EFFECT OF GROWTH REGULATORS ON DIRECT IN VITRO CULTURE AND CLONAL PROPAGATION OF WITHANIA SOMNIFERA

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Abstract

Indians frequently use the well-known medicinal plant *Withania somnifera* (L) Dunal to treat a wide range of clinical conditions. Asgand, a common chemical, has long been employed in the Unani and Ayurvedic medical systems, either alone or in conjunction with other therapies. It is mentioned in Discords' book "Kitabul-Hashaish" (78 AD). *Withania somnifera* is a plant that has anti-inflammatory (Mohallil-e-Warm), sedative (Musakkin), alterative (Muaddil), and aphrodisiac (Muqawwi-e-Bah) characteristics. Asgand is frequently discovered in the roots and leaves of this plant. A concerted effort has been made to look into a variety of topics related to the medication, including phytochemical and antibacterial research, keeping in mind the therapeutic properties of *Withania somnifera*. Since this plant is in danger of extinction owing to overuse, in vitro tissue culture is one of the greatest ways to preserve it and generate several Withania plants (from any part, fragment, or cell of plants due to totipotency) in a small space and at any time of year.

Keywords: *Withania,* Asgand, Medicinal, *In vitro,* Totipotency, Mass propagation, etc,.

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INTRODUCTON

Withania somnifera, a herb is a type of medicinal plant that belongs to the *Solanaceae* family and is recognized in Sanskrit as "Ashwagandha". It is a perennial plant with numerous therapeutic purposes in each typical and modern-day medicine (Das *et al*., 2010). The species is additionally seen as 'Indian Ginseng' due to the recovery residences of the roots (Andallu and Radhika, 2000; Winters, 2006). *Withania somnifera L*. (Dunal) is a member of Solanaceae, also known for thousands of years by Ayurvedic practitioners. The plant contains an abundance of secondary metabolites, also known as bioreactors, such as alkaloids, flavonoids, lactones, and steroidal, all of which have beneficial properties and are found in ninety extraordinary Ayurvedic formulations. It possesses a wide spectrum of pharmacological houses, along with antimicrobial, anti-inflammatory, anti-stress, antitumor, neuroprotective, cardio protective, and lots of greater for use within the treatment of biological tactics. Since Ayurvedic times, the plant has been used to cure asthma, bronchitis, emaciation ulcers, sleeplessness, and dementia (Gaurav, *et al.*, 2015, 2016a, 2018; Gaurav and Kumar, 2019). It is used as a liver tonic and astringent. Anti-rheumatic, anti-arthritic, anti-stress, abortifacient, adapt genic, anti-inflammatory, anti-anxiety, immunomodulatory activity, anti-tumour, anti-depression, and aphrodisiac properties are among the various pharmacological actions of the plant (Devi *et al*., 1992). Major biochemical factors such as alkaloids and steroids are accountable for Ashwagandha's therapeutic benefits. Withanolides is main tropane alkaloids of the plant that posses anti-tumor activity (Sethi *et al*., 2014). *Withania* (Ashwagandha) is an effective herb that may be utilized in blends and supplements to produce a variety of benefits. It is reported as natural tonic and health ingredients in vedas and viewed as 'Indian Ginseng'in everyday Indian computing device of medicine (Gaurav et al., 2016b). In India, the anticipated yearly production of Ashwagandha roots is more than 1500 tonnes, with a demand of around 7000 tonnes, necessitating further planting and production. For over 4000 years, Ashwagandha, also known as Indian ginseng or winter cherry, has been utilized as a useful plant in Ayurvedic and indigenous medicine. It prefers dry stony soil with image voltaic to incomplete pigmentation. To reproduce it can be grown from seed indoors the early spring, or from greenwood cuttings internal the later spring (Deni and Bown., 1995). The roots, leaves, and fruits (berries) are all highly medicinal. Ashwagandha is a well-known Ayurvedic rejuvenative plant that is used in many tonics and formulations. It is the best rejuvenative since it helps maintain adequate feeding of the tissues, notably muscle and bones, while also supporting the adrenals and reproductive system's proper operation. It thrives in arid, subtropical climates. The primary Ashwagandha-producing states in India include Rajasthan, Punjab, Haryana, Uttar Pradesh, Gujarat, Maharashtra, and Madhya Pradesh. But the risks of fungal infections are very high in these plants. *Withania somnifera* possessing little white flowers that bloom mostly in the rainy and winter seasons and can mature into fruit in the winter. Many different biological approaches can be used to extract plant compounds from the roots, leaves, and branches of the plant. There is insufficient scientific evidence that it is safe or effective for treating any disease. Although antibiotics limits the emergence of most of the prevalent bacterial diseases which affected man and animals in epidemic proportions but at the same time their inadvertent and overuse results in the development of antibiotic resistance (Devi *et al.*, 1992).

MATERIAL & METHODS

Collection of Explants: Healthy young two months old *Withania somnifera* plants were taken from the Medicinal Plant Garden of School of Agriculture, S.G.R.R. University, Dehradun.

Surface Sterilization:

Surface sterilization is a technique which entails the immersion of explants into appropriate attention of chemical sterilant(s) or disinfectant(s) for a detailed time ensuing within the establishment of an infection-free culture.

Young, healthy nodal segments (2-3 cm) were cut from the mother plant and washed for 8-10 minutes with mild detergent (tween 20), then rinsed under running tap water to remove detergent. After that, the explants were treated with 0.4 percent Bavistin + 0.4 percent PVP for 45 and 30 minutes, respectively, before being washed with double distilled water. Explants were then surface sterilized for 1-4 minutes in Laminar air flow with 0.1 percent $HgCl_2$, and the surplus $HgCl_2$ was washed away with 4 to 5 washes with double distilled sterilized water.

Culture Media:

In vitro regeneration is a technique for growing plant cells, tissues, and organs on artificial nutrient media under controlled tissue culture conditions to achieve various multiplication goals,

as well as for the development of plant systems as bioreactors for the production of value-added compounds. *In vitro* regeneration of planted *Withania* has been examined due to the crop's significant commercial and economic value, as well as its possibility to be improved through genetic manipulation (Murashige and Skoog., 1962). In vitro plant regeneration may be performed via somatic embryogenesis or organogenesis.

For MS media, four stock solutions were prepared as follows

Stock I	macronutrients	10x
Stock II	micronutrients	100x
Stock III	Fe-EDTA	100x
Stock IV	Vit. And AA	100x

MS medium anticipation can be accomplished by diluting stock I, II, III, and IV with two-thirds volume of demineralized double distilled water, then adding my inositol (0.01 percent WV) and sucrose (3 percent w\v). Plant growth regulators (PGR) in the required amount were added to the medium and thoroughly dissolved using a magnetic stirrer and final required volume was perpetuate with the help of double distilled water. The pH was adjusted to 5.7-5.8 by using of HCl or NaOH. For the preparation of solid medium 0.6-0.8% (w\v) agar powder was dissolved by heat (Gaurav *et al*., 2015).

Medium and Glassware Sterilization:

Autoclaving at 15psi (1.04 kg/cm^2) pressure at 121^{0}C for 20 minutes steam sterilized all tissue culture medium and containers. Thermolabile substances were sterilized separately by filtration (0.22µm Millipore) before being added to the autoclaved media and properly blended after it was cooled at 40-45^{0}C. The media was then dispensed into radiation-sterilized autoclave culture tubes to solidify (Gaurav *et al*., 2015).

Culture Conditions:

The cultures vessels were incubated in a growth room. The growth room temperature was maintained at 25 ± 2°C and light intensity of 100 µEM-2 sec-1 (1000 lux) was provided using florescent tubes. A 16-hour light/8 -hour dark cycle was maintained.

Inoculation:

Young, healthy nodal segments (2-3 cm) of *Withania somnifera* were inoculated in solid MS media containing 0.5-3.0 mg/l of BAP, 1.0mg/l Kinetin and 1.5mg/l NAA were incubated for *in vitro* germination.

Shoot Induction and *In vitro* Morphogenesis:

After a few weeks, the regenerated shoots and apical meristem were aseptically removed and inoculated (under LAF) onto MS media supplemented with various hormones to commence a fresh cycle of multiplication using BAP (0.5, 1.0, 1.5, 2.0, 3.0 mg/l), KIN (1.0 mg/l), and NAA (1.5 mg/l). The healthy shoot buds were removed and placed on an MS basal medium to remove any remaining cytokine. After one week, the shoot buds were removed from MS basal media and transplanted to various rooting media, including varying BAP/KIN (mg/l) concentrations. The remaining agar was washed away after complete rooted, and the seedlings were placed to containers containing a 1:1 mixture of sterilized sand and clay. The acclimatized plants were transferred to pots containing regular garden soil after two weeks and kept in a greenhouse under natural day length circumstances. The beakers were gradually removed after 3-4 weeks, and the plant pots were exposed to partial shade for a brief time, with the time spent in the natural environment increasing at the same time. Finally, the plants were returned to their natural habitat.

Primary Hardening:

This is a method wherein the tissue culture plants advanced in synthetic media are habituated to grow in natural environment.

The culture bottles were emptied of 4-6 week old rooted shoots. They were moved into a container containing coco peat and soil in a 1:1 ratio and housed in the Green House after rinsing away the agar with water. After acclimatization in the Green House for 4-6 week, they were transferred to net house for assessment of secondary hardening.

Secondary Hardening:

The tissue cultures plant life developed in synthetic media are conditioned to grow in natural environments in this way. After primary hardening, plants underwent secondary hardening. To do this, poly bags containing a 1:1:1 mixture of soil, farmyard manure and sand were employed and the plants were grown 6-8 weeks inside a shaded place.

RESULTS

The explants (apical meristem & nodal section) of *Withania somnifera* (L.) Dunal were cultured on MS basal medium supplemented with different concentrations of BAP (0.5-3.0 mg/L), Kinetin (1.0 mg/L). The length was measured on a weekly basis for the first 1–2 weeks to see how they developed. After 5-6 days of inoculation, shoots began to appear, and the greatest results (optimum conc. for maximum shoot formation in the shortest period) were obtained with the BAP conc. 1.5 mg/L. Other concentrations (1.0 mg/L Kinetin) exhibited initiation after some time, however the responses were less effective. Apical meristem & nodal section of *W. somnifera* were then inoculated on MS basal media supplemented with BAP (1.5 mg/L).

Table 1. Effect of growth regulators on shoot induction in *Withania somnifera.*

S.NO	Medium + Growth hormones mg/l	% of shoot induction	No. of shoots per culture (Mean±SE)	Average shoot length in cm. (Mean±SE)
1	MS+0.5 BAP	70%	1.33±0.33	1.23±0.09
2	MS+1.0 BAP	75%	3.00±0.58	2.23±0.09
3	MS+1.5 BAP	78%	3.67±0.88	2.60±0.06
4	MS+2.0 BAP	60%	1.67±0.33	1.40±0.06
5	MS+0.5 KN	40%	1.33±0.33	1.50±0.06
6	MS+1.0 KN	50%	1.67±0.33	1.63±0.03
7	MS+3.0 BAP+1.0 KN	45%	1.33±0.33	1.37±0.09
8	MS+1.5 BAP+1.5 NAA	75%	3.00±0.58	2.27±0.07

All the values are expressed as Mean ±SE (n=3)

Subculture

After 15 days, the healthy shoot buds were removed from the initiation media and transferred to MS multiplication medium (MS1, MS2, MS3, MS4) supplemented with different concentration of hormone (MS1 (1.0 mg/L BAP), MS2 (2.0 mg/L BAP), MS3 (3.0 mg/L BAP + 1.0 mg Kinetin), MS4 (1.5 mg/L BAP + 1.5 mg/L NAA). The MS3 media had the most shoot regeneration.

Table 2. Effect of various hormone concentrations on shoot regeneration

Multiplication Media	No. of explants Inoculated	No. of shoots proliferation/ explants (Mean±SE)	No. of contamination	% Survival rate
MS1 media	10	3.67±0.33	-	40%
MS2 media	10	4.67±0.33	2	50%
MS3 media	10	5.67±0.33	-	70%
MS4 media	10	6.67±0.33	1	60%

1. MS1 (1.0 mg/L BAP); 2. MS2 (2.0 mg/L BAP); 3. MS3 (3.0 mg/L BAP+1.0 mg/L Kinetin); 4. MS4 (1.5 mg/L
BAP+1.5 mg/L NAA)

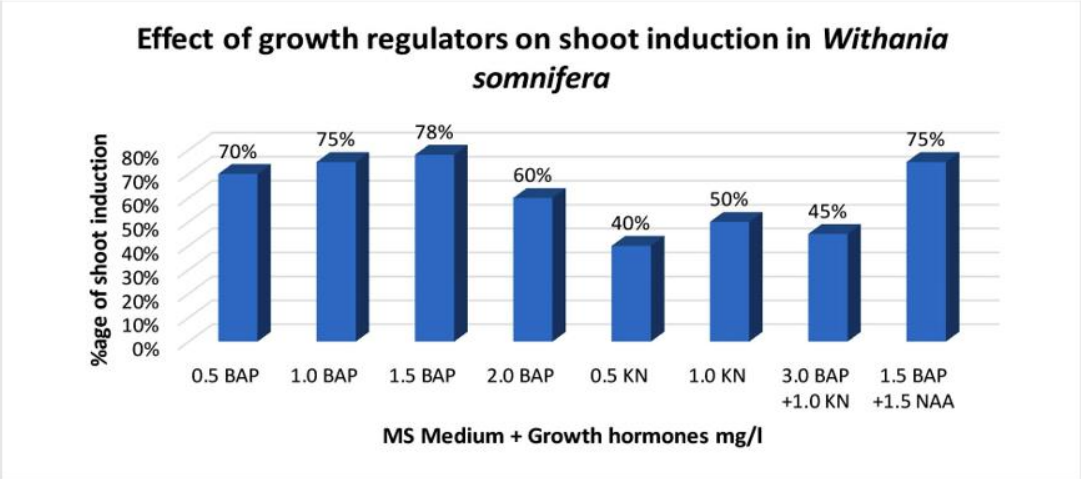

Figure 1 (Table 1). Effect of growth regulators on shoot induction in *Withania somnifera*

Figure 2 (Table 1). *In vitro* regeneration of shoot from nodal explant in *W. somnifera* in MS medium

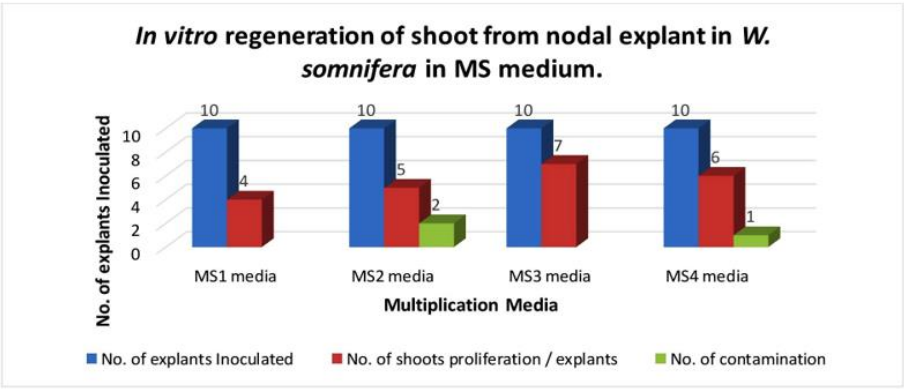

Figure 3 (Table 2). *In vitro* regeneration of shoot from nodal explant in *W. somnifera* in MS medium

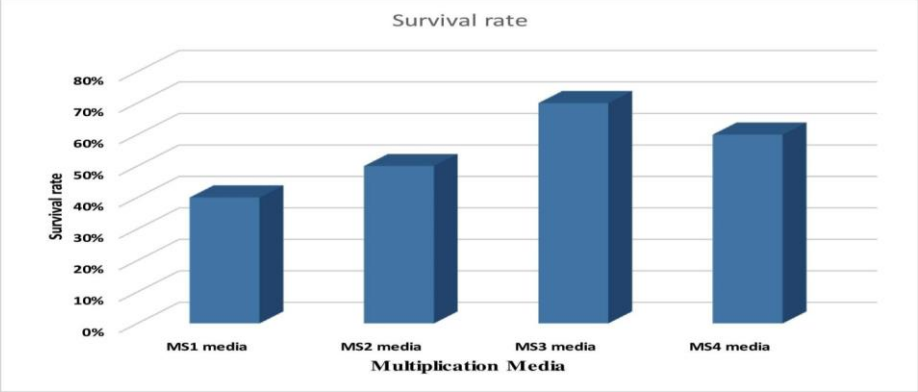

Figure 4 (Table 2). Survival rate

Figure 5 (Table 2). Effect of various hormone concentrations on shoot regeneration [MS3 (3.0 mg/L BAP+1.0 mg/L Kinetin)]

DISCUSSION

In vitro tissue culture is a useful biotechnological method for rapidly growing disease-free plants in order to preserve the genetic material of medicinal, uncommon, and exotic plants. *Withania somnifera* is an endangered therapeutic plant, (Kanungo and Sahoo, 2011; Gaurav *et al*., 2016b) categorize by the International Union for Conservation of Nature and Natural Resources (IUCNNR) (Supe and Roymon., 2006). As a result, a technique for propagating deemed worthy, medicinal plant was needed to meet the growing demand. As a result, the current study focuses on *in vitro* tissue culture of *Withania somnifera* for mass reproduction because very small parts of plant tissue organs are used as beginning vegetative tissues, which takes a short amount of time and is not affected by the season, whereas the traditional procedure takes a long time. *Withania somnifera* plant was successfully reproduced from nodal explants. It is possible to preserve *Withania somnifera* under tissue culture conditions using *in vitro* generated germplasm. An enhanced technique for rapid *in vitro* multiplication of the species has been designed to fulfil the goal, as it is a requirement of the study. A *Withania somnifera* high frequency regeneration process, has been standardized in this work, is easy, efficient, and time saving. Results indicated that the combinations of cytokinins and auxins in MS medium were most affective for shoot multiplication. Shoot buds were generated immediately from nodal explants, and many propagules were formed in clusters within six weeks of culture, demonstrating remarkable sprouting capacity. Explants of cotyledons were used to study how different combinations of cytokinins and auxins, BAP, kinetin and NAA, and cytokinins alone could drive the development of the shoot (BAP). In the current study, it was discovered that auxins (NAA) combined with cytokinins (BAP and KIN) were more successful at promoting shoot development than cytokinins (BAP and KIN) alone. Jhankare *et al*., 2011 has Discovered that isolated cotyledon explants of *Withania* genotypes were cultivated in the presence of BAP+2, iP, BAP+NAA, KIN+2,4-D, and KIN+NAA, they showed a strong organogenetic potential. The most effective combination of BAP (1.5 mg/l) and NAA (1.5 mg/l) for adventitious multiple shoot induction and shoot proliferation was found to be BAP (1.5 mg/l) plus NAA (1.5 mg/l) while 0.5mg/l of BAP and KIN was found less effective. It has been proven that Ashwagandha is an effective medicinal plant to cure several human diseases like other important medicinal plants (*Mentha, Nardostachys jatamansi, Adhatoda vasica, Mucuna puriens, bubble bush, Nyctanthes arbor)* (Bhoora, *et al.*, 2015; Pant, *et al.*, 2020, 2021, 2022; Sharma et al., 2022; Saini et al., 2021, 2022; Verma et al., 2021; Rawat et al., 2021).

CONCLUSION

Finally, the approach discussed here could be used to conserve and mass propagate *Withania somnifera* from nodal explants. Because of these numerous, unique, and desirable qualities of *Withania somnifera* mentioned above, we deemed it worthy.

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