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# DIRECT PLANT REGENERATION FROM NODAL EXPLANTS OF *WITHANIA SOMNIFERA* (L.) DUNAL-AN ENDANGERED MEDICINAL PLANT

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## ABSTRACT

A high frequency direct plant regeneration protocol has been developed for *Withania somnifera* using nodal segments as explants. MS medium when supplemented with BAP 4.0 mg $l^{-1}$ and IAA 2.0 mg $l^{-1}$ took least days for primordial appearance (5.34), multiple shoot initiation (5.45) and produced maximum shoot length (6.8 cm). The maximum response percentage (85) and number of shoots per culture (25) was found in MS medium supplemented with BAP 1.0 mg $l^{-1}$ and IAA 2.0 mg $l^{-1}$ while fresh weight of shoot (4.72g) and dry weight of shoot (0.37g) were found maximum in MS medium supplemented with 0.5 mg $l^{-1}$ BAP and 2.0 mg $l^{-1}$ IAA. Highest percentage of rooting (71.5), dry weight of root (0.42 g), plant height (8.52cm) and minimum days to root initiation (3.18) was recorded in MS medium supplemented with 5.0 mg $l^{-1}$ IBA while number of roots per shoot (25.6), root length (8.38cm), fresh weight of root (1.39g) and weight of plantlets (5.91g) was in IBA 4.0 mg $l^{-1}$.

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## INTRODUCTION

*Withania somnifera* (L.) Dunal, belonging to the family Solanaceae is one of the important medicinal plants in the field of pharmacology having enormous medicinal and aromatic properties and has been included in ancient text of Ayurveda (Sharma *et al.,* 2010). It is commonly known as Winter cherry or Indian ginseng or Ashwagandha. It contains several alkaloids, withanolides, a few flavanoids and reducing sugars (Ganzera *et al.,* 2003). This medicinally important plant species has been depleted from their 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). *W. somnifera* can be propagated both by sexual and asexual method. Commercially, *Withania* is propagated by seeds because of the lack of natural ability for vegetative propagation (Sen and Sharma, 1991), but the seed viability is limited to one year (Rani and Grover, 1999). Long duration of seed storage make the seeds futile and hence, low germination percentage (Farooqi and Sreeramu, 2004). This resulted in the adulteration of plant materials, making the plant endangered (Antonisamy and Manikam, 1999). The conventional propagation method cannot meet the increasing demand of this plant used as raw material for the preparation of pharmaceutical products. Type of explant and growth regulators concentration affects the morphogenic response in the culture medium (Mahato *et al.*, 2009). Micropropagation of *Withania somnifera* by different explants such as shoot tips (Sen and Sharma, 1991), auxiliary meristems (Roja and Heble 1991) auxiliary leaves, auxiliary shoot and hypocotyls and root segments (Rani and Grover, 1999) has been found that the rate of plant regeneration per explant is not sufficiently high for practical application. It is noted that in all of the above studies plants were regenerated via morphogenesis from callus induced from the explants. One disadvantages of such process is the recalcitrance of the callus to differentiation (Pan *et al.,* 1995) which is negatively affected the regeneration efficiency. So, it is necessary to establish a reliable, simple, efficient and rapid technique for the propagation and mass multiplication of *W. somnifera* to conserve the plant species and also to provide a constant supply of plant materials for the pharmaceutical industries round the year, irrespective of seasonal constraints. Alternatively, direct regeneration of Ashwagandha through *in vitro* technology can be used in which direct organogenesis occur as an alternative because the advantage of tissue culture technology lies in the production of high quality planting material on a year round irrespective of the season and weather. Therefore, the present study was undertaken to standardize the *in-vitro* protocol for direct regeneration of Ashwagandha by using nodal explants.

## MATERIALS AND METHODS

### Explants preparation

Actively growing and healthy shoot material of *W. somnifera*, nodal explants were about 2-3 cm in length cut from 3 month old plants growing in the Horticultural

Farm of the university. Nodal segments were excised from the plants and washed thoroughly with tap water, immersed in 1% mild detergent (cleaning agent) for 2-3 min. and washed thoroughly with distilled water. Subsequently, these were surface sterilized with 0.1% mercuric chloride solution for 1-2 min. and again washed well in distilled water to remove the traces of mercuric chloride.

**Multiple shoot induction and proliferation**

For culture establishment, initiation of shoots and multiplication, MS basal medium supplemented with macro and micro-elements, 3% (w/v) sucrose and 0.8% (w/v) bacteriological grade agar as gelling agent along with different concentrations of cytokinin, BAP (0.5 - 4.0 mg/l) in combination with auxin, IAA (2.0 mg/1) was used. The pH of the medium was adjusted to 5.8 using 1 N NaOH before being autoclaved at 121°C for 20 min. All the cultures were maintained at 25 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 20 ìmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps (2 × 40 W, Phillips, India).

The shoots were transferred into the root initiation medium. MS media supplemented with five different concentrations of auxin, indole-3-butyric acid (IBA) (1.0 – 5.0 mg/l) were used as growth regulators. The shoots were separated and placed vertically on rooting medium containing IBA for rooting of shoots.

**Acclimatization**

Plantlets with well developed roots were transferred to plastic cup containing autoclaved perlite, sand and vermicompost (1:1:1) and maintained for four weeks in culture room. Then the plantlets were transferred to poly cups containing garden soil and vermicompost (1:1) and were maintained in a shade net house.

**Statistical analysis**

The experiments were designed in Completely Randomized Design (CRD). In each treatment 50 seeds were inoculated @ 5 seeds per jam bottle and each treatment was replicated four times. The statistical analysis was done by employing the O.P Stat software packages and the means were compared using Duncan's multiple range test (DMRT) at the 0.05% probability level.

## RESULTS AND DISCUSSION

**Shoot induction and multiplication**

Direct shoot regeneration was attempted from the stem nodal explants of *Withania somnifera* using MS basal medium supplemented with various concentration and combination of BAP and IAA (Table-1 & Fig. 1). All the concentrations of BAP (0.5-3.0 mg/l) alone or in combination with IAA (1.5 and 2.0 mg/l) promoted adventitious shoot buds via direct organogenesis after five weeks of culture. Similar result was found by (Arumugam and Gopinath, 2011). The average minimum time (5.34 days) required for shoot primordial appearance was observed on MS media supplemented with 4.0 mg/l BAP in combination with 2.0 mg/l IAA and maximum days (6.80) needed on MS basal medium with 0.5 mg/l BAP and 2.0 mg/l IAA which was significant in respect to the other treatments. It was noticed that the time required for shoot initiation decreased with the increased concentration of BAP while the concentration of NAA remained same in all the combinations of BAP used. The explants cultured on MS medium without growth regulators failed to induce multiple shoots but each explant grew and developed. The highest percentage (86%) of shoot proliferation was noticed on MS basal medium supplemented with 1.0 mg/l BAP + 2.0 mg/l IAA which was significantly correlated with the other responses

**Table 1: Effect of BAP and IAA on *in-vitro* shoots induction and multiplication of Withania**

| Treatment | Treatment combination | Days to primordial appearance | Regeneration percentage | Days to multiple shoot initiation | Number of shoots per explant | Shoot length (cm) | Fresh weight of shoot (g) | Dry weight of shoot (g) |
|---|---|---|---|---|---|---|---|---|
| $MB_{0.5}IA_2$ | MS+ BAP 0.5 mg $l^{-1}$ + IAA 2.0 mg $l^{-1}$ | 6.80 | 81 | 6.90 | 12 | 5.9 | 4.72 | 0.37 |
| $MB_1IA_2$ | MS+ BAP 1.0 mg $l^{-1}$ + IAA 2.0 mg $l^{-1}$ | 6.50 | 86 | 6.50 | 25 | 4.6 | 3.36 | 0.29 |
| $MB_2IA_2$ | MS+ BAP 2.0 mg $l^{-1}$ + IAA 2.0 mg $l^{-1}$ | 6.05 | 82 | 6.18 | 18 | 3.6 | 2.23 | 0.11 |
| $MB_3IA_2$ | MS+ BAP 3.0 mg $l^{-1}$ + IAA 2.0 mg $l^{-1}$ | 5.65 | 78 | 6.00 | 16 | 4.2 | 2.84 | 0.23 |
| $MB_4IA_2$ | MS+ BAP 4.0 mg $l^{-1}$ + IAA 2.0 mg $l^{-1}$ | 5.34 | 75 | 5.45 | 12 | 6.8 | 4.12 | 0.27 |
| S E m± | 0.16 | 1.039 | 0.247 | 1.291 | 0.230 | 0.161 | 0.053 | |
| C D at 5% | 0.499 | 3.317 | 0.787 | 4.121 | 0.733 | 0.515 | NS | |

**Table 2: Effect of IBA on *in-vitro* rooting induction and growth of Withania plantlets**

| Treatment | Treatment combination | Percent rooting | Days to root initiation | Number of roots per shoot | Root length (cm) | Fresh weight of root (g) | Dry weight of root (g) | Weight of plantlets (g) |
|---|---|---|---|---|---|---|---|---|
| $MIB_1$ | MS+ IBA 1.0 mg $l^{-1}$ | 23.6 | 5.40 | 4.7 | 5.5 | 0.97 | 0.09 | 3.13 |
| $MIB_2$ | MS+ IBA 2.0 mg $l^{-1}$ | 36.2 | 5.05 | 8.5 | 6.2 | 1.14 | 0.12 | 3.61 |
| $MIB_3$ | MS+ IBA 3.0 mg $l^{-1}$ | 55.2 | 4.15 | 13.9 | 7.00 | 1.28 | 0.16 | 5.19 |
| $MIB_4$ | MS+ IBA 4.0 mg $l^{-1}$ | 67.8 | 3.50 | 25.6 | 8.38 | 1.39 | 0.21 | 5.91 |
| $MIB_5$ | MS+ IBA 5.0 mg $l^{-1}$ | 71.5 | 3.18 | 16.5 | 6.65 | 1.15 | 0.42 | 4.84 |
| SE m± | | 1.262 | 0.321 | 1.159 | 0.278 | 0.067 | 0.042 | 0.265 |
| CD at 5% | | 4.027 | 1.023 | 3.7 | 0.888 | 0.212 | 0.135 | 0.846 |

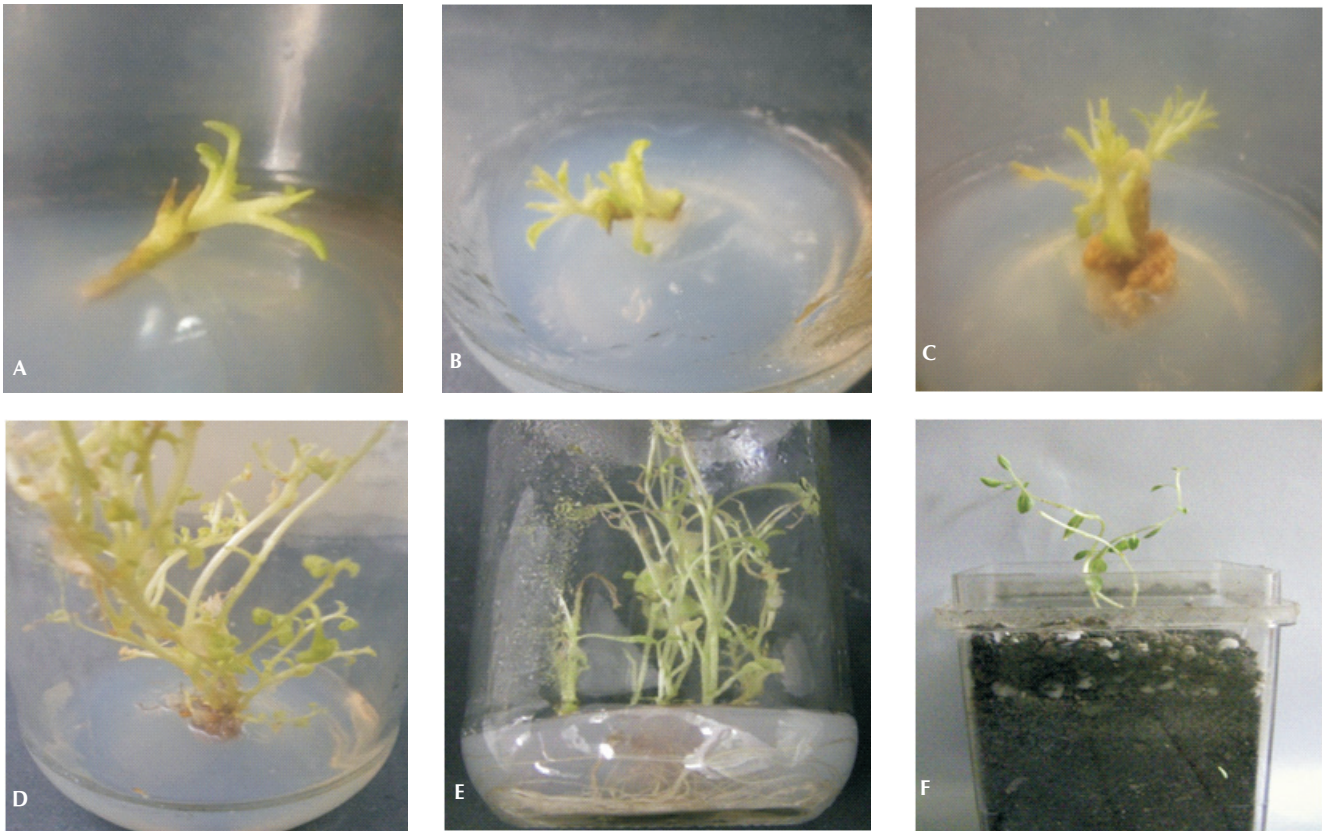


**Figure 1: *In vitro* plant regeneration from nodal explant of Aswagandha (A to F). A. Initiation of multiple shooting from nodal explant B. Multiple shoot differentiation C. Multiple shooting stage D. Regenerated shoots after subculture E. *In vitro* regenerated plantlets with profuse rooting F. Plantlets in hardeing media**

recorded. The result of shoot initiation approximately decreased with the increased BAP concentration and it was lowest (75%) with BAP 4.0 mg/l. This might be due to the toxic effect of higher concentration of growth regulator cytokinin on *Withania somnifera* shoots. Similar result on shoot differentiation found by Supe *et al.* (2011), Kumar *et al.* (2011); Soni *et al.* (2011) and Arumugam and Gopinath (2011). A progressive degree of shoot bud differentiation was observed at lower concentrations of BAP alone or combination with IAA while, at higher concentrations led to decrease in shoot induction. The results obtained were agreed to the observations reported by Govindaraju *et al.* (2003), Joshi and Padhya (2010), Kulkarni *et al.* (2006), Sivanesan (2007) and Supe *et al.* (2011).

The mean number of shoots per culture (25) was observed highest on MS basal medium supplemented with 1.0 mg/l BAP + 2.0 mg/l IAA which was significantly correlated with the other treatment recorded (Table 1 & Fig. A). The number of shoot per culture was minimum both at highest and lowest concentration of BAP used. The finding was coincided with the observations of Kumar *et al.* (2011) and Soni *et al.* (2011). The shoot length was significantly increased with the increased concentration of BAP when the IAA concentration remains same. The maximum mean length (6.80 cm) of the shoot was observed on MS basal medium supplemented with 4.0 mg/l BAP+ 2.0 mg/l IAA which was significantly higher than the other treatments followed by shoot length (5.90 cm) recorded with 0.5 mg/l BAP in combination with 2.0 mg/l IAA. The minimum days (5.45) required for multiple shoot initiation was found on MS basal medium supplemented with 4.0 mg/l BAP + 2.0 mg/l IAA. Similar result of shoot multiplication was obtained by Sharma *et al.* (2009), Sivanesan (2007), Kumar *et al.* (2011) and Chatterjee and Ghosh (2012). The positive effect of BA on bud proliferation and multiple shoot formation has been reported for several medicinal and aromatic plant species such as *Chlorophytum borivilianum* (Purohit *et al.* 1994), *Eclipta alba* (Franca *et al.*,1995), *Ocimum* sp. (Patnaik and Chand, 1996). By increasing the concentration of BA beyond the optimal level, a gradual reduction in the number of shoots was also reported for several medicinal plants including *Withania somnifera*. The maximum average fresh and dry weight was recorded on MS basal medium supplemented with 0.5 mg/l BAP+ 2.0 mg/l IAA and 4.0 mg/l BAP+ 2.0 mg/l IAA, respectively.

**Root induction and growth**

The elongated shoots were excised and implanted on MS medium fortified with different levels of IBA (1.0-8.0mg/l). The data presented in Table 2 showed that the highest (71.7%) rooting response was observed on MS basal medium supplemented with 5.0 mg/l IBA which was significantly higher than the other observations recorded. The result of root initiation was increased with the increased IBA concentration and it was lowest (23.6%) with IBA 1.0 mg/l. Similar results

were obtained by Supe *et al*. (2011), Kumar *et al*. (2011), Meheta and Mahato (2012). The average minimum days (3.18 days) needed for root emergence was observed on MS basal medium supplemented with 5.0 mg/l IBA and maximum days (5.40) noted on MS basal medium with 1.0 mg/l IBA. The time required for root initiation was decreased with the increased concentration of IBA. The mean number of roots per shoot (25.6) was observed highest on MS basal medium supplemented with 4.0 mg/L IBA followed by media containing 5.0 mg/l IBA. The numbers of root per culture were maximum when higher concentration of IBA used. The number of root was observed lowest when the shoots were culture on MS media supplemented with IBA 1.0 mg/L. Similar results were obtained by Joshi and Padhya (2010), Rani and Grover (1999), Sharma *et al*. (2010) and Valizadeh *et al*. (2009). The maximum mean length (8.38cm) of root was observed on MS basal medium supplemented 5.0 mg/L IBA which was significantly higher than the other treatments. The average fresh and dry weight was maximum on MS basal medium supplemented with 4.0 mg/L and 5.0 mg/L IBA, respectively. The weight of plantlet (5.91g) was observed highest on MS basal containing IBA 5.0 mg $L^{-1.}$

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