International Journal of Current Science Research
www.drbgrpublications.in

Volume: 2; Issue: 1; January-2016; pp 252-260. ISSN: 2454-5422

IN VITRO PROPAGATION OF ASHWAGANDHA (Withania somnifera) AND HPLC ESTIMATION OF WITHANOLIDES FOR NEUROLOGICAL DISEASES

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Abstract

Neurological ataxia have high social influence and are important aspect determining the prolonged healthy age and the vitality in the modern times. Some of the well known neurological ataxia are Alzheimer's, Huntington's, Parkinson's, amyotrophic lateral sclerosis and Creutzfeldt-Jakob's. According to the the United Nation's health agency, World Health Organization, mental and neurological disorders ranging from depression to Alzheimer's currently strike 400 million people universally and are set to rush in the next two decades. Non- availability of drugs for the prophylaxis and regimen of these disorders throws a confrontation for the researchers. The plentiful natural molecules of plant origin and their modification has yielded turnout not only the new lead molecules for drug discovery and development but also the nutraceuticals. Historically, Withania sominifera commonly known as Ashwagandha is being used as neurotonic for anxiety and neurological disorders. In the current study, culture conditions such as phytohormone and temperature were optimized for both direct and indirect organogenesis to obtain 61-80 shoots from nodal explants of Withania somnifera. The in-vitro cultured plantlets were rooted and reconcile for field trial conditions. The methanolic extract processed from the roots of these plantlet were subjected to HPLC for discosure of compounds.

Keywords: Withania, neurological disorders, Micropropagation, Organogenesis,Neurotonic, Withanolides.

Introduction

Aswagandha (Withania somnifera) is an ornamental and medicinal plant. It is used as a medicinal plant from the ancestor era. Parts of this plant used as a medicine for curing different diseases. Aurveda describes its medicinal use for human beings. The Sanskrit name “ashva” meaning horse and “gandha” meaning smelling was given to this plant due to the smell of the roots resembling a sweating horse. Currently around 200 traditional medicinal formulae are prepared in Ayurveda, Sid-dha and Unani systems using this plant. All the plant parts are credited with medicinal properties. Parts of plant used for curing different types of diseases. Property of curing a disease is depending upon the phytochemical/active compound composition of plant part. Different plant parts contain different types of active compounds. As a medicine all parts of this plant are used (Naveen gaurav 2013).

The genus Withania is restricted and related to the old World; rather it closely belongs to the genus Physalis, the gooseberries. Withania possesses a natural occurrence, most probably in the drier and humid areas, spread from the Mediterranean region to throughout tropical region of Africa to South Africa and also from the Cape Verde Islands and Canary region to the Arabia and Middle East region like India, southern China and Sri Lanka (Naveen gaurav *et al* 2015). Neurodegenerative ataxia such as Parkinson's disease (PD), alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS) (Chiti and Dobson 2006) and Creutzfeldt-Jakobdisease (CJD) (Chakrabarti *et al* 2009) are some of the well known age related ataxia. Inspite the great number of ongoing analysis, neurodegenerative ataxia remain non-curable. The drugs presently available for dementia, such as donepezil, an acetylcholine inhibitor are efficacious in the temporary regimen of memory dysfunction, but do not prevent or reverse the underlying neurodegeneration (Elizabeth *et al* 2007). Thus, in the present framework, there is an immediate need for a new bio-chemical entity(s) with zero or low toxicity which can effectually act on diverse molecular targets of these neurological disorders. The plentiful 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. Analysis on the multiple and varied plant species of the natural molecules are available (Campos *et al* 2010). Withania somnifera

(Solanaceae) also commonly known as ashwagandha is a well known herb in the ayurvedic and indigenous medical systems for 3000 years (Devi and Sharada 1992; Jaffer *et al* 1988) The plant is being opressed for preparation of over 190-200 formulations used in the tregimen of many physiological disorders (Antonisamy and Manickam 1999; Mirjalili *et al* 2009). 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 (Ven Murthy *et al* 2010). Over opression and the reproductive failure have rendered the species accessible to complete annihilation (Antonisamy and Manickam 1999). The natural propagation of Withania occurs through seeds but chances of seed setting get restricted due to diocious 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 through callus formed from leaf. Further, the methanol extract processed and obtained from the roots of Withania were subjected to HPLC for Withanolides identification.

Materials and Methods

Collection and sterilization of of nodal and leaf explants Withania somnifera plant was obtained from Forest Research Institute (FRI) Dehradun, India. Both leaf explants and nodal leaf explants were washed with Cween 20 and sterilized with 0.1% HgCl2 for 4-6 min followed by thorough washing in distilled 4-5 times. A rapid procedure for smooth and optimum propagation for Withania somnifera (a medicinally efficacious multipurpose plant), was done to involve in the study of the effect of temperature, physico-chemical treatments, photoperiod, growth regulators (IAA, IBA, 2–4 D and BA) and storage on product ability (Naveen gaurav *et al* 2015).

Media for propagation

The media used for the present work was MS (Murashige and Skoog 1962) media. PGR (Plant Growth Regulator) concentrations of BAP (0-8 mg/l); IAA (1-2 mg/l); 2,4-D (0.75-3 mg/l); and BAP (4 mg/l) + IAA (1-2mg/l) were used to determine the suffice concentration for shooting and callusing. The shoots regenerated were transferredto 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 autoclaved at 15 psi, 121 ^{0}C for 20 min.

Culture condition

The culture tubes and flasks were incubated in the culture room maintained under 8h/16h (dark/light) photoperiod and temperature was maintain at around 25±2^{0}C.

Materials for root extract

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

Analytical method

HPLC estimation Withanolides performed on Shimadzu 10 AS HPLC system, equipped with Ultra-Violet detector. For detection Varian C18 RP column and the active 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, 2.5, 4.5 and 7.5 mg/l. It was observed that number of shoots regenerated in MS media containing BAP at concentration 0 to 7.5 mg/l was 24.4 to 68.1 having shoot length ranging between 2 to 7 cms (Table 1) after 60 days of culture.The highest shoot proliferation and shoot length (7 cm) was observed in BAP 4.5 mg/L. This result is parallel with the earlier reports on medicinal plants such as Portulaca (Ashok and Mudasir 2010), Zingiber (Balachnadran S.M., Bhat S.R. 1990) Sida (Sivanesan and Jeong 2007) Azadirachta (Arora et.al., 2010). In another experiment IAA alone and BAP was used in combination with IA A to induce callus from leaf explants. It was observed that IAA at 1 mg/l and IAA (2.5 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	24.4	2.6
2	2.5	27.3	3.35
3	4.5	68.1	7.15
4	7.5	22.5	1.87

Results recorded after 60d. The experiments were repeated thrice, each consisting of 10 replicates.

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	0.0460	0.0595
2	1	0.0627	1.37
3	2.5	0.0581	0.077
	Conc of IAA (mg/l) with BAP 4 mg/l	Initial Weight of the callus (gm)	Final Weight of the callus (gm)
4	0	0.055	0.083
5	1	0.063	0.095
6	2.5	0.069	1.630

Results recorded after 30 d.The experiments were repeated thrice, each consisting of 5 replicates.

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 (Table 2). Its been earlier reported that 2, 4 D together with BAP or kinetin induce callus from leaf explants (Minal Wani et al., 2010; Hasan et al., 2008).The plants were transferred to MS media without PGR for rooting. 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.

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	0.0570	0.086
2	1.5	0.0648	0.095
3	3.0	0.0536	0.058

Results recorded after 30 d. The experiments were repeated thrice, each consisting of 5 replicates.

In vitro propogation of shoot of *Withania somnifera* (Wild variety)

Aseptic *in vitro* cutting of multiple shoots of *Withania somnifera*

Root extract power of *Withania somnifera*

Bacterial & Fungal infected contaminated flasks

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