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Rapid regeneration of *Withania* somnifera (L.) a valuable Medicinal plant using Micropropagation Technology

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ABSTRACT
Withania somnifera (L.) is well known for its medicinal properties. Metabolites of this plant contain various bioactive compounds that possess therapeutic potential.Tissue culture is an important approach for the multiplication of plants for improved agronomic performance using different concentration and combination of plant growth regulator. The aim of the present work to explore the application of tissue culture to develop an efficient in vitro tissue culture protocol to obtain maximum plantlet regeneration that can have a huge impact in ex situ conservation of biodiversity. The present study focused on in vitromicro propagation of Withania somnifera(L) through tissue culture, regenerated plants after acclimatization were transferred to soil, and they showed 80% survival. Murashige &Skoog (MS) media with different combinations and concentration of growth promoters viz.Indole Acetic Acid (IAA), 2, 4-D (2, 4 dichlorophenoxy acetic acid), and (Benzyl Amino Purine (BAP) regulators are used. The maximum callus induction was observed in the MS media supplemented with 2, 4-D (3mg/l) in internodalexplant of Withania somnifera. The best response in shoots formation was observed in media supplemented with BAP (0.2mg/l).Root formation was observed in 0.3mg/l in half strength MS media.
Keywords:*Withania somnifera(L.), Micro propagation, Growth regulator, Tissue culture*

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INTRODUCTION

The medicinal plant *Withania somnifera* (L.) commonly known as Ashwagandha belongs to familySolanaceae is well known for its wide spectrum of therapeutic potential throughout the world. It is very popular from ancient times as used in various traditional systems of medicine.*Withania somnifera* (L.) contains secondary metabolites e.g. withanolide, which possess very useful pharmacological effects that include antibacterial, anti-inflammatory, and control against infection [8]. Roots andleaves contain many active components that possess anti-inflammatory, antitumor, immunosuppressive and antioxidant properties moreover for stimulating strength and vigour [3, 4, 10]. Because of its importance,the prerequisite of *Withania somnifera* has abruptlyincreasing. According to red list of threatened species, *Withania somnifera* proved to be 99.75% of the endangered medicinal plant [24] and als oincluded in the list of threatened species by the International Unionfor Conservation of Nature and Natural Resources due to depletion from itsnatural habitat [9, 20, 26].

Micropropagation by shoot culture and somatic embryo techniques supports various crop plant enhancement programmes and progressively these approaches are used for the protection of endangered plant species.Several preceding researches have been reported about the production of secondary metabolites e.g. with an olides from tissue culture of

Withania somnifera species [2, 29, 20, 19, 20, 21, 11]**.** Several researchers [20, 30, 18, 12, 19, 22] have demonstrated the vegetative propagation of in vitro regeneration of *Withania somnifera* species using different explants such as shoot, auxiliary meristems, auxiliary leaves, auxiliary shoot, hypocotyls, root segments, node, and internodes.

In present study, wehave practiced in vitro protocol for rapid regeneration of *Withania somnifera* and establishment of plants in the field conditions.The present work deals with in vitro plant growth of *Withania somnifera* through tissue culture for propagation and ex-situ conservation. Regenerated plants after acclimatization were transferred to soil and they showed 80% survival. Weeds free germination of *Withania somnifera* is more effective and beneficiary through tissue culture method. Control use of fertilizers in cultivated crops at the time of germination may be effect the quality of plant and seeds. The rapid multiplication of *Withania somnifera* by tissue culture techniques can help to resolve these glitches and the benefits are extensive in the field of agriculture.

MATERIAL AND METHODS

Plant material

Plant of *Withania somnifera* (L.) were collected from the Green Houseof Biotech Park, Lucknow India.

Selection and surface sterilization of explants

The plant materials were excised from the field grown parent plant and the explants were prepared through appropriate trimming of the plant parts. Experiments were carried out with *Withania somnifera* to standardize the best media and two different explants were tested, viz. Inter-nodal,and leaves to observe the better totipotency and response. The explants were then washed under running tap water for 2-3 hours. They were treated with surfactant (teepol) for 10 minutes after then washed with salvon for 10 minute. After this the explants were washed with 70% ethyl alcohol for 30 seconds. Leaf explants were then treated 1-2 minute with 0.1% w/v $HgCl_2$ solution and nodal explants were with treated $HgCl_2$ for 2-4 minutes. However, the duration of the treatment was varied according to the nature and the source of the parent plant. Surface sterilized explants were then washed thoroughly with sterilized distilled water at least 4-5 times to get rid of even traces of $HgCl_2$ solution.

Implantation and Inoculation of explants

Experiments were carried out with *Withania somnifera* to standardize the best media and three different explants were tested, viz. Inter-nodal,and leaves to observe the better toti-potency and response. The basal medium in which the explants exhibit regeneration was further supplemented with various concentrations and combinations of growth regulators viz., cytokinin, auxin and Gibberellins, depending on the type of experiments carried out to study their effects on in vitro response of different explants.The surface sterilized explants were further trimmed to the required size by excising the cut end of the treated explants on a sterile petriplates. They were then implanted onto the culture medium. Nodal explants were implanted vertically with axillary bud pointing upwards, leaves, and inter-nodal explants horizontally on to the medium for callus induction response.

Incubation condition

The inoculated cultures were carefully labeled and transferred to the culture racks in the culture room. Cultures were kept for 16 hrs under the high intensity (4000 lux) white cool, fluorescent light. The temperature and relative humidity in the culture room varied between 24-30^{o}C and 60-70%, respectively.

Rooting and acclimatization

Shoots (7-8 cm long) with sufficient rooting were transferred to ½ strength MS liquidmedium with filter paper raft support for hardening, for two weeks. These well-developed rooted plantlets were then transferred to sterile vermiculite and covered with poly bags for 2 weeks to retain moisture. They were irrigated with ¼ strength MS medium without sucrose and kept less than 16 hr light and 25±2°C. Further, they were transferred to soil and normal growth of the potted plants was kept in room condition for 1 week.

RESULTS

Callus induction

The formulation of the best nutrient media, selection of right explants and roleof plant growth regulators either in single or in combination is a prerequisite for exploitation of tissue culture technology in ex situ conservation strategies. Keeping this background in mind a series of experimentation has been carried out in a medicinally important plant, *Withania somnifera*. The data was recorded after regular interval of six days. Callus induction response in leaves and internodes of *Withania somifera* media supplemented with, 2-4D (2mg/l) showed average response (++) as shown in table 1 and (3mg/l) showed maximum callus induction (++++) in internodal of *Withania somnifera* as shown inthe table 2.

Table 1: Callus induction from leaf explant in *Withania somnifera*

PGR 2,4-D	No.of replicate Withania Leaves	Days for callus induction					Remark
		8th day	14th day	20th day	26th day	32ndday	
MS0	4/4	+	++	++	++	+	
1mg/l	3/3	-	-	+	+	+	
2mg/l	2/2	-	+	++	++	++	Average response
3mg/l	2/2	+	+	+	+	+	
4mg/l	1/1	-	-	+	+	+	

Table 2: Callus induction from internodal explant in *Withania somnifera*

PGR 2,4-D	No.of replicate *Withania* Internodal	Days for callus induction					Remark
		8th day	14th day	20th day	26th day	32nd day	
MS0	2/2	-	-	-	-	+	Leaf formed
1mg/l	3/4	-	+	++	++	++	
2mg/l	2/2	-	+	++	++	+++	
3mg/l	4/5	+	++	++	+++	++++	Extremely good
4mg/l	1/6	-	+	++	++	++	

NOTE: -(+=response, ++=average, +++=good, ++++=extremely good)

Shoots regeneration

Shoots regeneration is took place and the callus multiplied. The data was recorded after regular interval of 10, 20 and 30 days as shown in (Fig. 1), (table 3, 4, and 5) respectively. The best response in shoots formation was observed in media supplemented with BAP (0.2mg/l) after 10 and 20 day (table 3 and 4) but after 30 days maximum no. of shoot formation was observed inBAP (0.1mg/l) as shown in table 5.

Table 3: Shoots regeneration in *Withania somnifer a*after 10 days

PGR BAP	No. of replicate	After 10 days		Remark
		Percentage (%) response	No. of shoots form	
MS0	6	4/6 (66.6%)	2/6	
0.1mg/l	6	3/6 (50%)	3/6	1 get contaminated
0.2mg/l	6	4/6 (66.6%)	3/6	Best response
0.3mg/l	6	2/6 (33.3%)	1/6	
0.5mg/l	6	4/6 (66.6%)	2/6	

Table 4: Shoots regeneration in *Withania somnifera* after 20 days

PGR BAP	No. of replicate	After 20 days		Remark
		Percentage (%) response	No. of shoots form	
MS0	6	5/6(83.3%)	3/6	
0.1mg/l	5	5/6 (83.3%)	5/6	
0.2mg/l	6	6/6 (100%)	5/6	Best response
0.3mg/l	6	4/6 (66.6%)	3/6	1 get contaminated
0.5mg/l	6	3/6 (50%)	3/6	

Table 5: Shoots regeneration in *Withania somnifera*after 30 days

PGR BAP	No. of replicate	After 30 days		Remark
		Percentage (%) response	No. of shoots formed	
MS0	6	4/6(66.6%)	2/6	
0.1mg/l	6	5/6 (83.3%)	3/6	**Best response**
0.2mg/l	6	2/6 (33.3%)	1/6	
0.3mg/l	6	3/6 (50%)	2/6	
0.5mg/l	6	1/6 (16.6%)	1/6	

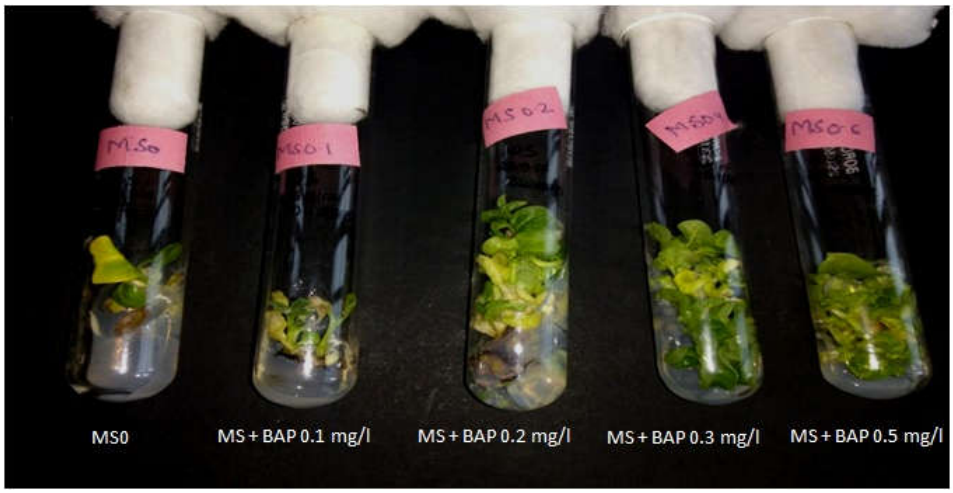

Figure 1. Regeneration of shoots in *Withania somnifera*

Root induction and acclimatization

The best result and root formation was observed in 0.3mg/l in half strength MS media as shown in (Fig. 2 A), (table 6 and 7).*Withania somnifera.*Shoots (8-9 cm long) with sufficient rooting were transferred to ½ strength MS liquidmedium with filter paper raft support for hardening, for two weeks. These well-developed rooted plantlets were then transferred to sterile vermiculite and covered with polybags for 2 weeks to retain moisture. They were irrigated with ¼ strength MS medium without sucrose and kept less than 16 hr light and 25±2°C. Further they were transferred to soil and normal growth of the potted plants was kept in room condition for 1 week. Hardening response was as good as 80%. The plants started growing and survive (Fig. 2 B).

Table 6: Root inductionsin *in vitro* developed shoots of*Withania somnifera on full strength MS media*

PGR IAA	No. of explant	Percentage (%)response	Length of roots (cm)	No. of shoots	Remark
MS0	7	2/7 (28.5%)	1 cm	2	
0.1mg/l	8	5/8 (62.5%)	1 cm	5	
0.3mg/l	8	7/8 (87.5%)	2 cm	7	**Best response**
0.5mg/l	6	5/6 (83.33%)	1.5 cm	5	
0.7mg/l	7	5/7 (71.42%)	2.5 cm	5	

Table 7: Rooting inductionin *in vitro* developed shoots of*Withania somnifera* on half strengthMS media

PGR IAA	No. of explant	Percentage (%)response	Length of roots (cm)	No. of shoots	Remark
1/2 M.S	7	4/7(57.14%)	1 cm	4	
0.1mg/l	6	4/6(66.66%)	2.5 cm	4	
0.3mg/l	6	6/6(100%)	1.2 cm	6	**Best response**
0.5mg/l	7	5/7(71.42%)	2 cm	5	
0.7mg/l	7	6/7(85.71%)	1.5 cm	6	

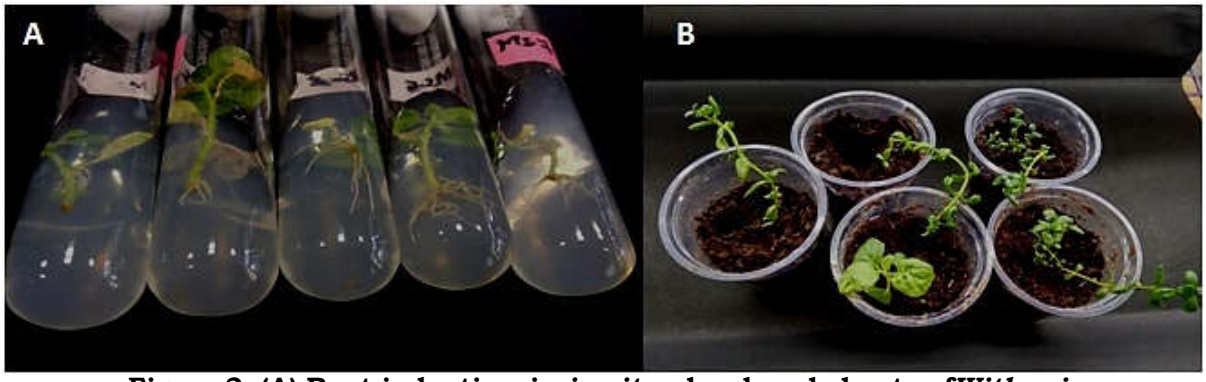

Figure 2. (A) Root inductionsin *in vitro* developed shoots of*Withania somnifera*.(B)acclimatized *Withania somnifera*

DISCUSSION

Withania somnifera is medicinally important crop of agriculture field but effects of weeds are suppressing the growth and rate of germination of this plant in cultivated and agriculture field. Weeds are suppressing the rate germination of *Withania somnifera* and uses of the weedicides and fertilizers are very expensive and costly. For now, there is no doubt about the role of tissue culture in improving agricultural production. The plant tissue culture is desirable in order to satisfy production demands, which has been developed for the mass propagation of medicinal plants were supported by [28, 29], the conservation of particular and endangered species [14, 15]. Development of an effective tissue culture protocol to acquire maximum plantlet regeneration that has a tremendous importance inconservation of biodiversity. The formulation of the best nutrient media, selection of right explants and role of plant growth regulators is a required for utilization of tissue culture technology in conservation of endangered plant species. It has been reported that treatment of shoots with GA_3 increases the length of shoots during multiplication or prior to rooting [5]**.** The combinations of BA and kinetin hormonals inclined morphogenetic response in the in vitro shoots. Sen and Sharma [20] were the first to describe in vitro shoot multiplication followed byrhizogenesis from aseptically germinated seedlings cultured on BAP supplemented MS medium.

It has been observed that multiple shoots were obtained via morphogenesis from axillary bud derived callus [18]. Shoot elongation was observed with adventitious shoot buds obtained from young leaf explants of Chile pepper on the MS medium supplemented with BAP and GA_3 [6]. *In vitro* regeneration of *Withania somnifera* species using different explants was reported by Shukla *et al.*, [22]. It has been studied thatleaf explants*of Withaniasomnifera* cultured on MS medium supplemented with IAA and BAPregenerate directlywithout superseding callus phase [1]. *In-vitro* propagation through organogenesis usingvarious explants and multiple shoot differentiation was accomplished on MS medium containingBAP was reported [10].Govindraju *et al.,* [7] have reported direct regeneration from leaf explants for the sameplant, but adventitious buds were formed when MS medium was supplemented with BAP(1.0-3.0 mg/l) along with IAA (0.5 mg/l) and Coconut milk (10%). Plantlets were transferred to pots containing sandand soil mixture, acclimatized in a culture room and finally rooted plants were transferred tosoil [23]**.** Sivanesan and Murugesan [25] for the same plant reportedthat IBA was efficient in inducing roots (100%) in regenerated shoots obtained from nodalexplants. The callus cultures of *Withania somnifera* from axillary leaves, axillary shoots,hypocotyls, and root segments on MS medium supplemented with 2 mg/l, 2,4-D and0.2mg/l kinetin reported [17]. Maximum root induction and proliferation wasfound in *Stevia rebaudiana*, when the medium is supplemented with 0.5 mg/l NAA [18]. Mass propagation of *Withania somnifera*using nodal and shoot tip explants and production of secondary metabolites on callus of different ploidy level in *Withania somnifera* was reported [15, 22]**.**

Withania somnifera is medicinally importantcrop of agriculture and requirement of this plant increases as contains bioactive compound for therapeutic uses. The plant tissue culture is desirable in order to satisfyproduction demands, which has been developed for the mass propagation of medicinal plantswere supported by [27,28]**.** Tissue culture in

Withania somnifera will be for mass production of planting materials, production of virus-freeplants, plant breeding purposes, conservation, and multiplication of crops and livestock.Additionally, this standardized callus induction and proliferation protocol used in further research for mass propagation of *Withania somnifera*via indirect regeneration methods. Plant tissue culture is an integral part of molecular approaches to plant improvement and acts as transitional whereby advances made by the molecular biologists in gene isolation and modification are transferred to plant cells.

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