DEVELOPMENT OF MULTIPLE SHOOT CULTURE OF ASHWAGANDHA *(Withania somnifera)* FOR *IN VITRO* ALKALOID STIMULATION STUDIES

By

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(2015-09-013)

THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Development of multiple shoot culture of ashwagandha *(Withania somnifera)* for *in vitro* alkaloid stimulation studies"** is a bonafide record of research work done by me during the course of research and that the thesis have not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Place: Vellayani

Date: 11/01/2021

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CERTIFICATE

Certified that this thesis entitled **"Development of multiple shoot culture of ashwagandha *(Withania somnifera)* for *in vitro* alkaloid stimulation studies"** is a record of research work done independently by Ms. Malavika M. R. (2015-09-013) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Place: Vellayani

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Sl No.	Title	Appendix No.
1	Murashige and Skoog (MS) medium	Appendix I

LIST OF ABBREVIATIONS

BAP	6- benzyl amino purine
BA	6- benzyl adenine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole -3- acetic acid
Kn	Kinetin
MS	Murashige and Skoog
GA_3	Gibberellic acid
IBA	Indole -3- butyric acid
NAA	α- naphthalene acetic acid
PGRs	Plant Growth Regulators
HPLC	High pressure liquid chromatography
$HgCl_2$	Mercuric chloride
FW	Fresh weight
DW	Dry weight.
%	Per cent
µg	Micro gram
µM	Micro molar
ppm	Parts per million
°C	Degree Celsius
cm	Centimeter
nm	Nanometer
et al.	And other co workers
h	Hour
g	Gram

g-1	Per gram
mg	Milli gram
ml	Milli litre
l	Litres
mins	Minutes

1. INTRODUCTION

Ashwagandha (*Withania somnifera* (L.) Dunal), commonly known as 'Indian ginseng' or 'winter cherry', is a member of the family Solanaceae. For more than 3,000 years this plant has been used as an essential herb in the Ayurvedic and indigenous medicinal systems. It is known as an aphrodisiac drug all over the world. The plant is found in India, Pakistan, Afghanistan, Palestine, Egypt, Jordan, Morocco, Sri Lanka, Spain, Canary Island, Eastern Africa, Congo, etc., (Kokate *et al*., 1996). Ashwagandha is widely distributed in Maharashtra, Gujarat, Rajasthan, Madhya Pradesh, Orissa, Uttar Pradesh, Punjab plains extending to the mountain regions of Punjab, Himachal Pradesh and Jammu. Withanolides (steroidal lactones with ergostane skeleton) are the major biochemical constituents of ashwagandha. In addition, various groups of withanosides, glycowithanolides, sitoindosides, alkaloids, saponins, amino acids, phenolic compounds, flavonoids and several other secondary bioactive metabolites have been isolated and characterized in this plant with broad-spectrum therapeutic activity (Elsakka *et al.,* 1990; Mishra and Singh, 2005).

The pharmacological studies carried out in ashwagandha have indicated its antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hematopoietic, anti-aging, anxiolytic and antidepressant activities. Also, it was found to affect various neurotransmitter receptors (Pattipati *et al*., 2003). It was reported by Jayaprakasam *et al.,* (2003), that the standard drug for cancer chemotherapy, doxorubicin was found less effective compared to a particular extract of this plant having withaferin A. Also, it was reported that the anti-inflammatory and immunomodulatory properties of ashwagandha root extracts are likely to contribute to its chemopreventive action (Prakash *et al*., 2002).

Ashwagandha is propagated commercially by seeds because of the lack of natural ability for vegetative propagation, but the seed viability is limited to one year making the long term storage futile (Sen and Sharma, 1991; Rani and Grover,

1999). However, the seed propagation is not satisfactory, since percentage of germination is low, due to the presence of certain inhibitory compounds in the fruit and high risk of catching various diseases (De Silva and Senarath, 2009). Hence, *in vitro* seed germination method using MS medium was found to be the best alternative for the rapid germination of ashwagandha.

The withanolide content in field grown plants are generally low i.e., about 0.001 to 0.5% of dry weight. However, multiple shoot cultures of ashwagandha have been proven to be effective in the bio accumulation (5 fold increase) of withanolide content (Ray and Jha, 2001; Satyajit *et al.,* 2011). So, the present study was aimed to develop a rapid and effective protocol for shoot multiplication that can be used to generate large number of multiple shoots within a short period of time. Thus, this protocol can be referred for enhancing the accumulation of alkaloids. Since the multiple shoots were developed from the explants derived from *in vitro* grown seedlings, an effective protocol for seed germination was also established, which could be used to produce more number of seedlings within short time duration. Hence the present study was undertaken with the following objective:

- To establish multiple shoot cultures of ashwagandha (*Withania somnifera* (L.) Dunal) for *in vitro* alkaloid stimulation studies.

2. REVIEW OF LITERATURE

A significant portion of current chemotherapeutics and conventional medicines come from higher plants (Zenk, 1978). The overuse of such wild medicinal plants has contributed to the gradual depletion of natural resources (Heble *et al*., 1985). Several authorities have warned of widespread deforestation, indiscriminate collection and vicious misuse of natural medicinal herbal flora (Akerele *et al*., 1991; Thakur, 1993) and they expressed the urgent need for alternative means of propagation, including biotechnology, to be established in order to provide the industry with an abundance of raw materials rich in active ingredients and to allow the conservation of rare and exotic herbal resources.

The healing effects of medicinal plants and the adverse effect of synthetic drugs as discovered by scientific studies are the basis for the use of plant-based medicines for disease prevention and cure throughout the world. There has been an unparalleled explosive growth in the herbal industry since the last three decades due to increasing consumer awareness and constructive strategies for preserving people's good health and well-being. The herbal plants are projected to have a global market worth around US$ 82 billion per annum and the demand is growing at a rapid rate. Recent data indicate that the amount of essential oil and aroma chemicals traded worldwide in 2011 alone from medicinal and aromatic plants was US 7.8 billion (Rao *et al*., 2012). Such facts indicate that herbal industry will be one of the global economy's major driving forces. Nevertheless, along with the rapid growth of the industry; the supply of the raw materials has not increased and in effect, results in a rapid depletion of the genetic pool of many important medicinal plants from their natural habitat. These factors have given an impetus to the development of alternative routes at genuine and industrial level for the production of bio-molecules without being dependent on filed medicinal plant cultivation and disturbing wild wealth.

2.1 *In vitro* production of Bioactive Compound

Three decades ago, large-scale cultivation methods for plant cells have been developed to produce secondary metabolites for antibiotics and amino acids, just as microbial and fungal fermentation. In 1983, the first procedure of plant cell culture was marketed in Japan for the production of shikonin and anthraquinones, commonly used in Japan for anti-inflammatory properties (Flores *et al*., 1987). With a few notable exceptions, undifferentiated plant cultures show their parent plant's level of patterns of secondary metabolite development characteristics.

Biotechnological tools are important for the multiplication and genetic enhancement of medicinal plants through the adoption of techniques such as *in-vitro* regeneration, cell / root culture growth and rapidly growing shoot cultures for secondary metabolite production (Bourgaud *et al*., 2001). For the *in-vitro* production of useful secondary metabolites, cell and organ cultures are an attractive alternative (Oksman-Caldustry and Inze, 2004). *In vitro* plant propagation has enormous potential for producing high-quality plant-based medicines (Murch *et al*., 2000). There are numerous reports on the regeneration of different medicinal plants through callus culture (Tripathi *et al*., 1996). Zenk (1978) has reported the successful establishment of cell lines capable of producing high yield of secondary compounds in cell suspension cultures. The deposition of secondary products in cultures of plant cells depends on the composition of the culture medium and the environment (Stafford, 1986). Ravishankar and Grewal (1991) have reported the influence of media constituents and nutrient stress for the production of diosgenin from callus cultures of *Dioscorea deltoidea.*

The key chemical factors involved in the scaling-up of secondary metabolite production are the availability of nutrients. In *Atropa belladonna* (Kwok and Doran, l995), the effect of depletion of carbon, nitrogen oxygen and hydrogen in the medium on the biomass and alkaloid production were studied. Bais *et al.*, (1999) and Zabetakis *et al*., (1999) have investigated the impact of exogenous

supplementation of certain biotic and abiotic elicitors to induce the production of secondary metabolites.

2.2 Ashwagandha – The candidate species

The candidate species *Withania somnifera* (L.) Dunal is naturally propagated by seeds and broadcast method of seed sowing has been suggested for its commercial cultivation (Nigam and Kandalkar, 1995). Ashwagandha is increasingly becoming a popular adaptogenic herb and is available throughout the western world as a dietary supplement. Roots and leaves are used in a number of preparations for their anti-inflammatory, anticonvulsive, antitumor, immunosuppressive and antioxidant properties besides for promoting vigor and stamina (Praveen *et al.*, 2010). Ayurvedic practitioners have used the roots of this plant for centuries with success as a tonic to increase vitality and longevity, as well as to treat health conditions as diverse as tumors and arthritis (Singh *et al*., 2010). Recently Jain *et al*., (2012) has given an extensive review of biotechnological intervention studies of *Withania somnifera* by several authorities.

2.3 Bioactive compounds in Ashwagandha

The plant is chemically very complex and more than 80 compounds are known from it (Van Wyk *et al*., 2000). The biologically active chemical constituents are alkaloids (ashwagandhine, cuscohygrine, anahygrine, tropine etc), steroidal compounds, including ergostane type steroidal lactones, withaferin A, withasomniferin-A, withasomidienone, withasomnidienone, withasomniferols, withanone etc. The constituents of ashwagandha roots are the steroidal alkaloids and steroidal lactones. They belong to a class of constituents called the withanolides (Elsakka *et al*., 1990, Mishra *et al*., 2000) with the main active chemical constituent withaferin A (Lavi *et al*., 1965). The reported alkaloids are anaferine; isopelletierine; tropine; pseudotropine; 3α- tigloyloxtropine and number of withanolides including withaferine-A; withanolide N and O; withanolide D and withanolide p; withanolide Q and R, 14α- hydroxyl steroids and withanolides G,

H, I, J, K and U (Kirson and Glotter, 1980). Seven new withanolide glycosides called withanosides I, II, III, IV, V, VI and VII were isolated and identified (Matsuda *et al*., 2001). Much of the pharmacological activity of aswagandha has been attributed to two main withanolides namely withaferin A and withanolide D.

Presently, withanolides, the active metabolites of ashwagandha have been commercially obtained by solvent extraction of roots, stems and leaves of the plant. Low yield from the natural source, genotypic and chemotypic variations, heterogeneity in content, long gestation period (3-4 years) between planting and harvesting, and uneconomical chemical synthesis are major constrains in industrial withanolide production (Praveen *et al.,* 2010).

2.4 Various biotechnological interventions of Ashwagandha

Withanolide A has drawn the attention due to its strong neuro pharmacologic properties of promoting neurite outgrowth and synaptic reconstruction (Sangwan *et al.,* 2007). Therefore it could be beneficial in neurologic disorders like Alzheimer's disease and Parkinson's disease, convulsions, cognitive function impairment, *etc*. Withanolide A, isolated from the roots of ashwagandha, (Liffert *et al.,* 2013) has been proved to possess strong pharmacological properties with regard to axonal outgrowth, regeneration of neurites and recovery of damaged synapses in mice. Root-specific production of withanolide A, in root cultures, especially hairy root cultures, and its scaling up via bioreactor is the most important way of producing *in vitro* cultures. But withanolide A has not been detected in *Agrobacterium rhizogene*- transformed hairy roots of ashwagandha (Banerjee *et al.,* 1994; Ray and Jha, 1999). Withanolides spotted in the cultures of aswagandha were identified to be produced primarily by the aerial parts. Withanolide A biogenesis was thus investigated in shoot cultures of ashwagandha which are the tissue culture complements of the indigenous plant's aerial parts (Dhar *et al.,* 2015).

Shoot cultures with specific plant growth regulators were established using explants from two experimental lines -*RSSelection-1* (*RS-Sel-1*) and *RSSelection-2* (*RS-Sel-2*) of ashwagandha (*W. somnifera* (L.) Dunal) on MS medium. Shoot cultures established from *RS-Sel-1* with benzyl aminopurine (BAP) 1.00 ppm and KN 0.50 ppm shown to have the highest concentration of withanolide A (14.3mg per 100g fresh weight and 238mg per 100g dry weight, i.e., 0.24%) in the green shoots. Investigations on the yield of green shoot cultures (0.24% DW) were much more comparable to the yields of *in vivo* grown plant's dried roots. Solid mass of shoot cultures from *RS-Sel-1* also recorded the maximum withanolide A production (3.7mg per 100g fresh weight; 46.2mg per 100g dry weight) with BAP 1.00 ppm and kinetin 0.50 ppm. In addition, RS-Sel-1 has demonstrated superior biogenesis / accumulation of withanolide A compared to RS- Sel-2.

The production of withanolides, a group of cytotoxic steroids was investigated (Suffness and Douros, 1982) in ashwagandha cell and shoots cultures. Internodal stem segments were used to establish multiple shoot cultures and cell cultures. No detectable amounts of withanolides were found in cell cultures. During an incubation time of 7 days, the shoot cultures provided approximately 4 fold growth and yielded 0.32 per cent dry wt. of total withanolides. The distribution of the withanolides in the culture of the shoots was different from that recorded in the plant leaves (Nittala and Lavie, 1981). The shoot cultures did not produce the main component of the plant withaferin A. Thin layer chromatography and high performance liquid chromatography have detected withanone, withanolide G, withanolide I, withanolide E, and trace quantities of 4-/3-hydroxywithanolide E. Extracts from the shoot culture showed cytotoxic activity against suspension culture of the mouse fibroblast L.929.While high concentrations of withanolides were metabolised by the shoot cultures, there were major qualitative differences between the plant and cultures.

Multiple shoot cultures of ashwagandha were developed from single shoot tip explants and explored their capacity for the development of two principle withanolides namely withaferin A and withanolide D. Shoot tips produced on MS

medium supplemented with BA (1 mg l±1) induced 10.0 ± 1.15 microshoots/ explants and shoot cultures produced both withanolides (withaferin A = 0.04 %, withanolide D = 0.06%). Addition of MS (solid) agar medium with 4% sucrose enhanced production of both withaferin A (0.16%) and withanolide D (0.08 %). Reducing the agar concentration to 0.16% has shown increment in the number of microshoots induced /explant to 25.5. MS liquid medium with 10% coconut milk encouraged a maximum increase in biomass (27 fold); number of microshoots induced (37.6 ± 1.45) as well as production of withaferin A (0.14%) (Ray and Jha, 2001).

Through high performance liquid chromatography (HPLC), the distribution of withanolide A in different organs of ashwagandha was examined (Praveen *et al.*, 2010). The quantitative expression of withanolide A in various organs studied was distinct and the accumulation was 386, 342, 272, 206, 102, 56, 35 and 23 µg g^{-1} DW in shoot tips, leaves, nodes, whole plant, internodes, roots, flowers and seeds respectively. Gradually, the amount of withanolide A declined from the aerial parts, i.e. from young leaves to root.

For mass propagation, regeneration from leaf explants of ashwagandha was examined on Murashige and Skoog's medium augmented with Kinetin (Kn) and 6-benzylaminopurine (BAP) alone or in combination. On the abaxial side, in the presence of Kn and BAP (4 µM), shoot buds were stimulated from the midrib. These shoot buds developed into shoots on the same medium. Rooting of these shoots was induced in 0.5 µM of IBA (Joshi and Padhya, 2010).

An effective procedure for *in vitro* plant propagation is established through direct adventitious shoot proliferation from leaf explants of ashwagandha by Kumar *et al.,* (2011). MS medium with 1.5mg/l BAP and 1.5mg/l IAA was identified to be the best for maximum *in vitro* response (i.e., 100% shooting and 68 number of shoots/explant). In MS medium fortified with 0.15mg / l GA_3 and 5mg / l IBA respectively, effective *in vitro* shoot bud elongation and rooting were found. Rooted plants were hardened and transplanted into earthen pots and during

transplantation; 80-90 per cent survival was seen.

Multiple shoots were attempted to trigger directly from shoot tips by inoculating on BAP augmented MS medium (0.5-3.0mg / l) and KN (0.5-3.0mg / l) alone or in combination while explants grown on MS medium without growth regulators were not able to stimulate multiple shoots and each explant grew into a single shoot. Among the three combinations tested viz., (MS+BAP, MS+KN and MS+BAP+KN), the MS medium augmented with BAP+KN treated explants achieved higher response (shooting and shoot number per explants) than those treated with BAP and KN alone. It was evident from this analysis that BAP (2mg / l) + KN (2mg / l) was significantly more successful in triggering shoot organogenesis (100 and 54.0±0.20) and shooting percentage and number of shoots per explant, respectively.

In order to optimize an effective medium for mass multiplication of shoots from nodal explants, the effect of different media on the number and length of shoots was assessed (Baba *et al.,* 2013). The highest numbers of shoots were found in the medium containing high concentration of BAP (2.0 to 3.0 mg/l). When subcultured in the same fresh medium after 15 days period, this medium showed about 15 to 20 numbers of shoots per culture. The length of the shoots ranged from 4 to 5cm. It was not successful in the media with KN and low concentration of BAP. At higher concentration of BAP along with KN, however, rapid propagation and elongation of shoots were observed. MS basal medium supplemented with lower concentration of BAP (0.5 and 1.0 mg/l) and KN i.e. medium M1, M2, M3 and M4 produced 5 to 9 shoots with shoot length 2 to 3 cm, while MS with BAP (2.0 to 3.0 mg/l) with KN (0.5 mg/l) produced maximum number of shoots (15 to 20) with 4 to 5 cm in length. The incorporation of auxin such as NAA at a concentration of (0.5 mg / l) facilitated shoot multiplication.

In vitro micropropagation or multiplication of ashwagandha *viz.* apical shoot proliferation was established by the use of various concentrations of plant hormones within short term (Radhika *et al.,* 2013). Shoots were triggered in MS

basal medium using the apical meristem of ashwagandha augmented with specific concentrations of 6- Benzyl amino purine (BAP) (0.15, 0.20 and 0.25 mg/L). Apical meristem explants showed induction of shoots within 6-7 days of transfer, with optimal concentration of BAP (0.25 mg/l) and it was found to be most efficient for multiple shoot development within 1-2 weeks.

The shoot buds were excised from the initiation media after 15 days and moved to the MS multiplication medium (MS1, MS2, MS3, MS4) with different concentration of hormones MS1 (1 mg/L BAP + 0.3 mg/L IAA), MS2 (0.5 mg/L Kinetin + 0.1 mg/L IAA), MS3 (0.5BAP mg/L + 0.1mg IAA) and MS4 (1 mg/L Kinetin + 0.3 mg/L IAA). MS3 medium displayed the maximum number of shoot regeneration. The established shoots were successfully rooted on the basal MS medium along with activated charcoal and Indole 3-butyric acid (IBA) (optimum concentration - 1mg/L).

Leaf, cotyledon, hypocotyl and epicotyl explants of ashwagandha were used to establish callus by Arumugam and Gopinath, (2013). MS medium with different concentrations of 2,4-D, BAP and NAA were used for callus induction. The highest percentage of callus induction (94.33 ± 1.20%) was found to be developed from the leaf explants in MS media with 2,4-D 3.0 mg/l. The highest number of multiple shoots (85.67 ± 0.88%) was found to be developed from the leaf callus in MS media with 4.0 mg/l BAP. The calli which had shown shoolets were moved to the rooting medium containing 10.0 mg / l NAA to produce multiple roots (89.33 ± 0.88%). The rooted plantlets were relocated to small polythene bags, which contain sterilized cow dung, sand and red soil (1: 2: 3) and kept in a mist house. After acclimation in the mist house the revived plantlets were hardened in the greenhouse and transferred to soil, which had shown 85 percent survival rate.

Another effective and high frequency plantlet regeneration method (Rathore *et al.*, 2016) is through direct organogenesis in *W. coagulans* using *in vitro*-derived leaves as explants. In MS medium with 4.44 μM 6-benzyl aminopurine (BAP),

73.7±4.3% explants responded and produced 11.4±0.9 shoot buds per explants. The replenished shoot buds elongated (6.7±0.22 cm) in MS medium with 1.11 μM BAP and 0.57 μM indole-3-acetic acid (IAA). The elongated shoots rooted both *in vitro* and *ex vitro* conditions. The regenerates were acclimatized by steady and incremental exposure to various regimes of temperature and relative humidity. The leaf- regenerates were examined for genetic stability using RAPD and ISSR molecular markers and confirmed true-to-type. The *in vitro* regeneration system created would be useful for genetic restoration program as the over exploitation and fertility problems forced *W. coagulans* on the brink of total extinction.

The pure compound of Withaferin-A is commercially obtained from the aerial parts of ashwagandha plants but the yield is not adequate for the current market conditions. The synthesis method of withaferin A is complicated and is not commercially feasible. Therefore, enhanced development of withaferin A through *in vitro* cell culturing is extremely desirable. A study on establishment of ashwagandha multiple shoot cultures and the standardization of a liquid shoot culture method for dramatic growth of multiple shoots, as a cost efficient system for the development of withaferin A in an elite accession (AGB002) of ashwagandha was carried out by Mir *et al.,* (2014). Withaferin-A (WS-3), has been proven to be an effective anti-cancer molecule and in this study, a liquid culture system for shoot proliferation, biomass accumulation and withaferin-A production was established.

In a study with nodal explants cultured on MS semisolid medium supplied with different concentration of 6- benzyl adenine (BA) and kinetin (Kn) showed varied responses. The largest number of regenerated shoots per explant (35±3.25) and the overall average shoot length (5.0 ± 0.25 cm) were reported on MS medium with BA (5.0 μM). The shoots were further propagated in half and full strength MS liquid medium with the same concentration BA. The shoots cultured on MS half strength liquid medium fortified with 4 gL^{-1} FW (fresh weight) shoot inoculums mass extracted from 5 week old nodal explants of ashwagandha showed

highest deposition of biomass and withaferin A content in 5 weeks. Withaferin A was generated in relatively larger quantity (1.30 % and 1.10 % DW) in shoots cultures in half and full strength MS liquid media compared to natural field grown plants (0.85 % DW). A significant amount of the withaferin A was also metabolized in the culture medium. Effective proliferation of shoots in liquid media and the synthesis of withaferin A *in vitro* opens new opportunities for both the bioreactor scale-up and compound's mass production (Mir *et al.,* 2014).

Increasing attention has been paid to the development of bioactive metabolites from *in vitro* cultures in recent decades. This method offers significant potential as an effective option for the production of high-value low-volume phytochemicals (Mulabagal *et al.,* 2004). Ahuja *et al.*,(2009) developed multiple shoot cultures of selected AGB002 and AGB025 accessions available in IIIM's Ashwagandha Germplasm Repository, Jammu, with various hormone combinations , BAP, IAA, IBA, NAA and 2, 4-D in varying quantities, either separately or in different combinations (0.5-1.0 mg/L). Repeated subcultures were maintained at every 4 weeks and well adapted proliferative shoot culture lines formed on MS medium with BAP (1.0 mg / L). After six subcultures, four weeks old shoot cultures were used for analysis of withanolides and glycowithanolides. The HPLC study found four glycowithanolides eluted in order of Withanoside IV (WSG-3), Physagulin D (WSG-P), Withastraronolide (WSC-O), and Withanoside VI (WSG- 3A). Both lines of the shoot culture acquired all four glycowithanolides but in variable amounts. WSG-3 content was reported as highest. It was found that there was a significant variation in glycowithanolide content between the parent and *in vitro* regenerated shoots while examining data for the parent plant and the corresponding shoot culture lines.

Multiple shoot cultures of medicinal herbs do provide reliable site of biomolecular input, increased product yields, survival and development of shoot cultures and provide a better option over intact plants (Rao *et al.,* 2002). In fact, the shoot cultures have i) a homogeneous and genetically consistent substance, ii) can be

dispersed in large amounts at any time iii) create a good uniform system for feeding trials with potential biosynthesis precursors under regulated conditions for improved production yield and (iv) include a perfect uniform framework for genomic dissection of the pathways. This study was the first to establish the production of glycowithanolides in ashwagandha based on *in vitro* shoot culture. It indicates that implementing *in vitro* culture conditions won't have any impact on the biosynthetic pathway for the glycowithanolides.

Since ashwagandha is mostly propagated by seeds and the survival rate of vegetative propagation is very poor, these fast and effective regeneration procedures could be used to produce chosen harvested varieties on a large scale. Such direct methods of regeneration, which reduce the genetic instability commonly experienced during callus mediated regeneration, may help to generate a large variety of selected ashwagandha superior chemo types which have strong demand in the present Indian market.

2.5 Elicitation

Secondary metabolites develop in plant cells in response to diverse biotic stresses (e.g. pathogens or insects) and abiotic stresses (e.g. temperature, salinity, water, radiation, heavy metal, and minerals) (Ramakrishna and Ravishankar, 2011). Such varying stress conditions were referred to as "elicitors'' (Dornenburg and Knorr, 1995), and elicitation was commonly used for the overproduction of secondary metabolites in plant cell and organ cultures (Dornenburg and Knorr, 1995; Ramakrishna and Ravishankar, 2011). The fungal, bacterial, and yeast origin elicitors (e.g., polysaccharides, glycoproteins, inactivated enzymes, purified crudlan, xanthan, and chitosan) and heavy metal salts have been known to stimulate the excessive production of secondary metabolites. Signaling molecules like methyl jasmonate and salicylic acid were also generally used to produce secondary metabolites in cell cultures and organ cultures (Kim *et al.,* 2004; Dong *et al.,* 2010; Shohael *et al.,* 2008; Thanh *et al.,* 2005; Yu *et al.,* 2002). Other significant factors affecting the effective development of biomass and secondary

metabolite aggregation are elicitor concentrations, period of exposure, and age or stage of the culture at the time of elicitor treatment.

Similarly, jasmonates have been used to elicit an increase in the accumulation of taxol in the cell cultures of *Taxuschinensis* (Ketchum *et al.,* 1999), saikosaponins in the root cultures of *Bupleurum falcatum* (Aoyagi *et al.,* 2001), and eleutherosides in the embryo cultures of *Eleutherococcus senticosus* (Shohael *et al.,* 2007).

Adventitious root cultures of ashwagandha, derived from leaf derived callus were treated with methyl jasmonate and salicylic acid separately by Sivanandhan *et al.*, 2012. In the two varieties collected from Kolli hills (Eastern Ghats) and Cumbum (Western Ghats) of Tamil Nadu, India, the biomass accumulation, culture age, elicitation period, and culture duration were optimized for higher withanolides production. Salicylic acid (SA) has improved the production of major withanolides (withanolide A, withanolide B, withaferin A, and withanone) as well as minor constituents (12-deoxy withastramonolide, withanoside V, and withanoside IV) in the Kolli hills variety among the two elicitors. The treatment of root biomass (11.70 g FW) on 30-day-old adventitious root cultures with 150 µM SA for 4 h elicitor exposure period resulted in the production of 64.65 mg g^{-1} dry weight (DW) withanolide A (48-fold); 33.74 mg g^{-1} DW withanolide B (29-fold); 17.47 mg g^{-1} DW withaferin A (20-fold); 42.88 mg g^{-1} DW withanone (37-fold); 5.34 mg g^{-1} DW 12-deoxy withastramonolide (nine fold); 7.23 mg g^{-1} DW withanoside V (seven fold); and 9.45 mg g^{-1} DW withanoside IV (nine fold) after 10 days of elicitation (40th day of culture) when compared to untreated cultures.

Sivanandhan *et al.,* (2014) studied the impacts of *Gracilaria edulis* and *Sargassum wightii* extracts for the production of biomass and withanolides in the ashwagandha multiple shoot suspension culture. Addition of 40 per cent *G. edulis* extract in MS liquid medium for 24 h exposure time in the culture reported the highest aggregation of biomass [62.4 g fresh weight and 17.82 g dry weight (DW)]

and withanolides production (withanolide A 0.76 mg/g DW; withanolide B 1.66 mg/g DW; withaferin A 2.80 mg/g DW and withanone 2.42 mg/g DW) after 5 weeks of culture, which were 1.45–1.58 fold higher than control culture. This naturally available *G. edulis* extract treated multiple shoot suspension culture procedure provides a possible alternative for optimum development of biomass and withanolides using shake-flasks.

The effect of cytokinins and conditions of culture including medium volume, harvest time and elicitation with abiotic elicitors (Salicylic acid / Methyl Jasmonate) has been analyzed in the multiple shoot culture of ashwagandha for proper development of biomass and withanolides (Sivanandhan *et al.,* 2013). Shoot inoculum mass (2 g l^{-l} FW) elicited at 100 µM of salicylic acid in the with 0.6 mg l^{-l} BA and 20 mg l^{-l} spermidine at 4 h exposure time during the 4th week in 20 ml liquid medium with higher withanolide generation (withanolides A [8.48 mg g^{-l} DW], withanolides B [15.47 mg g^{-l} DW], withaferin A [29.55 mg g^{-l} DW] and withanone [23.44 mg g^{-l} DW]), which were 1.14 to 1.18 fold higher compared to the elicitation with methyl jasmonate at 100 µM after 5 weeks of culture. The cultures elicited by salicylic acid did not show much difference in the accumulation of biomass compared to control. This cytokinin stimulation and multiple shoot culture protocol elicited with salicylic acid provide a great opportunity for optimum production of withanolides using liquid cultures.

Ashwagandha's leaf and root extracts were found to increase the glucose uptake in myotubes and adipocytes in a dose dependent manner, more active the with the leaf extract than the root (Gorelick *et al.,* 2015). Leaf extracts increased insulin secretion in basal pancreatic beta cells but no increase in insulin secretion in the stimulated cells. Six withanolides isolated from ashwagandha were tested for anti-diabetic activity based on glucose uptake in skeletal myotubes. Comparing with the control withaferin A was found to have an increase in the glucose uptake, with 10 µM producing a 54% increase, suggesting that withaferin A is at least partially responsible for ashwagandha's anti-diabetic activity. Elicitors were supplemented to the root growing

solutions, affected the physiological state of the plants, altering membrane leakage or osmotic potential. Methyl salicylate and chitosan increased withaferin A content by 75% and 69% respectively, and extracts from elicited plants increased the glucose uptake to a higher extent than that of the non-elicited plants, demonstrating a correlation between increased content of withaferin A and anti-diabetic activity.

Rangaraju *et al.,* 2019 have studied two varieties of ashwagandha Jawahar Ashwagandha-20 (JA-20) and Arka Ashwagandha (AA). The seeds were raised in *in vitro* conditions and morphological parameters were observed in both varieties. The adventitious roots were established from leaves of the two varieties under *in vitro* conditions. The effects of the media strength, amount and the combination of auxins (IAA and IBA) for adventitious roots multiplication using suspension culture were observed. The suspension cultures were kept for 30 days incubation, after that the root biomass was measured. The HPLC analysis of major withanolides in leaves and adventitious roots was done. Higher content of total withanolide 1.621 mg/g, withaferin A content of 1.362 mg/g and root yield of 4.066 g from 0.1g inoculum in 30 days was observed in Arka Ashwagandha variety as compared to Jawahar Ashwagandha-20 with a total withanolide content of 1.156 mg/g, *w*ithaferin A content of 0.930 mg/g on dry weight basis and root mass of 3.71g from 0.1g of inoculum in 30 days. Thus this study helped in identifying an elite cultivar of ashwagandha and a standard protocol for mass multiplication and proper growth of adventitious root in hormone-free media.

2.6 Quantification of withanolides by spectroscopical analysis

Modern analytical methods, such as chromatographic methods, are expensive and time-consuming, where as a UV spectroscopic method is simple, fast, sensitive, reliable, and efficient, resulting in short-term results. There are many reports for the quantification of alkaloid content in species of ashwagandha based on spectroscopic analysis. Root extracts of ashwagandha were used as per standard protocols to examine the secondary metabolites and quantify some of the active constituents such as alkaloids, flavonoids, saponins, and volatile oil. Preliminary

phytochemical analysis of cold and hot ethanol, methanol and aqueous extracts has shown the existence of alkaloids, saponins, flavonoids, steroids, tannins, proteins, reduced sugar and coumarins and the lack of quinones or anthraquinones. The total alkaloid, flavonoid contents were found to be 0.81±0.01%, 14.43±0.40% and total saponin content was (Foaming Index) FI<100 respectively. The substantial volatile oil content in the fresh root of ashwagandha has not been defined. The observations are consistent with the presence of biologically active constituents in ashwagandha polar extracts, which can be useful in validation and recognition of this herb (Zhang *et al.*, 2002).

Lini *et al.,* (2014), attempted to exploit the effect of light and moisture stress on the development of withanolide in *Withania somnifera* (L.) Dunal. Experiments on pot culture were carried out by introducing different levels of shade (25%, 50% and 75% shade) and water stress (75%, 50% and 25% field capacity) along with control under optimal conditions. The exposure of plants to these abiotic stressors has been proven to improve the development of withanolide for duration of 30 days, with a maximum accumulation under 75 percent shade condition. Plant growth and translocation pattern analysis have also shown beneficial improvements under low light conditions. In phytomedical enrichment programs, examining the importance of abiotic stressors such as low light and moisture stress, as associated with secondary metabolite production, is of significant relevance. Withanolide estimation was conducted as per the procedure described by Mishra, (1994). The concentration of withanolide was expressed as mg/g.

Sreevidya and Mehrotra (2003) developed a fast, quick and simple spectrophotometric method to estimate total alkaloids precipitated by the Dragendorff reagent (DR) in plant materials. It was based on the formation of yellow bismuth complex in nitric acid medium with thiourea. The yellow-colored complex formed obeys Lambert-Beer’s law in the concentration range of 0.06–50 µg/mL with maximum concentration at 435 nm. This method was used to measure, the percentage of alkaloids and some plant materials containing alkaloids

(*Withania somnifera, Berberis aristata, Solanum nigrum*, and *Piper longum*). The method was compared with other methods. It can be used by industries handling herbal medicines for a regular study of industrial samples for standardizing alkaloid- containing plant materials and for pharmaceutical products containing alkaloids.

A clear, responsive, and quick spectrophotometric method was used to determine total alkaloids in medicinal plants by Trivedi *et al.,* 2016. The method was based on the alkaloid reaction with bromocresol green (BCG) which forms a yellow product. The low yield and high market value of the pharmaceutically relevant alkaloids have prompted research in improved alternative production methods such as the use of tissue and cell culture. Therefore, the study aimed trial to alkaloid production *in vivo* (plant leaves, stems and roots) and *in vitro* callus culture conditions. The results showed that the callus leaf extract showed highest alkaloid content followed by root extract of mother plant. The callus stem sample has also more alkaloid content than stem of mother plant. In *in vivo* plant parts root showed highest alkaloid content. The alkaloid content varied with respect to the parts analysed. Spectrophotometric determination of total alkaloids with bromocresol green is a simple and sensitive method and does not need very special equipment. The proposed method has the advantage of being less time consuming, with the assay requiring an average of 1 hr. Therefore the method described in this study can be used for determination of a special group of alkaloids.

3. MATERIALS AND METHODS

The present study on the "Development of multiple shoot culture of ashwagandha (*Withania somnifera* (L.) Dunal) for *in vitro* alkaloid stimulation studies" was conducted during the period 2019-2020 at the Department of Plant Biotechnology and Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The materials used and methods followed are described below.

3.1 MATERIALS

3.1.1 Plant material

Seeds of ashwagandha, variety Arka ashwagandha procured from IIHR Bangalore were used for the study. The seeds procured were used for various treatments of seed germination. The explants derived from one month old *in vitro* grown seedlings were used for subsequent treatments for multiple shoot induction and maintained for 1 month to observe the response. Based on the response of these explants, a treatment that has shown the best response was adjudged as the best procedure for multiple shoot induction needed for the alkaloid stimulation studies of ashwagandha.

3.1.2 Chemicals and Glass/plasticwares

The chemicals and solvents used to prepare MS medium were from the company M/S Merck India, Mumbai. The MS medium (readymade, without agar) was also used for media preparation from Hi Media India, Karnataka. The sterilizing agents used for the surface sterilization of the seeds were from M/S Merck India, Mumbai and S.D. fine Chem. Ltd. India, Bombay. The plant growth regulators (PGRs), vitamins and other fine chemicals used in germination and shoot multiplication media were from Sigma Chem. Co., USA. All glasswares used for tissue culture work were from Borosil, India Ltd. except culture bottle with polypropylene cap (Excel Glasses Ltd., Alleppey and Steriliq Polypacks,Cochin).

3.1.3 Equipments

For the MS medium preparation microwave oven (Panasonic, Japan) was used for dissolving the agar. Later the medium, glasswares, forceps and scalpels (Hindustan Surgicals India, Tamil Nadu) were sterilized in an autoclave (Nat Steel Equipments Pvt Ltd India, Bombay,). The wet glasswares were dried using a hot-Air Oven (Oven Universal, India). The inoculation of the seeds and explants into respective medium was done using air flow cabinet (SteriClean Systems India, Chennai). Finally the culture bottles were incubated in the tissue culture stands (Symbiosis, India) for further studies.

3.1.4 Nutrient media for Tissue culture

Full and Half – strength MS (Murashige and Skoog, 1962) medium were used for the tissue culture experiments (Appendix I). Half-strength MS medium consisted of half- strength of macro, microelements and vitamins and full concentration of sucrose (3%) and myoinositol (100mg/l). The pH of MS medium was adjusted to 5.8 before adding agar (0.6% w/w) for solid medium and autoclaved at 121 ºC and 108 KPa for 18 mins.

3.1.5 Incubation Conditions

The cultures were incubated in culture bottles with a specified medium and maintained in culture room at 25 ± 2 ºC with a relative humidity of 50-60 % and 16 h photoperiod at a photon flux density of 50-60 µEm-2s-1 using daylight fluorescent tubes (Philips India Ltd India, Mumbai). In the culture room, seed cultures were incubated under the dark condition for seed germination during the initial period of 5- 6 days and after that, they were transferred to a light condition in the culture room.

3.2 METHODOLOGY

3.2.1 Establishment of aseptic seedlings

The seeds of the Arka Ashwagandha variety were collected from IIHR Bangalore. The seeds were kept in an airtight container and stored in the refrigerator at 20ºC. In treatment S_1 seeds were soaked in water overnight, next day they were surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated to half strength MS media without hormones. In S_2 seeds were soaked in 250ppm GA_3 overnight, next day they were surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media without hormones. In S_3, the seeds were soaked in tender coconut water for 1hour and then soaked in 250ppm GA_3 overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3. In treatment S_4 the seeds were soaked in water overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3. In S_5 the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 5 minutes and then inoculated in half strength MS media with 0.3% GA_3. In S_6 the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.05% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3% GA_3. In S_7 the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.05% $HgCl_2$ treatment for 5 minutes and then inoculated in half strength MS media with 0.3% GA_3.

The cultures were incubated in a culture room at 25 ± 2 ºC with a relative humidity of 50-60 % and 16 h photoperiod at a photon flux density of 50-60µEm-2s-1 from daylight fluorescent tube (Philips India Ltd, Mumbai). In the culture room, seeds were incubated under dark during the initial period of 5- 6 days and after that, they were transferred to light.

After germination was noticed, number of days taken for the seeds to germinate from the day of inoculation was noted, after observing for two weeks percentage of germination was determined by counting total number of seeds germinated to the total number of seeds inoculated, after that an average of the percentage values got from all the culture bottles were calculated. The seedlings were also observed every week and measured the length of 10 seedlings from each culture bottle and take an average value of the shoot length using centimeter scale, after that a final average of all these values were calculated to determine the shoot length of the seedlings.

3.2.2 Initiation and Establishment of Shoot Cultures

Nodal, leaf and shoot tip explants (0.5-1cm) were excised from 4 weeks old *in vitro* grown seedlings and inoculated onto the full strength - MS solid medium supplemented with different combinations of growth regulators:

- MS without hormones
- MS+1mgNAA+3mgKn
- MS+1.5mgNAA+1mgKn
- MS+1mgNAA+1.5mgKn
- MS+0.5mgBA+0.1mgIAA
- MS+2mgBA
- MS+2.5mgBA
- MS+3mgBA+1mgKn

The cultures were incubated under a 16 h photoperiod for 4 weeks and data were recorded at regular intervals. After 4 weeks of shoot culture initiation, the cultures were observed for one week's interval and determined the number of cultures showing the shoot induction, the percentage of shoot induction were calculated

with the total number of shoots induced per explants to the total number of explants inoculated, average shoot number of the multiple shoots were determined by counting the total number of multiple shoots induced in 10 explants of all the culture bottles and took an average of these values.

Finally, the average shoot length was also evaluated by measuring the approximate length of multiple shoots induced in 10 explants of all the culture bottles, they were taken out from the culture bottles and kept in the laminar air flow under sterile condition. A sterile twine thread was used to measure the length of the multiple shoots, position of the thread showing the length of the shoots were marked and the length was determined from that marked position on the thread with a centimeter scale. Thus an approximate length of multiple shoots induced in 10 explants of all the culture bottles was determined and an average value was calculated. After that these cultures were maintained by subculturing at a two weeks interval and observing the responses.

4. RESULTS

The study entitled "Development of multiple shoot culture of ashwagandha *(Withania somnifera)* for *in vitro* alkaloid stimulation studies" was conducted at Department of Plant Biotechnology and Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram, during 2019 -2020. The primary objective of this study was to establish multiple shoot cultures of ashwagandha (*W. somnifera*) for *in vitro* alkaloid stimulation studies. The results obtained from the study are summarized below.

4.1 Establishment of Aseptic Seedlings

The seeds of ashwagandha were subjected to different treatments as indicated in Table (1) and the *in vitro* cultures were maintained in the culture room at 25 ± 2^0C temperature, 60 ± 10% relative humidity in dark condition till germination was noticed. After germination they were transferred to light condition (25 ± 2^0C temperature, 60 ± 10% relative humidity, 16h photoperiod) in the culture room for proper growth of the seedlings. The effect of these treatments on seed germination was observed based on different parameters *viz.* days of germination, percentage of germination and shoot length of seedlings.

Table No.1: Details of various seed treatments adopted to enhance *in vitro* seed germination of ashwagandha

Treatments	Pretreatment of seeds	Seeds were treated with 5% Tween 20 for 5 minutes	$HgCl_2$ treatment	Media treatment
S_1	Overnight soaking in water		0.1% for 10 minutes	Half MS without hormones
S_2	Overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS without hormones
S_3	Tender coconut water treatment for 1hour then overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS with 0.3ppm GA_3
S_4	Overnight soaking in water		0.1% for 10 minutes	Half MS with 0.3ppm GA_3
S_5	Overnight soaking in water		0.1% for 5 minutes	Half MS with 0.3ppm GA_3
S_6	Overnight soaking in water		0.05% for 10 minutes	Half MS with 0.3ppm GA_3
S_7	Overnight soaking in water		0.05% for 5 minutes	Half MS with 0.3ppm GA_3

4.1.1 Time period taken to induce *in vitro* seed germination in various treatments

The time taken to induce germination in seeds of ashwagandha was observed in all the treatments mentioned in Table 1. The observations on the effect of different *in vitro* seed germination treatments in the time duration taken to induce germination is given in the table (2) below:

Table No.2: Effect of different treatments of seed germination on time period for germination

Treatments	Pretreatment of seeds	Seeds were treated with 5% Tween 20 for 5 minutes	$HgCl_2$ treatment	Media treatment	Days of germination
S_1	Overnight soaking in water		0.1% for 10 minutes	Half MS without hormones	No germination found
S_2	Overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS without hormones	No germination found
S_3	Tender coconut water treatment for 1hour then overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	14^{th} day
S_4	Overnight soaking in water		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	No germination found
S_5	Overnight soaking in water		0.1% for 5 minutes	Half MS with 0.3ppm GA_3	6^{th} day
S_6	Overnight soaking in water		0.05% for 10 minutes	Half MS with 0.3ppm GA_3	No germination found
S_7	Overnight soaking in water		0.05% for 5 minutes	Half MS with 0.3ppm GA_3	No germination found

Out of these seven treatments, S_3 and S_5 treatments resulted in the germination of seeds. In S_3 (seeds were soaked in tender coconut water for 1hour and then in 250ppm GA_3 overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes, 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated to half strength MS media with 0.3 ppm GA_3), the induction of germination was found on 14^{th} day of inoculation. But in the case of S_5 (seeds soaked in water overnight and next day treated with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated to half strength MS media with 0.3 ppm GA_3), the induction was found on 6^{th} day of inoculation, that is within one week of inoculation, germination was found in the dark condition. After that the seeds were transferred to light condition. The treatments S_1, S_2, S_4, S_6 and S_7 did not show any induction of germination. Thus S_5 was identified as the treatment for faster seed germination.

4.1.2 Effect of different *in vitro* seed germination treatments on percentage of germination

Second parameter observed was the percentage of seed germination in these treatments. The effect of different *in vitro* seed germination treatments on percentage of germination is given in the table (3) below:

Table No.3: Effect of different treatments of seed germination on percentage of germination

Treatments	Pretreatment of seeds	Seeds were treated with 5% Tween 20 for 5 minutes	$HgCl_2$ treatment	Media treatment	Percentage of seed germination (%)
S_1	Overnight soaking in water		0.1% for 10 minutes	Half MS without hormones	0
S_2	Overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS without hormones	0
S_3	Tender coconut water treatment for 1hour then overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	40
S_4	Overnight soaking in water		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	0
S_5	Overnight soaking in water		0.1% for 5 minutes	Half MS with 0.3ppm GA_3	100
S_6	Overnight soaking in water		0.05% for 10 minutes	Half MS with 0.3ppm GA_3	0
S_7	Overnight soaking in water		0.05% for 5 minutes	Half MS with 0.3ppm GA_3	0

Among these treatments, S_3 has shown only 40% of germination. In this treatment even though germination was induced the development of seedlings was very poor and slow. Maximum germination rate of 100% was found in the treatment S_5. The treatments S_1, S_2, S_4, S_6 and S_7 did not show any response. It was also observed that, not all seeds germinate at the same time. But after *in vitro* transfer of the germinated seeds to another culture bottle with fresh medium and keeping the non-germinated seeds under light condition in tissue culture stands, germination was noticed in those seeds also within 2-3 days. Thus S_5 was identified as the best treatment for *in vitro* seed germination (100%) of ashwagandha.

4.1.3 Effect of different *in vitro* seed germination treatments on shoot length of seedlings

Another parameter observed was the shoot length of the seedlings in these treatments. The effect of different *in vitro* seed germination treatments on shoot length of seedlings is given in the table (4) below:

Table No.4: Effect of different treatments of seed germination on shoot length of seedlings

Treatments	Pretreatment of seeds	Seeds were treated with 5% Tween 20 for 5 minutes	$HgCl_2$ treatment	Media treatment	Shoot length of seedlings (cm)
S_1	Overnight soaking in water		0.1% for 10 minutes	Half MS without hormones	-
S_2	Overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS without hormones	-
S_3	Tender coconut water treatment for 1hour then overnight soaking in 250ppm GA_3		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	3.75
S_4	Overnight soaking in water		0.1% for 10 minutes	Half MS with 0.3ppm GA_3	-
S_5	Overnight soaking in water		0.1% for 5 minutes	Half MS with 0.3ppm GA_3	5.87
S_6	Overnight soaking in water		0.05% for 10 minutes	Half MS with 0.3ppm GA_3	-
S_7	Overnight soaking in water		0.05% for 5 minutes	Half MS with 0.3ppm GA_3	-

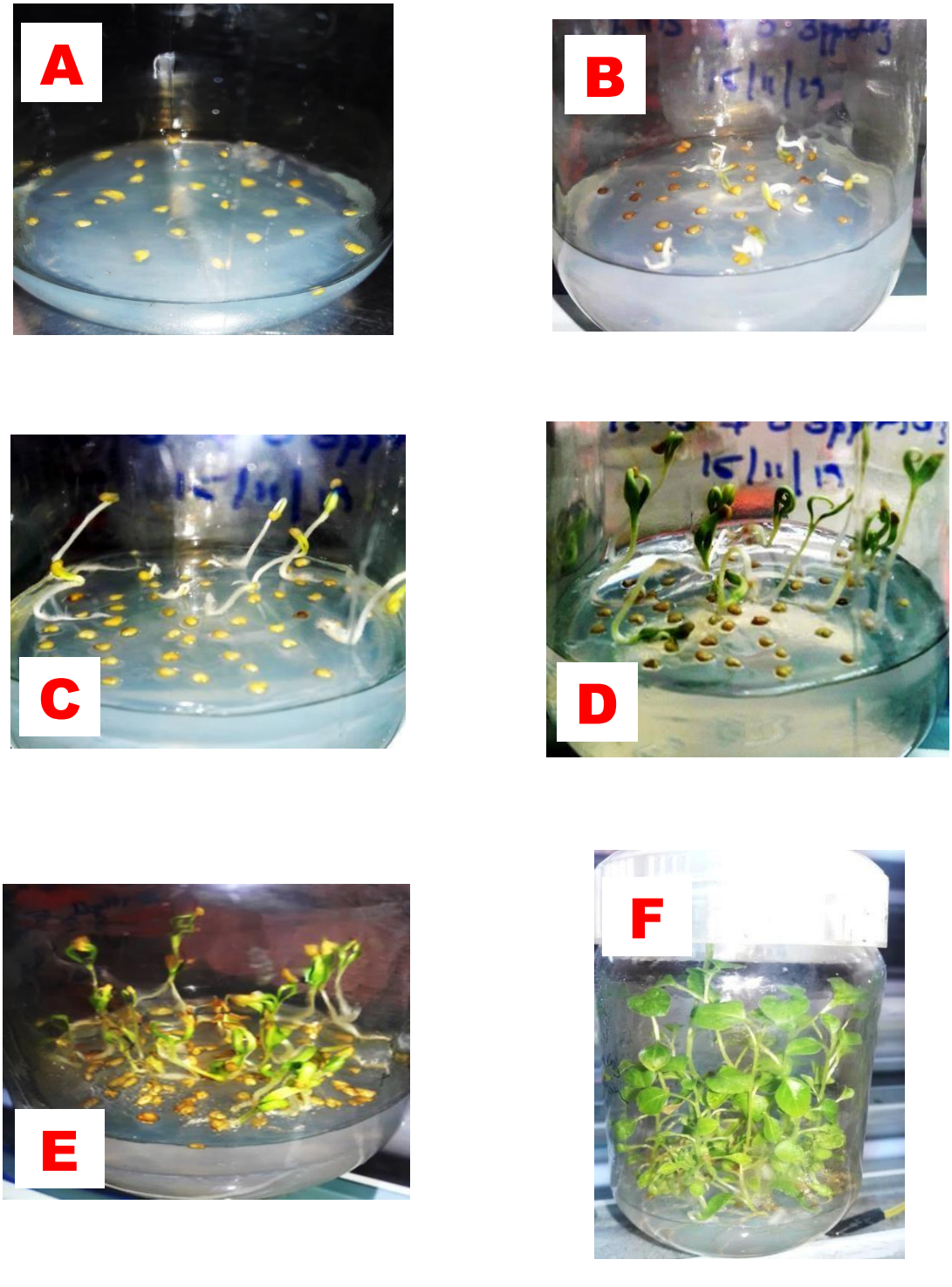

Plate: 1 Stages of seed germination of *Withania somnifera* in half MS with 0.3 ppm GA_3 (Treatment S_5) A: Seeds inoculated in MS media B: Seeds at 6^{th} day of inoculation C: At 12^{th} day of inoculation D: At 18^{th} day of inoculation E: At 24^{th} day of inoculation F: Two month old seedlings

Since germination was seen in S_3 and S_5, average shoot length of these treatments were noted. In treatment S_3, the average shoot length was about 3.75 cm (in 1 month) and in S_5 treatment, the average shoot length was 5.87 cm (in 1 month). Compared to S_3, the treatment S_5 has shown rapid development of the seedlings within a short period of time.

Other than the observations mentioned above another important advantage of treatment S_5 observed was that, after subculturing the germinated seedlings to fresh medium the old culture bottles with non-germinated seed were again kept in the culture before discarding them. Surprisingly seeds which didn't show the germination at the first time started showing the germination within 3 to 4 days after subculture. Thus after observing all these parameters, treatment S_5 was identified as the best one for *in vitro* seed germination of ashwagandha. Hence S_5 treatment was used to raise the *in vitro* grown mother plants in the present study.

4.2 Initiation and Establishment of Shoot Cultures

For establishing multiple shoot cultures, various explants were taken from one month old *in vitro* seedlings. The explants used were nodes, leaves and shoot tips. These explants were subjected to various treatments as shown in Table (5) for establishing shoot cultures.

The treatments used are shown in the table (5) below:

Table No.5: Different treatments used for multiple shoot induction of ashwagandha

Treatments	**Media**	**Response of explants after 1 month of inoculation**		
		Shoot tip	**Nodal segment**	**Leaf**
T_0	MS without hormones	No response	No response	No response
T_1	MS+1mgNAA+3mgKn	Shoot buds were found	Shoot buds were found	Shoot buds were found
T_2	MS+1.5mgNAA+1mgKn	Shoot buds were found	Shoot buds were found	Shoot buds were found
T_3	MS+1mgNAA+1.5mgKn	No response	No response	Shoot buds were found
T_4	MS+0.5mgBA+0.1mgIAA	Shoot buds were found	Multiple shoots were found	Callus found
T_5	MS+2mgBA	Shoot buds were found	Multiple shoots were found	Callus found
T_6	MS+2.5mgBA	No response	No response	Shoot buds were found
T_7	MS+3mgBA+1mgKn	Shoot buds were found	Shoot buds were found	Callus found

Different explants (nodes, leaves and shoot tips) were subjected to the treatments mentioned above (Table 5). These explants were excised (0.5-1 cm) from *in vitro* grown seedlings and inoculated in the culture bottles with specific media (Table 5) under *in vitro* conditions for establishing multiple shoots and they were maintained in controlled condition (25 ± 2^0C temperature, 60 ± 10% relative humidity and 16h photoperiod) in the tissue culture room. The effect of different treatments on these explants was noted constantly and the response was found after 30 days of inoculation.

4.2.1 Effect of different shoot multiplication media on shoot induction of leaf explants

The leaf explants were excised from the *in vitro* grown seedlings and placed with the adaxial surface touching the MS media supplemented with growth hormones. The effect of different treatments on leaf explants are given in the tables (6.1 & 6.2) below:

Table No.6.1: Effect of different shoot multiplication media on number of cultures and number of shoots/explants in leaf explants

Treatments	Media	Number of cultures showing shoot induction	Number of shoots/explant
T_0	MS without hormones	-	-
T_1	MS+1mgNAA+3mgKn	1±0.330	3±0.512
T_2	MS+1.5mgNAA+1mgKn	1±0.330	3±0.512
T_3	MS+1mgNAA+1.5mgKn	1±0.330	3±0.512
T_4	MS+0.5mgBA+0.1mgIAA	-	-
T_5	MS+2mgBA	7±0.578	8±0.650
T_6	MS+2.5mgBA	-	-
T_7	MS+3mgBA+1mgKn	-	-

Table No.6.2: Effect of different shoot multiplication media on shoot induction of leaf explants

Treatments	Media	Average shoot length (cm)	Percentage shoot induction (%)	Callusing
T_0	MS without hormones	-	-	-
T_1	MS+1mgNAA+3mgKn	0.25±0.050	10±0.350	-
T_2	MS+1.5mgNAA+1mgKn	0.25±0.050	10±0.320	-
T_3	MS+1mgNAA+1.5mgKn	0.25±0.050	10±0.320	-
T_4	MS+0.5mgBA+0.1mgIAA	-	-	+
T_5	MS+2mgBA	0.75±0.200	12±0.482	-
T_6	MS+2.5mgBA	-	-	+
T_7	MS+3mgBA+1mgKn	-	-	+

T_0 (treatment) was the control used in which the leaf explants were inoculated to full strength MS media without any growth regulators. It did not show any response in the leaf explants. In treatments T_1, T_2 and T_3, shoot induction was found after 30 days of inoculation, but the number of cultures showing multiplication (1±0.330), number of shoots per explant (3±0.512) and percentage were very poor (10%) and the average shoot length in these treatments were 0.25 cm. In the treatments T_4, T_6 and T_7, no shoot inductions were found, but in these treatments there was callus induction. Compared to rest of the treatments, T_5 showed better shoot induction (12%; the number of cultures showing multiplication-7±0.578; number of shoots per explant-8±0.650), after 30 days of inoculation with an average shoot length of 0.75±0.200cm. Even though shoot induction was found in T_5, the development of shoots was very poor and slow.

4.2.2 Effect of different shoot multiplication media on shoot induction of shoot tip explants

The shoot tip explants were excised (0.5-1 cm) from the *in vitro* grown seedlings and inoculated in MS medium supplemented with growth hormones. The effect of different treatments on shoot tip explants is given in tables (7.1 & 7.2) below:

Table No.7.1: Effect of different shoot multiplication media on number of cultures and number of shoots/explants in shoot tip explants

Treatments	Media	Number of cultures showing shoot induction	Number of shoots/explant
T_0	MS without hormones	-	-
T_1	MS+1mgNAA+3mgKn	1±0.330	3±0.512
T_2	MS+1.5mgNAA+1mgKn	1±0.330	3±0.512
T_3	MS+1mgNAA+1.5mgKn	-	-
T_4	MS+0.5mgBA+0.1mgIAA	5±0.412	5±0.220
T_5	MS+2mgBA	8±0.250	10±0.560
T_6	MS+2.5mgBA	-	-
T_7	MS+3mgBA+1mgKn	5±0.420	5±0.970

Table No.7.2: Effect of different shoot multiplication media on shoot induction of shoot tip explants

Treatments	Media	Average shoot length (cm)	Percentage shoot induction (%)	Callusing
T_0	MS without hormones	-	-	-
T_1	MS+1mgNAA+3mgKn	0.25±0.050	10±0.350	-
T_2	MS+1.5mgNAA+1mgKn	0.25±0.050	10±0.320	-
T_3	MS+1mgNAA+1.5mgKn	-	-	-
T_4	MS+0.5mgBA+0.1mgIAA	0.5±0.540	16±0.210	-
T_5	MS+2mgBA	1±0.350	33±0.07	-
T_6	MS+2.5mgBA	-	-	-
T_7	MS+3mgBA+1mgKn	0.5±0.560	16±0.235	-

T_0 (treatment) was used as a control in which the shoot tip explants were inoculated in full strength MS media without any growth regulators. This failed to induce multiple shoots. T_3 and T_6 treatments also failed to induce shoot cultures. In treatments T_1, and T_2 shoot induction was found after 30 days of inoculation, but the number of cultures showing multiplication (1±0.330), number of shoots per explant (3±0.512), percentage were very poor (10%) and the average shoot length in these treatments were 0.5 cm. Treatments T_4 and T_7 showed 16% shoot induction (the number of cultures showing multiplication-5±0.412, number of shoots per explant-5±0.220; the number of cultures showing multiplication-5±0.420, number of shoots per explant-5±0.970), with an average shoot length of 0.5 cm. Maximum shoot induction was shown by shoot tip explants (33%; the number of cultures showing multiplication-8±0.250, number of shoots per explant-10±0.560) in treatment T_5 (MS+ 2mg BA). Average shoot length was also maximum (about 1±0.350cm) in T_5. Even though shoot induction was found in T_5, the development of the shoot was not satisfactory in this treatment.

4.2.3 Effect of different shoot multiplication media on shoot induction of nodal explants

The nodal explants were excised (0.5-1 cm) from the *in vitro* grown seedlings and inoculated in MS media supplemented with growth hormones. The effect on nodal explants is given in the tables (8.1 & 8.2) below:

Table No.8.1: Effect of different shoot multiplication media on number of cultures and number of shoots/explants in nodal explants

Treatments	Media	Number of cultures showing shoot induction	Number of shoots/explant
T_0	MS without hormones	-	-
T_1	MS+1mgNAA+3mgKn	5±0.420	5±0.320
T_2	MS+1.5mgNAA+1mgKn	5±0.321	4±0.870
T_3	MS+1mgNAA+1.5mgKn	-	-
T_4	MS+0.5mgBA+0.1mgIAA	8±0.520	10±0.750
T_5	MS+2mgBA	20±0.570	15±0.670
T_6	MS+2.5mgBA	-	-
T_7	MS+3mgBA+1mgKn	8±0.540	10±0.860

Table No.8.2: Effect of different shoot multiplication media on shoot induction of nodal explants

Treatments	Media	Average shoot length (cm)	Percentage shoot induction (%)	Callusing
T_0	MS without hormones	-	-	-
T_1	MS+1mgNAA+3mgKn	0.5±0.541	16±0.245	-
T_2	MS+1.5mgNAA+1mgKn	0.3±0.324	16±0.287	-
T_3	MS+1mgNAA+1.5mgKn	-	-	-
T_4	MS+0.5mgBA+0.1mgIAA	1±0.258	33±0.641	-
T_5	MS+2mgBA	1.85±0.750	80±0.05	-
T_6	MS+2.5mgBA	-	-	-
T_7	MS+3mgBA+1mgKn	1±0.254	33±0.624	-

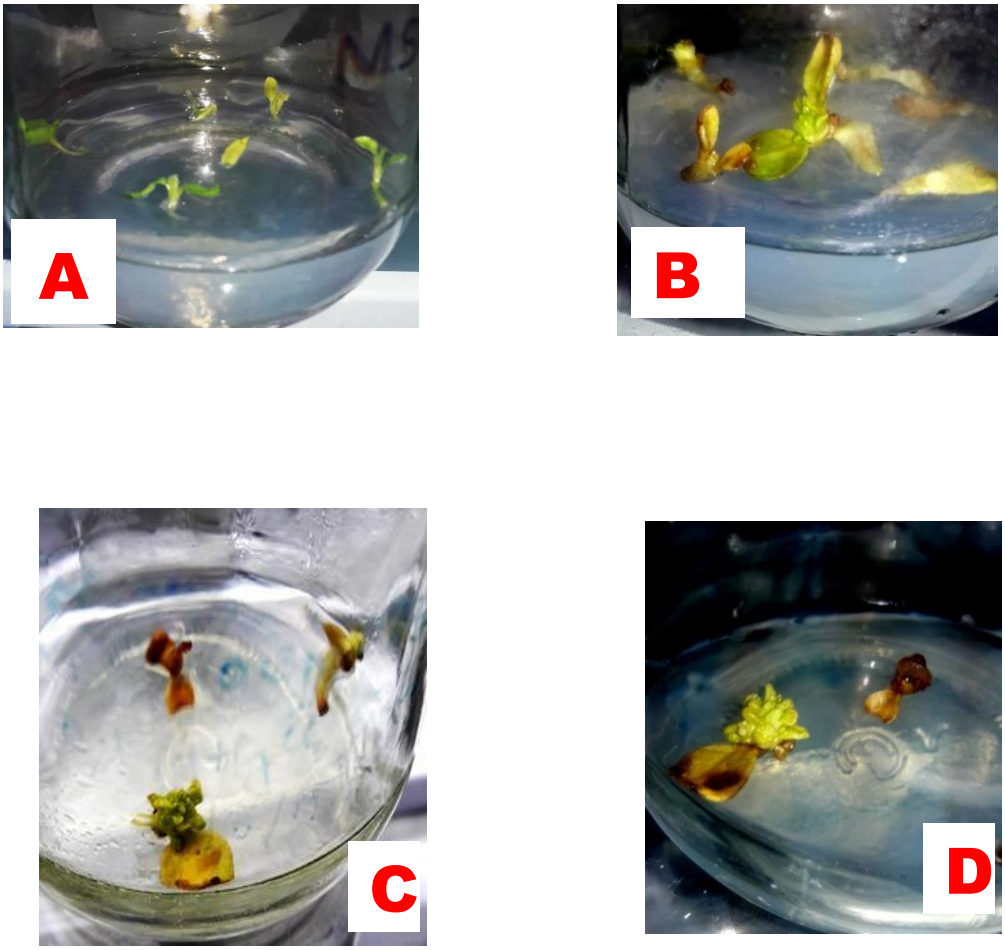

Plate: 2 Stages of shoot induction in nodal explants in treatment T_5 (MS+2mg BA) A: Nodal explants inoculated in MS media B: Shoots induced after 30 days of inoculation C: After 1st subculture (4th day of inoculation) D: After 30 days of 1st subculture E: After 2nd subculture (6th of inoculation)

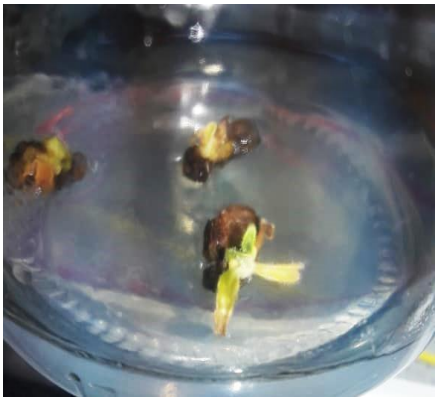
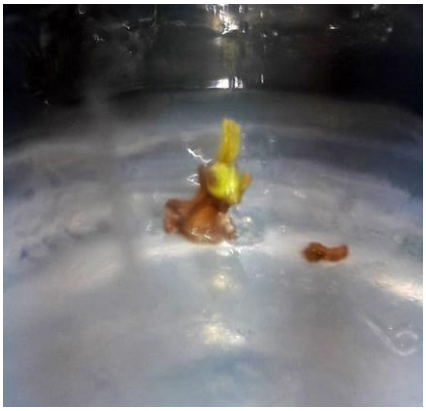


Plate: 3 Multiple shoots generated in treatment T_5 (MS+2mg BA)

T_0 (treatment) was used as a control, in which the nodal explants were inoculated to full strength MS media without any growth regulators. This did not show any induction of shoots. In treatments T_1 and T_2, shoot induction was found after 30 days of inoculation, but the number of cultures showing multiplication (5±0.420; 5±0.321), number of shoots per explant (5±0.320; 4±0.870) and percentage of shoot induction were not satisfactory (16%). Compared to T_2 (average shoot length - 0.3±0.324cm), explants in T_1 have shown better growth (average shoot length - 0.5±0.541cm) of shoots. Treatments T_3 and T_6, failed to show any response. But in treatments T_4 and T_7, the nodal explants showed 33% of shoot induction after 30 days of inoculation (the number of cultures showing multiplication-8±0.520, number of shoots per explant-10±0.750; the number of cultures showing multiplication-8±0.540, number of shoots per explant-10±0.860); with an average shoot length of 1 cm. Maximum shoot induction of 80% was noted in treatment T_5, after 30 days of inoculation (the number of cultures showing multiplication-20±0.570, number of shoots per explant-15±0.670) with an average shoot length of 1.85±0.750cm. After observing the effects of different shoot multiplication media on leaf, shoot tip and nodal explants; nodal explants are found to show maximum response (80%) in inducing multiple shoots with the treatment T_5 (MS+ 2mg BA). In shoot tip explants (33%) and leaf explants (12%) also maximum response was noticed with T_5 treatment even though the percentage of shoot induction was poor. Thus T_5 (MS + 2mg BA) was found to be the effective treatment for establishing multiple shoots. Maximum development of multiple shoots (based on the average shoot length) was also shown by nodal explants (1.85±0.750cm) in T_5 itself.

Thus in the light of above data, it was clear that all the three explants had shown maximum shoot development in treatment T_5 (MS + 2mg BA). A comparison of all the parameters observed in the three explants was made to determine which of the three explants is showing maximum shoot development in treatment T_5 (MS + 2mg BA). The comparison is given in the table below (table no.9).

Table No. 9: Comparison of effect on different explants in treatment T_5 (MS + 2mg BA)

Parameters	Explants		
	Leaf	**Shoot tip**	**Node**
Number of cultures showing shoot induction	7±0.578	8±0.250	20±0.570
Number of shoots/explant	8±0.650	10±0.560	15±0.670
Percentage of shoot induction (%)	12±0.482	33±0.07	80±0.05
Average shoot length (cm)	0.75±0.200	1±0.350	1.85±0.750

From this table (table no.9) it's clear that nodal explants (Number of cultures showing shoot induction-20±0.570; Number of shoots/explant-15±0.670; Percentage of shoot induction-80±0.05%; Average shoot length-1.85±0.750cm) were showing maximum shoot development in treatment T_5 (MS + 2mg BA), compared to shoot tip (Number of cultures showing shoot induction-20±0.570; Number of shoots/explant-15±0.670; Percentage of shoot induction-80±0.05%; Average shoot length-1.85±0.750cm) and leaf explants (Number of cultures showing shoot induction-20±0.570; Number of shoots/explant-15±0.670; Percentage of shoot induction-80±0.05%; Average shoot length-1.85±0.750cm).

5. DISCUSSION

Withania somnifera Dunal, (Ashwagandha) is one of the most popularly used plants, reported to have anti-inflammatory, anti-arthritic, antitumor, antioxidant, immunomodulatory, and hepato protective effects (Al-Hindawi *et al.,* 1989; Ziauddin *et al.,* 1996; Rasool *et al.,* 2000, Winters, 2006; Harikrishnan *et al.,* 2008 and Bhattacharya *et al.,* 2002). It is used to treat stress, strain, fatigue, pain, skin diseases, diabetes, gastro intestinal diseases, rheumatoid arthritis and epilepsy (Sandhu *et al.,* 2010). It is enlisted as an important herb in the Indian Ayurvedic system of medicine, Unani and Chinese traditional medicinal systems. ashwagandha is mainly adapted to xeric and drier regions of tropical and subtropical domains, ranging from the Canary Islands, the Mediterranean region and Northern Africa to South west Asia (Mirjalili *et al.,* 2009). Ashwagandha is reported to have a wide-range of low molecular weight secondary metabolites mainly, terpenoids, flavonoids, tannins, alkaloids and resins. It has been widely experimented for its chemical components that include compounds of diverse chemical structures like withanolides, alkaloids, flavonoids and tannin (Elsakka *et al.,* 1990; Jamal *et al.,* 1991; Jamal *et al.,* 1995). Of these, withanolides are accredited with generally recognized remedying effects. The vast medicinal properties of withanolides lead to the unsystematic collection of this plant in bulk quantities by herbalists, Ayurvedic and Unani companies. Thus systematic cultivation and micro propagation is needed for this species. Under these circumstances, it is necessary to develop strategies for rapid mass propagation of this species to meet up the market demand and also to protect from extinction. Several efforts have been made for regeneration and mass propagation of ashwagandha under culture conditions (Kulkarni *et al.,* 1996). Callus induction, root organ culture, plantlet regeneration, and withanolide production in multiple shoots and roots have been reported in ashwagandha (Rani *et al.,* 2003; Ray and Jha, 2001; Sabir *et al.,* 2008; Wadegaonkar *et al.,*2006).

Ashwagandha is propagated commercially by seeds, but the seed viability is limited to one year (Sen and Sharma, 1991; Rani and Grover, 1999). However, the seed propagation is very poor, since the percentage of germination is low, because of certain inhibitory compounds present in the fruits and high risk of various diseases

(De Silva and Senarath, 2009). This resulted in the over exploitation of plant materials, making the plant endangered (Manickam *et al.,* 2000). However, the increasing demand of this plant cannot be fulfilled by the conventional propagation methods for the preparation of therapeutic drugs. An alternative protocol for propagation through *in vitro* seed germination and seedling development is important because of poor viability of stored seeds and less available knowledge about seed germination of ashwagandha. The method of *in vitro* seed germination can be utilized for the rapid propagation and conservation of ashwagandha.

In the present study, multiple shoots were established from the explants derived from one month old *in vitro* grown seedlings. The seeds of *W. somnifera* (Arka Ashwagandha) were subjected to different treatments for *in vitro* seed germination. For control, the seeds were inoculated in full MS media without hormones. But no germination was observed in the control. In a study conducted on seed germination of ashwagandha by Sharma *et al.,* (2016) also, the germination rate in the treatment MS media without hormones was very much poor. In another experiment conducted by Gopichand *et al.,* (2017) on *Hedychium spicatum* to study the effect of growth hormones on seed germination and plant growth the percentage of seed germination was very low in the control treatment having no hormones. Similar effect was noted by Al-Saedi (2019) in a study conducted to know the effect of different concentrations of growth hormones on seed germination and callus induction of *Atropa belladonna* wherein the treatment without hormones did not induce any germination.

Seed germination and seedling growth are known to be controlled by exogenous hormones. Using growth regulators in pre-treatment, plays an important role in regulating germination and vigour (Raghav and Kasera, 2012). Gibberellins are reported to induce seed germination in a wide range of plant species (Thomas *et al.,* 2005). In the present study no germination was found in treatment S_2, where GA_3 was incorporated in the pre-treatment. But in the treatment S_3, GA_3 was added in the pre-treatment and half strength MS media and 40% germination was found within 14 days of inoculation. The seedlings showed 3.75 cm shoot length after one month of germination, even though the development of the seeds was taking more time.

GA_3 induces the germinating seed cells to generate molecules of mRNA that code for hydrolytic enzymes. Gibberellic acid is a very potent hormone whose natural occurrence in plants controls their development. In two ways, GA_3 exerts its effect, first by growing embryo growth potential and secondly by inducing hydrolytic enzymes (Govindaraju *et al.,* 2003).Since the importance of GA_3 was evident, in the present study, 0.3ppm GA_3 was supplemented in full strength MS media in few treatments *from* S_3 to S_7. Not only in ashwagandha, but in various other medicinal plants also there are reports indicating the importance of GA_3 in seed germination. Sharma *et al.,* (2016) conducted an experiment to know the effect of different concentrations of GA_3 on seed germination and seed development of ashwagandha and reported that the highest germination percentage (92.67%) was found in MS medium supplemented with 3.0 mg l^{-1} GA_3 along with 3.0 mg l^{-1} Kn and the least germination percentage (68.53%) was observed in MS medium containing no growth regulators. An improvement in seed germination of *Asparagus sprengeri* with application of GA_3 was evidenced, but its concentration beyond optimum dose was found to cause reduction in germination percentage (Dhoran and Gudadhe, 2012).

In a study by Younesikelaki *et al.,* (2016), the effect of various surface sterilization treatments on seed germination of *Althaea officinalis* was observed.

In that study maximum percentage of germination was observed in 0.2% of $HgCl_2$ treatment for 7 minutes within 12 days after inoculation. But in the present study, maximum percentage of germination was observed in treatment S_5, in which treatment with 0.1% $HgCl_2$ for 5 minutes was given and the least time taken to induce germination (germination on 6^{th} day) was also with this treatment.

Arumugam and Gopinath (2013) reported, *in vitro* regeneration of ashwagandha using different explants. The explants used were leaves, cotyledons, hypocotyls and epicotyls. MS media supplemented with different combinations of 2,4-D, BAP and NAA were used to induce callus formation. Similarly, different explants derived from *in vitro* grown seedlings were used by Shukla *et al.*, (2010) and mainly they were shoot tip, nodal leaf and root segment for *in vitro* mass propagation of ashwagandha via shoot cultures. Likewise in the present study also nodal, shoot tip and leaf explants were derived from one month old *in vitro* grown seedlings. For induction of multiple shoots they were subjected to treatments incorporated with different concentrations and combinations of plant hormones.

In the present study, different explants were inoculated in full strength MS media without hormones (which served as control) and they were observed for 30 days after inoculation. But no induction of shoots was observed in that case. Similarly, Kumar *et al.*, (2011) have done shoot induction studies in ashwagandha (in which the leaf explants with various combinations of BA, Kn and IAA were used) and reported that there was no response of shoot induction in the treatment without growth regulators.

Radhika and Amutha (2013) obtained, 85% shoot induction after 15 days of inoculation with the treatment (MS+0.5mg BA+ 0.1mg IAA) in ashwagandha. Similar results as in the present study has been observed by Ambasta (2015) who reported 20% shoot induction with nodal explants of ashwagandha in the medium MS+1mg NAA +3mg Kn within 20 days of inoculation, only 10% shoot induction was noticed in the leaf explants using the medium MS+1mgNAA+3mgKn and

15% of shoot induction in leaf explants after 25 days of inoculation using the medium MS+1.5mgNAA+1mgKn in an experiment carried out for the *in vitro* propagation.

Maximum percentage of shoot induction observed in the present study was in treatment T_5, where the explants were inoculated in full strength MS media supplemented with 2mg BA. Out of the three explants used, nodal explants have shown 80% of shoot induction after 30 days of inoculation. Similarly 85% shoot multiplication was observed in the study conducted on ashwagandha by Baba *et al.,* (2013) for the same treatment (MS+ 2mg BA). The average shoot length noticed was also about 1-2 cm. Another study conducted for the regeneration of ashwagandha (Pandey *et al.,* 2013) revealed 90% of shoot induction in MS+2mg BA treatment in nodal explants and concluded that nodal explants treated in BA have given the best response in shoot multiplication.

Micropropagation of *Gynura procumbens* was carried out by Keng *et al.,* (2009) using nodal explants and combinations of BA and NAA to induce multiple shoots and reported that BA (a cytokinin) played an important role in the induction of multiple shoots and was very effective in shoot proliferation. Also shoot regeneration of *Bacopa monniera* was studied using leaf, nodal and intermodal explants and the explants were treated to various concentrations of BA and more number of multiple shoots was obtained in nodal explants with 2mg BA (Karataş *et al.,* 2014).

6. SUMMARY

An investigation on "Development of multiple shoot culture of ashwagandha (*Withania somnifera*) for *in vitro* alkaloid stimulation studies" was conducted at the Department of Plant Biotechnology and Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram, during 2019-2020.

W. somnifera (L.) Dunal, the winter cherry, is an important medicinal plant. It has been in use in Ayurvedic and other systems of native medicine for over 3,000 years. It is also known as the 'Indian ginseng' for its curative medicinal properties. It is a valuable constituent in traditional ayurvedic drug preparations against many diseases like, hiccup, female disorders, cough, rheumatism, dropsy etc. Different parts of this plant are useful in the treatment of conditions like inflammation, tuberculosis etc. and exhibit magnificent antitumor and anti-bacterial activities. Due to the unsystematic collection of bulk amounts of this plant by local herbalists, this plant species is on the brink of extinction. In these instances` it is important to develop techniques for rapid *in vitro* mass propagation of this species to cope with the current market demands and also for protection from the loss of genetic diversity.

The primary objective of the study was to establish multiple shoot cultures of ashwagandha (*Withania somnifera* (L.) Dunal) for *in vitro* alkaloid stimulation studies. The seeds of ashwagandha, variety Arka ashwagandha procured from IIHR Bangalore were used for the study. The seeds were subjected to various treatments to enhance seed germination under *in vitro* conditions.

Among the different treatments studied, S5 (treatment in which the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 5 minutes and then inoculated in half strength MS medium with 0.3% GA_3) was found to be the best for seed germination (100%). But the treatment S_1 (seeds soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$

treatment for 10 minutes and then inoculated to half strength MS medium without hormones) failed to induce germination. Treatment S_2 (seeds soaked in 250ppm GA_3 overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS medium without hormones) also failed to induce any germination. But in the treatment S_3, 40% of germination was found on the 14^{th} day of inoculation and the seedlings showed an average shoot length of 3.75 cm in 28 days. In this treatment S_3, the seeds were soaked in tender coconut water for 1hour and then soaked in 250ppm GA_3 overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS medium with 0.3 ppm GA_3. The growth of the seedlings was very slow and the yield was also very poor in treatment S_4 (in which the seeds were soaked in water overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS medium with 0.3 ppm GA_3). In treatment S5, the time of exposure of the seeds to $HgCl_2$ treatments was reduced to 5 minutes and the rest was the same as above. In S_6, the concentration of $HgCl_2$ was reduced to 0.05% but the time of exposure of the seeds to $HgCl_2$ treatments was 10 minutes. In S_7, the concentration of $HgCl_2$ and time of exposure of the seeds to $HgCl_2$ treatments were reduced as 0.05% and 5 minutes respectively. The treatments S_4, S_6 and S_7 failed to give any response. Compared to the rest of the treatments, S_5 has shown not only maximum germination rate but also faster development of the seedlings.

Nodal, leaf and shoot tip explants were taken from one month old *in vitro* grown seedlings and subjected to different shoot multiplication media for shoot induction. Maximum response was found in treatment T_5 (MS+2mgBA) with 80% shoot induction in nodal explants, 33% shoot induction in shoot tip explants and callus induction was found in case of leaf explants. The explants were inoculated in the MS medium without any hormones T_0 (control) and this treatment failed to show any response. Later various explants (shoot tip, leaf and node) were

subjected to different combinations of growth regulators and observations were made. In the treatments T_1 (MS + 1mg NAA + 3mg Kn), T_2 (MS + 1.5mg NAA + 1mg Kn), T_3 (MS + 1mg NAA + 1.5mg Kn), T_4 (MS + 0.5mg BA + 0.1mg IAA), T_6 (MS + 2.5mg BA) and T_7 (MS + 3mg BA + 1mg Kn) shoot inductions and callus formations were found in these explants. But the shoot formation and its development was not as expected. The development was too slow and was not satisfactory. Among these treatments, T_5 (MS+2mgBA) was found to exhibit both maximum shoot induction and faster development of the multiple shoots. Shoot induction was found after 30 days of inoculation. Among the three explants used, nodal explants showed maximum shoot induction (80%; the number of cultures showing multiplication-20±0.570, number of shoots per explant-15±0.670) with an average shoot length of 1.85±0.750cm in treatment T_5 (MS+2mgBA).

Thus in the present study, the use of nodal explants from *in vitro* derived seedlings and subjecting them to treatment T_5 (MS+2mgBA) is adjudged as the best protocol for establishing multiple shoots which can be used for *in vitro* propagation and alkaloid stimulation studies of ashwagandha. Further studies about the pathway of secondary metabolite production, *in vitro* stimulation studies for the uniform bioaccumulation for the large scale production of alkaloids and efficient mass propagation techniques will unravel the problems of extinction and achieving the current market demands of ashwagandha.

7. REFERENCES

Ahuja, A., Kaur, D., Sharada, M., Kumar, A., Suri, K. A., and Dutt, P. 2009. Glycowithanolides accumulation in *in vitro* shoot cultures of Indian ginseng (*Withania somnifera* Dunal). *Nat. Prod. Commun.* 4(4): 254-298.

Akerele, O., Heywood, V., and Synge, H. 1991. Medicinal Plants: policies and priorities. *Conserv. Med. Plants* 1(4): 3-11.

Al-Hindawi, M. K., Al-Deen, I. H., Nabi, M. H., and Ismail, M. A. 1989. Anti-inflammatory activity of some Iraqi plants using intact rats. *J. Ethnopharmacol.* 26(2): 163-168.

Al-Saedi, N. J. J. 2019. Effect of different concentrations of plant hormones on seed germination and callus induction of *Atropa belladonna* in *in vitro* conditions. *Plant Arch.* 19(1): 1268-1274.

Ambasta, S. K. 2015. Effect of various growth hormone concentration and combination on protocol optimization for *in vitro* rapid propagation of *Withania somnifera*. *Indian J. Sci. Technol*. 9(28): 1-6.

Aoyagi, H., Kobayashi, Y., Yamada, K., Yokoyama, M., Kusakari, K., and Tanaka, H. 2001. Efficient production of saikosaponins in *Bupleurum falcatum* root fragments combined with signal transducers. *Appl. Microbiol. Biotechnol.* 57(4): 482–488.

Arumugam, A., and Gopinath, K. 2013. *In vitro* regeneration of an endangered medicinal plant *Withania somnifera* using four different explants. *Plant Tiss. Cult. Biotechnol.* 23(1): 79-85.

Baba, I. A., Alia, A., Saxena, R. C., Itoo, A., Kumar, S., and Ahmad, M. 2013. *In vitro* propagation of *Withania Somnifera* (L.) Dunal (ashwagandha) an endangered medicinal plant. *Int. J. Pharm. Sci. Invent.* 2(3): 6-11.

Bais, H. P., Sudha, C. G., and Ravishankar, G. A. 1999. Putrescine influences growth and production of coumarins in hairy root cultures of Witloof Chicory (*Cichorium intybus* L.). *Plant Growth Regul*. 18: 159-165.

Banerjee, S., Naqvi, A., Mandal, S., and Ahuja, P. 1994. Transformation of *Withania somnifera* (L) Dunal by *Agrobacterium rhizogenes*: infectivity and phytochemical studies. *Phytother. Res.* 8(4): 452–455.

Bhattacharya, S. K., Bhattacharya, D., Sairam, K., and Ghosal, S. 2002. Effect of *Withania somnifera* glycowithanolides on a rat model of tardive dyskinesia. *Phytomedicine,* 9(2): 167-170.

Bourgaud, F., Gravot, A., Milesi, S., and Gontier, E. 2001. Production of plant secondary metabolites: a historical perspective. *Plant Sci.* 161: 839- 851.

Constabel, F., Gaudet-La-Prairie, P., Kurz, W. W., Kutney, J. P. 1982. Alkaloid production in *Catharanthus roseus* cell cultures. *Plant cell Rep.* 1(1): 139-142.

De Silva, M. A. N., and Senarath, W. T. P. S. K. 2009. *In vitro* mass propagation and greenhouse establishment of *Withania somnifera* (L.) Dunal (Solanaceae) and comparison of growth and chemical compounds of tissue cultured and seed raised plants. *J. Natn. Sci. Foundation Sri Lanka.* 37(4): 249-255.

Devi, P. U. 1996. *Withania somnifera* Dunal (Ashwaganda): Potential plant source of a promising drug for cancer chemotheraphy and radio sensitization.

Indian J. Exp. Biol. 34(4): 927-932.

Devi, P. U., and Sharada, A. C. 1992. *In vivo* growth inhibitory effect of *Withania somnifera* (Ashwaganda) on a transplantable mouse tumour, Sarcoma 180. *Indian J. Exp. Biol.* 30(3): 169- 172.

Devi, P. U., Sharada, A. C., Solomon, F. E., Kamath, M. S. 1992. *In vivo* growth inhibitory effect of *Withania somnifera* (Ashwagandha) on a transplantable mouse tumor Sarcoma 180. *Ind. J. Exp. Biol.* 30:169-172.

Dhar, N., Razdan, S., Rana, S., Bhat, W. W., Vishwakarma, R., and Lattoo, S. K. 2015. A decade of molecular understanding of withanolide biosynthesis and *in vitro* studies in *Withania somnifera* (L.) Dunal: prospects and perspectives for pathway engineering. *Front. Plant Sci.* 6: 1031-1045.

Dhoran, V. S., and Gudadhe, S. P. 2012. Effect of plant growth regulators on seed germination and seedling vigour in *Asparagus sprengeri* Regelin. *Int. Res. J. Bio. Sci.* 1(7): 6-10.

Dong, J., Wan, G., Liang, Z. 2010. Accumulation of salicylic acid induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture. *J. Biotechnol*. 148: 99–104.

Dornenburg, H., and Knorr, D. 1995. Strategies for improvement of secondary metabolite production in plant cell cultures. *Enz. Microb. Technol*. 17: 674–684.

Elsakka, M., Grigorescu, E., Stănescu, U., Stănescu, U., and Dorneanu, V. 1990. New data referring to chemistry of *Withania somnifera* species. *Rev. Med. Chir. Soc. Med. Nat. Iasi.* 94(2): 385-387.

Flores, H, E., Hoy, M. W., and Pickard, J. J. 1987. Secondary metabolites from root cultures. *Trends in Biotechnol*. 5(4): 64-69.

Gopichand, R. L., Maurya, A. K., Agnihotri, V. K., and Singh, R. D. 2017. Effect of growth hormones on seed germination and plant growth: with chemical components of *Hedychium spicatum* Ham. ex. Smith. *Ornam. Med. Plants* 1: 1-14.

Gorelick, J., Rosenberg, R., Smotrich, A., Hanuš, L., and Bernstein, N. 2015. Hypoglycemic activity of withanolides and elicitated *Withania somnifera*. *Phytochem.* 116: 283-289.

Govindraju, B. S., Rao, R., Venugopal, R. B., Kiran, S. G., and Rao, S. 2003. High frequency plant regeneration in Ashwagandha [*Withania somnifera* (L.) Dunal]. *Plant Cell Biotechnol. Mol. Biol.* 4(2): 49-56.

Harikrishnan, B., Subramanian, P., and Subash, S. 2008. Effect of *Withania somnifera* root powder on the levels of circulatory lipid peroxidation and liver marker enzymes in chronic hyperammonemia. *J. Chem.* 5(4): 872-877.

Heble, M. R., and Staba, E. J. 1985. Multiple shoot cultures: A viable alternative *in vitro* system for the production of known and new biologically active plant constituents. *Prim. Sec. Metabolism Plant Cell Cult.* 6(5): 281-289.

Jain, R., Kachhwaha, S., and Kothari, S. L. 2012. Phytochemistry pharmacology and biotechnology of *Withania somnifera* and *Withania coagulans*: A review. *J. Med. Plants Res.* 6: 5388-5399.

Jamal, S. A., Choudhary, M. I., and Asif, E. 1991. Two withanolides from *Withania somnifera*. *Phytochemistry,* 30(11): 3824-3826.

Jamal, S. A., Qureshi, S., Ali, S. N., and Choudhary, M. I. 1995. Bioactivities and structural studies of withanolides from *Withania somnifera*. *Chem. Heterocycl. Com*. 31(9): 1047-1059.

Jayaprakasam, B., Zhang, Y., Seeram, N. P., and Nair, M. G. 2003. Growth inhibition of human tumour cell lines by withanolides from *Withania somnifera* leaves. *Life Sci*. 74: 125-132.

Joshi, A. G., and Padhya, M. A. 2010. Shoot regeneration from leaf explants of *Withania somnifera* (L.) Dunal. *Notulae. Scientia. Biologicae.* 2(1): 63-65.

Kang, S., Jung, H., Kang, Y., Yun, D., Bahk, J., Yang, J., Choi, M. 2004. Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. *Plant Sci.* 166(9): 745–751.

Karataş, M., and Aasim, M. 2014. Efficient *in vitro* regeneration of medicinal aquatic plant water hyssop (*Bacopa monnieri* L. Pennell). *Pak. J. Agri. Sci.* 51(3): 667-672.

Kartha, K. K., Leung, N. L., Pahl, K. 1980. Cryopreservation of strawberry meristem and mass propagation of plantlets. *J. Amer. Soc. Hort. Sci*. 105: 481-484.

Keng, C. L., Yee, L. S., and Pin, P. L. 2009. Micropropagation of *Gynura procumbens* (Lour.) Merr. an important medicinal plant. *J. Med. Plants Res.* 3(3): 105-111.

Ketchum, R. E. B., Gison, D. M., Croteau, R. B., and Shuler, M. L. 1999. The kinetics of taxoid accumulation in cell suspension cultures of Texus following elicitation with methyl jasmonate. *Biotechnol. Bioeng*. 63(4): 97–105.

Kim, Y. S., Hahn, E. J., Murthy, H. N., Paek, K. Y. 2004. Adventitious root growth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. *Biotechnol. Lett.* 26: 1619–1622.

Kirson, I., and Glotter, E. 1980. 14α-hydroxy steroids from *Withania somnifera* (L) Dunal. *J. Chem. Res. Synop*. 10: 338-339.

Kokate, C. K., Purohit, A. P., and Gokhale, S. B. 1996. *Pharmacognosy*. 4th Edn., Nirali Prakashan, Pune, India pp: 133.

Kulkarni, A. A., Thengane, S. R., and Krishnamurthy, K. V. 1996. Direct *in vitro* regeneration of leaf explants of *Withania somnifera (*L.) Dunal. *Plant Sci.* 119(2): 163-168.

Kulkarni, S. K., and Dhir A. 2008. *Withania somnifera*: An Indian ginseng. *Prog. Neuropsychopharmacol. Biol. Psychiatry.* 32: 1093-1105.

Kumar, O. A., Jyothirmayee, G., and Tata, S. S. 2011. *In vitro* plant regeneration from leaf explants of *Withania somnifera* (L) Dunal (Ashwaganda)-an important medicinal plant. *Res. Biotechnol.* 2(5): 85-120.

Kwok, K. H., and Doran, P. M. 1995. Kinetic and stochiometric analysis of hairy roots segmented bubble column reactor *Biotechnol. Prog*. 11(2): 429-435.

Lavi, D., Glotter, E., and Shro, Y. 1965. Constituents of *Withania somnifera* Dun,

The structure of Withaferin A. *J. Chem. Soc*. 30: 7517-7531.

Liffert, R., Hoecker, J., Jana, C. K., Woods, T. M., Burch, P., Jessen, H. J., Neuburger, M., and Gademann, K. 2013. Withanolide A: synthesis and structural requirements for neurite outgrowth. *Chem. Sci*. 4(7): 2851-2857.

Lini, J., Manju, R. V., Roy, S., Viji, M .M., and Edison, L. K. 2014. Stress induced enhancement of secondary metabolite production in *Withania somnifera* (L). *Int. J. Trop. Agric*. 32(4): 617-620.

Majumdar, D. N. 1955. *Withania somnifera* Dunal. Part II: alkaloidal constituents and their chemical characterization. *Ind. J. Pharm*. 17(2): 158-161.

Manickam, V. S., Mathavan, R. E., and Antonisamy, R. 2000. Regeneration of Indian ginseng plantlets from stem callus. *Plant Cell. Tiss. Org. Cult.* 62(3): 181-185.

Matsuda, H., Murakami, T., Kishi, A., and Yoshikawa, M. 2001. Structures of withanosides I, II, III, IV, V, VI, and VII, new withanolide glycosides, from the roots of Indian *Withania somnifera* and inhibitory activity for tachyphylaxis to clonidine in isolated guinea-pig ileum. *Bio. Org. Med. Chem*. 9: 1499–1507.

Mir, B. A., Khazir, J., Hakeem, K. R., Koul, S., and Cowan, D. A. 2014. Enhanced production of withaferin-A in shoot cultures of *Withania somnifera* (L) Dunal. *J. Plant Biochem. Biotechnol:* 23(4): 430-434.

Mirjalili, M. H., Moyano, E., Bonfill, M., Cusido, R. M., and Palazón, J. 2009. Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. *Molecules*, 14(7): 2373-2393.

Mishra, L., Singh, B., and Dagenais, S. 2000. Scientific basis for therapeutic use of *Withania somnifera* (Ashwagandha): A review. *Altern. Med. Rev.* 5(2): 335-346.

Mishra, S. N. 1994. Colorimetric method for estimation of total withanolides. In *10th All India Workshop Report on Medicinal and Aromatic Plants held at Trichur*, pp. 379-81.

Mulabagal, V., and Tsay, H. S. 2004. Plant cell cultures-an alternative and efficient source for the production of biologically important secondary metabolites. *Int. J. Appl. Sci. Eng*. 2(1): 29-48.

Murashige, T. 1974. Plant propagation through organ cultures. *Annu. Rev. Plant Physiol*. 25: 135-166.

Murch, S. J., KrishnaRaj, S., and Saxena, P. K. 2000. Tryptophan is a precursor for melatonin and serotonin biosynthesis in *in-vitro* regenerated St. John's wort (*Hypericum perforatum* L. cv. Anthos) plants. *Plant Cell Rep.* 19: 698-704.

Murthy, H. N., Lee, E. J., and Paek, K. Y. 2014. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell Tiss. Org.* 118(1): 1-16.

Nag, K., and Hasan, Z. U. 2013. Uses of Wild Medicinal Herbs and Ecology of Gardens of District Bhopal city, Madhya Pradesh (India). *Int. J. of Pharm. & Life Sci.* 4(6): 2437-2439.

Nigam, K. B., and Kandalkar, V. S. 1995. Ashwagandha, In: Chadha, K. L., and Rajendra, G. (eds), *Advances in Horticulture Vol.11- Medicinal and*

Aromatic Plants. Malhotra Publishing House, New Delhi, India, pp. 337-344.

Nittala, S. S., Lavie, D. 1981. Chemistry and genetics of withanolides in *Withania somnifera* hybrids. *Phytochemistry,* 20(8): 2741-2748.

Oksman-Caldentey, K. M., and Inzé, D. 2004. Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. *Trends in Plant Sci*. 9: 433–440.

Pandey, V., Mishra, M. K., Atri, N., and Misra, P. 2013. *In vitro*-seed germination of different chemotypes of *Withania somnifera*. *Int. J. Tech. Res. Appl*. 1(5): 1-6.

Pattipati, S., Amanpreet, S., and Shrinivas, K. 2003. Effect of *Withania somnifera* root extract on Haloperidol induced Orofacial Dyskinesia: Possible mechanism of action. *J. Med. Food* 6(6): 107-114.

Prakash, J., Gupta, S., and Dinda, A. 2002. *Withania somnifera* root extract prevents DMBA-induced squamous cell carcinoma of skin in Swiss albino mice. *Nutr. cancer* 1(6): 91-97.

Praveen, N., Naik, P. M., Manohar, S. H., and Murthy, H. N. 2010. Distribution of withanolide a content in various organs of *Withania somnifera* (L.) Dunal. *Int. J. Pharma. Bio Sci.* 1(3): 125-136.

Radhika, R., and Amutha, K. 2013. *In vitro* micropropagation of *Withania somnifera* (L.) Dunal by different concentrations of growth regulators. *Int. J. Pharma. Bio. Sci*. 4: 1161-1167.

Raghav, A., and Kasera, P. K. 2012. Seed germination behaviour of *Asparagus racemosus* (Shatavari) under *in vivo* and *in vitro* conditions. *Asian J. Plant Sci. Res.* 2(4): 409-413.

Ramakrishna, A., and Ravishankar, G. A. 2011. Influence of abiotic stress singles on secondary metabolites in plants. *Plant Sign Behav.* 6: 1720– 1731.

Rangaraju, S., Lokesha, A. N., and Aswath, C. R. 2019. Improved production of withanolides in adventitious root cultures of *Withania somnifera* by suspension culture method. *Biosci.Biotech.Res.Comm.* 12(1): 73-79.

Rani, G., and Grover, I. S. 1999. *In vitro* callus induction and regeneration studies in *Withania somnifera*. *Plant Cell Tiss. Org. Cult.* 57(1): 23-27.

Rani, G., Virk, G. S., and Nagpal, A. 2003. Callus induction and plantlet regeneration in *Withania somnifera* (L.) Dunal. *In vitro* cellular & developmental biology- *Plant* 39(5): 468-474.

Rao, M. V., Lee, H., Creelman, R. A., Mullet, J. E., Davis, K. R., 2000. Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. *Plant Cell* 12(7): 1633–1646.

Rao, R. B. R., Rajput, D. K., Nagaraju, G., and Adinarayana, G. 2012. Scope and potential of medicinal and aromatic plants products for small and medium enterprises. *J. Pharmacognosy*, 3(4): 112-114.

Rao, S. R., and Ravishankar, G. A. 2002. Plant cell cultures: chemical factories of secondary metabolites. *Biotechnol. Adv.* 20(2): 101-153.

Rasool, M., Latha, L. M., and Varalakshmi, P. 2000. Effect of *Withania somnifera* on lysosomal acid hydrolases in adjuvant-induced arthritis in rats.

Pharm. Pharmacol. Commun. 6(4):187-190.

Rathore, M. S., Mastan, S. G., Yadav, P., Bhatt, V. D., Shekhawat, N. S., and Chikara, J. 2016. Shoot regeneration from leaf explants of *Withania coagulans* (Stocks) Dunal and genetic stability evaluation of regenerates with RAPD and ISSR markers. *S. Afr. J. Bot*. 102: 12-17.

Ravishankar, G. A., and Grewal, S. 1991. Development of media for growth of *Dioscorea deltoidea* cells and *in-vitro* diosgenin production: Influence of media constituents and nutrient stress. *Biotechnol. Lett*. 13: 125-30.

Ray, S., and Jha, S. 1999. Withanolide synthesis in cultures of *Withania somnifera* transformed with *Agrobacterium tumefaciens*. *Plant Sci.* 146:1–7.

Ray, S., and Jha, S. 2001. Production of withaferin A in shoot cultures of *Withania somnifera*. *Planta. Medica*. 67(5): 432-436.

Sabir, F., Sangwan, N. S., Chaurasiya, N. D., Misra, L. N., Tuli, R., and Sangwan, R. S. 2008. Rapid micropropagation of *Withania somnifera* L. accessions from axillary meristems. *J. Herbs Spices Med. Plants,* 13(4): 123-133.

Sandhu, J. S., Shah, B., Shenoy, S., Chauhan, S., Lavekar, G. S., and Padhi, M. M. 2010. Effects of *Withania somnifera* (Ashwagandha) and *Terminalia arjuna* (Arjuna) on physical performance and cardiorespiratory endurance in healthy young adults. *Int. J. Ayurveda Res.* 1(3): 144-164.

Sangwan, R. S., Chaurasiya, N. D., Lal, P., Misra, L., Uniyal, G. C., Tuli, R., Sangwan, N. S. 2007. Withanolide A bioregeneration in *in vitro* shoot cultures of ashwagandha (*Withania somnifera* Dunal), a main medicinal plant in Ayurveda. *Chem. Pharm. Bull.* 55(5): 1371-1375.

Satyajit, K., and Santi Lata, S. 2011. Direct organogenesis of *Withania somnifera* L. from apical bud. *Int. Res. J. Biotechnol*. 2(3): 58-61.

Sen, J., and Sharma, A. K. 1991. Micropropagation of *Withania somnifera* from germinating seeds and shoot tips. *J. Plant Cell Tiss.Org.* 26(2): 71-73

.

Shamsa, F., Monsef, H., Ghamooshi, R., and Verdian-rizi, M. 2008. Spectrophotometric determination of total alkaloids in some Iranian medicinal plants. *Thai. J. Pharm. Sci*. 32(5): 17-20.

Sharma, S., Gantait, S. S., and Thokchom, R. 2016. *In vitro* seed germination and seedling development of *Withania somnifera* (L.) Dunal. *Afr. J. Agric. Res.* 11(17): 1552-1558.

Shohael, A. M., Murthy, H. N., Lee, H. L., Hahn, E. J., and Paek, K. Y. 2008. Increased eleutheroside production in *Eleutherococcus sessiliflorus* embryogenic suspension cultures with methyl jasmonate treatment. *Biochem. Eng. J*. 38(3): 270–273.

Shrivastava, S., and Dubey, P. K. 2007. *In vitro* callus induction and shoot regeneration in *Withania somnifera* Dunal. *Int. J. Biotechnol*. *Biochem.* 24: 10-19.

Shukla, D. D., Bhattarai, N., and Pant, B. 2010. *In vitro* mass propagation of *Withania somnifera* (L.) Dunal. *Nepal J. Sci. Tech.* 11: 101-106.

Singh, A. K., Varshney, R., Sharma, M., Agarwal, S. S., and Bansal, K. C. 2006. Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *J. Plant Physiol.* 163(3): 220-223.

Singh, G., Sharma, P. K., Dudhe, R., and Singh, S. 2010. Biological activities of *Withania somnifera*. *Ann. Biol. Res.* 1(3): 56-63.

Sivanandhan, G., Arun, M., Mayavan, S., Rajesh, M., Jeyaraj, M., Dev, G. K., Manickavasagam, M., Selvaraj, N., and Ganapathi, A. 2012. Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. *Appl. Biochem. Biotechnol.* 168(3): 681-696.

Sivanandhan, G., Mariashibu, T. S., Arun, M., Rajesh, M., Kasthurirengan, S., Selvaraj, N., Ganapathi, A. 2011. The effect of polyamines on the efficiency of multiplication and rooting of *Withania somnifera* (L.) Dunal and content of some withanolides in obtained plants. *Acta. Physiol. Plant* 33(3): 2279–2288.

Sivanandhan, G., Rajesh, M., Arun, M., Jeyaraj, M., Dev, G. K., Arjunan, A., Manickavasagam, M., Muthuselvam, M., Selvaraj, N., and Ganapathi, A. 2013. Effect of culture conditions, cytokinins, methyl jasmonate and salicylic acid on the biomass accumulation and production of withanolides in multiple shoot culture of *Withania somnifera* (L.) Dunal using liquid culture. *Acta. Physiol. Plant.* 35(3): 715-728.

Sivanandhan, G., Selvaraj, N., Ganapathi, A., and Manickavasagam, M. 2014. Improved production of withanolides in shoot suspension culture of *Withania somnifera* (L.) Dunal by seaweed extracts. *Plant Cell Tiss. Org. Cult.* 119(1): 221-225.

Sivanesan, I. 2007. Direct regeneration from apical bud explants of *Withania somnifera* Dunal. *Indian J. Biotechnol*. 6(4): 125-127.

Sreevidya, N., and Mehrotra, S. 2003. Spectrophotometric method for estimation of alkaloids precipitable with Dragendorff's reagent in plant materials. *J. AOAC Int*. 86(6): 1124-1127.

Stafford, A. 1986. Plant Cell Biotechnology: A perspective. *Enzyme Microbial. Tech.* 8: 578- 597.

Suffness. M., and Douros, J. 1982. Current status of NCI plant and animal product program. *J. Nat. Prod.* 45: 1-4.

Takayama, S., Misawa, M., 1981. Mass propagation of *Begonia* and *hiemalis* plantlets by shake cultures. *Plant and Cell Physiol*. 22(6): 461-467.

Teli, N. P., Patil, N. M., Pathak, H. M., Bhalsing, S. R., and Maheshwari, V. L. 1999. *Withania somnifera* (Ashwaganda); regeneration through meristem culture. *J. Plant Biochem. Biot.* 8(2): 109-111.

Thakur, R. S. 1993. Medicinal plants-biotechnological and modern biological approaches. In: Srivastava, H. C. (ed.), *Biotechnological Applications for Food Security in Developing Countries*, pp: 309-333.

Thanh, N. T., Murthy, H. N., Yu, K. W., Hahn, E. J., and Paek, K. Y. 2005. Methyl jasmonate elicitation enhanced synthesis of ginsenoside by cell suspension cultures of *Panax ginseng* in 5-l balloon type bubble. *Appl. Micorbiol. Biotechnol.* 67:197–201.

Thomas, S. G., Rieu, I., and Steber, C. M. 2005. Gibberellin metabolism and signaling. *Vitam. Horm.* 72: 289-338.

Tripathi, A. K., Sukla, Y. N., and Kumar. S. 1996. Ashwagandha [(*Withania somnifera*, Dunal (Solanaceae)]: A status report. *J. Med. Aromat. Plants*

8: 46-62.

Trivedi, I., Jha, V. K., Ambasta, S. K., Trivedi, M. P., Prasad, B., and Sinha, U. K. 2016. Quantitative spectrophotometric estimation of total alkaloids in *Withania somnifera* L. *in vivo* and *in vitro*. *Int. J. Appl. Biol. Pharm. Technol.* 7(2): 254-257.

Van Wyk, B. E., and Gericke, N. 2000. *People's plants: A guide to useful plants of Southern Africa*. Briza publications, 351p.

Verma, A. K., Singh, R. R., and Singh, S. 2012. Improved alkaloid content in callus culture of *Catharanthus roseus*. *Bot. Serbica,* 36(2): 123-130.

Vinotha, S. 2015. Phytochemical Screening of Various Extracts of Root of *Withania Somnifera* (L) Dunal. *Arch. Bus. Res.* 3(2): 47-56.

Wadegaonkar, P. A., Bhagwat, K. A., and Rai, M. K. 2006. Direct rhizogenesis and establishment of fast growing normal root organ culture of *Withania somnifera* Dunal. *Plant Cell Tiss. Org. Cult.* 84(2): 223-225.

Winters, M. 2006. Ancient medicine, modern use: *Withania somnifera* and its potential role in integrative oncology. *Altern. Med. Rev.* 11(4): 24-34.

Younesikelaki, F. S., Ebrahimzadeh, M. H., Desfardi, M. K., Banala, M., Marka, R., and Nanna, R. S. 2016. Optimization of seed surface sterilization method and *in vitro* seed germination in *Althaea officinalis* (L.)-an important medicinal herb. *Indian J. Sci. Technol*. 9(28): 1-6.

Yu, K. W., Gao, W., Hahn, E. J., Paek, K. Y. 2002. Jasmonic acid improving ginsenoside accumulation in adventitious root culture of *Panax ginseng* C. A. Meyer. *Biochem. Eng. J.* 11(6): 211–215.

Zabetakis, I., Edwards, R. O., and O'Hagan, D. 1999. Elicitation of tropane alkaloid biosynthesis in transformed root cultures of *Datura stramonium*. *Phytochemistry*, 50(3): 53-56.

Zenk, M. H., El-Shagi, H., and Schulte, U. 1978. Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. *Planta. Med*. 33(4): 79-101.

Zhang, X. and World Health Organization, 2002. Traditional medicine strategy 2002 2005.

Ziauddin, M., Phansalkar, N., Patki, P., Diwanay, S., and Patwardhan, B. 1996. Studies on the immunomodulatory effects of Ashwagandha. *J. Ethnopharmacol*. 50(2): 69-76.

8. APPENDICES

APPENDIX I

Murashige and Skoog (MS) medium

Components	Quantity (mg/l)
NH_4NO_3	1650
KNO_3	1900
$CoCl_26H_2O$	0.0250
KH_2PO_4	170
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.26
KI	0.83
H_3BO_3	6.2
$Na_2MoO_32H_2O$	0.25
$CaCl_2.6H_2O$	332.2
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.6
$MnSO_4.4H_20$	16.9
$MgSO_4$	180.7
Nicotinic acid	0.5
Pyridoxin-HCl	0.5
Thiamine	0.1
Glycine	2.0
Myoinositol	100
Sucrose	30 g/l
pH	5.8

DEVELOPMENT OF MULTIPLE SHOOT CULTURE OF ASHWAGANDHA *(Withania somnifera)* FOR *IN VITRO* ALKALOID STIMULATION STUDIES

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ABSTRACT OF THESIS

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9. ABSTRACT

The study entitled "Development of multiple shoot culture of ashwagandha *(Withania somnifera)* for *in vitro* alkaloid stimulation studies" was conducted at the Department of Plant Biotechnology and Department of Plant Physiology, College of Agriculture, Vellayani, Thiruvananthapuram, during 2019-2020. The primary objective of the study was to establish multiple shoot cultures of ashwagandha (*Withania somnifera*) for *in vitro* alkaloid stimulation studies. The seeds of *Withania somnifera*, variety Arka ashwagandha procured from IIHR Bangalore were used for the study. The seeds were subjected to various treatments to enhance seed germination under *in vitro* condition.

Among the different treatments studied, S_5 (the treatment in which the seeds were soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 5 minutes and then inoculated in half strength MS media with 0.3% GA_3) was found to be the best for seed germination (100%). But the treatment S_1 (seeds soaked in water overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated to half strength MS media without hormones) failed to induce germination. Treatment S_2 (seeds soaked in 250ppm GA_3 overnight, surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media without hormones) also failed to induce any germination. But in the treatment S_3, 40% of germination was found on 14^{th} day of inoculation and the seedlings showed an average shoot length of 3.75 cm in 28 days. In this treatment S_3, the seeds were soaked in tender coconut water for 1hour and then soaked in 250ppm GA_3 overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1% $HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3.The growth of the seedlings was very slow and the yield was also very poor in the treatment S_4 (in which the seeds were soaked in water overnight and the next day surface sterilized with 5% Tween 20 for 5 minutes followed by 0.1%

$HgCl_2$ treatment for 10 minutes and then inoculated in half strength MS media with 0.3 ppm GA_3).In treatment S_5, the time of exposure of the seeds to $HgCl_2$ treatments was reduced to 5 minutes and the rest was the same as above. In S_6, the concentration of $HgCl_2$ was reduced to 0.05% but the time of exposure of the seeds to $HgCl_2$ treatments was 10 minutes. In S_7, the concentration of $HgCl_2$ and time of exposure of the seeds to $HgCl_2$ treatments were reduced as 0.05% and 5 minutes respectively. The treatments S_4, S_6 and S_7 failed to give any response. Among these treatments, treatment S_5 has shown maximum germination rate (100%) with an average shoot length of 5.87 cm. Germination was found within 6 days of inoculation. Compared to the rest of the treatments, S_5 has shown not only maximum germination rate but also faster development of the seedlings.

Nodal, leaf and shoot tip explants were taken from one month old *in vitro* grown seedlings and subjected to different shoot multiplication media for shoot induction. The explants were inoculated in plain MS media without any hormones in treatment T_0 and this treatment failed to show any response. In the treatment T_1 (MS+1mgNAA+3mgKn) shoot multiplication response of different explants *viz.* shoot tip (10%), leaf (10%) and nodal explants (16%) were very poor. Similarly in treatment T_2 (MS+1.5mgNAA+1mgKn) also the response of shoot tip (10%), leaf (10%) and nodal (16%) explants were not satisfactory. In the treatment T_3 (MS+1mgNAA+1.5mgKn), leaf explants showed shoot induction (10%) but there was no response in both shoot tip and nodal explants. But in the treatments T_4 (MS+0.5mgBA+0.1mgIAA) and T_7 (MS+3mgBA+1mgKn), 33% of shoot induction was found in both cases of shoot tip and nodal explants and callus development was found in case of leaf explants. Leaf explants showed 12% shoot induction in treatment T_6 (MS+2.5mgBA) and no shoot induction was found in both shoot tip and nodal explants. Maximum response was found in treatment T_5 (MS+2mgBA) with 80% shoot induction in nodal explants, 33% shoot induction in shoot tip explants and callus development was found in case of leaf explants.

Among the treatments, T_5 (MS+2mgBA) was found to exhibit both maximum shoot induction and faster development of the multiple shoots. Shoot induction was found after 30 days of inoculation. Among the three explants used, nodal explants showed maximum shoot induction (80%; the number of cultures showing multiplication-20±0.570, number of shoots per explant-15±0.670) with an average shoot length of 1.85±0.750cm in treatment T_5 (MS+2mgBA). Hence in the present study, the use of *in vitro* derived nodal explants and subjecting them to treatment T_5 (MS+2mgBA) is adjudged as the best protocol for establishing multiple shoots which can be used for *in vitro* propagation and alkaloid stimulation studies of *Withania somnifera*.