ISSN 2278- 4136

ZDB-Number: 2668735-5

IC Journal No: 8192

Volume 2 Issue 2

Online Available at www.phytojournal.com

Journal of Pharmacognosy and Phytochemistry

# Feasibility studies on *in vitro* mass-scale propagation of Indian Ashwagandha (*Withania somnifera)* cultivars for commercial purpose

Pooja Patel, R. Krishnamurthy*

1. Maliba Research Centre for Excellence in Tissue Culture, Maliba Campus, C. G. Bhakta Institute of Biotechnology, Maliba Campus, Uka Tarsadia University, Bardoli, Dist. Surat, Gujarat, India-394350
[E-mail:krishnamurthy@utu.ac.in ; krisnashanti@gmail.com; Tele: +91 9825349279]

The explants with higher regeneration potential raised through *in vitro* germination of seeds of two genotypes of *Withania somnifera* namely: WS-100 and WS-134 were cultured on MS basal media fortified with 0.6mg/ml BAP and 0.4 mg/ml IAA. Culture medium MS + 0.2 mg/l BAP + 30.0 gm/l sucrose + 7.5 gm/l agar, induced callus in higher frequencies in both the varieties. While in multiple shooting, maximum shoots were observed in WS 100 with MS+0.2 mg/lit BAP+0.2 mg/lit IAA and in WS 134 with MS+0.3mg/l BAP + 0.2 mg/l IAA. Higher *in vitro* rooting response was achieved on rooting medium MS + 5.0 mg/1 IBA + 25.0 gm/1 sucrose + 7.5 gm/1 agar in WS 100 and in WS 134 with MS +0.6 mg/l BAP+ 2.5 mg/1 IBA + 25.0 gm/1 sucrose + 7.5 gm/1 agar. In terms of *in vitro* genotypic response, genotype WS-100 was found significantly superior to WS-134 for the most of the attributes investigated. Regenerated plantlets were established successfully in the field after primary and secondary hardening. The present investigation brought out possibility of mass-scale *in vitro* (micropropagation) production of Indian Ashwagandha cultivars.

*Keyword*: *Withania somnifera* cultivars, Micropropogation and MS basal Medium.

## 1. Introduction

*Withania somnifera* (L.) Dunal, is an erect, evergreen, perennial shrub and member of Solanaceae family is a widely used medicinal plant considered as aphrodisiac and rejuvenating and useful in the treatment of inflammatory, anti tumour agent[1], it is well known for years as an important drug in Ayurvedic formulation. Root of the plant *Withania somnifera* (Ashwagandha) reportedly exhibit antioxidant, immunomodulatory and haematopoietic properties[2].

Ashwagandha roots used in both Ayurveda and Unani medicines. In ayurvedic system of medicine, there is several products where Ashwagandha used as single plant based formulation and therefore, there is huge demand for root raw material in industries necessiating its cultivation in large scale. Ayurvedic Industries prepares Ashwagandha root with having quality of less branched roots, low fibre and high carbohydrate contents. Roots are prescribed as medicines for hiccups, several female disorders, bronchitis, rheumatism, dropsy, stomach and lung inflammation, and skin diseases. The roots used in medicines prescribed for curing disability and sexual weakness in males[3]. According to red list of threatened species, 44 plant species are critically endangered, 113 endangered and 87 vulnerable. *W. somnifera* proved to be 99.75% of the endangered medicinal plants[4,5]. As over harvesting of *W. somnifera,* the plant is going to

be endangered condition in the Southern India[6]. The active pharmacological components of *Withania somnifera* constituents are withanolides (Steroidal lactones with ergostane skeleton) and alkaloids[7]. The active constituent of Indian *Withania somnifera* are withaferin-A and withanolide-A, both are present in leaves and roots of the plant, used as a source of drugs. Total alkaloid content in the root of the Indian cultivars has been reported to be between 0.13 and 0.31%, which showed antitumor and radio sensitizing effects in animal models[8]. It also possesses anti-stress, immunomodulatory, anti-oxidant and antibacterial activity[9,10,11].Due to high medicinal value, this plant is collected from wild and used as raw material for bulk manufacturing in medicinal industries, leading to over exploitation and it has now become an endangered plant species. The main problems for commercial cultivation of Ashwagandha in India, is that this crop is raised through direct seed broad casting along with monsoon rains as 6 months rainfed root crop and therefore, there is tremendous variatious on root growth, root morphology, root yield and active ingredients among plants of the same cultivar. Hence, tissue culture plants through micropropagation are desirable. Micropropagation of *Withania somnifera* employs different explants such as shoot tips, auxiliary meristems, auxiliary leaves, auxiliary shoot and hypocotyl and root segments has been demonstrated[12]. There is also limitation in commercializing Ashwagandha crop through seeds, as the maximum seed viability retain only upto one year. Due to poor viability of stored seed and lack of standardized protocol for *in vitro* multiplication using recommended cultivars, the present study was carried out to standardize mass scaling- up Tissue Culture plants of Indian Ashwagandha cultivars.

*Withania somnifera* can be propagated by sexual (seeds) method. Seed propagation, however, is not always satisfactory, since the heterogenetically the strain produces a great deal of variation. Alternatively multiplication through cuttings give rise to less ramified plants and is consequently less productive than plants obtained from seeds. The requirement of *Withania somnifera* has sharply raised due to its popularity owing to a large scale unrestricted exploitation. This medicinally important plant species has now, been depleted from their natural habitat and hence included in the list of threatened species by The International Union for Conservation of Nature and Natural Resources[13]. Keeping this fact in view, the present investigation was focused for developing *in vitro* regeneration of plants from the two Indian Ashwagandha Cultivars.

(a) (b)

**Fig 1: (a)** *Withania somnifera* cultivar WS-100, **(b)** Seeds of *Withania somnifera* cultivars WS-100

## 2. Materials and Methods

### 2.1 Collection of Plants

Seeds of *Withania somnifera* of two different cultivars (WS-100 & WS-134) of India procured from the Regional Medicinal Plants Center, Agricultural University campus, Anand, India.

### 2.2 Surface Sterilization

The seed explants were washed thoroughly with 30% (v/v) commercial Sodium hypochloride (5.25%v/v) for 10-12 min, 5% v/v of Isopropyl alcohol (IPA) for 30 Sec. Subsequently they were sterilized by tween-20(2drops/100ml) and then with 0.1% $HgCl_2$ solution for 1-2 minutes and again washed well in distilled water for 3-4 times to remove the traces of $HgCl_2$ (Mercuric chloride).

### 2.3 Culture Media

The all seeds were implanted on sterile medium consisting of salts and vitamins of MS medium[14], supplemented with 0.8% (w/v) Agar and 3% (w/v) Sucrose.

### 2.4 Culture Conditions

All cultures were maintained at 25±2°C under 16 hrs photoperiod at a photosynthetic (2000-2500) lux, provided by cool daylight fluorescent lamps (Philips).

### 2.5 Inoculation

Seeds of both the cultivars of *Withania somnifera* were kept for *in vitro* germination in MS media containing 0.1-0.6 mg/l of BAP and 0.1-1.0mg/l GA3. (Figure.1, A & B)

### 2.6 Initiation

After germination the plant were transferred to MS basal medium containing BAP-0.1mg/l and kept in the growth room for 4 weeks (Figure 2, C & D).

### 2.7 Shoot Multiplication

Shoots were subcultured in the MS media supplemented with the range of BAP 0.1 1.0 mg/l and IAA 0.1 0.6 mg/l in order to produce maximum number of multiple shoots. (Figure 3, E & F).

### 2.8 Rooting

After multiple shooting, the shoots were transfered into the rooting medium along with different concentration of NAA and IBA for developing of roots (Figure 4, G & H).

### 2.9 Primary Hardening

4-6 week old rooted shoots were removed from the culture bottles. After washing away the Agar with water, they were transferred into protray containing cocopet and soil of 1:1 ratio and were kept in the Green House (Figure 5, I & J). After acclimatization in the Green House for 4-6 week, they were transferred to Net House for secondary hardening.

### 2.10 Secondary Hardening

After primary hardening, plants were transfered for secondary hardening, to poly bags containing the combination of Soil, FYM(Farm Yard Manure) and Sand in the ratio of 1:1:1 (Figure 6, K & L).

## 3. Result and Discussion

### 3.1 Callus Induction

Callus initiation was observed in both the cultivars of *W. somnifera* within 3 to 4 weeks on MS media supplemented with BAP (0.6mg/l). The morphology of callus is recorded (Table 1).It was observed that cultivar WS 134 produced callus of pale green colour and fragile, whereas WS 100 witnessed pale yellow colour and hard callus. The shoot induction from callus was not attempted due to changes of plant variation in callus raised plants.

**Table 1.** Callus morphology of Ashwagandha cultivars after 3-4 weeks

| Observation | WS 100 | WS 134 |
|---|---|---|
| Colour<br>Texture | Pale Yellow<br>Hard | Pale Green<br>Fragile |

### 3.2 Multiple Shoot Induction

Multiple shoots developed from the shoot cuttings of *in vitro* seed raised plants on MS medium with 0.2 mg/ml IAA and various combination of BAP (0.1 1.0 mg/l). Both cultivars WS-100 and WS-134 have showed maximum of 8-9 shoots/explants (80-90% response) with 0.2 mg/l IAA at 4 weeks.

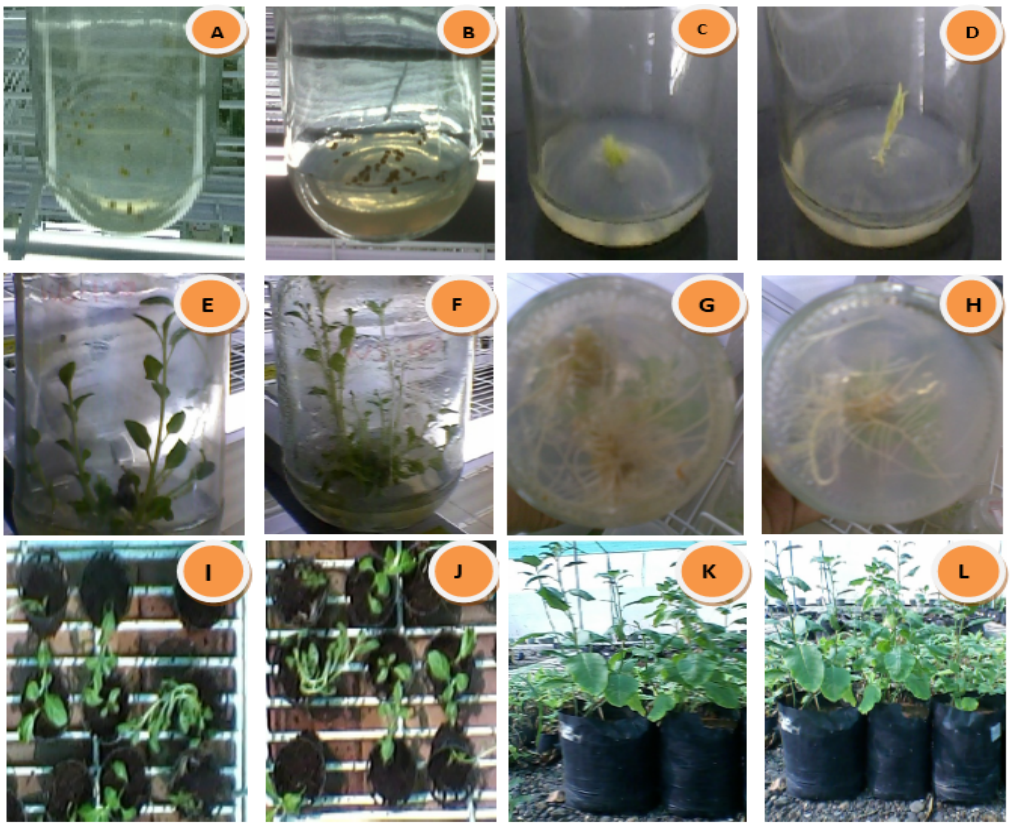


**Fig 2:** *In vitro* germination of seeds of Ashwagandha cultivars WS-100 (A) and WS-134 (B) on MS media; Shoot growth of *in vitro* seed raised plants of WS-100 (C) and WS-134 (D); Multiple shooting of WS-100 (E) and WS-134 (F), on MS media; Root induction of WS-100 (G) and WS-134 (H); Plantlets of WS-100 were exposed to primary hardening in portray to acclimatization for 4-6 weeks in Net House (I, J); Primary hardening plants of WS-100 (K) and WS-134 (L) were transferred to polybags for secondary hardening in Green House.

**Table 2:** Effect of different cytokinins in combination with IAA (0.2 mg/l) on shoot multiplication of *Withania somnifera* cultivars on MS medium at 4 weeks.

| Plant growth regulators (mg/l) | | Percentage response | | Number of shoots/explant | |
|---|---|---|---|---|---|
| BAP | IAA | WS 100 | WS 134 | WS 100 | WS 134 |
| 0 | 0 | 0 | 0 | 00 | 00 |
| 0.1 | 0.2 | 20 | 15 | 2.1 ± 0.4 | 1.10±0.2 |
| 0.2 | 0.2 | 90 | 80 | 9.9 ± 0.1 | 8.5±0.3 |
| 0.4 | 0.2 | 75 | 65 | 7.4 ± 1.1 | 6.9±0.8 |
| 0.6 | 0.2 | 70 | 60 | 7.6 ± 0.2 | 6.5±0.4 |
| 0.8 | 0.2 | 60 | 55 | 6.1 ± 0.3 | 5.5±0.4 |
| 1.0 | 0.2 | 55 | 45 | 5.8 ± 0.4 | 5.2±0.8 |

### 3.3 Root Induction

The elongated shoots were separated and transferred to different concentration of rooting hormones with MS basal medium and kept under light in the growth room. Observations on root formation recorded at 4 weeks. Maximum rooting of 80-90% (4-5 roots/shoot) was recorded with 0.4 NAA+ 0.4 IBA mg/l (Table 3).

**Table 3:** Effect of various auxins on root formation in *Withania somnifera* cultivars on MS at 4 weeks.

| Auxin conc. (mg/l) WS100 | WS134 | Percentage rooting WS 100 | WS134 | Number of roots/shoot WS 100 | WS134 | Basal callus WS100 | WS134 | Growth of Root WS 100 | WS134 |
|---|---|---|---|---|---|---|---|---|---|
| **NAA** | **NAA** | | | | | | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | - | - | - | - |
| 0.2 | 0.2 | 25 | 20 | 1.15 ± 0.3 | 1.01±0.2 | + | + | + | + |
| 0.4 | 0.4 | 35 | 25 | 1.90 ± 0.1 | 1.19±0.4 | + | + | ++ | + |
| 0.6 | 0.6 | 40 | 35 | 2.83 ± 0.2 | 2.15±0.3 | + | + | +++ | +++ |
| **IBA** | **IBA** | | | | | | | | |
| 0.2 | 0.2 | 40 | 35 | 1.25 ± 0.1 | 1.10±0.2 | - | - | + | - |
| 0.4 | 0.4 | 55 | 45 | 2.20 ± 0.4 | 2.10±0.3 | - | - | ++ | + |
| 0.6 | 0.6 | 60 | 55 | 2.95 ± 0.2 | 2.25±0.4 | - | - | +++ | ++ |
| **NAA + IBA** | | | | | | | | | |
| 0.2 + 0.4 | | 30 | 25 | 1.20 ± 0.1 | 1.10±0.2 | - | - | + | - |
| 0.4 + 0.4 | | 90 | 80 | 5.10 ± 0.2 | 4.75±0.5 | - | - | +++ | ++ |
| 0.6 + 0.4 | | 75 | 65 | 3.80 ± 2.7 | 2.90±1.5 | + | - | ++ | + |
| 0.2 + 0.6 | | 60 | 55 | 2.75 ± 0.1 | 2.25±0.3 | + | - | + | - |
| 0.4 + 0.6 | | 70 | 60 | 3.25 ± 0.4 | 3.15±0.2 | - | - | ++ | + |
| 0.6 + 0.6 | | 55 | 45 | 2.01 ± 0.2 | 1.70±0.3 | + | - | + | - |

*Symbol indication: - = no growth; + = low growth; ++ = optimum growth; +++ = maximum growth; ± SD of 5 replications

### 3.4 Primary and Secondary Hardening

Plantlets with fully expanded leaflets and well developed roots were removed from culture bottle and washed thoroughly to remove all traces of agar and transferred to Soil, FYM and Sand in 1:1:1 ratio mixture and kept in a controlled condition for one month (Figure 2, K & L).Both the cultivars showed 100 survival and at the end of one month, the plant were transferred to the field. Growth measurements were taken and presented in the Table 4.The growth measurements of tissue culture plant of WS-100 and WS-134 at the end of one month witnessed 15-20 cm root length with 28-32 cm shoot height and 3 4 side branches/plant. In the earlier studies, it was attempted to produce tissue culture plants using shoot tips[12] and axillary buds[8] as explant sources of wild plant of Ashwagandha. Indian Ayurvedic Industry prepares to buy Ashwagandha roots of cultivated source materials as the wild grown plants exhibited more fibrous and low carbohydrate content of roots with much branched root system having perennial habit. Whereas, the cultivars WS-100 and WS-134 selected in the present study is of 6-7 months crop duration, having annual habitat. However, in the present study, the tissue culture methods standardized with commercial cultivars of Indian Ashwagandha making the micropropogation method feasible tool for large scale production of Ashwagandha cultivars. Furthermore, the technology will provide uniformity among plants and also quality.

**Table 4:** Morphological features of two *in vitro* raised cultivars of *W. somnifera* at one month after transferred to green house

| Features | WS-100 | WS-134 |
|---|---|---|
| Diameter of stem | 0.3 | 0.2 |
| Colour of stem | green | green |
| Length of leaves | 12 cm | 10 cm |
| Height of stem | 32 cm | 28 cm |
| Side branches per plant | 3 | 4 |
| Length of root | 20 cm | 15 cm |
| Colour of root | off white | off white |

## 4. Conclusion

It was observed that among two cultivars of Ashwagandha tested for mass-scale propagation using tissue culture (micropropagation) method, the cultivar WS-100 exhibited faster growth when compared to WS-134. Therefore, cultivar WS-100 could be considered for commercial cultivation of tissue culture plants. Bulk production by tissue culture method of Ashwagandha is ideal because of the variability among seed grown plants in commercial cultivation. Moreover, large number of plants required as the plant spacing normally followed at 15 cm× 15 cm or 30 cm×30 cm as a densely population crop. The dense population in commercial cultivation is prepared in order to avoid or reduce more branching of shoot system as the root is the only official commercial parts. In commercial cultivation, the crop reported to produce 500-600 kg dry root yield/ha.

## 5. Acknowledgement

We are thankful to the management of Maliba Campus, UKa Tarsadia University, Bardoli, Gujarat for providing financial support for this work. Help rendered by Mr. Sunil Patil, Senior Laboratory Supervisor, Tissue Culture Laboratory is greatly acknowledged.

## 5. References

1. Naidu PS, Singh A and Kulkarni SK, Effect of *Withania somnifera* root extract on haloperidolinduced orofacial dyskinesia: Possible mechanism of action. J. Med. Food, 2003; 6: 107-114.
2. Mishra LC, Singh BB and Dagenais S. Scientific basis for the therapeutic use of *Withania somnifera* (Aswagaandha), a review. Altern. Med. Rev.2000; 5:334-346.
3. Joshi C, Gajbhiye N, Phurailatpam A, Geetha KA and Maiti S. Comparative morphometric, physiological and chemical studies of wild and cultivated plant types of *Withania somnifera* (Solanaceae). Curr. Sci.2010; 99: 644-650.
4. Siddique NA, Bari MA, Pervin MM, Nahar N and Banu LA. *et al.*, (2005). Screening of Endangered Medicinal Plants Species by Questionnaire Survey in Barind Tract in Bangladesh. Pakistan Journal of Biological Sciences. 2005;8 (12):1783-1793.
5. Rahman M. Red data Book of Vascular plants. Bangladesh National Herbarium. Dhaka, Bangladesh,2001
6. Manickam VS, Mathavan RE and Antonisamy R. Regeneration of Indian ginseng plantlets from stem callus. Plant Cell Tissue Organ. Cult.2000;62: 181-185.
7. Elsakka M, Grigorescu E, Stanescu U, Stanescu U, Dorneanu V. New data referring to chemistry of *Withania somnifera* species. Rev Med Chir Soc Med Nat Iasi.1990; 94:385–387.
8. Sharma S, Sharma MC and Kohli DV. In vitro micro propagation of medicinally important roots and axillary bud combination. Journal of Optoelectronic and biomedical materials 2009;4: 379-381.
9. Kupchan SM, Doskotch RW, Bollinger P, Muphail AT, Sim GA and Saenz RJ. The isolation and structure elucidation of a novel steroidal tumor inhibitor from *Acnistus arborescens.* J. Am. Chem. Soc,1965; 87:5805.
10. Devi PU, Sharada AC ,Solomon FE and Kamath MS. *In vitro* growth inhibitory effect of *Withania somnifera* (Ashwagandha) on a transplantable mouse tumor sarcoma 180. Ind. J. Exp. Biol 1992;30:169-172.

11. Devi PU, Sharada AC and Solomon FE. Antitumor and radiosensitizing effects of *Withania somnifera* (Ashwagandha) on a transplantable mouse tumor sarcoma 180. Ind. J. Exp. Biol 1993;31:607-611.
12. Sen J and Sharma AK. Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. Plant Cell, Tissue and Organ culture. 1991;26:71-74.
13. Kavidra NT, Neelesh CS, Vaibhav T and Brahma D. Micropropagation of *Centella asiatica* (l.) a valuable medicinal herb. Plant Cell, Tissue and Organ culture 2000;62:175- 179.
14. Murashige T, Skoog F.A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant 1962;15:473-497.