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IN VITRO PROPAGATION OF *Withania somnifera* AND ESTIMATION OF WITHANOLIDES FOR NEUROLOGICAL DISORDERS

SAHANA RAO, TEESTA V.K., AVNISH BHATTRAI, KRITHIKA KHUSHI AND SAVITHRI BHAT*

Department of Biotechnology, BMS College of Engineering, Bull temple road, Bangalore- 560019, India.
*Corresponding Author: Email- savithri.bhat@gmail.com, savithri.bt@bmsce.ac.in

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Abstract- Neurodegenerative disorders have great social impact and are important factors determining the maximum healthy age and the mortality in the modern times. Some of the well known neurological disorders are Parkinson's, Alzheimer's, Huntington's, amyotrophic lateral sclerosis and Creutzfeldt-Jakob' s. According to the World Health Organization (WHO), the UN's health agency, mental and neurological disorders ranging from depression to Alzheimer's currently strike 400 million people globally and are set to surge in the next two decades. Non- availability of drugs for the prophylaxis and treatment of these disorders throws a challenge for the researchers. The abundant natural molecules of plant origin and their modification has yielded not only the new lead molecules for drug discovery and development but also the nutraceuticals. Historically, *Withania somnifera* commonly known as Ashwagandha is being used as neurotonic for anxiety and neurological disorders. In the present study, culture conditions such as hormone and temperature were optimized for both direct and indirect organogenesis to obtain 60-80 shoots from nodal explants of *Withania somnifera*. The *in-vitro* cultured plantlets were rooted and acclimatized for field conditions. The methanolic extract prepared from the roots of these plantlet were subjected to HPLC for detection of compounds. These extract are being further characterized for the presence and detection of withanolides via Mass spectroscopy.
Key words- Ashwagandha, Micropropagation, Neurotonic, Organogenesis, Withanolides.

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Introduction

Neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) [9] and Creutzfeldt-Jakobdisease (CJD) [8] are some of the well known age related disorders. Despite the great number of ongoing investigations, neurodegenerative disorders remain incurable. The drugs currently available for dementia, such as donepezil, an acetylcholine inhibitor are efficacious in the temporary treatment of memory dysfunction, but do not prevent or reverse the underlying neurodegeneration [11]. Thus, in the present context, there is an urgent need for a new bio -chemical entity(s) with zero or low toxicity which can effectively act on diverse molecular targets of these neurological disorders.

The abundant natural molecules of plant origin and their modification has yielded not only the new lead molecules for drug discovery and development but also the nutraceuticals. Reviews on the multiple and varied plant species of the natural molecules are available [6]. *Withania somnifera* (Solanaceae) also commonly known as ashwagandha is a well known herb in the ayurvedic and indigenous medical systems for 3000 years [10,13] The plant is being exploited for preparation of over 200 formulations used in the treatment of various physiological disorders [1,15]. It is used therapeutically as an adaptogen for patients with nervous exhaustion, insomnia, and debility due to stress and as an immune stimulant in patients with low white blood cell counts [19]. Over exploitation and the reproductive failure have rendered the species vulnerable to complete extinction [1]. The natural propagation of *Withania* occurs through seeds but chances of seed setting get limited due to unisexual nature of flowers. *In-vitro* propagation of this plant will not only provide a means of disease free healthy clones for extraction of pure drugs, but also a solution for its extinction. In the present study, we report direct organogenesis

through nodal segment and indirect via callus formed from leaf. Further, the methanol extract prepared from the roots of *Withania* were subjected to HPLC for Withanolides.

Materials and Methods

Collection and sterilization of of nodal and leaf explants

Withania somnifera plant was obtained from GKVK (Gandhi Krishi Vignan Kendra), Bangalore, India. Both nodal and leaf explants were washed with tween 20 and sterilized with 0.1% Hg Cl2 for 5 min followed by thorough washing in sterilized water 4-5 times.

Media for propagation

The media used for the present work was MS [16] media. PGR (Plant Growth Regulator) concentrations of BAP (0-8 mg/l); 2,4-D (0.75-3 mg/l); IAA (1-2 mg/l and BAP (4 mg/l) + IAA (1-2mg/l) were used to determine the optimum concentration for shooting and callusing. The shoots regenerated were transferred to MS media without any PGR for rooting. Rooted plants were transferred to Liquid MS media for 10 days and shifted to soil thereafter. The media was sterilized at 15 psi, 121 degree for 20 min.

Culture condition

The culture tubes and flasks were incubated in the culture room maintained under 16h/8h (light/dark) and temperature was maintained at around 25±2^{o}C.

Materials for root extract

HPLC grade methanol used to extract Withanolides from the roots of the *Withania somnifera* in the ratio of 1:10, 1:25, 1:50.

Analytical method

HPLC estimation Withanolides performed on Shimadzu 10 AS HPLC system, equipped with UV detector. For estimation Varian C18 RP column and the mobile phase with the mixture of Acetonitrile:Methanol:Orthophosphoric acid (55:45:1) was used. The HPLC was run at 1350 psi and sample detected at 224 nm.

Results and discussion

The multiple shoot regeneration from nodal explants was assessed on MS media containing BAP at 0, 2, 4 and 8 mg/l (Fig. 1a, 1b) It was observed that number of shoots regenerated in MS media containing BAP at concentration 0 to 8 mg/l was 20 to 60 (Fig. 1c) having shoot length ranging between 2 to 7 cms (Table 1) after 60 days of culture.The highest shoot proliferation (67) and shoot length (7 cm) was observed in BAP 4 mg/L. This result is parallel with the earlier reports on medicinal plants such as *Portulaca* [3], *Zingiber* [4] *Sida* [18] *Azadirachta* [2]. Increase in the concentration of BAP reduced shoot proliferation drastically. Bhau and Wakhlu observed that high concentration of BAP resulted in decreased in shoot number in mulberry. High concentration of cytokinin have been reported for the reduction in shoot bud induction frequency in *Bacopa*.

In another experiment IAA alone and BAP was used in combination with IA A to induce callus from leaf explants (Fig. 1d &1e). It was observed that IAA at 1 mg/l and IAA (2 mg/l) with BAP (4 mg/l) increased weight of the callus to more than 20 times in 30 days (Table 2).

Table 1- Effect of MS medium containing different concentration of BAP on multiple shoots formation and shoot length from nodal explants of Withania somnifera.

Sr. No.	Conc. of BAP mg/l	Number of shoots per explants	Shoot length (cm)
1	0	24.5±2.3	2.5±0.5
2	2	27±1.0	3.35±0.15
3	4	67.5±2.5	7.25±0.25
4	8	22.5±2.0	1.85±0.35

Results recorded after 60d. The experiments were repeated thrice, each consisting of 10 replicates. Values represent the mean± SE.

Table 2- Effect of MS medium containing different concentration of IAA +BAP or IAA alone on callus formation from leaf explants of Withania somnifera.

Sr. No.	Conc of IAA (mg/l)	Initial Weight of the callus (gm)	Final Weight of the callus (gm)
1	0	0.045 ±0.001	0.0595±0.0015
2	1	0.0625±0.0025	1.37±0.155
3	2	0.058±0.002	0.077±0.0055
	Conc of IAA (mg/l) with BAP 4 mg/l	**Initial Weight of the callus (gm)**	**Final Weight of the callus (gm)**
4	0	0.054±0.004	0.082±0.002
5	1	0.062±0.001	0.092±0.0019
6	2	0.0695±0.0015	1.61±0.02

Results recorded after 30 d.The experiments were repeated thrice, each consisting of 5 replicates.Values represent the mean± SE.

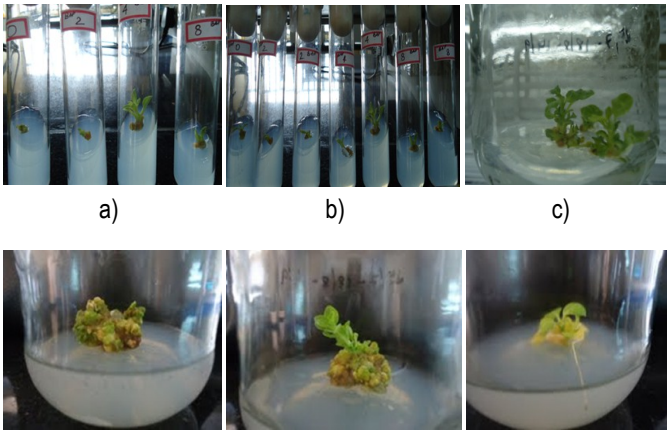
Table 3- Effect of MS medium containing different concentration of 2,4 D on callus formation from leaf explants of Withania Somnifera

Sr. No.	Conc. Of 2, 4D mg/l	Initial Weight of the callus (gm)	Final Weight of the callus (gm)
1	0.75	0.054±0.004	0.085±0.005
2	1.5	0.0645±0.0045	0.095±0.0048
3	3.0	0.053±0.001	0.056±0.002

Results recorded after 30 d. The experiments were repeated thrice, each consisting of 5 replicates. Values represent the mean± SE.

The combination of auxin and cytokinin have been reported for callus induction form leaf explants (Castillo et al., 2000., Shu et al., 2005., Vidya et al., 2005).The third set of experiment comprises of 2, 4 D (0.75 to 3 mg/l)for induction of callus from leaf explants and the increase in the callus weight was observed at 1.5 mg/l of 2,4 D. Its been earlier reported that 2, 4 D together with BAP or kinetin induce callus from leaf explants (Wani et al., 2010., Hasan et al., 2008).The plants were transferred to MS media without PGR for rooting (Fig. 1f)

The methanol extracts of roots were prepared and subjected to HPLC with Acetonitrile: Methanol :Orthophosphoric acid (55:45:1) as mobile phase and the peak for Witharferin was obtained at retention time between 2.8 and 3.0 min. (Fig. 2). The present report will have relevance in microcloning of the important medicinal herb in India besides its implication in genetic engineering and pharmaceutical industry.



a) b) c)

d) e) f)

Fig. 1- Organogenesis in *Withania somnifera* a) and b) Initial shooting in 0- 8 mg/l BAP from nodal explants (10 days); c) Shoot proliferation from the nodal explants in the presence of 4 mg/l BAP d) and e) Indirect organogenesis; formation of callus and regeneration of shoots from callus; f) Rooting from the shoots regenerated in the presence of MS media without PGR.

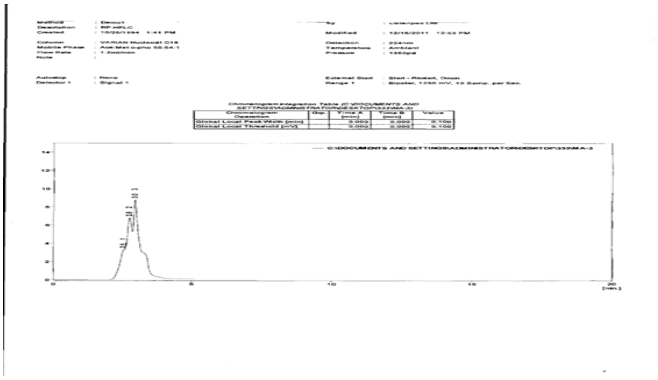


Fig. 2- HPLC analysis of withanaloides from the root extract of *Withania somnifera* prepared in methanol.

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