8-10 days to render uniform germination and then they were placed in light. The intensity of light was 2000 lux, 16 h/day. The temperature was maintained for germination at 25±2ºC and 60% humidity for *in vitro* growth and development. After germination the seedlings (25-30 days old) were transferred into 150 ml conical flask (Borosil, India) containing half strength MS medium and maintained for another 25-30 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 121ºC for 20 min. The nodal explants were taken from 50-60 days old seedlings in cultured flasks for further *in vitro* studies (Fig. 1). When the nodal explants from earlier or after 50-60 days old seedlings were used as explants, callus formation was observed in all the treatments, but there was no or a less number of shoot bud formation was observed. Hence the nodal segments of 50-60 days old seedlings of *W. somnifera* were used as source of explants.

Fig. 1. *In vitro* raised seedlings of *Withania somnifera* used as a source of explants (a) 25-30 days old *In vitro* raised seedlings in moistened cotton; (b) 50-60 days old *In vitro* raised seedlings in half strength MS medium.

2.4 Tissue Culture Media and Preparation

The medium for indirect regeneration-MS medium [27] containing 0.8% agar (w/v) and 3% sucrose (w/v) with different hormones like auxins (2,4-D: 2,4-dichlorophenoxy acetic acid, IAA: indole-3-acetic acid, NAA: naphthalene acetic acid and IBA: indole-3-butyric acid), cytokinins (BAP: 6-benzyl amino purine and Kn: kinetin) and gibberellic acid (GA_3) were used individually or in combination with different concentrations. The nodal explants from *in vitro* seedlings were individually inoculated in MS medium supplemented with various concentrations of auxin (2,4-D) alone and in combination with cytokinin (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 used to find the optimum concentration for callus induction. Then the callus were transferred into shoot induction medium containing

different concentrations of BAP alone and in combination with IAA to find the optimum concentration for multiple shoot development.

The stock solutions of macro and micronutrients, iron chelates and vitamins were prepared separately and stored in amber coloured bottles at 4ºC. The growth regulators were dissolved separately in respective solvents and made up with double distilled water. The stock solutions were pipetted out and final volume of the medium was made up with double distilled water with or without addition of carbohydrate sources and growth regulators/organic additives based on the nature of the experiment. 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 5.6-5.8 using 1N NaOH or 1N HCl solutions and then autoclaving at 1.06 kg cm^{-2} (121ºC) 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.

2.5 Subculture, Shoot Elongation and Root Induction

The nodal explant derived callus with emerging shoots was subcultured in MS medium supplemented with BAP (0.5–2.0 mg/l) alone and BAP (1.0 mg/l) and in combination with IAA (0.1–0.4 mg/l). The explants were transferred to fresh medium with the same composition at 4-week intervals during the subculture.

In vitro shoots with a height of 1.0 cm and above were excised from the nodal explant derived callus and transferred to MS medium containing 3% sucrose and 0.8% agar fortified with different concentrations of GA_3 (0.5–2.0mg/l) for shoot elongation. Cultures were maintained under white fluorescent light at a photon flux of 30 µmol m^{-2} s^{-1} for a 16/8 h photoperiod at 25±2ºC.

The elongated shoots (2-8cm 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 the same conditions of the above.

2.6 Hardening 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 then transferred to small cups containing sterile soil: sand: vermiculite (1:2:1, v/v/v). The small cups were covered with transparent plastic bags to retain humidity and were maintained under a 16/8 h photoperiod at 25±2ºC. After 3-4 weeks well grown plants were transferred to the field.

2.7 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.

3. RESULTS AND DISCUSSION

3.1 Nodal Explants and Indirect Regeneration

Nodal explants collected from 50-60 days old seedlings produced adventitious shoots through indirect regeneration. Tables 1 and 2 shows callus induction and shoot regeneration from nodal explants. Callus initiation from nodal explants was noticed at 20–25 days after inoculation. Different concentrations of auxin (2,4-D, 0.5–3.0mg/l) alone and in combination with cytokinin (Kn, 0.1–0.5mg/l) were used for callus induction. Better callusing response (80.0%) was recorded on MS medium supplemented with 2, 4-D (2.0 mg/l) and Kn (0.2 mg/l) (greenish compact; Fig. 2).

Table 1. Callus induction from 50-60 days old nodal 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	-	18	30.0	Brown compact
1.0	-	20	33.3	Brown compact
1.5	-	24	40.0	Brown compact
2.0	-	32	53.3	Brown compact
2.5	-	26	43.3	Brown compact
3.0	-	21	35.0	Brown compact
2.0	0.1	34	56.7	Greenish compact
2.0	0.2	48	80.0	Greenish compact
2.0	0.3	42	70.0	Greenish compact
2.0	0.4	38	63.3	Greenish compact
2.0	0.5	35	58.3	Greenish compact

** Out of 60 explants inoculated*

The callus was transferred into medium containing various concentrations of BAP (0.5–2.0 mg/l) alone and in combination with IAA (0.1–0.4mg/l) to test the regeneration potential. Shoots were initiated from nodal explants derived calli when cultured in the medium supplemented with different concentrations of BAP either alone or in combination with various concentrations of IAA (Fig. 2). BAP at 1.0 mg/l produced 5.6±0.58 shoots per callus and 8.4±0.54 shoots per callus were formed on the medium containing BAP (1.0mg/l) and in combination with IAA (0.2mg/l) in 4 weeks, respectively. With repeated subcultures on the same medium at intervals of 4 week, each nodal explants produced 16.8 ± 0.89 shoots on medium containing BAP (1.0 mg/l) alone and 31.4±2.17 shoots on medium containing BAP (1.0mg/l) and in combination with IAA (0.2mg/l) in the second subculture.

The callus growth induction, and subsequent differentiation through organogenesis is accomplished by the differential application of growth regulators and the control of culture conditions. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. There are many reports on the regeneration of various medicinal plants via callus culture. In our previous study, *In vitro* plant regeneration of *W. somnifera* through direct and indirect regeneration protocols were established for multiple shoot development from epicotyl explants of 50-60 days old seedlings and this protocol produced a higher

number of shoots within a short period [28]. Indirect regeneration of *W. somnifera* and comparative analysis of withanolides in *In vitro* and greenhouse grown plants were reported [29]. Role of plant growth regulators on *In vitro* regeneration and mass propagation of *W. somnifera* was reported [30-32]. In this study, the nodal explants produce more number of shoots than epicotyl explants (data not shown) through indirect shoot regeneration. Similarly, the plant regeneration and mass propagation of *W. somnifera* through *In vitro* callus culture was reported [33].

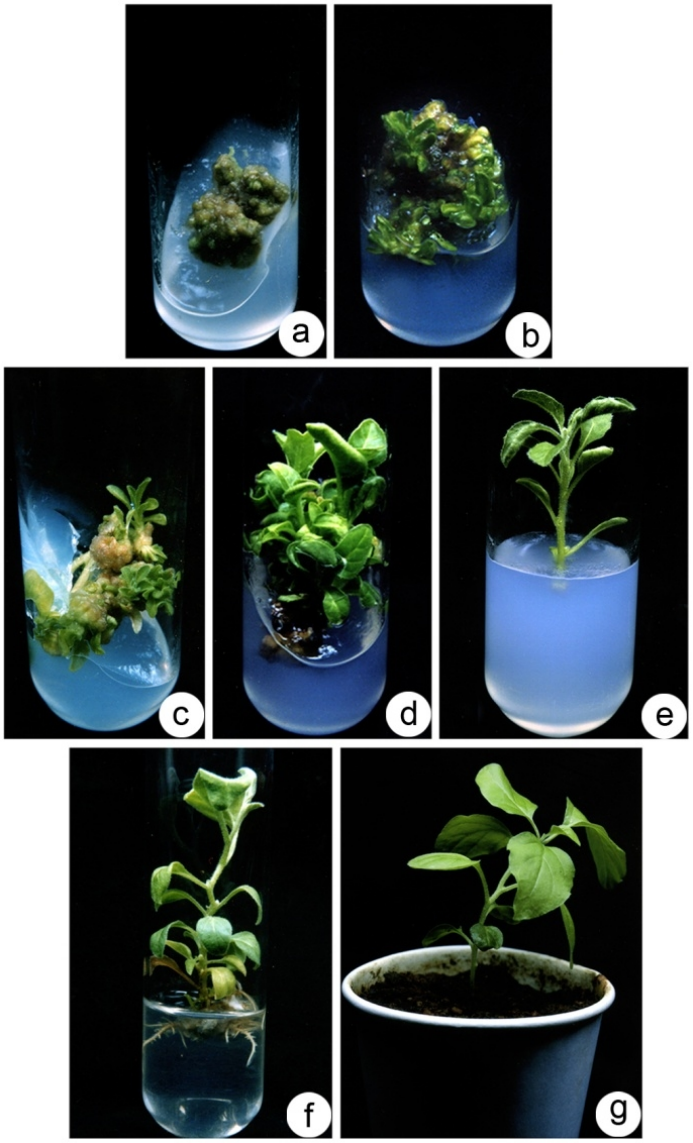

Fig. 2. Regeneration of shoots from 50-60 days old nodal explants derived callus of *Withania sominifera* on MS medium (a) Greenish compact nodular callus 2,4-D (2.0 mg/l) + kinetin (0.2 mg/l); (b-d) Initiation of adventitious shoots and proliferation from nodal explant derived callus BAP (1.0 mg/l) + IAA (0.2 mg/l); (e) Shoot elongation GA_3 (1.0 mg/l); (f) Rooting of shoot IBA (0.8 mg/l); (g) Acclimatized plant

In the present study, the synergistic effect of IAA and in combination with BAP on promotion of shoot organogenesis and multiplication was observed, which is in agreement with previously reported in *Alpinia golonga* [34] and *Valariana wallichi* [35]. Rao *et al.* [36] and Zimmy and Lorz [37] observed that the composition of nutrient media was found to be an important factor in enhancing regeneration frequency in many plant species. In the present study, the subcultures improved the efficiency of shoot regeneration from explant. Similar results were also recorded in our previous study [28]. The *in vitro* shoot propagation from axillary meristems, petiole and leaf explants of *W. somnifera* were reported [38-41].

Antonisamy and Manickam [42] reported that the *in vitro* method for rapid regeneration using nodal explants of *W. somnifera*. Nodal explants of *W. somnifera* differentiated shoot buds on MS, SH and B_5 media containing different concentrations and combinations of auxin and cytokinin by direct regeneration and the regenerated plantlets were transferred to the field with 87% of survival rate [43]. In the present study, similar type of results was observed by indirect regeneration and the regenerated plantlets were transferred to the field with 98% of survival rate. So, this is an efficient protocol for indirect regeneration of *W. somnifera* using nodal explants.

3.2 Elongation and Rooting of Shoots

When shoots reached a height of 1.0 cm or above, they were separated from the callus and then transferred to MS medium supplemented with different concentrations of GA_3 (0.5 – 2.0 mg/l) for shoot elongation. MS medium with GA_3 produced optimum elongation of shoots, which were healthy and normal. The MS medium containing GA_3 at 1.0mg/l showed maximum shoot elongation response (96.7%) with a mean shoot length of 6.8±0.65cm (Table 3 and Fig. 2). Similar response was observed and reported in the previous study using epicotyl explants of *W. somnifera* [28]. At higher GA_3 concentrations (>1.0 mg/l), basal callusing and subsequent inhibition of shoot elongation were observed.

The elongated shoots were transferred to root induction medium containing different concentrations of NAA (0.2–1.0mg/l), IAA (0.2–1.0mg/l) and IBA (0.2–1.0mg/l). Addition of IBA to rooting medium improved rooting efficiency of shoots. The percentage of shoots that produced roots increased with increasing IBA concentrations (Table 4). More than 96% of shoots produced roots (7.2±0.45 roots/shoot) with a mean root length of 4.0±0.45 cm at 0.8 mg/l IBA (Fig. 2). IAA at 0.8 mg/l resulted in the next higher percentage (85%) of rooting followed by NAA at 0.8mg/l (66.7%). However, the number of roots produced per shoot was low in IAA and NAA treatments. The roots were very thin and delicate in both IAA and NAA amended MS medium. Various auxins were successfully employed for rooting of shoots in many other plant species. IAA was also used to induce rooting of shoots in *Momordica dioica* [44]. In the present study, auxins were successfully used in inducing roots from regenerated shoots. More than 96% of shoots produced roots in MS medium containing IBA (0.8 mg/l) followed by IAA (0.8 mg/l) and NAA (0.8 mg/l).

Similarly, regenerated shoots obtained from the axillary shoot base callus were rooted on MS medium containing IBA (2.0 and 4.0mg/l) or IBA (2.0mg/l) with IAA (2.0mg/l) [18]. Manickam et al. [45] reported that the IBA (9.84 μM) induced roots from shoot explants of *W. somnifera*. In the present study, maximum rooting (96.7%) was achieved when the shoots were cultured in IBA (0.8mg/l) amended medium. Similar results were observed in which maximum shoots (87%) rooted when cultured on MS medium containing 2.0mg/l IBA [46].

Table 2. Effect of different concentrations of BAP alone and in combination with IAA on shoot regeneration from 50-60 days old nodal explants derived callus of *W. somnifera* on MS medium

Growth Regulators (mg/l)		No. of cultures forming multiple shoots*	Shoot multiplication (%)	Initial culture		First subculture		Second subculture	
BAP	IAA			No. of shoots / callus	Height of shoots (cm)	No. of shoots / culture	Height of shoots (cm)	No. of shoots / culture	Height of Shoots (cm)
0.5	-	28	46.7	3.3±0.33a	0.9±0.05a	6.6±0.45a	1.0±0.09ab	10.3±0.09a	0.9±0.05a
1.0	-	41	68.3	5.6±0.58d	1.1±0.09bc	10.7±0.89c	1.3±0.14c	16.8±0.89d	1.4±0.14c
1.5	-	36	60.0	4.7±0.40c	1.0±0.09b	9.2±0.85b	1.1±0.09b	14.2±0.72c	1.2±0.09b
2.0	-	30	50.0	4.0±0.36b	0.8±0.05a	7.4±0.54a	1.0±0.05a	12.0±0.80b	1.0±0.05a
1.0	0.1	36	60.0	5.8±0.45d	1.2±0.09cd	10.5±0.89c	1.0±0.05ab	16.9±0.89d	1.4±0.14c
1.0	0.2	58	96.7	8.4±0.54f	1.4±0.14e	22.3±1.39f	1.5±0.09d	31.4±2.17g	2.0±0.18e
1.0	0.3	50	83.3	7.5±0.46e	1.3±0.09de	15.4±0.94e	1.4±0.14c	23.2±1.12 f	1.6±0.14d
1.0	0.4	42	70.0	6.0±0.36d	1.3±0.05d	12.7±0.63d	1.1±0.06b	21.3±1.16e	1.3±0.09bc

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≤ 0.05)

** Out of 60 explants inoculated*

Table 3. Effect of GA_3 on shoot elongation of regenerated shoots of *W. somnifera*

GA_3 (mg/l)	No. of cultures responding elongation*	Shoot elongation response (%)	Mean shoot length (cm)	Mean no. of nodes
0	10	16.7	2.0±0.14a	2.8±0.14a
0.5	36	60.0	3.0±0.49b	5.0±0.36b
1.0	58	96.7	6.8±0.65d	10.0±0.72d
1.5	40	66.7	5.1±0.45c	7.4±0.45c
2.0	32	53.3	2.8±0.34b	4.7±0.45b

Data shown are mean ± SD of six experiments each experiment consisted of 10 replicates values with the same letter within colums are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level (p≤ 0.05)

** Out of 60 explants inoculated*

Table 4. Effect of NAA, IAA and IBA on root induction from regenerated shoots of *W. somnifera*

Plant growth regulators (mg/l)		No. of cultures producing roots*	Cultures producing roots (%)	Mean no. of roots / shoot	Mean root length (cm)
NAA	0.2	19	31.7	1.9±0.09a	1.4±0.09a
	0.4	24	40.0	3.1±0.18b	1.7±0.14b
	0.6	30	50.0	4.2±0.36cd	2.4±0.22d
	0.8	40	66.7	4.6±0.25def	3.0±0.22e
	1.0	28	46.7	3.8 ± 0.36c	2.4±0.18d
IAA	0.2	23	38.3	3.2±0.27b	1.8±0.14b
	0.4	34	56.7	4.3±0.27d	1.9±0.12bc
	0.6	42	70.0	5.0±0.45fg	2.8±0.24e
	0.8	51	85.0	5.5±0.45h	3.5±0.32f
	1.0	36	60.0	4.8±0.36ef	2.4±0.27d
IBA	0.2	32	53.3	4.5±0.22de	1.8±0.12b
	0.4	40	66.7	5.3±0.36gh	2.2±0.19cd
	0.6	46	76.7	6.4±0.36i	3.6±0.31f
	0.8	58	96.7	7.2±0.45j	4.0±0.45g
	1.0	44	73.3	5.5±0.54h	3.4±0.31f

Data shown are mean±SD of six experiments each experiment consisted of 10 replicates values with the same letter within colums are not significantly different using Duncan's Multiple Range Test (DMRT) at 5% level ($p \leq 0.05$)
** Out of 60 explants inoculated*

3.3 Hardening of Plants

Well-rooted plants (4.0–6.0 cm height) obtained from rooting medium were transferred to small cups filled with sterilized soil: sand: vermiculite (1:2:1 v/v/v) for hardening (Fig. 2). These plants were maintained under 80% relative humidity in the growth chamber at 25±2ºC. Later, 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. Ninety eight percent of plants survived in the hardening process (data not shown) and these plants were established successfully in the experimental field. This protocol yielded a higher number of plantlets within a period of 100-110 days. The protocol developed in this study offers a simple and improved *in vitro* method to regenerate *W. somnifera* from nodal explants.

4. CONCLUSION

In this study, an efficient protocol was developed for indirect regeneration of *W. somnifera* using nodal explants. In conclusion, the rapid propagation is possible through indirect *In vitro* plant regeneration in *W. somnifera* species, because that have long germination time and low levels of seed germination. So, this *In vitro* indirect plant regeneration protocol is usefull to produce a large number of plants from a single nodal explant of *W. somnifera* in a short period of time. Furthermore, this standardised indirect regeneration protocol will be used in the production and mass propagation of transgenic plants of *W. somnifera*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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