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Micropropagation of Ashwagandha (*Withania somnifera*)

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ABSTRACT

In the present work *in vitro* propagation of a multipurpose medicinal plant, *Withania somnifera* was done. Direct regeneration of nodal explants and their multiplication have been optimized using cytokinin BAP (0.5-4.0 mg/l) and combination of BAP (0.5 mg/l) + NAA (0.5-3.0 mg/l) respectively. MS media with nodal explants supplemented with BAP (2.0 mg/l) produced maximum average number of shoots (2±0.37) and average shoot length was found to be 2.8±0.15 cm. Best initiated shoots then sub cultured for shoot multiplication, an improved shoot multiplication in terms of average number of shoots (5.3±0.41) and average shoot length 6.5±0.12 cm was observed on MS media in combination with BAP (0.5 mg/l) + NAA (1.5 mg/l). Maximum average number (12± 0.20) and average length 9.8±0.26cm of roots were observed on MS media supplemented with IBA (2.0 mg/l) out of different IBA (1-5 mg/l) concentrations were taken in to consideration during the study. Regenerated plantlets were successfully transferred to greenhouse condition.

KEY WORDS: *WITHANIA SOMNIFERA*, ASHWAGANDHA, MICROPROPAGATION, NODAL EXPLANT, BAP, NAA, IBA.

INTRODUCTION

Application of biotechnology for conservation of important plant species has been given priority under circumstances, in particular when many valuable plant genetic resources are getting decimated rapidly from natural flora (Kumar *et al*., 2013). 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).Many study revealed that cultivation of medicinal plants especially high value medicinal plants is creating new dimension in the field of agriculture. Indian herbal industry is at blooming stage. However, cultivation

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of medicinal plant is not easy. It is a challenging task because very little knowledge of seed biology. Efforts have not been made to search elite specimen and their propagation.

Withania sominifera is a green shrub found throughout the drier parts in India, Baluchistan, Pakistan, Afganistan, Shri Lanka, Congo, South Africa, Egypt, Morocco, and Jordan. In India, it is widely grown in the provinces of Madhya Pradesh, Uttar Pradesh, plains of Punjab andnorthwestern parts of the India like Gujarat and Rajasthan *Withania sominifera* (L.) Dunal, commonly called Indian ginseng is a member of the family Solanaceae, growing up to a height of 30-150 cm. It is considered as important medicinal plant in the Ayurvedic and indigenous medicinal system of India. It has many medicinal properties like anti-inflammatory, anticancer, antistress, anti-ageing, immune-modular, adaptogenic and shows the free radical scavenging activity It is used for treatment of tuberculosis, rheumatism, inflammatory conditions and cardiac diseases. It is also useful as abortificient, amoebicide, anodyne, bactericide, contraceptive and spasmolytic. The roots are also used as sedative for senile debility and for the prevention and inhibition of Alzheimer's disease (Sivanesan, 2007, Rout *et. al.,* 2011, Udaykumar *et al.* 2013 and Darwesh *et al.*, 2014).

Recently, Dharajiya *et. al.,* (2014) have analyzed the antibacterial activity of various solvents viz. aqueous, Hexane, ethyl acetate and methanol extracts of stem of *Withania somnifera* was evaluated against gram negative bacteria *Escherichia coli, Serratia marcescens*, *Pseudomonas aeruginosa* and gram positive bacterium *Bacillus cereus* by agar well diffusion method.

Traditionally *W. somnifera* is propagated from seeds, but the mature and healthy seeds are not always available for germination. The viability period of seeds is very short and their germination is also poor. The provision of alternative sources of *Withania somnifera* by encouraging its cultivation will go a long way in reducing their heavy dependence on the wild populations. Conventional propagation methods have proved to be inadequate to meet this challenge. Large scale production through plant *in vitro* regeneration will provide a means of putting the plant onto the market at lower prices.

Many earlier studies have reported *in vitro* propagation of Ashwagandha by using different explants, such as shoot tips (Hadeer *et al.*, 2014; Rani *et al.,* 2014; Baba *et al.,* 2013; Sivanesan, 2007; Ray and Jha, 2001and Roja and Heble, 1991), axillary bud (Rani and Grover, 1999), hypocotyl (Kulkarni *et al.* 2000), cotyledon (Kumar *et al.* 2013), leaf (Joshi and Padhya, 2010 and Kulkarni *et al.* 1996), seed (Supe *et al.*, 2006), cotyledonary leaf segments (Rani *et al.* 2003), callus of leaf (Arumugam and Gopinath 2013), shoot tip and root (Shrivastava and Dubey, 2007;) and the nodal areas, (Kumar *et al.,* 2011). The present study was done to determine the effect of growth hormones on shoots initiation, multiplication, rooting and hardening of Ashwagandha to standardize the micro propagation technique in Ashwagandha, as very less literature available for plant regeneration through nodal explant.

MATERIAL AND METHODS

The plant material of Ashwagandha was collected in plastic bags from Dhanwantari Medicinal and Aromatic plants project, Mahatma Phule Krishi Vidyapeeth, Rahuri. The plant material was propagated in College of Agricultural Biotechnology, Loni, Tal. – Rahata, Dist. – Ahmednagar, Maharashtra for further use.

Young nodal segments were selected as explants and were washed under running tap water for 15 minutes. Later immersed in 1% tween-20 for 1 minute and washed with sterile double distilled water for 2-3 times. The primarily surface sterilized nodal segments then rinsed in 70% ethanol for 1 minute under laminar air flow hood and washed with sterile double distilled water for 2-3 times. Finally rinsed with 0.1% mercuric chloride ($HgCl_2$) for 7-8 minutes and washed with sterile double distilled water for 4-5 times to remove all the surfactants.

One by one explants were placed on the filter paper to soak up the extra water. The nodal explants were then cut from both ends. Finally with the help of forcep the explants were inoculated on the surface of the MS media in such a way that ¾ part of the nodal explants would be in contact with MS media. The culture bottles containing explants inoculated on MS media supplemented with respective different conc. of BAP and was incubated at 25±3°C under white fluorescent light (2000 lux) for 16/8 hours light and dark conditions. In total 6 weeks of initiation period one subculture was done in 3rdweek and data for average shoot number per explants and average shoot length was recorded at the end.

The best initiated grown shoots were then transplanted on MS media supplemented with different concentrations of BAP and NAA. Again the data for average shoot number per explant and average shoot length was recorded after 6 weeks of incubation period.

For complete plant development regenerated shoots were excised and transferred to MS medium supplemented with different concentrations of IBA and data of average number of roots and average length of roots (cm) was recorded after 6 weeks of inoculation in growth chamber. Each treatment was repeated trice and statistical analysis were done by calculating the standard error (SE) for the treatments.

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).

RESULTS AND DISCUSSION

During initiation of explants it has been observed that nodal explants cultured on MS media devoid of growth hormone (Cytokinin) failed to induce the initiation of explants (Table 1). Out of all concentrations of BAP (0.5-4.0 mg/l) used for shoot initiation BAP (2 mg/l) promoted the shoot initiation i.e. average number of shoot (2±0.37) and average length of shoot (2.8±0.15cm) (Fig. 1-2). Irshad et al., (2013) reported that axillary and apical buds gave maximum response with respective to the initiation of explant when inoculation on MS media fortified with 1mg/l BAP. They found 75% response with 1-3 number of shoot/explant and shoot length was 2 cm. Darwesh et al., (2014) also found similar results i.e. number of shoot 2.57 and shoot length/explant 3.09 but with hormonal concentration as 2.0 mg/l BAP and 0.1 mg/l NAA.

Prominent *in vitro* response (average shoot number 5.3±0.41 and average shoot length 6.5±0.12cm) was observed of best initiated shoots cultured on MS media augmented with BAP (0.5 mg/l) + NAA (1.5 mg/l) (Table 2).. Increasing concentration of BAP and NAA resulted in gradually increased in *in vitro* response of shoots, while further increased concentration was found to be directly proportional to poor response of the shoot multiplication (Fig. 3). Rani et al., (2014) observed the different concentration of NAA and BAP were showing the best result for shoot elongation and direct shoot regeneration at 0.5 mg/l BAP with 3.0 mg/l and 2.0 mg/l NAA. Similar results were found by Rout et. al., (2011) when different growth hormone tested in augmentation with MS media for shoot elongation. 2.0 mg/l BAP with 1.0 mg/l NAA was found to be best eliciting 82.3% shoot induction with highest shoot number 4.8 shoots/callus and shoot length was 4.3 cm.

Finally initiated elongated shoots were excised and implanted on MS media augmented with IBA (1-5 mg/l) (Table 3). The optimum root induction i.e. average number of roots (12±0.20) and average root length (9.8±0.26cm) was obtained on MS media supplemented with 2 mg/l IBA (Fig. 4). The rooting results were found to be consonance with results obtained by Sivanesan (2007); observed that half strength MS media containing 2.0 mg/l IBA was found to be the best. It produced 100% rooting with 16 roots/shoot and length was 10.5cm. Kumar et al., observed optimum rooting i.e. 72% with root length 11 cm and number of roots/shoot was 25 on full strength MS media supplemented with 5.0 mg/l IBA.

At the end, well rooted plants 6.0 - 8.0 cm height obtained from rooting medium were transferred to plastic tea cups for hardening (Fig. 5-6). Afterward, individual cups with single plant were transferred to Polyhouse and 75% relative humidity was maintained. Overall, 90% of plants survived in the hardening process (data not shown) and these plants were established successfully in the experimental field.When regenerated plantlets were transferred to the field, 87% survival rate was obtained by Deshmukh *et al.* (2012).Arumugam *et al.*, (2013) observed the regenerated plants were successfully hardened and acclimated 85% of plantlets survived well under natural conditions after transplantation.

The production of Ashwagandha roots through conventional methods of cultivation (seed) is less than the requirement due to numerous reasons viz. poor yield, takes long time, poor viability of seeds, susceptibility of the seeds and seedlings to fungal infections like seedling mortality and blight, leaf blight, seed rotting (Misra *et al.,* 1997). This medicinally significant plant species has been depleted from its natural habitat and is now included in the list of endangered species (Kanungo and Sahoo, 2011; Patel and Krishnamurthy 2013) by the International Union for Conservation of Nature and Nat-

Table 1: Effect of BAP on shoot initiation from nodal explant.

Sr. No.	MS medium + BAP(mg/l)	Avg. shoot number (mean±SE)	Avg. shoot length (cm) (mean±SE)
1.	MS medium + 0.0 (control)	-	-
2.	MS medium + 0.5	1 ± 0.28	1 ± 0.26
3.	MS medium + 1.0	1.3 ± 0.05	1.6 ± 0.43
4.	MS medium + 1.5	1 ± 0.20	1.4 ± 0.25
5.	MS medium + 2.0	2 ± 0.37	2.8 ± 0.15
6.	MS medium + 2.5	1.3 ± 0.11	1 ± 0.20
7.	MS medium + 3.0	1.3 ± 0.41	1.2 ± 0.11
8.	MS medium + 3.5	1 ± 0.20	1.6 ± 0.15
9.	MS medium + 4.0	1.6 ± 0.41	2.3 ± 0.34

FIGURE 1 TO 6: The successive stages of *in vitro* propagation of *Withania somnifera*. (Fig. 1) Direct regeneration of shoot from nodal segments as a explants of ashwagandha cultured on Murashige and Skoog media. (Fig. 2) Initiation of the multiple shoot formation (Fig. 3-4). Development of more number of multiple shoot. (Fig. 5-6) Root formation from regenerated shoot.

ural Resources (Kavidra*et al.*, 2000; Supe*et al.*, 2006). The rapid multiplication of Ashwagandhaby tissue culture techniques can help to solve these problems and the benefits are extensive in the agricultural world.

In vitro propagation of Ashwagandha has been achieved by using nodal segment as explant. The explant was initiated on MS media supplemented with different concentration of BAP (0.5 to 4.0 mg/l) resulted in best response on BAP (2.0mg/l) produced in terms of average number of shoots 2±0.37 and average shoot length was 2.8±0.15cm. The best initiated shoots then transferred for multiplication on MS medium supple-

Table 2: Effect of BAP and NAA on shoot multiplication of Ashwagandha.

Sr. No.	MS medium +BAP(mg/l) + NAA (mg/l)	Avg. shoot number (mean±SE)	Avg. shoot length (cm) (mean±SE)
1.	MS medium + 0.5 + 0.5	2.6 ± 0.25	3.4 ± 0.28
2.	MS medium + 0.5 + 1.0	1.6 ± 0.30	2 ± 0.32
3.	MS medium + 0.5 + 1.5	5.3 ± 0.41	6.5 ± 0.12
4.	MS medium + 0.5 + 2.0	4 ± 0.43	4.7 ± 0.23
5.	MS medium + 0.5 + 2.5	2.6 ± 0.36	2.3 ± 0.23
6.	MS medium + 0.5 + 3.0	2.5 ± 0.32	2.1 ± 0.11

Table 3: Effect of IBA on rooting of Ashwagandha.			
Sr. no	**MS medium + IBA (mg/l)**	**Avg. root number (mean±SE)**	**Avg. root length (cm) (mean±SE)**
1.	MS medium + 1.0	6.6 ± 0.37	5.3 ± 0.20
2.	MS medium + 2.0	12 ± 0.20	9.8 ± 0.26
3.	MS medium + 3.0	5.3 ± 0.15	4.7 ± 0.43
4.	MS medium + 4.0	3.6 ± 0.25	4 ± 0.32
5.	MS medium + 5.0	3.3 ± 0.36	3.7 ± 0.28

mented different concentration of BAP in combination with NNA. After six weeks of incubation BAP and NAA (0.5mg/l+ 1.5mg/l) produced maximum average number of shoot 5.3±0.41 and average shoot length 6.5±0.12cm. MS medium supplemented with IBA 2.0mg/l produced maximum average of number roots 12±0.20 and average root length was 9.8±0.26cm. After rooting, rooted plantlets were successfully established in primary and secondary hardening. After 30 days of inoculation on rooting medium, the rooted plantlets were removed from the culture tube and washed with distilled water. These *in vitro* derived plantlets were transferred to plastic pots containing mixture of sand and FYM in 1:1 ratio for 18 days for hardening. It was concluded from this study that plant regeneration from nodal explant of *W. somnifera* offered a great Potential in agriculture and this in genetic transformation of this important species. The protocol can be exploited for *in vitro* generating new genetic variability and production of bioactive constituents from the plant.

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