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Research Article

Stress induced somatic embryogenesis: An alternative technique to enhance production of withaferin A in Indian ginseng (*Withania somnifera* L.) Dunal

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Abstract

Withania somnifera commonly known as Ashwagandha is being used as neurotonic for anxiety and neurological disorders. Conventional propagation of Withania through seeds limits the seed setting and results in variations in withanolides production. In the present study, we report differentiation of somatic embryos from nodulated embryogenic callus of leaf explants on Murashige and Skoog medium supplemented with N6-benzylaminopurine (4 mg /l) and IAA (2mg/l) in the absence of exogenous calcium. The microscopic details of embryogenic calli upon staining with toluidine blue showed differentiation of calli into pro embryos , globular and heart shaped embryos. Both, TLC and HPLC analysis revealed increase in withaferin A content in methanolic extracts of these embryos. In-vitro propagation of Withania somnifera via somatic embryogenesis provides solution for its extinction and reduces the variations in withanolide content amongst Withania plants.

Keywords: *Ashwagandha, somatic embryogenesis, withaferin A, calcium stress, neurodegeneration*

Introduction

Despite the great number of ongoing research, certain age related neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), Amyotrophic lateral sclerosis (ALS)(F. Chiti and C.M. Dobson, 2006) and Creutzfeldt-Jakobdisease (CJD) (O. Chakrabarti *et al*, 2009) remain incurable. The drugs currently available are used in the treatment of memory dysfunction temporarily and do not cure or reverse the degenerated neurons to healthier ones (Elizabeth Hohnadel *et al*, 2007). The alkaloids present naturally in plant have been used in prevention of several such related cases (H.C. Campos *et al*, 2010) and withanlides found in *withania somnifera* is one of them.

Withania somnifera(Ashwagandha) , a solanaceous herb is well known for its medical importance in ayurveda (P. U. Devi and Sharada,1992 and H. J. Jaffer *et al*,1988).Over 200 formulations of *Withania* is used in the treatment of various physiological and nervous disorders (M. H. Mirjalili *et al*, 2009 and M.R. Ven Murthy *et al*, 2010). Withaferin A, a major component of *Withania somnifera* is a steroidal lactone and a potent inhibitor of angiogenesis. It inhibits both NF-κB and Sp1 transcription factor activity(Prasanna Kumar *et al*, 2009) and down regulates VEGF gene expression (R. Mohan *et al*, 2004 and Prasanna Kumar *et al*, 2009).

The reproductive failure and continuous exploitation of *Withania somnifera* have rendered the species vulnerable to its complete extinction (R. Antonisamy and Manickam, 1999). *In-vitro* propagation of this plant via somatic embryogenesis will provide a solution for its extinction and production of disease free healthy clones .This will also reduce the variations in withanolide content between the batches of *Withania* plants. There are reports on *in vitro* propagation via organogenesis and somatic embryogenesis of *Withania somnifera* from vegetative tissues. (Sen and Sharma, 1991;Rani and Grover, 1999; Kulkarni *et al*, 2000; Sahana Rao *et al*, 2012). For the first time, we report induction of somatic embryogenesis in the absence of exogenous calcium and its effect on withaferin A content in *Withania somnifera.*

Materials and Methods

Collection and sterilization of leaf explants

Withania somnifera plant was obtained from GKVK (Gandhi Krishi Vignan Kendra), Bangalore, India. Leaf explants were washed with tween -20 and sterilized with 0.1% Hg Cl_2 for 5 min followed by thorough washing in sterilized water 4-5 times.

Media for organogenesis and somatic embryogenesis

The media used for the present work was MS (Murashige and Skoog, 1968) supplemented with IAA (0-2 mg/l) and BAP (4 mg/l) to determine the optimum concentration for indirect organogenesis. The shoots regenerated were

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transferred to MS media without PGR(Plant growth regulator) for rooting (Sahana *et al*, 2012). For induction of embryogenic callus, MS media without exogenous calcium containing IAA (2mg/l) and BAP (4mg/l) was used. The media was sterilized at 15 psi, 121 degree for 20 min.

Culture conditions

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^{0}C.

Histological observation and withanolides extraction

Embryogenic callus was sectioned and stained in toluidine blue (0.1g in 70% ethanol) and observed under microscope.HPLC grade methanol was used to extract withanolides from the dried organogenic IAA (2mg/l and BAP 4mg/l with calcium) and embryogenic callus (IAA 2mg/l and BAP 4mg/l without calcium) of the *Withania somnifera* .For both TLC and HPLC 0.1gm of sample (organogenic and embryogenic) was dissolved in 1ml of methanol. Standard withaferin A compound was obtained from Natural remedies ,Bangalore, India.

Analytical methods

TLC plates were prepared with silica gel (40%) and mobile phase consisting of chloroform: methanol (90:10) was used for separation of withanolides. The HPLC estimation of withanolides was performed on Shimadzu 10 AS HPLC system, equipped with UV detector. The Varian C18 RP column and the mobile phase with the mixture of Acetoni-trile:Methanol:Orthophosphoric acid (55:45:1) was used. The HPLC was run at 1350 psi and sample detected at 224 nm.

Results and Discussion

As previously reported BAP alone or IAA and BAP in combination induced callus from leaf explants (Sahana et al, 2012). It was observed that IAA (2 mg/l) with BAP (4 mg/l) increased weight of the callus to more than 18 times in 30 days (Table 1). 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 plant regeneration and rooting occurred in PGR free MS media (Sahana et al, 2012).

In another set of experiment, MS media with and without exogenously supplied calcium was supplemented with BAP (4mg/l)and IAA(2mg/l) .Embryogenic callus was observed within 20 days of culture of leaf explants in MS media containing BAP and IAA without exogenous calcium (Fig 1A) .The embryogenic callus differentially stained with toluidine blue showed Pro embryo, globular and heart shaped embryos (Fig 1B). The conditions responsible for differentiation of callus into embryogenic callus are categorized into two parameters ; PGR types and concentrations and stress factors. The reports on stress factors induced embryogenesis are available in plants such as *Arabidopsis, camellia, Dianthus, Vigna* and *Zingiber (* Ikeda-Iwai *et al*, 2003; Aoshima, 2005; Karami *et al*, 2006; Begun *et al*, 2007; Lincy *et al*, 2009) respectively.

A wide range of abiotic stress such as drought, salinity, UV radiations act as elicitors and increase the production of alkaloid in in -vitro plant cultures (D. S. Seigler 1998 and F. Dicosmo and M. Misawa, 1985) .Certain physical factors such as wound and temperature and deficiency in nitrogen and phosphorous influence accumulation of phenylpropanoides (R.A. Dixon and N. Paiva , 1995; L. Chalker-Scott and L.H. Fnchigami, 1989) Apart from effecting signalling response levels, calcium is known to increase production of betalin in *Beta vulgaris*(N. Tuteja and S. Mahajan, 2007 ; B. Savitha , 2006).

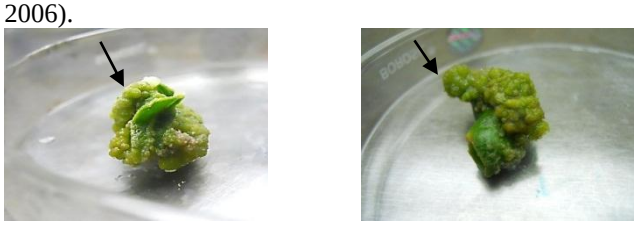

Fig 1A:Induction of somatic embryo from nodular embryogenic callus of leaf explants cultured in MS media conatining BAP 4mg/l and IAA 2mg/l without exogenous calcium .

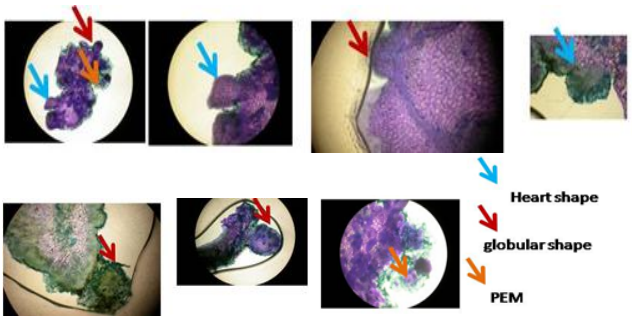

Fig 1B: Histoligcal observation of various stages of somatic embryos as formed from leaf explants cultured in MS media conatining BAP 4mg/l and IAA 2mg/l without exogenous calcium .

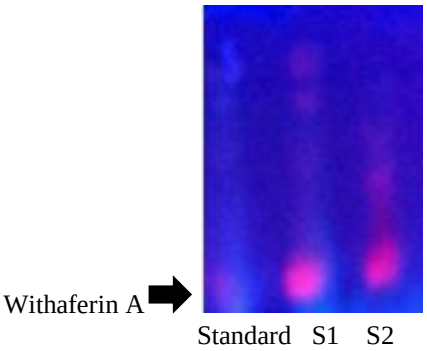

Fig 2:TLC profile of dried embryogenic callus formed from leaf explants cultured in MS media containing BAP 4mg/l and IAA 2mg/l without exogenous

calcium(S2).Lane 1: standard withaferin A and lane 2 (S1): dried organogenic callus formed leaf explants cultured in MS media containing BAP 4mg/l and IAA 2mg/l .

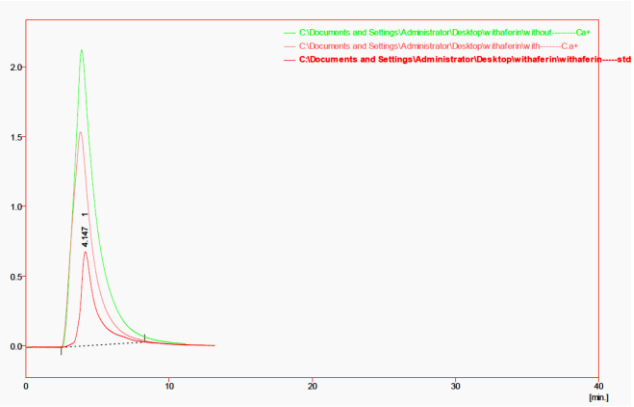

Fig 3:HPLC profile of dried embryogenic callus formed from leaf explants cultured in MS media containing BAP 4mg/l and IAA 2mg/l without exogenous calcium(green peak).Red peak: standard withaferin A and Orange peak: dried organogenic callus formed leaf explants cultured in MS media containing BAP 4mg/l and IAA 2mg/l .

Though several reports on stress induced embryogenesis and stress induced alkaloid production are available individually , the studies which co-relate stress induced somatic embryogenesis and alkaloid production are rare. There are few reports published in which these two techniques are applied such as, saponin in *Panax ginseng* (Asaka *et al*, 1994),naphthoquinone in *Plumbago rosea* (Komaraiah *et al*, 2004) , and others (Payne *et al*, 1991).In the present study, absence of exogenous calcium was used as stress factor to induce embryogenesis and estimate levels of withaferin A. The TLC (Fig 2) and HPLC (Fig 3) reports showed higher concentration of withaferin A in embryogenic callus formed in the absence of exogenous calcium. The peak for Withaferin in HPLC was obtained at retention time between 3 to 4.2 min. (Fig 3).

Table 1- Effect of MS medium containing different concentration of IAA+BAP or BAP alone on callus formation from leaf explants of *Withania somnifera*. Results recorded after 30 d.The experiments were repeated thrice, each consisting of 5 replicates.Values represent the mean± SE.

S.No	Conc of IAA (mg/l) with BAP 4 mg/l	Initial Weight of the callus (gm)	Final Weight of the callus (gm)
1	0	0.06±0.005	0.079±0.002
2	1	0.059±0.002	0.12±0.005
3	2	0.065±0.001	1.71±0.003

The present report will have relevance in production of clones via embryogenesis and uniformity in the concentrations of withaferin A produced in plants propagated through embryogenesis. Thus the technique of correlating stress induced embryogenesis and alkaloid production can be applied to important medicinal herbs in India besides its implication in genetic engineering and pharmaceutical industry.

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