

IN VITRO MASS PROPAGATION OF *WITHANIA SOMNIFERA* (L.) DUNAL AN IMPORTANT MEDICINAL PLANT OF BANGLADESH

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Abstract

An efficient *in vitro* regeneration system was developed for *Withania somnifera* (L.) Dunal through direct and indirect organogenesis from nodal and leaf segment explants. Highest direct shoot regeneration was recorded when nodal explants were cultured on MS with 2.0 mg/l BAP and 0.5 mg/l NAA where the average number of shoots per explants was 10.5. For indirect regeneration remarkable results on shoot initiation were observed when node and leaf segment callus were cultured on MS +1.0 mg/l BAP +0.5 mg/l NAA. It was observed that node callus showed best response for multiple shoot/explants (28.9) on MS +1.0 mg/l BAP +0.5 mg/l NAA. But the mean of shoots from the callus of leaf segment explants was found to be 15.1 on the same media. The best response towards root induction was observed on MS with 2.5 mg/l IBA. The well rooted plants were successfully acclimatized and transferred to soil.

Introduction

Withania somnifera L. an important medicinal plant belonging to Solanaceae is locally known as Ashwagandha. It is an important medicinal shrub distributed throughout the Bangladesh and South Asian countries (Negi *et al.* 2006). The leaves of this plant contain withanolides which are the active pharmaceutical ingredients like Withaferin-A that exhibits anti-bacterial and anti-tumor properties; roots contain a number of alkaloids like somniferine, withasomnine etc. which are prescribed for female disorders, rheumatism, dropsy, arthritis, sedative for senile debility and also inhibits Alzheimer's disease (Nigam and Kandalkar 1995).

W. somnifera (Ashwagandha) has been depleted from its natural habitat and is now included in the list of endangered species (Patel and Krishnamurthy 2013) by the International Union for Conservation of Nature and Natural Resources. Propagation of *W. somnifera* is primarily via seeds (Kattimani and Reddy 1999). However, conventional propagation of this plant by seeds is not reliable and is inadequate to meet commercial demands because of the low viability of the stored seeds (Siddique *et al.* 2004) and low seed germination rates (Vakeswaran and Krishnasamy 2003). Hence, there is a strong need for the development of an efficient regeneration system for the propagation, conservation and sustainable usage of *W. somnifera* clones within a reasonable time frame.

The development of plant tissue culture system can offer an opportunity for producing drugs from the leaves and roots of *W. somnifera* in the laboratory. Although earlier attempts have been made for the propagation of *W. somnifera* through tissue culture (Kulkarni *et al.* 2000; Manickam *et al.* 2000) but considerable effort is still required to conserve and also for commercial supply of the plant. Therefore, the present study was carried out to establish a suitable protocol for large scale *in-vitro* propagation of *W. somnifera* by direct and indirect shoot regeneration.

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Materials and Methods

The experiment was conducted at Plant tissue culture section, Biological Research Division, BCSIR Laboratories Dhaka. Seeds of *Withania somnifera* were collected from BCSIR medicinal garden. Germination capacity of the seeds of *W. somnifera* were tested on cotton bed and on MS media. Seeds were soaked into water for three hours and surface sterilized with a slight modification of Goswami *et al.* (2018) prior to *in vitro* germination experiment. Two types of explants (nodal segment and leaf segment) were collected from the *in vitro* germinated plants grown in MS media. Isolated explants were inoculated and cultured on MS media (Murashige and Skoog 1962) containing BAP, NAA, IAA, 2,4 -D and Kn singly or in combinations for direct and indirect (through callus) *in vitro* regeneration of shoots. *In vitro* regenerated shoots were sub-cultured regularly to fresh medium at an interval of 15 days for further multiplication. About 3- 4 cm long shoots were separated and cultured on rooting medium containing full and half strengths of MS without hormonal supplement or with different concentrations of IBA. All *in vitro* grown cultures were maintained under illumination on a 16 hrs photoperiod at $25 \pm 2^\circ\text{C}$. The well rooted plantlets were then transferred to plastic pot containing garden soil and compost in ratio of 2:1 and moist them adequately for proper hardening.

Results and Discussion

It is reported that the seed germination of *W. somnifera* is poor (Kumar *et al.* 2001). So, in the present experiment at first preliminary study on seed germination under controlled condition was conducted for *W. somnifera*. Germination of seeds were tested on cotton bed. Seeds were grown on cotton bed and on MS media under uniform controlled condition and data were recorded. First three days seeds were kept at 37°C incubator and after that germination experiment was maintained under illumination on a 16 hrs photoperiod at $25 \pm 2^\circ\text{C}$. Results depicted synergistic activity of cotton bed and MS media on seed germination indicated by poor seed germination (Fig. 1). Maximum percentage of seed germination was only 37 after 9-15 days. Similar findings were reported by Karnick (1978), Kumar *et al.* (2001), Kanungo and Sahoo (2011). Almost 35-40 days were required to obtain a full plant of *W. somnifera* with a length of 3-10 cm. Explants were collected from *in vitro* grown plants and nodal and leaf segments were used as explants for *in vitro* regeneration experiments. It was also found that around medium-aged explants were suitable for *in vitro* shoot induction. The explants were cultured on MS medium supplemented with various concentrations of BAP, IAA, NAA, 2,4-D, and Kn singly or in combinations to observe their effect on initiation and development of shoots. Among all the hormonal combinations used in this study, MS media supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA was found to be the most suitable for shoot initiation from the nodal explants of *W. somnifera*. Results of this experiment are presented in Table 1. Almost 90% of the explants showed regeneration initiation (Table 1) on this media (2.0 mg/l BAP and 0.5 mg/l NAA). Shoot initiation from nodal explants are presented in Fig. 2a. The mean number of shoots per explants was 10.5 (Fig. 2b) in this media (2.0 mg/l BAP and 0.5 mg/l NAA). Banu *et al.* (2017) reported that combinations of BAP and NAA showed better results from node explants in *Gynura*. Ghimire *et al.* (2010) also reported that BAP and NAA showed best response towards regeneration using petiole and leaf explants in *Withania*. Next to the best hormonal combination (MS+2.0 mg/l BAP and 0.5 mg/l NAA) maximum response towards shoot initiation (Fig.2c) was found on MS supplemented with 2.0 mg/l BAP, 1.0 mg/l NAA and 0.5 mg/l Kn (Table 1). Baba *et al.* 2013 also reported that shoot cultures were initiated on MS medium containing BA (0.5 – 2.0 mg/l) with (NAA 0.2-0.5 mg/l) in *W. somnifera*. Mollika *et al.* (2011) also reported similar findings in *Brassica*. It was observed that 15-23 days were required to start regeneration initiation using different media combination. Combinations of 2.0 mg/l BAP and 0.5 mg/l Kn also showed good response for shoot initiation from nodal explants.

Formation of multiple shoots and proliferation of such shoots using this media combination is presented in Fig. 2(d, e). Although among the various hormonal combinations used for regeneration, most of the cases direct organogenesis was observed; callus was also formed from the nodal explants (Table 1). Nayak *et al.* (2013) also reported direct organogenesis from cotyledonary node explants in *Withania*.

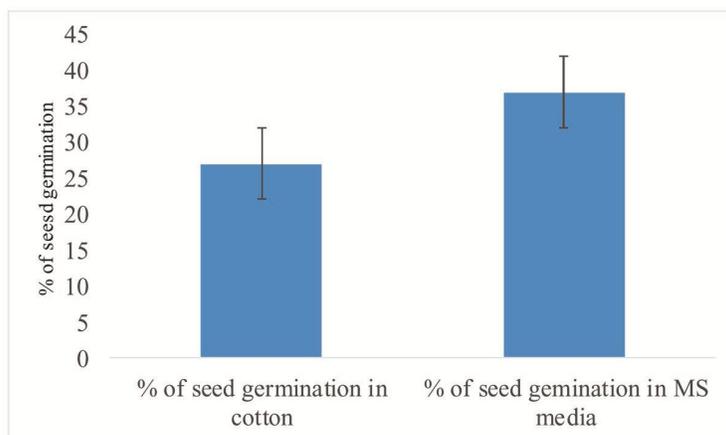


Fig.1. Experiment on seed germination of *W. somnifera* L.

Table 1. Effect of MS medium supplemented with different concentrations and combinations of hormones for direct shoot initiation from nodal explants of *W. somnifera* L.

Hormonal supplements (mg/l)					% of responsive explants towards shoot initiation	Days required to get response	Average no. of shoot/explants
BAP	NAA	Kn	IAA	2,4-D			
0.5	-	-	-	-	-	-	-
0.5	0.5	-	-	-	25	21-22	Only friable light yellow callus
1.0	-	-	1.0	-	54	10-13	Only friable light yellow callus
1.0	1.0	-	-	-	38	21-23	3.1
1.0	-	0.5	-	-	69	17-19	4
2.0	-	0.5	-	-	78	15-19	9
1.0	1.5	-	-	-	72	20-21	6.3
2.0	-	1.0	1.0	-	63	22-23	5.8
1.0	1.0	0.5	-	0.3	15	23-24	Only friable callus
-	-	0.2	-	2.0	30	15-19	Only friable whitish callus
2.0	0.5	-	-	-	90	15-18	10.5
2.0	1.0	0.5	-	-	87	16-18	7

On the other-hand, leaf segment explants showed callus formation. The best response was observed on MS media supplemented with 2.0 mg/l BAP, 1.0 mg/l NAA and 0.5 mg/l Kn. 87% of the explants which showed callus induction on this media combination (Table 2). Callus initiation from leaf explants are presented in Fig.2f. Jain *et al.* (2011) also reported callus induction using combinations of BAP and kn from shoot bud explants of *Withania*. Callus induction was also found in MS media supplemented with 1.0 mg/l BAP, 1.0 mg/l NAA, 0.5 mg/l kn and 0.3 mg/l 2,4-D. Valizadeh and Valizadeh (2009) also reported similar findings using leaf segments in *W. coagulans*. Callus induction was observed after four weeks on culture media. Very often callus

formation was found to occur using leaf segment explants but most of the cases these calli did not show any sign of shoot multiplication when they were sub-cultured on the same media combinations.

Table 2. Callus induction rate and shoot initiation rate after 4 weeks in culture on MS medium supplemented with different concentrations and combinations of hormones from leaf segment explants of *Withania somnifera* L.

BAP	Media combination				Texture of callus	% of callus formation	% of shoot initiation from callus
	NAA	Kn	IAA	2,4-D			
0.5	0.5	-	-	-	-	-	-
1	1	-	-	-	Slow growing whitish friable callus	45	33
1	1	1	-	-	Light yellow friable callus	56	40
2	.5	-	-	-	Light yellow friable callus	80	63
2	1	.5	-	-	Light yellow friable callus	87	76
1	-	-	1	-	Brown compact callus	72	Only callus
1	-	1	1	-	Whitish friable callus	78	50
2		0.5	0.5	-	Brown compact callus	70	27
2	-	1	1	-	Deep brown compact callus	50	Only callus
2	-	-	-	0.5	Slow growing brown compact callus	75	Only callus
2	-	-	-	1	Whitish compact callus	67	Only callus
2	-	-	-	2	Brown and white compact callus	80	Only callus

Table 3. Responses of callus towards multiple shoot formation on MS medium with low concentration hormonal combinations of BAP, NAA, 2, 4-D and Kn (mg/l).

Hormonal supplement (mg/l)					% of multiple shoot regeneration from node callus	% of multiple shoot regeneration from leaf segment callus	Days required to get response	Average no. of shoot/explants after 60 days	
BAP	NAA	IAA	Kn	2,4-D				Node callus	Leaf segment callus
1.0	-	1.0	-	-	-	-	-	-	-
1.0	0.2	-	0.2	-	73	50	7-9	13	6
1.0	0.5	-	0.5	-	77	65	5-9	23.7	5.5
1.0	0.5	-	-	-	87	84	5-10	28.9	15.1
1.0	-	0.2	0.2	-	70	56	7-9	17	7
1.0	-	0.2	-	-	58	27	6-10	8.1	6.6
1.0				0.5	61	43	10-12	5.5	3.0

In a separate set of experiment, special focus was given to produce multiple shoots from the callus developed from node and leaf segment explants. For this purpose, MS medium supplemented with low concentration and combinations of BAP, NAA, Kn, IAA, 2, 4-D were chosen in which callus were further sub-cultured. Remarkable result was observed when nodal callus and leaf segment callus were sub-cultured into MS +1.0 mg/l BAP +0.5 mg/l NAA (Table 3). Almost 5-9 days were required for shoot formation from node callus (Fig. 2g) and 8-10 days were required for shoot formation from leaf segment callus (Fig. 2h). In this combination best responses on multiple shoot formations were obtained (Fig. 2i, 2j.) and the mean number of shoots (28.9) were found from node callus after sixty days of culture (Table 3). Khan *et al.* (2018) also found that 2.0 mg/l BAP, 0.5mg/l IAA and 0.02mg/l NAA was best for the multiple shoot formation from nodal segments in *Rauvolfia serpentina*. Fatima *et al.* (2012) reported multiple shoot formation using BAP and NAA from node explants of *Withania*. But the mean of shoots

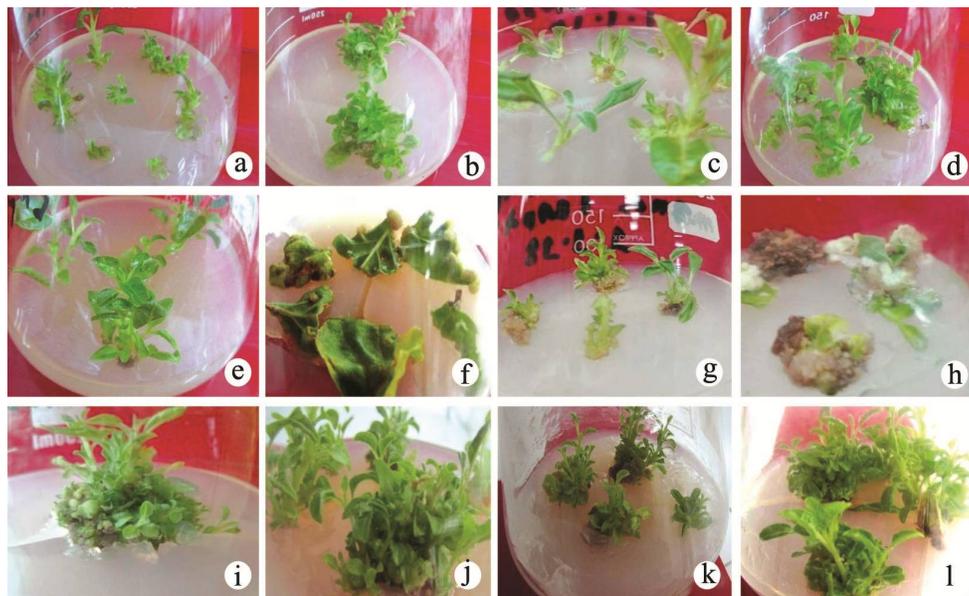


Fig. 2(a-l). Different stages of direct and indirect shoot regeneration of *W. somnifera* L. a. Shoot initiation from nodal explants on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA. b. Multiple shoot formation from nodal explants on MS with 2.0 mg/l BAP and 0.5 mg/l NAA. c. Shoot induction from nodal explants on MS medium supplemented with MS supplemented with 2.0 mg/l BAP, 1.0 mg/l NAA and 0.5 mg/l Kn. d. Formation of multiple shoot from nodal explants on MS with 2.0 mg/l BAP, 1.0 mg/l NAA and 0.5 mg/l Kn. e. Elongated multiple shoots of *W. somnifera* from nodal explants on the same media. f. Callus initiation from leaf segment explants of *W. somnifera* on MS with 2 mg/l BAP, 1 mg/l NAA and 0.5 mg/l Kn. g. Indirect shoot initiation from callus of node explants on MS medium supplemented with 1.0 mg/l BAP +0.5 mg/l NAA. h. Friable callus of leaf segment explants from which shoot initiated. i. Initiation of multiple shoots of *W. somnifera* from callus of node explants on MS with 1.0 mg/l BAP +0.5 mg/l NAA. j. Development of multiple shoots of *W. somnifera* from callus of node explants on the same media combination. k. Formation of multiple shoots from leaf segment callus on the same media combination. l. Developed multiple shoots from the leaf segment callus on MS+1.0 mg/l BAP + 0.5 mg/l Kn + 0.5 mg/l NAA.

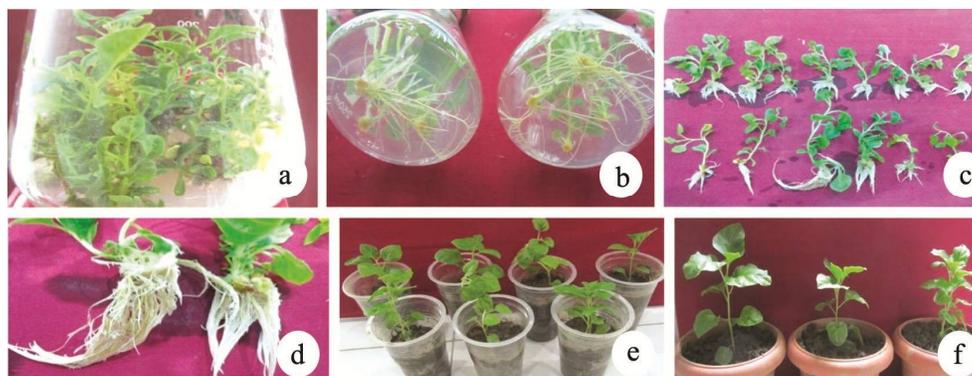


Fig. 3(a-f). Root induction and subsequent establishment of *in vitro* regenerated plants of *W. somnifera*. a. Fully matured elongated shoots of *W. somnifera* developed from node callus. b. Formation of roots at the base of the cut end of *in vitro* grown shoots on MS media supplemented with 2.5 mg/l IBA. c. Rooted plantlets of *W. somnifera* developed on MS with 2.5 mg/l IBA. d. Enlarged view of *in vitro* roots. e. Well-developed rooted plantlets transplanted into small plastic pots. f. Fully acclimatized *in vitro* generated plantlets of *W. somnifera* in larger clay pot.

from the callus of leaf explants was found to be 15.1 (Table 3) in this media combination (Fig 2k). Similar findings were also reported by Banu *et al.* (2017) using leaf explants in *Gynura*. When the callus was cultured on MS+1.0 mg/l BAP + 0.5 mg/l Kn + 0.5 mg/l NAA multiple shoots were formed with the mean number of shoots 23.7 and 5.5 (Fig. 2l) from node and leaf segment callus, respectively. Rapid multiplication and elongation of shoots were observed in higher concentrations of BAP with Kn in *Withania* reported by Baba *et al.* (2013).

After shoot regeneration for induction and development of roots rooting medium containing full and half strength of MS medium with different concentrations of IBA was tried. Best response was observed on MS with 2.5 mg/l IBA. Fully matured elongated shoots (Fig. 3a) were separated and cultured on freshly prepared rooting medium. In this hormonal combination roots were formed at the base of the cut end of *in vitro* grown shoots (Fig.3b). The rooted plantlets developed on MS+2.5 mg/l IBA are presented in Fig.3c. The mean no of roots per plantlets were found 27.3 (Fig. 3d) on MS with 2.5 mg/l IBA. Nayak *et al.* (2013) also reported root formation using half MS with IBA in *Withania*. The well-developed rooted plantlets were successfully transplanted into small plastic pots containing autoclaved soil (Fig.3e). The survived plantlets were transferred to larger clay pots (Fig.3f) and successfully established into the field.

Based on the above discussion it may be concluded that the *in vitro* mass propagation protocol developed in the present investigation could be used for the large-scale cultivation as well as conservation of endangered plant *W. somnifera*. Direct regeneration and indirect regeneration from the explants will be useful for genetic transformation experiments and will also help in metabolic engineering for bioactive withanolides production in the laboratory.

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