Agrobacterium rhizogenes MEDIATED TRANSFORMATION OF ASHWAGANDHA (*Withania somnifera* (L.) DUNAL)

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(2009-09-108)

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"*Agrobacterium rhizogenes* MEDIATED TRANSFORMATION OF ASHWAGANDHA (*Withania somnifera* (L.) DUNAL)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

Place: Vellayani

Date: 18/12/2014

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LIST OF ABBREVIATIONS AND SYMBOLS USED

BAP	6- benzyl amino purine
IAA	Indole -3- acetic acid
MS	Murashige and Skoog
GA_3	Gibberellic acid
IBA	Indole -3- butyric acid
MSB	Murashige and Skoog basal
NAA	α- naphthaleneacetic acid
PGR	Plant Growth Regulators
HPLC	High pressure liquid chromatography
TLC	Thin Layer Chromatography.
$HgCl_2$	Mercuric chloride
Fw	Fresh weight
Dw	Dry weight.
%	Per cent
µm	Micro meter
µl	Micro litre
@	At the rate of
ºC	Degree Celsius
cm	Centimeter
et al.	And other co workers
Fig.	Figure
g	Gram

g-1	Per gram
mg	Milli gram
ml	Milli litre
sec	Seconds
min	Minutes
R_f	Retention factor

INTRODUCTION

1. INTRODUCTION

Since time immemorial, plants are a primary source of food and medicines. Complex compounds derived from primary metabolism, such as terpenoids, alkaloids, steroids, glycosides, sterols, lignins, tannins, flavanoids, essential oils etc. which constitutes secondary metabolites serves as medicines. The search on therapeutically active compounds and its use are being increased with the advancement of biochemistry, phytochemistry, metabolic engineering, biotechnology and chemotaxonomy. It is presumed that secondary metabolite produced by plant are used to ward off predators, attract pollinators and in combating infectious diseases. Plant secondary metabolites are biosynthetically derived from primary metabolite by specific genetically controlled, enzymatically catalyzed reactions that lead to the formation of complex compounds. The production of these compounds is often less than 1% dry weight and depends greatly on the physiological and developmental stage of the plant. Many secondary metabolites exhibit impressive biological activities like antimicrobial, antibiotic, insecticidal, anti-cancerous activities. With the development of research on phytochemistry and pharmacology, the biological activity of many secondary metabolites were determined during the past few decades. It is estimated that only 20% of the plant flora has been studied and 60% of synthetic medicines owe their origin from plants. It is estimated that out of 70,000 plant species, nearly 8000species have found out to have medicinal value (Nag and Hasan, 2013).

It is estimated that, that approximately, 80% of the world's population in developing countries depends primarily on herbal medicine for basic primary healthcare needs (Allison, 1996; Anon., 1998). As per the analysis of WHO, the demand for medicinal plant raw material is growing at the rate of 15-20% annually. According to the recent estimate of WHO, the demand for medicinal plant is likely to increase from the current US $14 billion a year to US $5 trillion

in 2050. It is worthwhile to note that the export market of herbal species appears to be growing faster with the growing world demand.

The World Health Organization (WHO) finding is that, traditional medicines are widely used in India. The Indian systems of medicine use around 8,000 species of plants which include trees (33%), herbs (32%) shrubs (20%), climbers (12%) and epiphytes, grasses, lichens, ferns and algae put together (3%) (Agarwal and Ghosh, 1985). In India different indigenous systems, Ayurveda, Unani, Ampchi are practicing of which Ayurveda is more popular and rapidly developing.

It is important to note that approximately 60% of the medicinal plants used in the traditional systems of medicine (Ayurveda, Siddha, Unani) roots are the principle material for drug preparations (Agarwal and Ghosh, 1985). It is estimated that more than 90% of the plant species used by the industry is collected from the wild and more than 70% of the plant drugs involved destructive harvesting and very few are in cultivation. A number of root drug-yielding plants are facing threat and depletion in their habitats due to indiscriminate collection. Harvesting roots for medicinal use is unfavorable as it destroys the whole plant. Many medicinally active secondary metabolites are obtained by direct extraction from plants grown in natural habitat. However several factors can alter their yield. Development of biotechnological methods such as micropropagation, cell/root and hairy root cultures is one of the major solutions to circumvent these problems. Compared to the cell cultures, organized cultures, and especially root cultures, can make a significant contribution in the production of secondary metabolites and it offers unique opportunities for providing root drugs in the laboratory, without resorting to field cultivation. Growing roots as isolated organ *in vitro* with faster growth potential and equal or better synthesizing ability as that of field growing plants will have advantages.

The candidate species of the present study, *Withania somnifera* known *as* Ashwagandha, is commonly known as 'Indian ginseng' or 'winter cherry'. The plant has been reported from India, Pakistan, Afghanistan, Palestine, Egypt, Jordan, Morocco, SriLanka, Spain, Canary Island, Eastern Africa, Congo, Madagascar and South Africa. These areas represent wide variations of soil, rainfall, temperature and altitude (Kokate *et al*. 1996). In India, ashwagandha is widely distributed in north-western region, Maharashtra, Gujarat, Rajasthan, Madhya Pradesh, Orissa, Uttar Pradesh, Punjab plains extending to the mountain regions of Punjab, Himachal Pradesh and Jammu.

The plant has been used as an important herb in the Ayurvedic and indigenous systems of medicine for more than 3,000 years. It is well known world over as aphrodisiac drug. The pharmacological experiments conducted on *W. somnifera* revealed antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hematopoetic, anti-ageing, anxiolytic, antidepressive rejuvenating properties and also influences various neurotransmitter receptors in the central nervous system (Pattipati *et al*., 2003). The researchers revealed that a specific extract from the plant, withaferin A, was more effective in the inhibition than the common cancer chemotherapy drug, doxorubicin they used to compare it with (Jayaprakasam *et al.,* 2003). Studies revealed that the anti-inflammatory and immunomodulatory properties of *Withania somnifera* root extracts are likely to contribute to the chemo preventive action (Prakash *et al*., 2002).

The roots of the plant are categorized as rasayanas drugs in Ayurveda, which are reputed to promote health and longevity by augmenting defense against disease, arresting the ageing process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and creating a sense of mental wellbeing (Weiner and Weiner, 1994). In Ayurveda, the fresh roots are sometimes boiled in *milk*, prior to drying, in order to leach out undesirable constituents. The berries are used as a substitute for rennet, to coagulate milk in cheese making (Puri, 2003). The roots

and berries of the plant are used in herbal medicine. The species was considered as sedative (indicating its species name *"somnifera"* means "sleep-bearing" in Latin, but it has been also used for sexual vitality and as an adaptogen. In the traditional system of medicine Ayurveda, this plant is claimed to have potent aphrodisiac rejuvenative and life prolonging properties. It has general animating and regenerative qualities and is used among others for the treatment of nervous exhaustion, memory related conditions, insomnia, tiredness potency issues, skin problems and coughing.

The biochemical composition of *W. somnifera* has been widely studied and detected approximately 35 compounds in various laboratories. The importance of *Withania somnifera* is due to the presence of bioactive molecules collectively called as withanolides. Major withanolides like withaferin-A, withanolide-A have been demonstrated to possess significant and specific therapeutic action in carcinogenesis, Parkinson's disease and Alzheimer's disease (Choudhary *et al*., 2005; Jayaprakasam *et al*., 2003). The production of withanolides in plants is restricted to only a few species within Solanaceae with their most prodigal amounts and structurally diversified forms found in *W. somnifera* (Sangwan *et al*., 2007). The root is regarded as a tonic, aphrodisiac and is used in consumption, emaciation, debility, dyspepsia and rheumatism. A decoction of the root is used for colds and chills (Davis and Kuttan, 2000). The root extract of this species has recently been accepted as a dietary supplement in the United States.

Under *in vitro* condition, adventitious roots grow vigorously in phytohormone supplemented medium and have shown tremendous potential of accumulation of valuable secondary metabolites (Murthy *et al*., 2008). Adventitious roots induced by *in-vitro* methods showed high rate of proliferation and active secondary metabolism. Plant cell organ cultures are promising technologies to obtain plant-specific valuable metabolites (Verpoorte *et al*., 2002). Cell and organ cultures have a higher rate of metabolism than the field grown plants because the initiation of cell/organs and to a condensed biosynthetic cycle. Further, plant cell/organ cultures are not limited by environmental, ecological and

climatic conditions and cells/organs can thus proliferate at higher growth rates than whole plant in cultivation (Rao and Ravishankar, 2002).

Since the roots of *W. somnifera* contain a number of therapeutically applicable withanolides, harvesting roots is destructive for the plants and mass cultivation of roots *in vitro* will be an alternative technique for the large scale production of these secondary metabolites. The development of a fast growing root culture system would offer unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation (Sudha and Seeni, 2001). The development of *Agrobacterium rhizogenes* mediated hairy root cultures offers a remarkable potential for commercial production of a number of low-volume and high value secondary metabolites (Mehrotra *et al*., 2010). Besides, hairy root cultures provide an excellent experimental system to study the various aspects of biosynthesis of useful phytochemicals, such as enzymatic pathways, key intermediates, and critical regulation points (Fu *et al*., 2006) and the production of transgenic herbals (Piatczak *et al*., 2006). The hairy root culture systems revealed the pharmacological activities in addition to the enhanced production of bioactive molecules (Syklowska-Baranek *et al*., 2012) and the possibilities for utilization of artificial polyploidization for improving germplasm and breeding (He-Ping *et al*., 2011). Hairy root cultures are also an attractive experimental system, as they are long-term aseptic root clones, genetically stable with growth rates comparable to those of the fastest-growing cell suspension cultures (Lorence *et al*., 2004).

The commercial cultivation of *Withania somnifera* has two limitations, first, the plant to plant variation in the alkaloid quantity and yield, and secondly the long gestation period (4 to 5yrs) between planting and harvesting (Rani *et al*., 2003; Ciddi, 2006). Plants requirement for dry regions for active growth is yet another limitation. The increasing market demand of the drug causes overexploitation of natural populations. Therefore, there is need to develop approaches to ensure the availability of raw material of a consistent quality from

regular and viable source, thus circumventing the need of harvesting plants from wild. Development of root and hairy root culture is an ideal method to get the raw material from the laboratory without resorting to field cultivation. The genetically transformed ("hairy") roots produced by plant tissue after infection with the soil bacterium, (*Agrobacterium rhizogenes*) is a promising *in vitro* source of secondary metabolites.

In the present study considering the importance of the plant, an attempt was made to establish *Agrobacterium rhizogenes* mediated hairy root and normal root cultures of *Withania somnifera*. For the study, experiments were planned to standardize best explants from different sources (juvenile explants and shoot cultures), best *Agrobacterium rhizogenes* strain, medium, nutrient and physiological parameters for root induction, and establishment of root cultures in liquid medium. Confirmation of the transformation was done by PCR. The root cultures grown in shake flask cultures were carried out for secondary metabolite analysis. The major bioactive molecules present in this plant namely withaferin-A, withanolide-A analysis was done using TLC. Qualitative and quantitative analysis was done by HPLC.

REWIEW OF LITERATURE

2.REVIEW OF LITERATURE

Medicinal plants are an important source of a material for the pharmaceutical industry (Balandrin *et al*., 1985), comprising some 25% of prescribed drugs. Along with the biologically active metabolites, many other chemical compounds are produced commercially from medicinal plants, including insecticides, cosmetics, narcotics and aromatics (Bajaj *et al*., 1988). A major portion of the modern chemotherapeutics and traditional drugs are derived from higher plants (Zenk, 1978). Over exploitation of such wild medicinal plants has resulted in gradual depletion of the resources (Heble *et al*., 1985). Extensive deforestation activities, indiscriminate collection and ferocious exploitation of natural flora of medicinal herbs was warned out by many authorities (Akerele *et al*., 1991; Thakur, 1993) and they emphasized the urgent need for the development of alternative means of propagation including biotechnology so as to have abundant supply of raw material rich in active ingredients for the industry, and would enable conservation of the rare and endemic herbal wealth.

The therapeutic properties of medicinal plants and adverse effect of synthetic drugs as reveled by scientific studies are the basis for the world attention to utilize plant based drugs (herbal drugs) for the prevention and cure of diseases. Since last three decades, there was an unprecedented explosive growth in the herbal industry due to the growing awareness among the consumers and positive approaches to maintain good health and well-being of people. It is estimated that the herbal plants have global market worth about US$ 82 billion per annum and the demand is growing at a rapid pace. Recent data is noteworthy, that value of essential oil and aroma chemicals alone from medicinal and aromatic plants traded during 2011 in the world was US 7.8 billion (Rao *et al*., 2012). These facts indicate that herbal based industry will be one of the major driving forces of global economy. However, the availability of the raw materials has not been increasing along with the rapid growth of the industry and in turn, results rapid depletion of genetic resource of many valuable medicinal plants from its natural habitat. These factors have made an impetus to develop

alternative routes for the production of bio-molecules at genuine and industrial level without depending on filed cultivation of medicinal plants and disturbing wild wealth.

In vitro production of Bioactive Compounds

Three decades ago, the development of large-scale culture methods for plant cells could be used to produce secondary metabolites just as microbial and fungal fermentation, for antibiotic and amino acid. In 1983, the first plant cell culture process was commercialized in Japan for the production of shikonin, anthraquinones, extensively used in Japan for the anti-inflammatory properties (Flores *et al*., 1987). With a few notable exception, undifferentiated plant cultures rarely show the level of patterns of secondary metabolite production characteristics of their parent plant. Even in those cases where cell cultures can produce appreciable levels of compounds, their accumulation is dually incompatible with cell growth.

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in-vitro* regeneration, development of cell/root cultures and genetic transformations for fast growing transgenic root cultures for production of secondary metabolites (Barz and Ellis, 1981; Bourgaud *et al*., 2001). *In-vitro* production of valuable secondary metabolites, plant cell or organ culture is an attractive alternative to the extraction of whole plant material (Oksman-Caldustry and Inze, 2004). *In-vitro* propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch *et al*., 2000). There are many reports on the regeneration of various medicinal plants via callus culture (Tripathi *et al*., 1996). Successful establishment of cell lines capable of producing high yields of secondary compounds in cell suspension cultures has been reported by Zenk (1978). The accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, and on environmental conditions (Stafford, 1986). Ravishankar and Grewal, 1991 reported that the influence of

media constituents and nutrient stress influenced the production of diosgenin from callus cultures of *Dioscorea deltoidea.*

Secondary metabolite biosynthesis in transformed roots is genetically controlled but it is influenced by nutritional and environmental factors. To obtain high density root culture the culture condition should be maintained at the optimum level. Nutrient availability is the major chemical factors involved in scaling up. Carbon, nitrogen oxygen and hydrogen depletion in the medium along with the biomass increase and alkaloid production has studied in *Atropa belladonna* by (Kwok and Doran, l995). Effect of exogenous supplementation of Put has been investigated by Bais *et al*., 1999. Similarly other biotic and abiotic elicitors have been tried to induce the increased production of secondary metabolites (Zabetakis *et al*., 1999).

Normal and Transformed Root Culture

The genetic and biochemical changes which occur in many pathways involved in secondary metabolism are developmentally regulated (Signs and Flores, 1990). In view of the difficulties presented by undifferentiated plant cell cultures for secondary metabolites, tremendous efforts have been done to exploit the root cultures for the production of useful compounds. In 1934, Phillip White, the pioneer of modern plant tissue culture developed the first root culture in a medium consisting of mineral nutrients, sugars and vitamins (White, 1934). The potential of hairy roots for secondary metabolite production was realized by several laboratories in the mid of 80s. Since then many reported the production of these metabolites in a number of species. Transformed hairy root culture have been established successfully for the production of tropane alkaloids in *Hyoscyamus muticus* (Flores and Filner, 1985), betacyanin pigments from *Beta vulgaris* (Hamill *et al*., 1986), artemisinin from *Artemisia annuals* (Liu *et al*., 1998) and coumarins from *Cichorium intybus* (Bais *et al*., 1999).

The genetically transformed ("hairy") roots produced by plant tissue after infection with the soil bacterium, (*Agrobacterium rhizogenes*) is a promising *in*

vitro source of secondary metabolites. Integration and expression of T-DNA genes in host plant cells lead to development of the hairy roots, which can be excised and grown *in vitro* as hairy root cultures (Chilton *et al*., 1982) upon treatment with antibiotics such as carbencillin or ampicillin which eliminates excess bacteria. T-DNA contains four root locus (rot) genes that are responsible for increased sensitivity to endogenous auxins (Shen *et al*., 1988). The balance of endogenous hormones produced by the transformed cells results in the proliferation of roots at the best wound site. The hairy root cultures are proved to be genetically and biochemically stable than cell culture. Thus, the development of a fast growing root culture system would offer unique opportunities for producing root drugs in the laboratory without having to depend on field cultivation (Sudha and Seeni, 2001). When optimized for liquid cultures, hairy roots can be grown in industrial-scale bioreactors providing a convenient, abundant and sustainable source of phytochemicals (Ono and Tian, 2011).

Withania somnifera

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. 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 made extensive review of biotechnological interventions of *Withania somnifera* made by several authorites.

Bioactive compounds in *Withania somnifera*

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 steroidallactones, 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, a phytosteroid (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; withanolide p and 8; withanolide Q and R; withanolide y, 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 had been isolated and identified (Matsuda *et al*., 2001). Much of ashwagandha pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D.

Presently, withanolides, the active metabolites of *W. somnifera* have been commercially obtained by solvent extraction of roots and leaves of the plant. Low yield from the natural source, genotypic and chemotypic variations, heterogeneity in content, long gestation period (4–5 years) between planting and harvesting, and uneconomical chemical synthesis are major constrains in industrial withanolide production.

Biotechnological intervention on *Withania somnifera*

Biotechnological interventions were carried out to establish normal root cultures for the production of bioactive molecules in *W. somnifera* in various laboratories. Direct root initiation from leaf explants of *W. somnifera* was achieved on half-strength MS medium supplemented with 15 g/l sucrose, and

different concentrations of growth regulators (Wadegaonkar *et al*., 2006). The study conducted by Sharada *et al*., 2007 showed that production of withanolides was closely associated with morphological differentiation. During their study, five selected withanolides (withanone, withaferin A, withanolide A, withanolide B, withanolide E) were identified by HPLC-UV (DAD) - positive ion electrospray ionization mass spectroscopy in *W. somnifera* (L.) Dunal and tissues cultured *in-vitro* at different developmental phases. Adventitious root culture was established in the Jawahar variety of *W. somnifera* using MS basal medium supplemented with 0.5 (mg/l) IAA and 2.0 (mg/l) IBA. Root tips from germinated seedlings, maintained plants and adventitious roots were maintained in suspension medium (half-strength MSB medium supplemented with 3% sucrose) for a period of 1 to 6 months. Withanolide content in adventitious root sample was found to be superior compared to other roots at any given point of time during the 6 month growth period (Wasnik *et al*., 2009). The root cultures of *W. coagulans* were obtained from *in-vitro* germinated sterile plantlets. The roots grew in MS medium containing 0.25 mg/l IBA and 30 g/l sucrose. The root cultures synthesized withanolides of which withaferin A was the major compound. The productivity of withaferin A in the three-week-old cultured roots was 11.65 µg/g (AbouZid *et al*., 2010). Adventitious roots have induced from leaf explants of *W. somnifera* for the production of withanolide-A, which is having pharmacological activities. Adventitious roots were induced directly from leaf segments of *W. somnifera* on half -strength Murashige and Skoog (MS) semisolid medium (0.8% agar) with 0.5 mg/l IBA and 30 g/l sucrose showed higher accumulation of biomass (108.48 g/l FW and 10.76 g/l DW) and withanolide-A content (8.8 ± 0.20 mg/g DW) within five weeks (Praveen and Murthy, 2010). Leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). The highest callus induction rate (89.5%) and shoot regeneration rate (92 %) were obtained when 2 mg dm-3 BAP was combined with 0.5 mg dm-3 IAA. Three major withanolides (withaferine A, 12-deoxywithastramonolide and withanolide-A) were investigated in different plant organs from *in vitro* and greenhouse grown

plants (Dewir *et al*., 2010). Callus cultures failed to synthesize withanolides. Multiple shoot cultures synthesized significant levels of withanolides. Maximum callus proliferation was obtained on MS medium supplemented with 2.26 µM 2, 4-D (Rani and Grover, 2003). Callus culture were initiated from leaf, shoot tip and root explants on MS medium supplemented with 2,4-D in concentration of 2 mg/l and Kn in 0.2 mg/1 (Shrivastava and Dubey, 2007).

Murthy and Praveen (2012) investigated the effects of macro elements (NH_4NO_3), KNO_3), $CaCl_2$), $MgSO_4$) and KH_2PO_4)) and nitrogen source of MS medium on the accumulation of biomass and withanolide-A content on adventitious root culture. The highest accumulation of fresh and dry biomass (127.52 and 12.45 g /l) was recorded in the medium with 0.5× concentration of NH_4NO_3) and the highest production of withanolide-A was recorded in the medium with 2.0× KNO_3) (14.00 mg/ g DW). The adventitious root growth was greater when the concentration was higher than that of and the withanolide-A production was highest in the absence. Maximum biomass growth was achieved at ratio of 14.38:37.60, while withanolide-A production was greatest (11.76 mg \g DW) when the ratio was 0.00:18.80 mM. They claimed that results of this study are useful for scale-up processes.

Exogenous hormone influence on development of root culture was reported by Nandagopal *et al*., 2011. The leaf and hypocotyl explants from 25-day old *in vitro* raised seedlings were cultured on half-strength MS medium supplemented with different concentrations and combinations of IAA, IBA and NAA. Between the two explants, leaf explants responded well for both root induction and growth than the hypocotyl explants. A 0.5 mg/l of NAA and 0.1 mg/l of IBA combination induced the highest percentage of rooting from mature leaf and hypocotyl explants under total darkness. After three weeks, well-established roots were separated and 0.5 g fresh root tissue was subcultured in half-strength MS liquid medium supplemented with 0.2 mg/l NAA and 0.5 mg/l IBA, under continuous agitation at 110 rpm and in total darkness. The biomass of

the root increased to 5.80 g after 6 weeks of culture. The root culture was maintained up to 8 weeks.

Recently a similar study was done by Pradeepa *et al*., 2014 to find out the effect of sucrose and auxin concentration on induction of *in vitro* adventitious roots from the leaf explants of *Withania somnifera,* was achieved on full strength MS medium supplemented with 45 g/l sucrose, and different concentration of auxins. Basal medium supplemented with 1mg/l IBA and 1mg/l IAA achieved the maximum number of roots with 100 % response. The roots were transferred to full strength MS suspension medium supplemented with the above combination of auxins and incubated on an orbital shaker at 100 rpm at 25 ± 2 °C. Adventious roots were harvested after 10^{th} and 25^{th} day of inoculation, and growth index was found to be 0.06 and 30.27 respectively.

Sangwan *et al.,* 2008 have studied incorporation of ^{14}C from [2-^{14}C]-acetate and [U-^{14}C]-glucose into withanolide A in the *in vitro* cultured normal roots as well as native/orphan roots of *W. somnifera*. Analysis of products by thin layer chromatography revealed that these primary metabolites were incorporated into withanolide A, demonstrating that root-contained withanolide A is de novo synthesized within roots from primary isoprenogenic precursors. Therefore, withanolides are synthesized in different parts of the plant (through operation of the complete metabolic pathway) rather than imported.

Murthy *et al.* (2008) reported that MS-based liquid medium was superior for the growth of transformed roots compared to other culture media evaluated (SH, LS and N6), with MS-based medium supplemented with 40 g/l sucrose being optimal for biomass production. Cultured hairy roots synthesized withanolide A, a steroidal lactone of medicinal and therapeutic value. The concentration of withanolide A in transformed roots (157.4 µg/g DW) was 2.7 fold more than in non-transformed cultured roots (57.9 µg/g DW).

Morphology and withanolide production of *Withania coagulans* hairy root cultures was studied by Mirjalili *et al*., 2009. In this study leaf sections of *Withania coagulans* was inoculated with *Agrobacterium tumefaciens* strain C58C1 (pRiA4) induced transformed roots with the capacity to produce the most important bioactive compounds of withania species, withanolide A and withaferin A. The hairy roots obtained showed two morphologies: callus-like roots (CR) with a high capacity to produce withanolides and typical hairy roots (HR) with faster growth capacity and lower withanolide accumulation. The aux1 gene of pRiA4 was detected by PCR analyses in all roots showing callus-like morphology. However, this gene was only detected in 12.5% of the roots showing typical hairy root morphology. This fact suggests a significant role of aux genes in the morphology of transformed roots. Time course studies of withanolide production showed that withanolide A accumulated during the first part of the culture whereas the maximum accumulation of withaferin A occurred at the end of the culture period. Some transformed root lines, such as HR112 and CR26, showed considerable potential to produce withanolides in a scaled up bioreactor system, especially the important pharmaceutical compound withanolide A.

Murthy *et al.* (2008) reported establishment of hairy root cultures of *Withania somnifera* for the production of withanolide A. Transformation was done using *Agrobacterium rhizogenes*. Explants from seedling roots, stems, hypocotyls, cotyledonary nodal segments, cotyledons and young leaves were inoculated with *A. rhizogenes* strain R1601. Hairy roots were induced from cotyledons and leaf explants. The transgenic status of hairy roots was confirmed by PCR using *npt* II and *ro*l B specific primers and, subsequently, by Southern analysis for the presence of *npt* II and *rol* B genes in the genomes of transformed roots. Four clones of hairy roots were established; these differed in their morphology. The doubling time of faster growing cultures was 8-14 days with a 5 fold increase in biomass after 28 days compared to cultured, non-transformed seedling roots. MS-based liquid medium was superior for the growth of transformed roots compared to other culture media evaluated (SH, LS and N6),

with MS-based medium supplemented with 40 g/l sucrose being optimal for biomass production. Cultured hairy roots synthesized withanolide A, a steroidal lactone of medicinal and therapeutic value. The concentration of withanolide A in transformed roots (157.4 µg g/l DW) was 2.7 fold more than in non-transformed cultured roots (57.9 µg g/l DW).

Effect of elicitors on hairy roots was studied by Sivanandhan *et al*., 2013 in this study initial inoculums mass of 5 g FW were elicited separately with methyl jasmonate (MeJ) andsalicylic acid (SA) at various concentrations for different exposure times after 30 days of culture. Enhanced production of biomass (32.68 g FW and 5.54 g DW; 1.23-fold higher), withanolide A (132.44 mg/g DW; 58-fold higher), withanone (84.35 mg/g DW; 46-fold higher), and withaferin A (70.72 mg/g DW; 42-fold higher) were achieved from 40 day-old harvested hairy roots elicited with 150 lM SA for 4 h exposure time. The present study reported a higher production of withanolide A, withanone and withaferin A from the elicited-hairy roots of *W. Somnifera* under optimal inoculum mass, harvest time, elicitor exposure time and its concentration. These results will be useful for biochemical and bioprocess engineering for the viable production of withanolides in hairy root culture. Increased production of withanolide A, withanone, and withaferin A was found in hairy root cultures of *Withania somnifera* elicited with methyl jasmonate and salicylic acid.

Very recently, *Agrobacterium rhizogenes* mediated genetic transformation using nodal explants of *W. somnifera* had been reported by Udayakumar *et al*. (2014). They used *Agrobacterium tumefaciens* strain EHA105 that harbored a binary vector pGA492, which carrying kanamycin resistant (*npt*II), phosphinothricin resistant (*bar*) and an intron containing *β*-glucuronidase (*gus*-intron) genes. The sensitivity of nodal explants to kanamycin (300 mg l^{-1}) was determined for the selection of transformed plants. Transformation was confirmed by histochemical *β*-glucuronidase (*GUS*) assay and amplification of the *npt*II gene by polymerase chain reaction (PCR). PCR and southern blot analyses confirmed

the integration of *npt* II gene in the genome of *W. somnifera* and the transformation frequency of 3.16 % has been achieved.

Chitturi *et al*., 2010 established callus cultures of *W. somnifera* from leaves on MS media supplemented with dicamba (2 mg/l), Kinetin (0.1 mg/l) and sucrose (3% w/v). Effect of various precursors and elicitors on suspension cultures was studied. The addition of precursor's sodium acetate (50 mg/l) (~10 folds), mevalanolactone (50 mg/l) (~14 folds), squalene (50 mg/l) in colloidal form (~23 folds) and cholesterol (25 mg/l) in colloidal form (~30.5 folds) have shown significant increase in bioproduction of withaferin A. Among the various biotic and abiotic elicitors used *Verticillium dahliae* (5% w/v cells extract) (~10 folds) and copper sulphate (100 μm/l) (~2.5 folds) have shown moderate increase in the bioproduction of withaferin A. The effective precursors and elicitors were studied for optimization of day of addition and found that maximum bioproduction of withaferin A was seen in the cultures when precursors and elicitors were added to 3 day old suspension cultures.

Somatic embryogenesis and plantlet regeneration from leaf explants of *Withania somnifera* was reported by Sharma *et al*., 2010. Embryogenic callus was obtained from leaf explants on MS medium supplemented with 2, 4-dichlorophenoxy acetic acid (0.5-5.0 mg/l) and N6-benzylaminopurine (0.5-5.0 mg/l). High frequency of somatic embryo mass induction (10.89 ± 0.78) was noticed on 2, 4-D (0.5 mg/l), BAP (1.0 mg/l) along with casein hydrolysate (10.0 mg/l). This nodulated callus upon regular subculturing on the same medium and hormonal regime showed various typical stages of embryo development i.e. heart shaped, torpedo shaped and cotyledonary stages culminating into germination and maturity. Well-developed cotyledonary stage embryos were germinated on MS medium fortified with BAP (0.5 mg/l). Highest percentage of somatic embryo formed shoots on MS medium augmented with BAP (0.5 mg/l), but they did not show germination on MS medium without plant growth regulators. The shoots raised from somatic embryos were rooted on MS medium supplemented with

indole-3- butyric acid (1.0 mg/l). The survival rate after transplantation of plantlets was 55%. Plants produced were morphologically similar to mother plants.

A similar study has been carried out in Centre for Plant Biotechnology and Molecular Biology and Biochemistry Laboratory of the College of Horticulture, Kerala Agricultural University, Vellanikkara and the Biochemistry Laboratory of Aromatic and Medicinal Plants Research Station, Odakkali by Smini Varghese, 2006. She studied about the enhancement of secondary metabolite production using techniques such as addition of osmoregulants, precursor feeding and elicitation. The addition of osmoregulant like poly ethylene glycol (2.0 % and 5.0 %), methionine precursor (1 mM and 2 mM) and yeast extract (2.5 and 5.0 g/l) failed to enhance the withaferin A content, whereas the biotic elicitor *Aspergillus* homogenate elicited a positive influence on the biosynthesis of withafein A in the hairy root cultures.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study on the '*Agrobacterium rhizogenes*' mediated transformation of Ashwagandha (*Withania somnifera* (L.) Dunal)" was conducted during the period 2013-2014 at Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The materials used and method followed are described below.

3.1 MATERIALS

3.1.1 Plant material

Seeds from maturated ripened fruits of *Withania somnifera* were used to raise aseptic seedlings. The cotyledonary nodes of aseptic seedlings were used to initiate shoot cultures. Leaf and terminal shoot cuttings derived from shoot cultures were used to initiate normal root culture. Cotyledons and hypocotyls derived from aseptic seedlings were used to initiate hairy root culture.

3.1.2 Chemicals and Glass/plastic wares

a. Media chemicals, Solvents and Silica gel G-60 –Merck
b. Sterilizing agents: Merck (P.) Ltd. and S.D. fine Chem. Ltd. India, Bombay, India.
c. Plant growth regulators (PGRs), Vitamins, Authentic samples and other fine chemicals: Sigma Chem. Co., USA.
d. Chemicals for molecular biology works: Taq DNA polymerase (Finzymes, Helsinki, Finland), agarose gel (Lonza) primers (IDT, Coralville, USA)
e. Glasswares: All glasswares used for tissue culture, molecular biology and phytochemical work were from Riviera glass Pvt. Ltd. Mumbai and Borosil, India Ltd. except culture bottle with polypropylene cap (Excel Glasses Ltd., Alleppey and Steriliq Polypacks, Cochin)
f. Plasticwares: PCR tubes, tips

g. TLC Plates: Glass TLC Plates were prepared by coating the plates with silica gel G-60 to a thickness of 0.3 mm. The plates were activated in an oven at 100-105 ºC. The readymade TLC plates (Merck aluminium sheets 20x20 cm silica gel 60 F 254) was also used for fine experiments.

3.1.3 Equipments

1. Rotavapor (Heidolph instruments, Germany)
2. Autoclave (Nat Steel Equipments Pvt Ltd, Bombay, India)
3. Air flow cabinet (Klenzaids, Bombay, India)
4. Hot-Air Oven (Oven Universal, India)
5. Gyratory Shaker (New Brunswick, USA)
6. Refrigerated incubator shaker (New Brunswick, USA)
7. UV-Vis Spectrometer: XP 3001 xplorer, USA
8. Gel documentation system (UVP EC3 Chemi HR 410 Imaging System, USA)
9. Micro centrifuge (Eppendorf, Germany)
10. Biophotometer (Eppendorf, Germany)
11. Thermocycler (Eppendorf ESP-S, Germany)
12. HPLC (Waters 600)

3.1.4 Nutrient media for Tissue culture and *Agrobacterium rhizogenes*

Full and Half – strength MS (Murashige and skoog, 1962) medium were used for all tissue culture experiments (Appendix II). Half-strength MS medium consists 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. The pH of the bacterial medium was adjusted to 7.0 before adding agar (9%). For the bacterial culture Yeast Mannitol Broth (YMB) (Hooykaas *et al*., 1977) broth and agar were used (Appendix I).

3.1.5 Incubation Conditions

Shoot 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 50-60 µEm-2s-1 from day light fluorescent tube (Philips India Ltd, Mumbai). In a culture room seeds for seed germination, and cultures for normal and hairy root initiation were incubated under dark condition during initial period of 2-3 weeks. Root suspensions were incubated in gyratory shaker under 16 h photoperiod. *Agrobacterium rhizogenes* strains were incubated under 30 ºC in incubator.

3.2 METHODOLOGY

3.2.1 Establishment of aseptic seedlings

The ripened fresh fruits of *Withania somnifera* were collected from the Medicinal Plant Gene Bank Garden of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), during the month of September-October. They were washed in detergent solution, 5 % (v/v) teepol for 5 min and removed the detergent by repeated washing under running tap water. The ripened fruits were surface-sterilized with 0.1 % (w/v) mercuric chloride ($HgCl_2$) for 10-14 mins. Thereafter, the fruits were repeatedly washed five to six times using sterile distilled water to remove the traces of $HgCl_2$. Later fruits were blotted on sterile coarse filter paper. The fruits were split opened and separated the seeds from placenta. Thereafter the seeds were again surface sterilized with 0.1 % $HgCl_2$ for 1 min. and washed with sterile distilled water for 3-5 min, decanted the water carefully using sterile spatula and the seeds were spread on a coarse filter paper to remove the traces of water. The seeds so treated (15-20) were inoculated onto full and half-strength MS basal solid medium supplemented with 0.5 mg/l GA_3 in 250 ml Erlenmeyer flasks.

3.2.2 Initiation and Establishment of Shoot Cultures

Cotyledonary nodal explants (0.5-1cm) were excised from 3-4 weeks old aseptic seedlings by removing the cotyledons and inoculated onto the full strength -MS solid medium supplemented with different concentrations (0.1-1.0 mg/l) of

BAP and 0.02-0.2 mg/l IAA dispensed in either culture tubes or 250 ml Erlenmeyer flasks. The cultures were incubated under 16 hr photoperiod for 8 weeks and data were recorded two weeks interval. After 6 weeks of shoot culture initiation, the regeneration potential of shoot tip, nodal segments in terms of frequency of response, shoot number and length were evaluated. The nodal segments were inoculated on with 4 weeks interval to agar-gelled multiplication medium to raise shoot cultures.

3.2.3. Initiation and Establishment of Normal root Cultures

Leaves and shoots from 3-4 weeks old aseptic shoot cultures were used for normal root induction. For leaf derived root culture, 3rd to 5th leaf from apical bud of the shoots were separated and transversely cut into two segments. Both terminal and basal segments were inoculated into half-strength MS solid medium supplemented with different concentrations of auxin, IBA and IAA (0.05–0.8 mg/l) in 250 ml Erlenmeyer flasks and incubated under dark. The explants were placed horizontally (dorsal surface of the leaf towards down) on the medium. Half strength MS medium without auxin was kept as control. To induce root on shoots, terminal shoot cuttings (2.5 to 3.0 cm) from 4 week-old shoot cultures were separated and inoculated into half-strength MS solid medium supplemented 0.2 mg/l IBA in 25x150 mm culture tubes and 250 ml Erlenmeyer flasks. The efficiency of shoot cuttings for normal root induction followed by repeated inoculation of the same shoot after harvesting the initiated roots was analyzed during 4 passages. Shoot were prepared for fresh inoculation after slicing the basal callused end along with induced roots.

To establish normal root suspension, roots, approximately 50 mg (Fw per flask) were segmented after 25 days of induction and were inoculated into half-strength MS liquid medium supplemented with different concentrations of IBA (0.2 mg/l, 0.5 mg/l, 0.8 mg/l) in 250 ml Erlenmeyer flask under 16 hr photoperiod in gyratory shaker. The cultures were kept under continuous agitation at 80-90 rpm. The growth of the root was assessed by determining root biomass in terms of Fw/Dw for a period of 30 days.

3.2.4. Initiation and establishment of Hairy root culture

3.2.4.1 Preparation of Bacterial strain

The *Agrobacterium rhizogenes* strains (A4, LBA-9402 and MTCC 532) were used to determine the most efficient strain for transformation. The bacterial cultures, stored at 4 ^{0}C, were activated by culturing in Yeast Mannitol Broth (YMB) liquid medium under 180 rpm at 28 ^{0}C. The overnight-grown cultures were streaked onto YMB solid medium and incubated under dark at 28 ^{0}C for 48 h to raise virulent cultures for the infection.

3.2.4.2. Pre-incubation of explants and infection procedure

The explants (cotyledons and hypocotyls) from 2-3 weeks old seedlings were pre-incubated on full strength MS basal solid (MSB) medium for 2–3 days prior to infection. The pre-incubated explants were wounded with a sterile scalpel and dipped into the overnight grown bacterial cultures for the induction of hairy roots. Wounds were made gently on the explants without bacteria and the treatments served as controls. The infected and control explants were placed on 15 ml half-MSB medium in petri dishes and incubated under dark for co-cultivation.

3.2.4.3. Hairy root induction and establishment

After 3 days of co-cultivation, the explants were rinsed with sterile distilled water, blotted on sterile filter paper and transferred onto fresh full-MSB medium containing 500 mg/l ampicillin individually and incubated under dark until the infected sites responded and thereafter cultured at 24 ± 2 ^{0}C under a 16 hr photoperiod. Three days after infection the explants were transferred onto fresh MSB medium containing the same concentration of antibiotics to eliminate *Agrobacterium* at 2-6 days intervals for 6 weeks. Before transferring explants were washed (3-6 wash) with sterile distilled water and blotted on a sterile filter paper (Whatman No. 1). They were incubated under dark until the infected sites responds. During the initial passages, a part of the parental explants was retained. And as the passages goes on, pieces of parental explants were removed slowly to finally get a clump of roots alone. When the bacteria-free roots were established,

they were transferred in to 250 ml Erlenmeyer flasks containing MSB liquid medium and kept on a gyratory shaker at 100 rpm.

3.2.4.4. PCR analysis

3.2.4.4.1. Plasmid Isolation Using Alkaline Lysis

Plasmid of *Agrobacterium rhizogenes* strain A4 was isolated using alkaline lysis method (Appendix III). Bacteria was freshly streaked onto YMB solid medium and incubated at 30 ºC overnight to get single colony. The single colonies were inoculated into YMB liquid medium dispersed in five culture tubes (5 replicates) and the cultures were incubated at 24 ºC overnight with continuous shaking at 130 rpm in refrigerated incubator shaker. Later one ml of the bacterial suspension from each tube was combined was spinned at 10,000 x g for 5 min. at room temperature. The pellet was retained by discarding the supernatant. Afterwards, it was resuspended in a total of 1 ml ice-cooled solution I (50 mM). The bacteria was fully resuspended and to get uniform suspension up and down pipetting was done. To the suspension 2 ml 0.2 N NaOH/1.0 % SDS was added at room temperature. Subsequently, it was mixed thoroughly by repeated gentle inversion without vortexing. To the lysate 1.5 ml ice-cold solution III was added. It was then mixed thoroughly by repeated gentle inversion without vortexing. The total solution was centrifuged at 15,500 x g for 30 minutes at 4 ºC. To the resulting supernatant 2.5 volume isopropanol was added to precipitate the plasmid DNA. It was thoroughly mixed by repeated gentle inversion without vortexing. Again it was centrifuged at 15,500 x g for 30 minutes at 4 ºC and the resulting supernatant was removed. The last obtained pellet contains the plasmid DNA.

The pellet was then rinsed in ice-cold 70 % EtOH and air-dried for 10 min. to allow the EtOH to evaporate. To dissolve the pellet dH_2O or Tris EDTA (TE) was added. After addition of 2 ul RNase A (10 mg/ml), the mixture was incubated for 20 min. at room temperature to remove RNA.

3.2.4.4.2. Genomic DNA isolation from normal and hairy root samples (3-day protocol)

3.2.4.4.2.1. First day

Genomic DNA was isolated from hairy root culture transformed by *A. rhizogenes* A4 strain and non-transformed (normal root) root from cultures as per modified CTAB (Cetyl trimethyl ethyl ammonium bromide) method described by Murray and Thompson, 1980 (Appendix IV). The normal roots of *Withania somnifera* used for the genomic DNA isolation was taken from the root suspension established in rooting medium (half- strength MS medium with 0.2 mg/l IBA). The normal root culture was treated as the control for the PCR analysis. Approximately 2 g of normal and transformed roots were harvested from culture media and washed well with distilled water. The roots were then dried by blotting on a filter paper and cut into pieces using surgical blade and finely ground using liquid nitrogen in a mortar and pestle. Extraction buffer (8-10 ml) warmed at 65 ºC for 5-10 min. and 1 % β-mercaptoethanol was added to the ground sample. The thoroughly mixed slurry was transferred to a labelled screw-cap centrifuge tube (30 ml) and incubated at 65 ºC in a water-bath for 1-2 hours with occasional mixing. Equal amount of chloroform-isoamyl alcohol mixture (24:1) was then added to the slurry and centrifuged at 10000 rpm for 5 min at 4 ºC. The supernatant transferred to a fresh tube and added 1/10th volume of CTAB/NaCl with equal volume of chloroform. Centrifuged again at 10000 rpm for 5 min at 4 ºC and transferred the clear supernatant to a fresh tube. To the supernatant, CTAB precipitation buffer (double the volume of supernatant) was added and incubated overnight at 37 ºC in a water-bath.

3.2.4.4.2.2. Second day

After incubation, the tubes were centrifuged at 8000 rpm for 10 min at 4 ºC, the pellet was collected and the supernatant was discarded. The pellet was resuspended by adding 1 ml of high salt TE buffer and transferred to glass tubes. To this suspension, 1 ml of isopropanol was added, incubated at -20 ºC for 30 min and centrifuged at 8000 rpm for 10 min at 4 ºC. The pellets were washed with 1

ml of 80 % ethanol by spinning at 10000 rpm for 5 min at 4 ºC and were resuspended in 0.5 ml of 1X TE. The solution was then transferred to 2 ml Eppendorf tubes and incubated for 10 min in a thermostat at 55 ºC with 2 µl of RNase. Equal volume of chloroform-isoamyl alcohol mixture was added and centrifuged at 10000 rpm for 5 min. The upper aqueous layer was collected, transferred into a fresh tube, added more than double the volume of 100% ethanol and 50 µl of 3 M sodium acetate and then kept for overnight precipitation at -20 ºC.

3.2.4.4.2.2.3 Third day

The tubes were centrifuged at 12000 rpm for 15 min at 4 ºC, collected the pellet and washed with 0.5 ml of 70% ethanol. Centrifuged again at 10,000 rpm for 5 min at 4 ºC, pellets resuspended in 100 µl 1X TE and the resuspended pellets collected and stored at -20 ºC. Five µl of isolated genomic DNA of samples were loaded into 0.8 % agarose gel and it was visualised using UV transilluminator (UVP)

3.2.4.4.2.3. Quantification of DNA

The quantity and quality of the isolated DNAs was assessed using Biophotometer (Eppendorf, Germany). The DNA sample was 50 times diluted by adding, 1 µl of isolated DNA sample to 49 µl of 1X TE or sterile distilled water and recorded the reading at absorbance at 260 nm and 280 nm after setting appropriate blank (Distilled water). The concentration (µg/µl) and absorbance at 260 nm, 280 nm and 260/280 nm were recorded.

3.2.4.4.3. PCR amplification of *rol* A and *rol* C gene from plasmid DNA (*Agrobacterium rhizogenes* strain A4) and DNA of normal and transformed roots

3.2.4.4.3.1. Materials Required:

1. Template DNA
2. Downstream oligonucleotide primer (Bangalore Genei Pvt. Ltd., India)
3. Upstream oligonucleotide primer (Bangalore Genei Pvt. Ltd., India)
4. Tag DNA polymerase

5. 10 x reaction buffer
6. 25 mM $MgCl_2$ (optional)
7. Nuclease-free water
8. dNTP mix (10 mM of each dNTP)

3.2.4.4.3.2 Components of PCR mixture

Components	Volume (µl)
1. Nuclease-free water	16
2. 10 X reaction buffer	2.5
3. dNTP mix(10 mM)	0.5
4. Taq DNA polymerase (2 U/µl)	0.5
5. Downstream primer (15 p moles)	1.5
6. Upstream primer (15 p moles)	1.5
7. Template DNA	2.5
	25 µl (Total volume)

The PCR amplification of the transformed, untransformed plant genomic DNA as well as the positive control, *A. rhizogenes* plasmid DNA was carried out using *rol* A and *rol* C primers. The primers for amplification of the *rol* A product were, forward—5'-AGA ATG GAA TTA GCC GGA CTA-3' and reverse—5' -GTA TTA ATC CCG TAG GTT GTT T-3' and for *rol* C, forward 5'-ATG GCT GAA GAC GAC CTGTGT T-3' and reverse 5'- TTA GCC GAT TGC AAA CTT GCT C-3', were used respectively. The reaction components were combined in a thin-walled PCR tube (Appendix V). The contents of the tube were mixed by a brief spin in a micro centrifuge (eppendorf). The tubes were placed in the thermo cycler and proceeded with the thermal cycling program as indicated below.

Initial denaturation for 1 min at 94 °C, followed by 34 cycles of denaturation for 1 min at 94 °C, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min.

3.2.4.4.3.3. Analysis of PCR products by agarose gel electrophoresis

3.2.4.4.3.3.1. Materials:

Agarose (Electrophoretic grade)	DNA loading dye
Standard DNA size marker	Gel running boat
Electrophoresis unit	Ethidium bromide stock solution

Agarose Gel Electrophoresis

The integrity and purity of the isolated DNA was analysed by subjecting it to agarose gel electrophoresis (Appendix VI). Agarose was used at 1.4 % level and melted in IX TAE. The samples were loaded by mixing a 10 µl aliquot of the total reaction with 2 ul of loading dye. Standard DNA size marker was loaded and the samples were run at 50 V till the dye reaches $3/4^{th}$ of the gel. The gel was removed from the tank and placed in ethidium bromide solution for 10 min. and then destained in distilled water and examined on UV transilluminator. The gel was again visualised using gel documentation system (UVP EC3 Chemi HR 410 Imaging System, USA) using VisionWorks LS Software.

3.2.5. Phytochemical analysis

3.2.5.1. Thin Layer Chromatography (TLC)

Hairy roots, normal roots and field roots of dry biomass, 2 g were extracted using methanol. The roots were air oven dried at 50 ºC for 3-4 hr before extracting.

3.2.5.2. Extraction and Separation of secondary metabolites

Total alkaloid and withanolide was separated from the roots of different samples followed by the method described by Yu *et al*., 1974. Root powder (2 g) from each treatment was extracted separately with 30 ml of methanol or twice in a conical flask under constant shaking (60 rpm). It was also extracted using soxhlet apparatus by running for 4-5 hours. The extracts were filtered using Whatmann No. 1 filter paper (disc was saturated with methanol). The extracts were

concentrated using rotavapor (Buchii) and weighed to find out the total crude extract.

The extracts thus obtained were made aqueous with equal volume of water and extracted with 20 ml of hexane for twice. The residual substance thus obtained was extracted with diethyl ether for twice. The ether extract was combined and washed with water, dried over an anhydrous sodium sulphate and concentrated, the extract was weighed to get the total withanolide content. Withanolides were confirmed by TLC using Liebermann Burchard reagent for steroids (Appendix VII).

3.2.5.3. Confirmation of withanolides by TLC

For TLC analysis, a small amount of withanolide fraction was dissolved in minimum quantity of diethyl ether and spotted on Silica gel G- 60 glass plates (10×10 cm) using capillary tube along with authentic sample of withanolide-A and withaferin- A. Chromatogram was developed using the solvent systems, hexane: chloroform: methanol (5:4:1), hexane: ethyl acetate: methanol (5.5:4:0.5), hexane: ethyl acetate (2:8), chloroform:methanol (9:1), chloroform: ethyl acetate: methanol (9:0.5:0.5), hexane:ethyl acetate:methanol (7.5:2:0.5). Withanolides were observed after spraying plates with Libermann Burchard reagent or dipping the plates in anisaldehyde reagent. Both these methods were followed by keeping in iodine chamber or drying in hot air oven at 90 ºC for 5 minutes or observing under UV (230 nm) for visualising the band formed. The presence of withanolides was determined by comparing the Rf values.

3.2.5.3. Separation of Alkaloid fraction

The residual aqueous methanolic solution after the ether extraction was acidified with 1% H_2SO_4 and subsequently basified with ammonia solution (ph 11.0). The basified solution was extracted three times with chloroform (10ml ×3). The combined chloroform extract was dried over anhydrous Na_2So_4 and concentrated to get total alkaloid extract.

3.2.5.4. High pressure liquid chromatography (HPLC)

HPLC was done from Sree Chithra Tirunal Institute for Medical Sciences and Technology, Poojappura, Thiruvananthapuram. For HPLC, 50 mg extract was dissolved in 1 ml of methanol and 10 µl from this stock was again made up to 1 ml. Standards of withaferin A and withanolide A (1 mg) was totally dissolved in 1 ml of methanol. Both samples and standard were passed through 0.22 µ fibers before injection. The samples and standard (20 µl each) were injected into a Waters 600 series pump with Reprobond C 18 column – 4.6×250 mm, 7725 Rheodyne injector, Mobile phase of Acetonitrile:water/DW-60/40, Column oven-35 °C, flow rate- 1.0 ml/min was maintained with 2487 UV detector at 225 nm (withanolide-A) and 254 nm (withaferin- A). The retention time was noticed and compared with that of standards.

RESULTS

4. RESULTS

The present study on the '*Agrobacterium rhizogenes* mediated transformation of Ashwagandha (*Withania somnifera* (L.) Dunal)' was conducted during the period 2013-2014 at Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The results obtained from the laboratory are summarized below.

4.1 ESTABLISHMENT OF ASEPTIC SEEDLINGS

Withania somnifera (Plate 1) seeds possess hard seed coat which makes it difficult to germinate easily. Surface sterilization of the ripened berries using 0.1% $HgCl_2$ for 12 minutes was favorable to obtain 84.5% infection free seeds. Various concentrations of GA_3 (0.1 mg/l –1.0 mg/l) were evaluated for their effect on the germination of seeds and best treatment was assessed based on the germination percent and time required for germination (days). Inoculation of seeds in media supplemented with all concentrations of GA_3 favored germination at varying degree. Among the various concentrations of GA_3, it was found that incorporation of GA_3 at 0.5 mg/l provided 93 % germination (Table 1). It also could shorten the period of germination from 10 days to 7 days. The seeds were germinated within 6 to 7 days in most of the treatments. Half- strength MS + 0.5mg/l GA_3 medium was best for seed germination when compared to half-strength strength MSB medium (control). The maximum length of hypocotyls obtained to be 2.40 cm (Plate 2) in half strength MS + 0.5mg/l GA_3 medium. When maximum seeds were germinated within 11 – 14 days under dark, the cultures were transferred to light condition for the fast normal growth. This was to avoid etiolation of the seedling.

4.2 INITIATION AND ESTABLISHMENT OF SHOOT CULTURES

Shoot cultures were initiated using cotyledonary nodal explants (0.5 - 1cm) derived from 6 weeks old aseptic seedlings. During the previous experiments in the laboratory 0.2 mg/l was found to be the best for shoot

Plate 1. Field grown plant of *Withania somnifera*

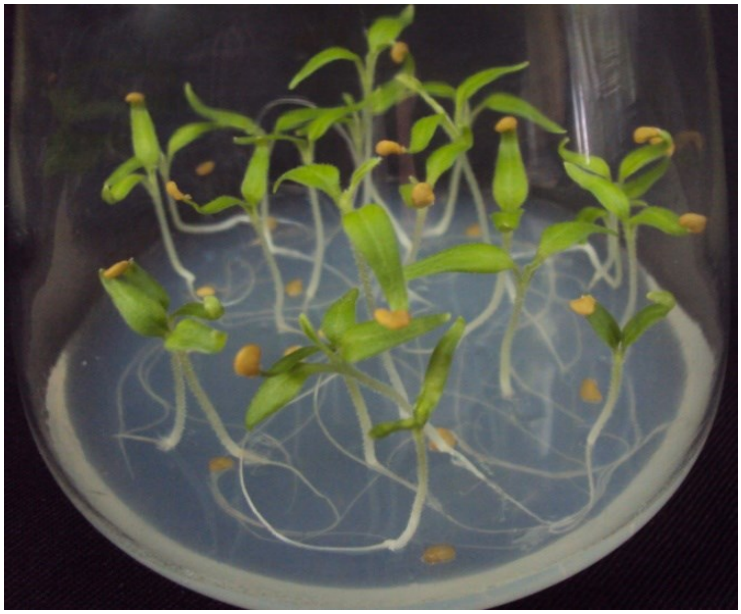


Plate 2. Four week –old seedlings grown in half strength MS + 0.5mg/l GA_3 medium

Table 1: Effect of GA_3 in half- strength MS medium on seed germination

Treatment (mg/l)	Frequency (%) (10 days)	Length of the seedling (cm)	Frequency (%) (20 days)	Length of the seedling (cm)
½ MSB (control)	7.25	0.6 ± 0.20	15.3	1.4 ± 0.33
½ MSB + 0.1 mg/l GA_3	11.45	1.85 ± 0.25	28.5	2.75 ± 0.20
½ MSB + 0.2 mg/l GA_3	30.75	1.32 ± 0.30	62.2	3.2 ± 0.30
½ MSB + 0.5 mg/l GA_3	52.50	2.4 ± 0.40	93	4.3 ± 0.10
½ MSB + 0.7 mg/l GA_3	25.46	1.25 ± 0.22	44.35	2.8 ± 0.23
½ MSB + 1.0 mg/l GA_3	13.04	1.45 ± 0.20	41.17	1.9 ± 0.21

initiation. Hence BAP at 0.2 mg/l was considered for the present experiment. In order to get rapid shoot multiplication, the explants were inoculated onto full strength MS solid medium supplemented with 0.2 mg/l BAP and IAA (0.02 mg/l, 0.05 mg/l and 0.1 mg/l) dispensed in Erlenmeyer flasks and culture tubes. Single axillary shoot bud (0.5 - 0.8 cm) initiation was noticed in all the treatments within 4 – 5 days in Erlenmeyer flask, while it was delayed in culture tubes. The multiple shoots initiation was observed after 15 days of growth (Plate 3). Thereafter the cultures were grown rapidly by producing extensive axillary branches and attained maximum robust growth at 25th day (Figure 1). Maximum growth in terms of multiple shoots and length of shoot was recorded after 25th day in the culture supplemented with 0.2 mg/l BAP and 0.05 mg/l IAA in Erlenmeyer flasks (Table 2). Shoot growth declined after 30 days and necrosis after 35th day. Hence the shoots were subcultured every 3weeks interval into fresh medium. No rooting was observed in any of the treatments but callusing was found towards the 3rd week.

4.3 INITIATION AND ESTABLISHMENT OF NORMAL ROOT CULTURES

Normal root initiation experiment preliminarily aimed to find out (1) the root initiation potential of terminal shoot cuttings (3.5 - 4 cm), terminal and basal segments of leaves derived from aseptic cultures (2) the effect of different concentration of IBA and IAA (0.05 mg/l, 0.1 mg/l, 0.2 mg/l, 0.5 mg/l, 0.8 mg/l) in Erlenmeyer flasks and culture tubes. For all the experiments for root initiation half- strength MS medium was used. The cultures of terminal shoot cuttings were only incubated under light. Leaves were kept under dark conditions.

Among different auxins at varying concentrations tried it was found that IBA was better auxin than IAA. Among different explants tried, terminal shoot cuttings were more efficient to produce normal roots than both terminal and basal segments of leaves. The effect of different auxin treatments are represented in Table 3 and Table 4. The shoots inoculated in half strength MS medium supplemented with 0.2 mg/l IBA initiated highest frequency(98%) of root initiation within 8-9 days in Erlenmeyer flask compared to culture tubes (Plate 4(4b) and 4(4c). In this medium shoots initiated a mean number 20.1 ± 3.2 of

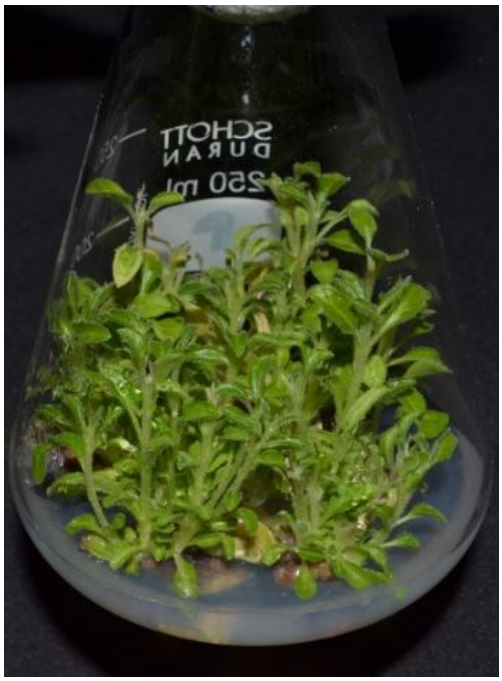
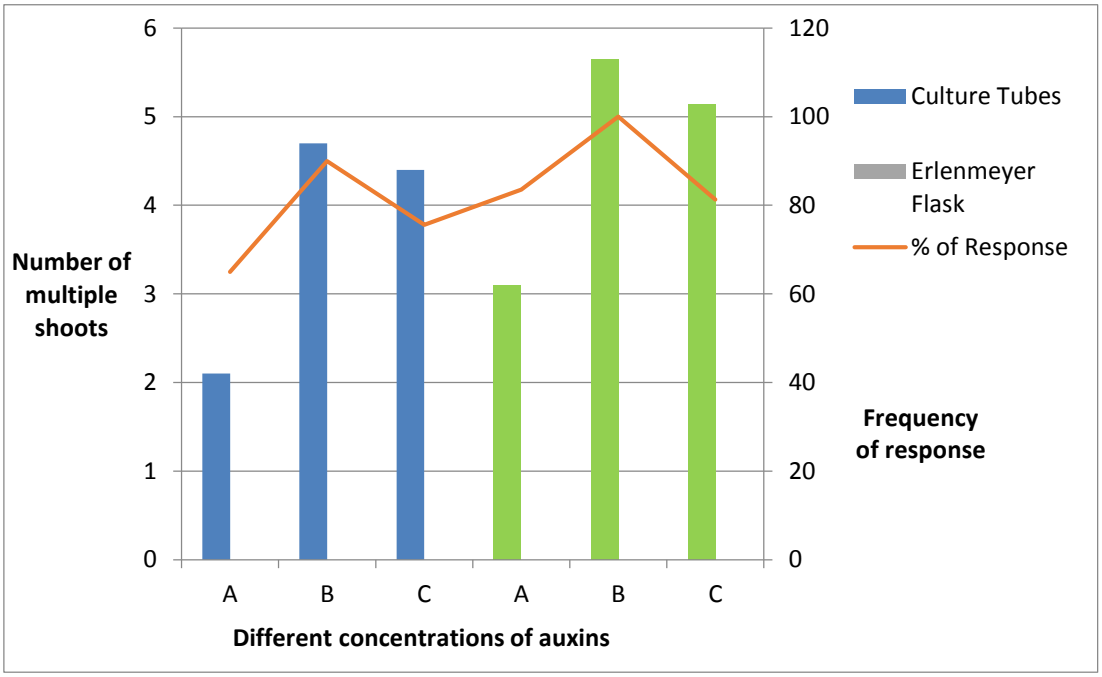


Plate 3. Four week - old shoot culture grown in full strength MS + 0.2 mg/l BAP + 0.05 mg/l IAA

4a. Erlenmeyer flask **4b. Culture tube**

Plate 4. (4a and 4b) Establishment of normal root culture



A: 0.2 IBA+ 0.02 IAA

B: 0.2 IBA+ 0.05IAA

C: 0.2 IBA+ 0.1 IAA

Figure 1: Effect of glassware and plant growth regulators on axillary shoot initiation on cotyledonary nodal explants

Table 2: Effect of glassware and plant growth regulators on axillary shoot initiation on cotyledonary nodal explants of *Withania somnifera.*

Glass ware	PGR (concentration -mg/l)		Frequency of response (%)	Mean no of shoots/explant	Mean Length of shoot/explant (cm)	Degree of callusing
	BAP	IAA				
Culture tube	0.2 mg/l	0.02 0.05 0.1	65 90 75.6	2.1 ± 0.2 4.7± 0.10 4.4± 0.40	4.2 ± 0.30 6.2 ± 0.75 4.7 ± 0.83	+ ++ +++
Erlenmeyer flask	0.2 mg/l	0.02 0.05 0.1	83.5 100 81.3	3.1 ± 0.5 5.65± 0.2 5.14 ± 0.5	5.3± 0.40 7.4± 0.2 5.8 ± 0.02	++ + +++

Table 3: Normal root induction from terminal shoot cuttings, basal and terminal segments of leaf of *Withania somnifera* grown on half-strength MS medium supplemented with IBA under dark condition

Explants	Auxins (mg/l) IBA	Frequency of response	Mean No. of roots (%)	Mean length of root (cm)	Degree of callusing
Terminal shoot cuttings	0.05	42.5	09 ± 2	2.2 ± 0.4	-
	0.1	55.4	12 ± 5	3.4 ± 0.3	-
	0.2	98	20 ± 3	5.4 ± 0.5	-
	0.5	32.5	10 ± 4	3.2 ± 0.2	++
	0.8	14.5	08 ± 1	2.5 ± 0.4	++
Basal segments of leaf	0.05	60.7	2 ± 2	1.3 ± 0.6	++
	0.1	74	5 ± 3	2.4 ± 0.8	++
	0.2	92	6 ± 1	3.5 ± 0.3	+
	0.5	45.3	1 ± 2	1.4 ± 0.3	++
	1	24	2 ± 2	1.3 ± 0.2	++
Terminal segments of leaf	0.05	25.3	1 ± 1	1.5 ± 0.4	++
	0.1	33.1	3 ± 3	2.5 ± 0.3	++
	0.2	56.5	3 ± 1	3.5 ± 0.3	+
	0.5	29.2	2 ± 2	2.3 ± 0.8	++
	1	15.2	1 ± 2	1.3 ± 0.9	++

Table 4: Normal root induction from terminal shoot cuttings, basal and terminal segments of leaf of *Withania somnifera* grown on half-strength MS medium supplemented with IAA under dark condition

Explants	Auxin (mg/l) IAA	Frequency of response	Mean No. of roots (%)	Mean length of root (cm)	Degree of callusing
Terminal shoot cuttings	0.05	16.2	6 ± 1	0.8 ± 0.6	++
	0.1	25.9	9 ± 2	1.2 ± 0.5	++
	0.2	35.3	11 ± 2	2.5 ± 0.7	++
	0.5	0	0	0	+++
	0.8	0	0	0	+++
Basal segments of leaf	0.05	0	0	0	+++
	0.1	22.2	1 ± 2	0.5 ± 0.2	++
	0.2	42.8	3 ± 1	0.8 ± 0.2	++
	0.5	0	0	0	+++
	1	0	0	0	+++
Terminal segments of leaf	0.05	0	0	0	+++
	0.1	0	0	0	+++
	0.2	14.2	1	0.2 ± 0.02	++
	0.5	14.2	1	0.2 ± 0.02	++
	1	0	0	0	+++

roots with a mean length of 5.4 ± 0.5 cm which was the best. The roots initiated on shoot cuttings were sturdy, thick and healthy compared to the leaf. Besides in this optimized auxin concentration callus formation at the cut basal end of the shoot was very minimal.

During the subsequent experiment efficiency of shoot cuttings for normal root induction followed by repeated inoculation of the same shoot after harvesting the initiated roots was analyzed for continuous 4 passages. Shoots were prepared for fresh inoculation after slicing the basal callused end along with induced roots. The data recorded for these repeated passages of the same shoot are represented in Figure 2. The results showed that, during the first and second passages the different parameters measured (frequency of response, root length, root number, callusing) were not marginally different from those observed during root initiation (Plate 5(5a and 5b)). When passage was shifted to third 60% of the shoots initiated roots. The roots formed during the third passage were short and thick (Plate 5(5c)). In the fourth transfer the frequency of response was marginally declined to 20% and root number was reduced (Plate 5(5d)). The callus formation at cut basal end was enhanced on shoots on fourth transfer and the roots became short.

Between the two segments of leaf inoculated, basal segments in half strength MS medium supplemented with 0.2 mg/l IBA in Erlenmeyer flask showed best response for root induction than terminal segments. The leaves incubated under dark responded within 10-12 days. Basal segments initiated in 0.2 mg/l IBA showed highest frequency(92%) with a mean number of roots, 6±1 and a mean length of 3.5±0.3cm on the other hand low frequency of (56.5%) of response were noticed on terminal leaf segments (Table 3 and table 4). Basal segment of roots are fast growing and produce many lateral roots mainly from the petiole of the leaf (Plate 6(6a)).

The callus formation from the cut end of the terminal segment was pronounced and it hindered the smooth initiation of roots (Plate 6(6b)). Roots induced by leaf were thin with lateral root production along the length of the root.

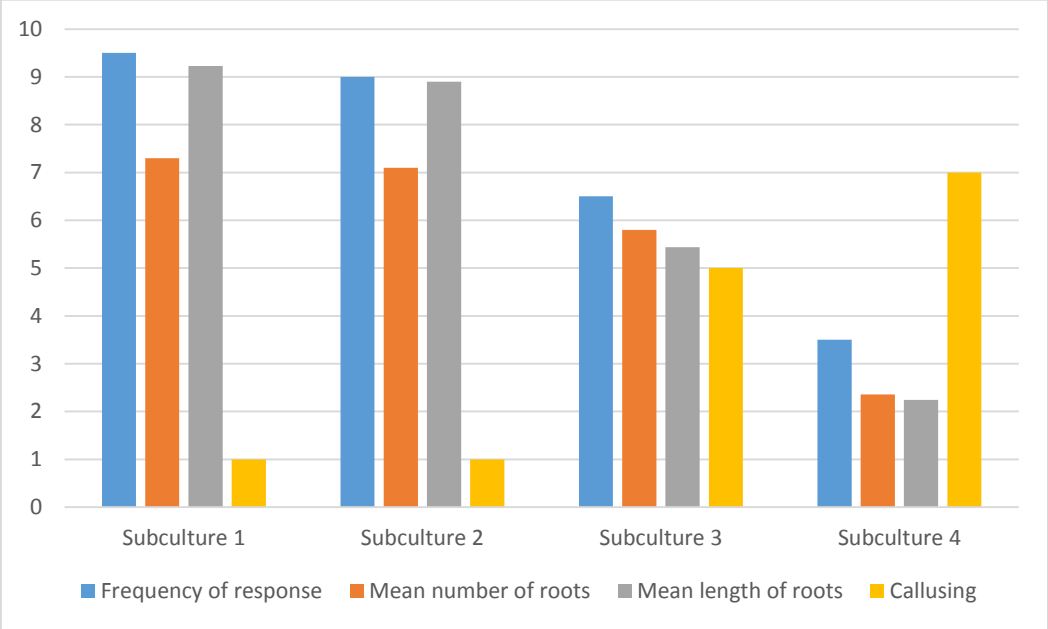
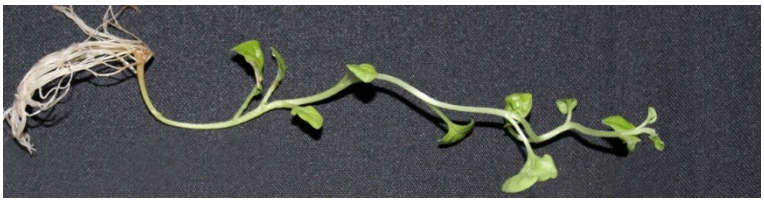


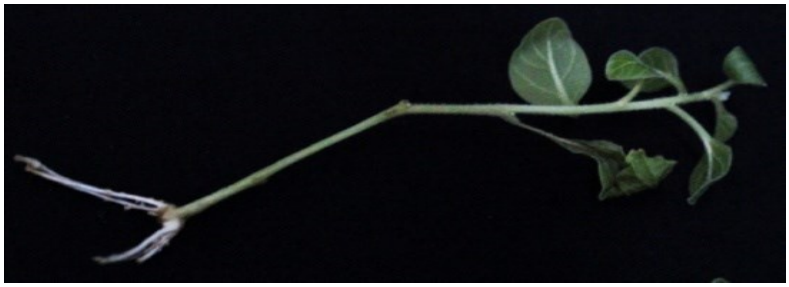
Figure 2: Effect of repetitive transfer of same shoots for normal root induction

5a. First subculture



5b. Second subculture

5c. Third subculture



5d. Fourth subculture

Plate 5. (5a, 5b, 5c and 5d) Repetitive transfer of same shoots for normal root induction

6a. Basal leaf segment

6b. Terminal leaf segment

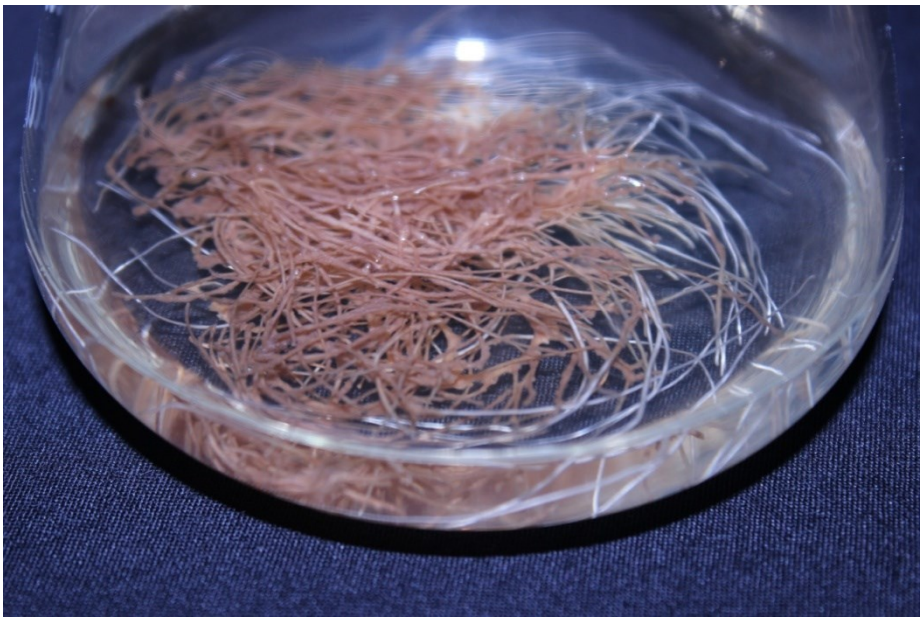
Plate 6. (6a and 6b) Induction of leaf from basal and terminal portion of the leaf

4.3.1 Establishment of normal root suspension

To establish normal root suspension, roots were segmented after 25 days of induction and approximately 50 mg (fw per flask) were inoculated into half-strength MS liquid medium supplemented with different concentrations of IBA (0.2 mg/l, 0.5mg/l, 0.8mg/l) in 250 ml Erlenmeyer flask under 16 h photoperiod in gyratory shaker. Normal root suspension was established separately for root derived from shoot and leaf segments. Among the different concentrations of IBA maximum root growth was recorded on half- strength MS liquid medium supplemented with 0.2 mg/l IBA was the best (Plate 7(7a)). Callusing was prominent when auxin concentration was increased. Growth was noticed on 4th day in liquid medium by the elongation of root tip and subsequent lateral root formation and further elongation. The time course of growth of normal root suspension for a period of 5 weeks is shown in (Figure 3). The fresh and dry root biomass of normal root increased after 10 days and reached maximum (3.4g/ 0.31g fw/dw) at 23rd day and thereafter it was declined. A six-fold increase was obtained when compared with initial inoculum of 50 mg roots. Roots grown in this optimized medium were healthy and viable. However, roots became brown, flaccid and brittle and noticed slight callus formation at the stationary phase of growth of 27-30 days. The actively growing root suspension were harvested (5 week interval) and used for phytochemical assays. Roots grown in medium with high concentration of IBA (0.8mg/l), lateral roots are short and callused and eventually turned brown (Plate 7(7b)). These callused roots were not subjected to phytochemical analysis.

4.4 INITIATION AND ESTABLISHMENT OF HAIRY ROOT CULTURE

The pre-incubated different explants (cotyledons, leaves, internode and hypocotyls) from 2-3 weeks old seedlings and leaf and internodes from shoot cultures infected with different *A. rhizogens* strains (A4, LBA 9402, MTCC 532) initiated hairy roots, based on the explant and type of strain. The pre-incubated explants were wounded. Transformation was expressed by the direct emergence of fine hair like roots without callus (Plate 8). Transformation efficiency of the *A.*



7a. Half- strength MS liquid medium + 0.2 mg/l IBA

7b. Half- strength MS liquid medium + 0.8 mg/l IBA

Plate 7. (7a and 7b) 4 week old normal root suspension cultures in half-strength MS liquid medium supplemented with different concentrations of auxin

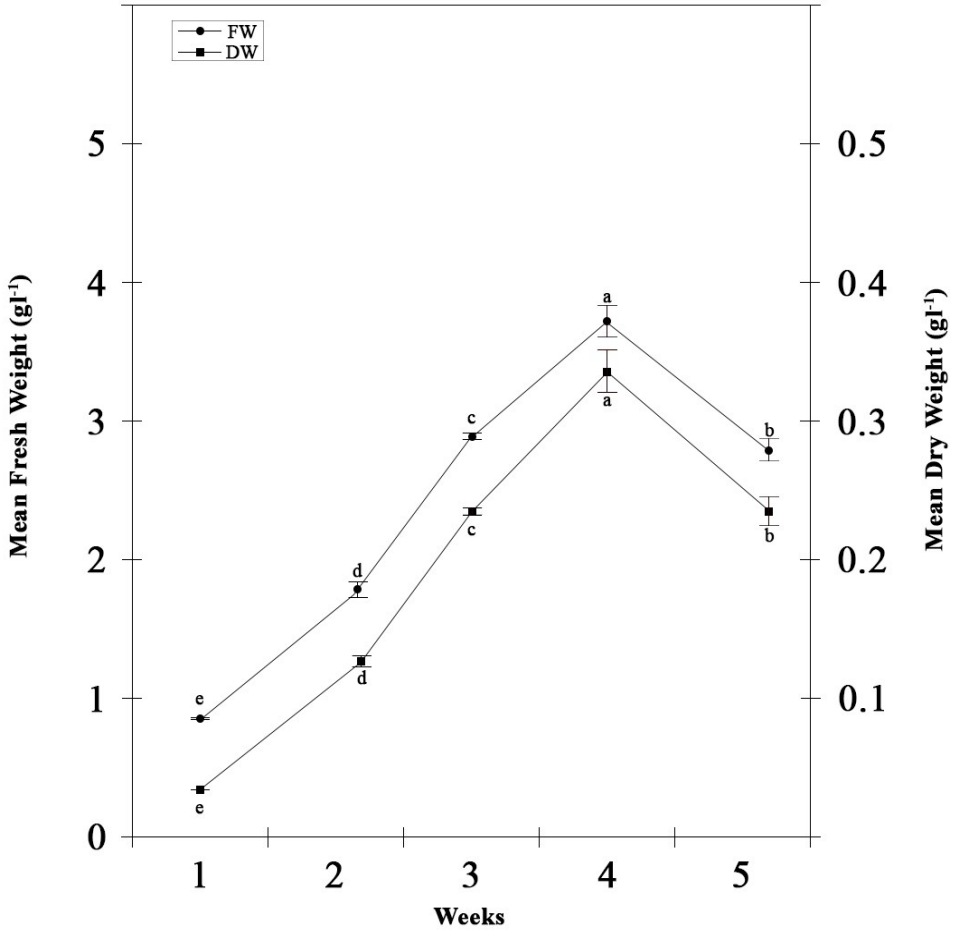


Figure 3. Time profile of normal root growth

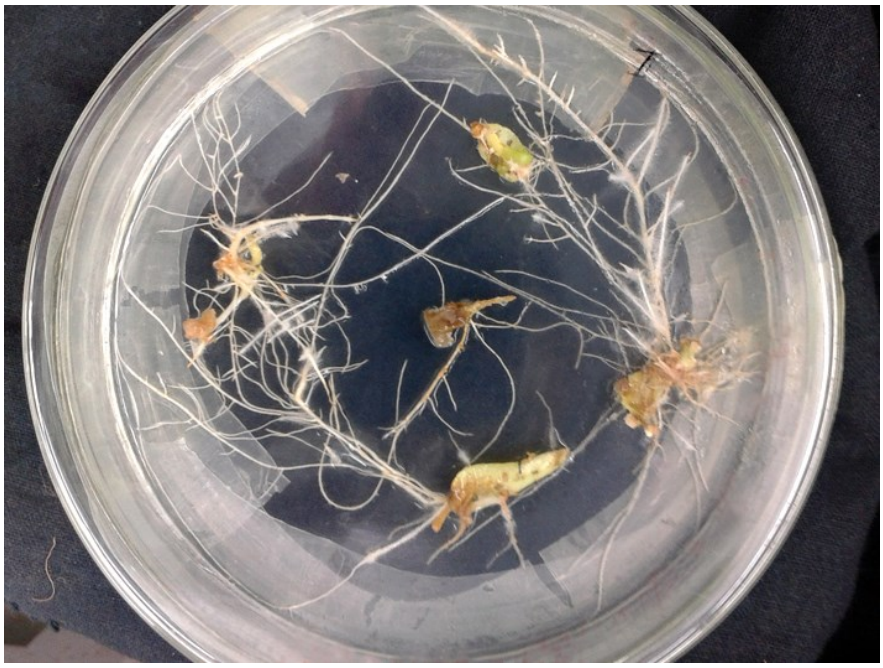


Plate 8. Hairy roots induced on cotyledons

rhizogenes strains and duration required for the root induction on various explants is given in Table 5. The type of explants and strain were an important factor responsible for the rapid induction of hairy root in the present system. Among the various explants infected, cotyledon was found to be the best explant for fast induction of roots with maximum frequency and number of hairy roots compared to other explants. Among the various *A. rhizogenes* strains used, A4 was the most virulent for hairy root induction followed by LBA 9402 and MTCC 532. A4 strain induced maximum frequency of transformation (82.3 ± 0.3%) within 10-12 days and roots were elongated rapidly by producing many lateral roots which tend to grow upwards, whereas MTCC 532 induced callus alone and LBA 9402 performed a low rate of transformation. The control explants did not produce roots.

Among other explants cotyledon responded quickly (Table 6). Of all the explants, the least responding was that of the internode and hypocotyl. The highest degree of callusing was shown by internodes. Very thin and scanty roots were produced by the explant without *Agrobacterium rhizogenes* infection (control). Small roots formed in the control culture were more in leaf explants. Approximately 20% of the cotyledonary explant induced 3-4 roots after 6 days. Upon repeated transfer to antibiotics, 500 mg/l ampicillin containing medium, 90% of the explants induced roots rapidly within 12-15 days (Plate 9). After successful passages roots were established in full strength MS solid medium without any supplementation of hormones (Plate 10(10a)).

4.4.1 Establishment of hairy root suspension

To establish hairy root suspension, roots were segmented after 25 days of induction and approximately 50 mg (fw per flask) were inoculated into full-strength MS liquid medium. In shake flask culture, growth of the hairy roots started on second day with an exponential phase during 7^{th} to 16^{th} day (Plate 10(10b). Maximum fresh/ dry weight was obtained on 15^{th} day and found to be slowly decreasing (4.7g/0.56g fw/dw). A nine-fold increase was obtained when compared with initial of 50 mg roots. The root started discoloration after 22-23

Table 5: Frequency of hairy root induction on juvenile explants derived from aseptic seedlings of *W. somnifera* followed by infection with different *A. rhizogenes* strains

Bacterial strain	**Explant type**	**Frequency of transformation (%)**	**Hairy root induction (days)**
A4	Cotyledon Leaves Hypocotyl internode	82.3 ± 0.3 70.5 ± 0.2 16.5 ± 0.7 19.2 ± 0.4	10-12 15-19 20-25 20-25
MTCC 532	Cotyledon Leaf Hypocotyl internode	32.1 ± 0.2 25.8 ± 0.4 12.5 ± 0.2 15.3 ± 0.2	36–38 30–32 35-38 33-35
LBA 9402	Cotyledon Leaf Hypocotyl internode	35.2 ± 0.3 30.4 ± 0.5 18.5 ± 0.3 14.2 ± 0.5	23-26 25-27 28-30 23-25

Table 6. Effect of explants on hairy root induction of *Withania somnifera* followed by infection with *Agrobacterium rhizogenes* strain A4

Explants	No. of roots	Frequency of transformation (%)	Length of root	Duration of transformation (days)	Degree of callusing
Cotyledon	17	90	11.5	10 -12	(+)
Leaf	11	80	6.5	15 – 19	(+)
Internode	4	66	0.8	20 -25	(++)
Hypocotyl	3	12	0.5	20-25	(+)

Plate 9. Hairy root initial growth in full strength MS solid medium after transfer to medium with antibiotics

10a. Full strength MS solid medium

10b. Full strength MS liquid medium

Plate 10. (10a and 10b) Hairy roots established in full strength MS solid medium and liquid medium

days and growth was decreasing. It was noticed that *Withania* hairy roots can not be run beyond 25th- 28th day. The time course of growth of hairy root suspension for a period of 30 days is shown in (Figure 4). The actively growing root suspension were harvested (2-3 week) and used for phytochemical assays.

4.4.2. Quantification of DNA

The quantity and quality of the isolated DNAs was assessed by diluting the sample 50 times by adding, 1µl of isolated DNA sample to 49µl of 1X TE or sterile distilled water and recorded the reading at absorbance at 260 nm and 280 nm (Table 7).

4.4.3. PCR amplification of *rol* A and *rol* C gene from *Agrobacterium* and DNA of normal and transformed roots

Hairy root and normal root (control) were used for total DNA extraction. Extractions were made from *invitro* grown cultures for both transformed and untransformed roots. Plasmid from *Agrobacterium rhizogenes* (A4) was isolated by using Alkaline Lysis method. The primers used for amplification of the *rol* A product were, forward—5'-AGA ATG GAA TTA GCC GGA CTA-3' and reverse—5' -GTA TTA ATC CCG TAG GTT GTT T-3' and for *rol* C, the forward 5'-ATG GCT GAA GAC GAC CTGTGT T-3' and reverse 5'- TTA GCC GAT TGC AAA CTT GCT C-3', respectively. Amplification were performed on 25 ng of genomic DNA in a final reaction volume of 25µl containing 1X PCR buffer, dNTP, primers and Taq DNA polymerase. Thirty-five cycles of amplifications were performed (94^{0}C, 1 min; 55^{0}C, 1min; 72^{0}C 1min). A predenaturation step of 4 min and a final extension step of 5 min were used. The amplification products were observed by electrophoresis on 1.2% agarose gel stained by ethidium bromide along with marker.

Molecular evidence for the transformed status of the hairy roots was obtained by PCR analysis. When observed under UV transilluminator a band 300 base pair and 550 base pair was confirmed in extracts from transformed roots and

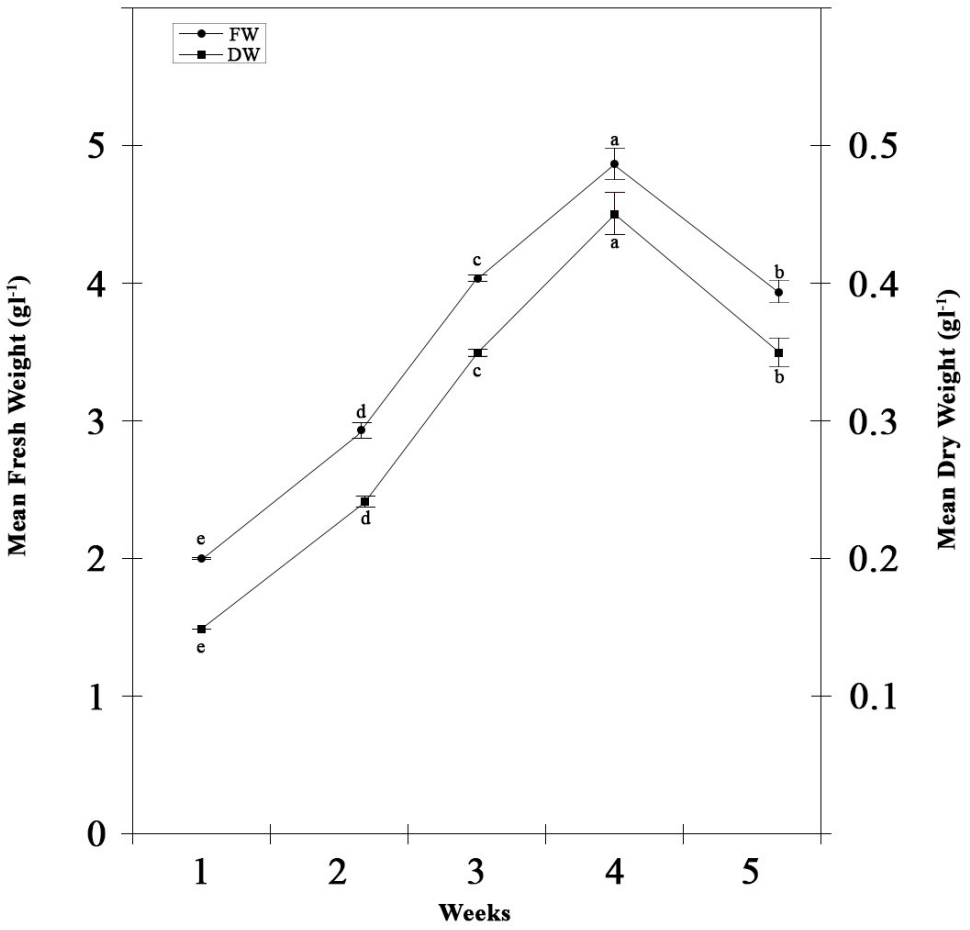


Figure 4. Time profile of hairy root growth in full-strength MS medium

Table 7. Concentration (µg/µl) and absorbance at 260 nm, 280 nm and 260/280nm of samples

Sample	**µg/µl**	**A_{260}**	**A_{280}**	**$A_{260/280}$**
Hairy root	0.020	0.008	0.003	2.74
Normal root	0.033	0.013	0.006	2.25

Agrobacterium rhizogenes (A4) using specific primer. Putatively transformed hairy root cultures of *W. somnifera* were positive for both the gene fragments, whereas no amplification was found from normal root (Negative control) DNA (Plate 11). Thus the PCR analysis showed that all the transformed root (hairy root) are able to express the *rol A* and *rol C* gene which is a clear indication of transformation of the explants of *Withania somnifera* with Ri plasmid.

4.5 PHYTOCHEMICAL ANALYSIS

4.5.1 Thin Layer Chromatography (TLC)

Extraction of samples using soxhlet was found to be effective than other methods. Two grams of the powder of hairy roots harvested from shake flask cultures yielded 0.461 g of total crude extract after methanolic extraction in soxhlet. Total withanolide content was about 0.198g in hairy root sample. Total crude extract and total withanolide of normal roots from shoots were 0.395 g and 0.121 g respectively and for leaf- derived normal roots from were 0.374 g and 0.108 g respectively. Field roots yielded a total crude extract of 0.423g and total withanolide content of 0.142 g. Chromatogram was developed using different solvent systems out of which hexane: ethyl acetate: methanol in the ratio 7.5:2:0.5 found to provide best separation. The presence of withanolide A and withaferin A were confirmed when the Rf value coincided with the compound in all the samples samples in the TLC (Plate 12 and Plate 13(13a)). Rf – 0.36 cm confirmed the presence of withaferin A and Rf- 0.59 cm confirmed the presence of withanolide A. The two standards were again confirmed by Co-TLC (Plate 13(13b)). In the case of detection of withanolides, dipping the plates in anisaldehyde reagent found to be better than spraying plates with Libermann Burchard reagent.

4.5.2 High performance liquid chromatography (HPLC)

Qualitative and quantitative detection of the compounds in various samples was done by using HPLC. The results indicated that authentic sample eluted a retention time of 3.8- 4.2 mins for withaferin-A and 4.2-4.8 mins in for

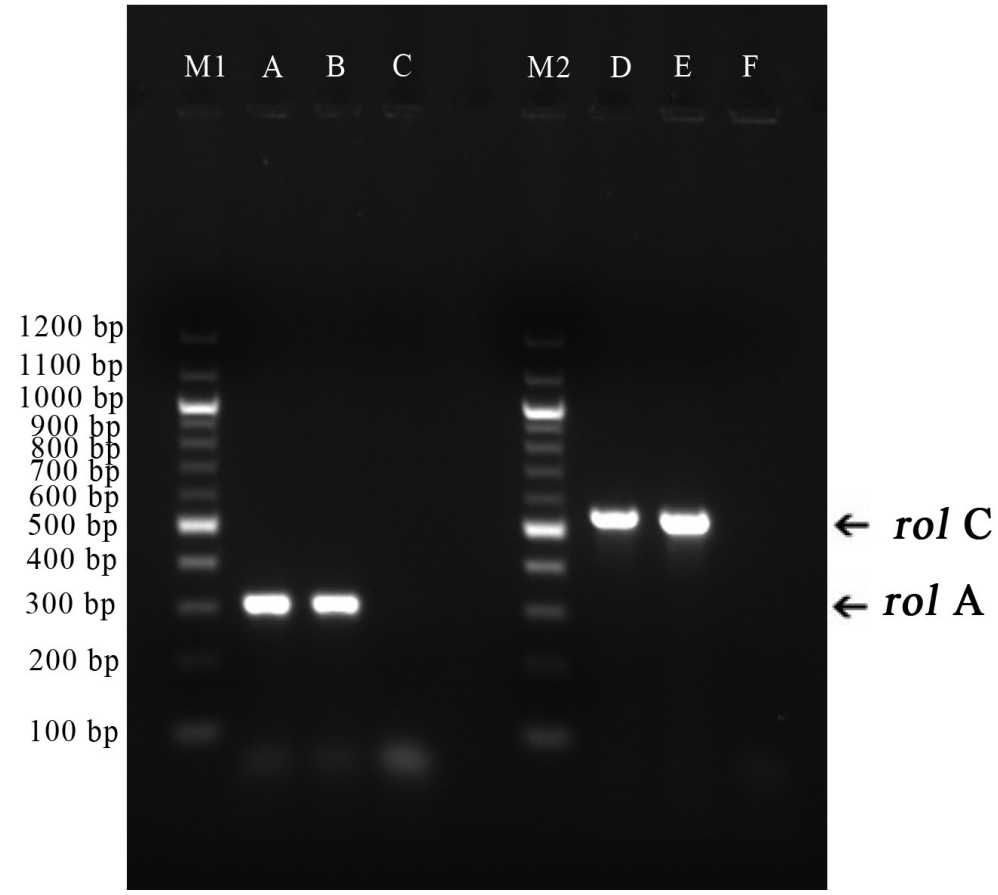
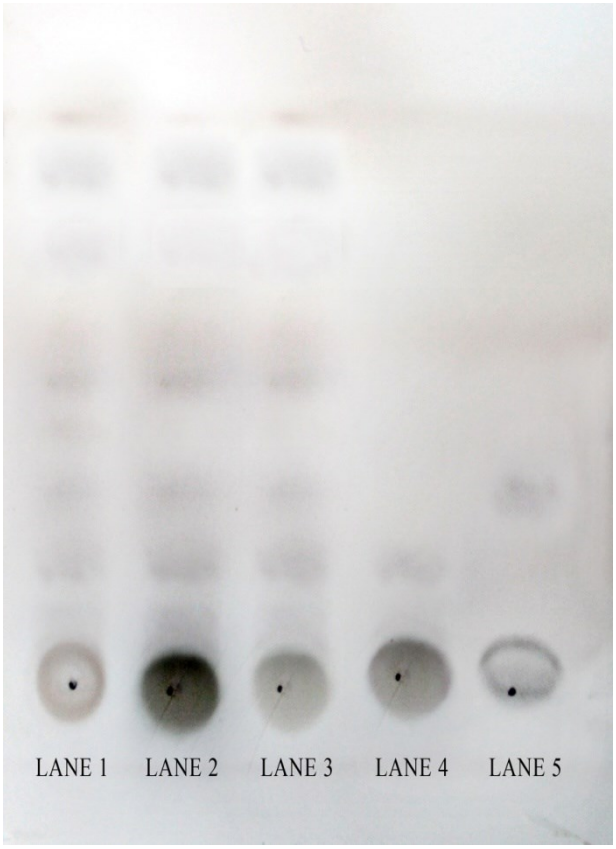


Plate 11. PCR analysis of hairy root culture of *W. somnifera* transformed by *A. rhizogenes* A4

lane M1 and M2—Marker (1200 bp), lane A and D—plasmid DNA (positive control), lane B and E-genomic DNA of hairy root culture showing amplified fragment of *rol A* (300 bp) and amplified fragment of *rol* C (550 bp), lane C and F-genomic DNA from normal root culture (negative control)



Lane 1: Field root

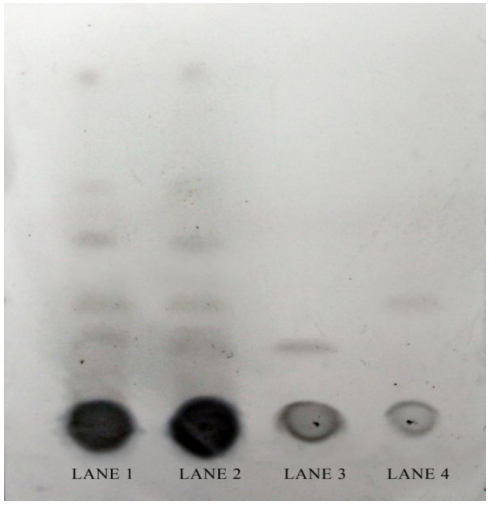
Lane 2: Hairy root

Lane 3: Normal root

Lane 4: Withaferin-A

Lane 5: Withanolide-A

Plate 12. TLC analysis



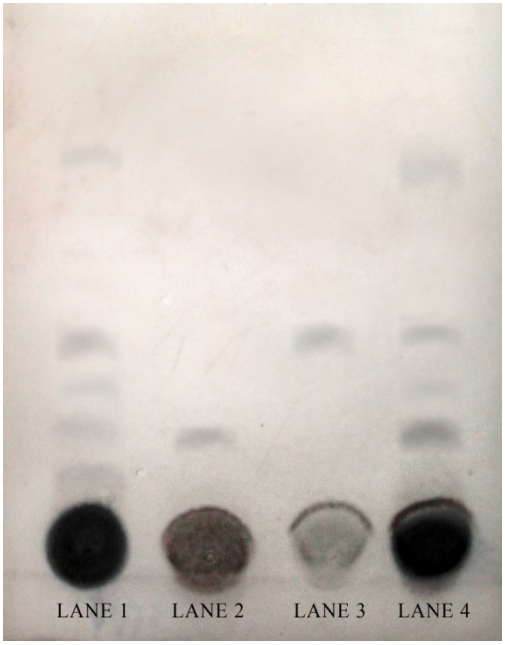
Lane 1: Normal root- shoot

Lane 2: Normal root- leaf

Lane 3: Withaferin-A

Lane 4: Withanolide-A

13a: TLC analysis



Lane 1: Normal root

Lane 2: Withaferin-A

Lane 3: Withanolide-A

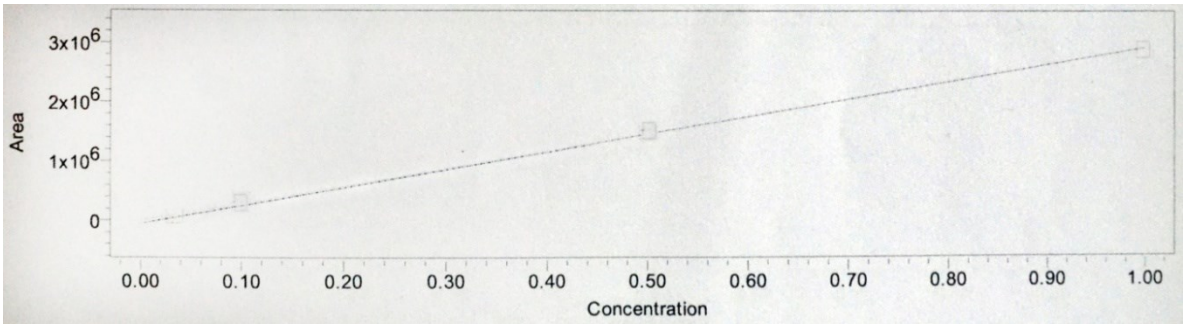
Lane 4: Normal root+Withaferin-A

13b: Co-TLC

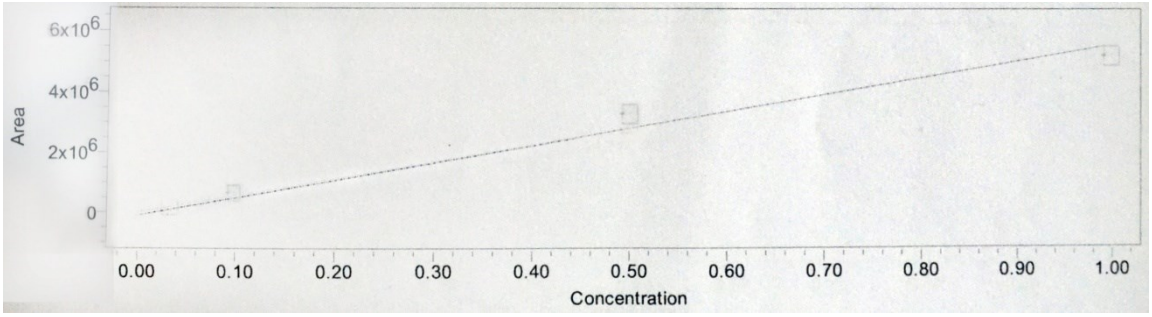
Plate 13. (13a and 13b) TLC chromatograms

withanolide-A. The retention time of peaks in the samples, (normal roots from shoot and leaf), hairy roots and field roots, were 3.88, 3.81, 3.89 mins. corresponding to standard Withaferin-A, and 4.55, 4.63, 4.57 for withanolide-A respectively. In the HPLC profile of all the samples analyzed both withaferin-A and withanolide-A was detected. The quantitative analysis done followed by the preparation of the calibration curve (0-1mg) (Figure 5(5a) and 5(5b)) detected that withaferin-A concentration was more while the concentration of withanolide- A was negligible in all samples (Table 8). Among the different *in vitro* samples analyzed concentrations of the compounds was slightly more in hairy root culture. The hairy root culture at peak period of growth (23 days) shown a concentration of 22.3 mg/g dw of withaferin- A and 2.35 mg/g dw withanolide-A (Figure 6(6a)). Between the normal root cultures both compounds was more in shoot derived cultures than leaf derived normal root cultures. The former at peak period of growth shown a concentration of 4.3 mg/g dw of withaferin- A and 1.65 mg/g dw of withanaloide-A (Figure 6(6b)) and latter shown a concentration of 2.75 mg/g dw withaferin- A and 0.35 mg/g dw of withanaloide-A (Figure 7). Comparing the compounds from field roots with the samples, concentration of withaferin- A, 8.55 mg/g dw and withanaloide-A, 3.85mg/g dw was more. The HPLC chromatogram of standards and different samples are shown in Figure 8, 9, 10, 11 and 12.

In all the *in vitro* roots the compounds production was negligible during the initial period of 1week. Accumulation of maximum concentration of the compounds was noticed when the hairy root culture reached its log phase during the 23rd day, which was greater than the amount found in the normal root cultures. The compounds production declined after 6 weeks, which corresponded with the slowing of growth. During the stationary phase of growth production of the compound was not increased (Figure 3 and 4).



5a. Withaferin-A

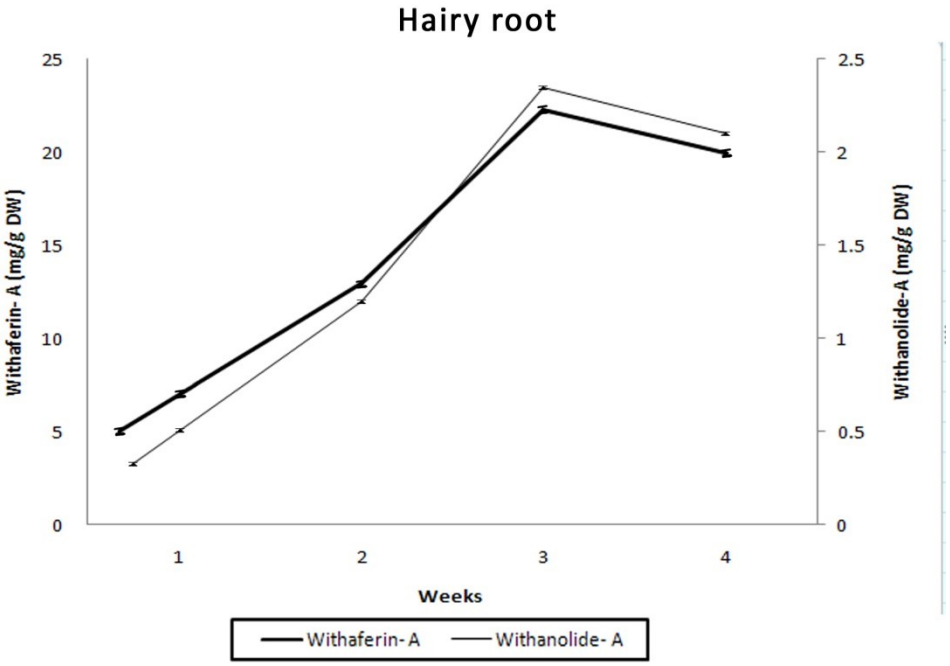


5b. Withanolide-A

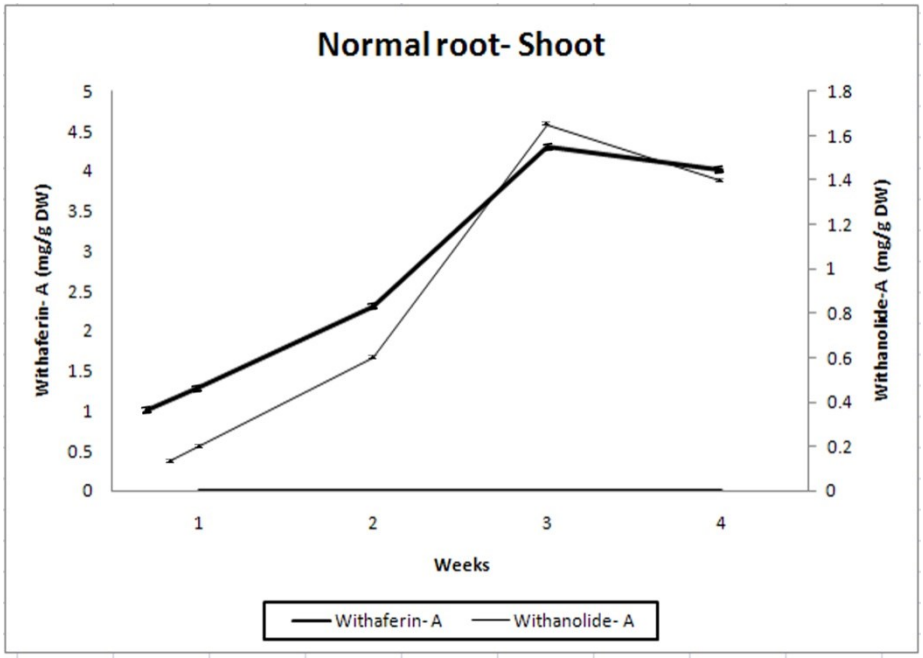
Figure 5. (5a and 5b): Calibration plot of withaferin-A and withanolide-A

Table 8. Concentration and retention time of different samples

	Hairy root	**Normal root-shoot**	**Normal root- leaf**	**Field root**
Withaferin- A (mg/g dw)	22.3	4.3	2.75	8.55
Concentration (mg/ml)	0.446	0.086	0.055	0.171
Retention time (min)	3.883	3.833	3.817	3.892
Withanolide-A (mg/g dw)	2.35	1.65	0.35	3.85
Concentration (mg/ml)	0.077	0.033	0.007	0.047
Retention time (min)	4.575	4.526	4.550	4.630



6a. Hairy root growth



6b. Normal root- shoot growth

Figure 6. (6a and 6b): Time profile of hairy root and normal root-shoot growth

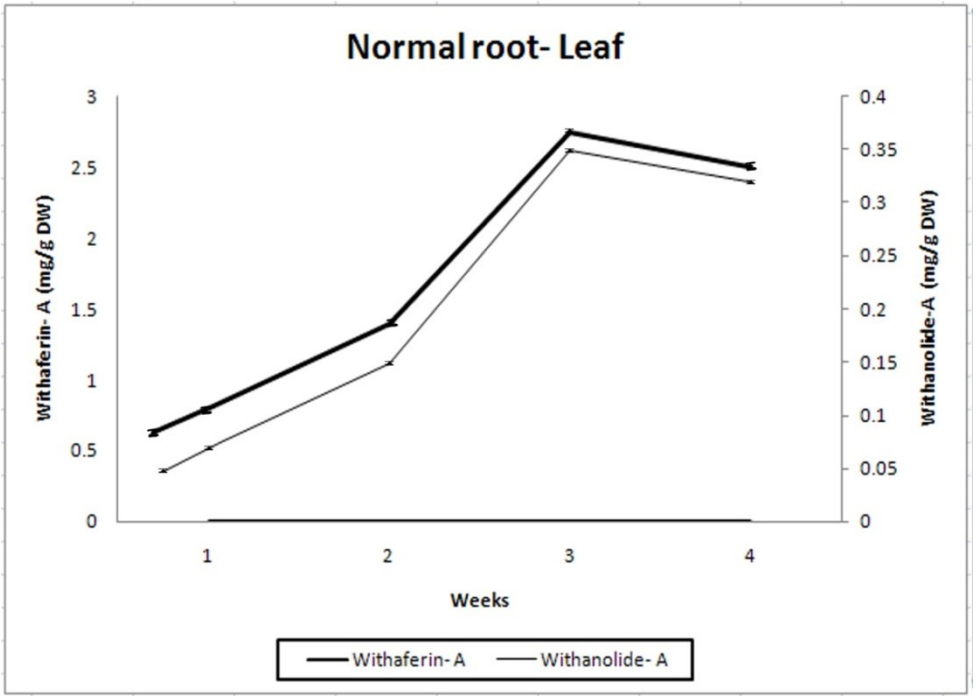
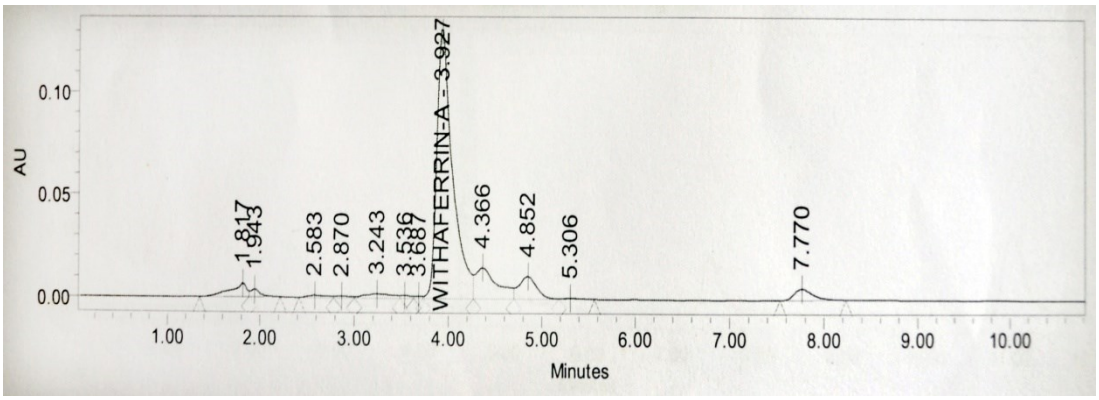
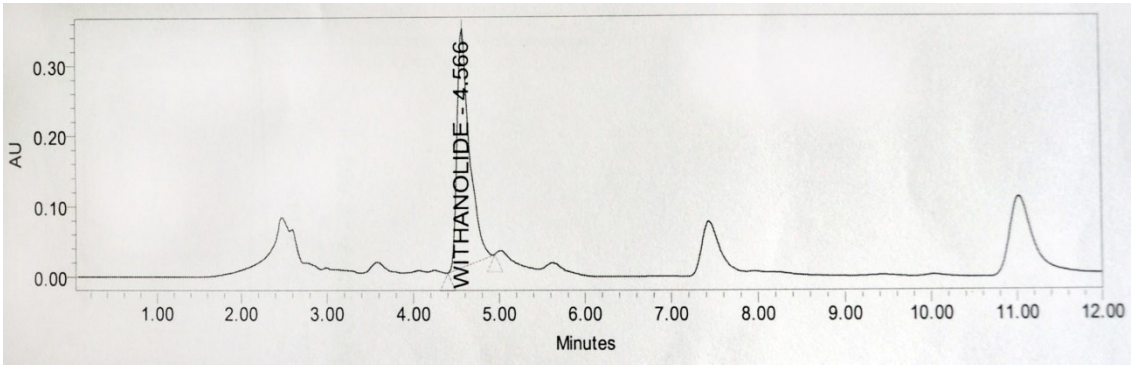


Figure 7. Time profile of normal root- leaf growth

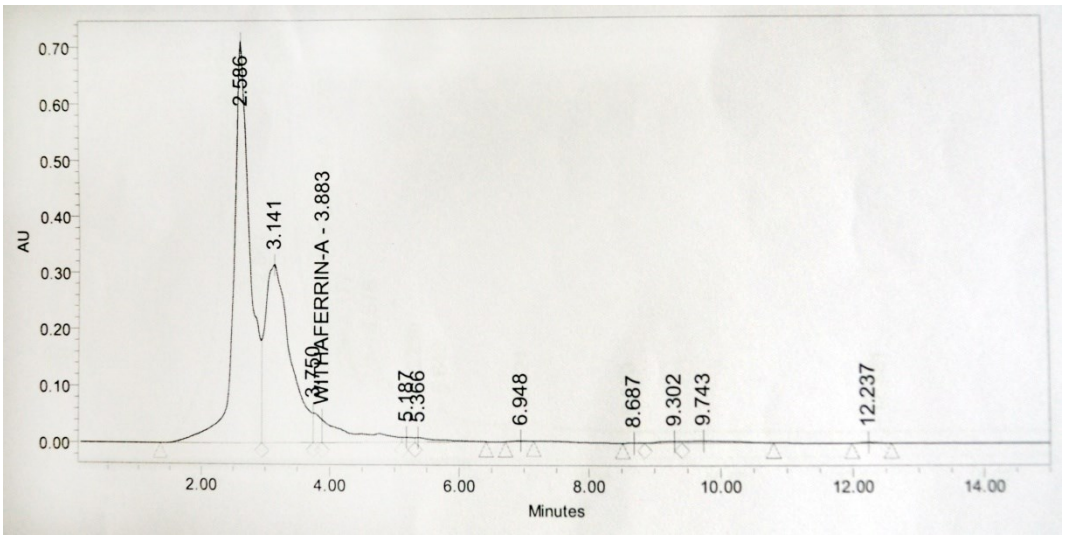


8a. Withaferin-A

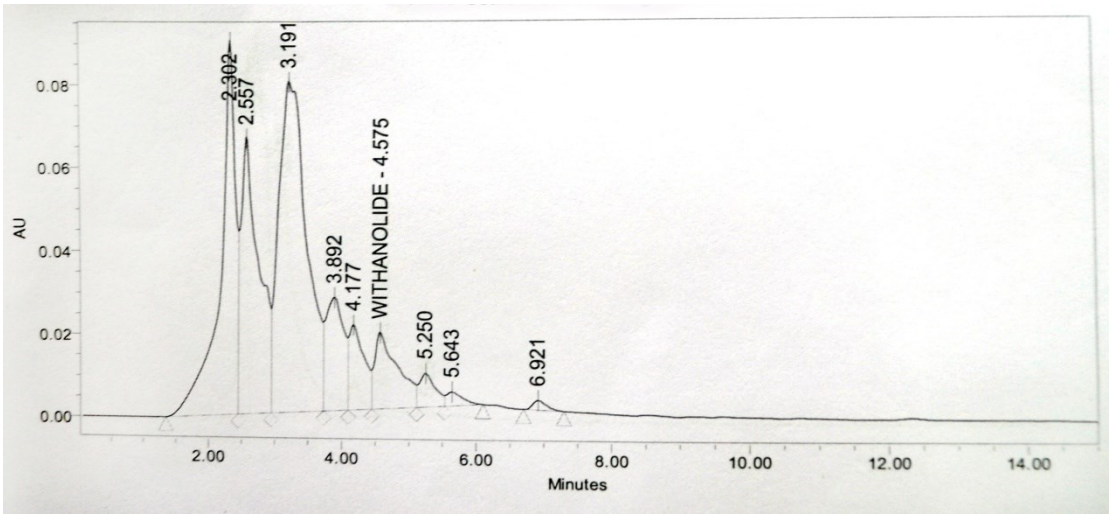


8b. Withanolide-A

Figure 8. (8a and 8b) Chromatograms of standards

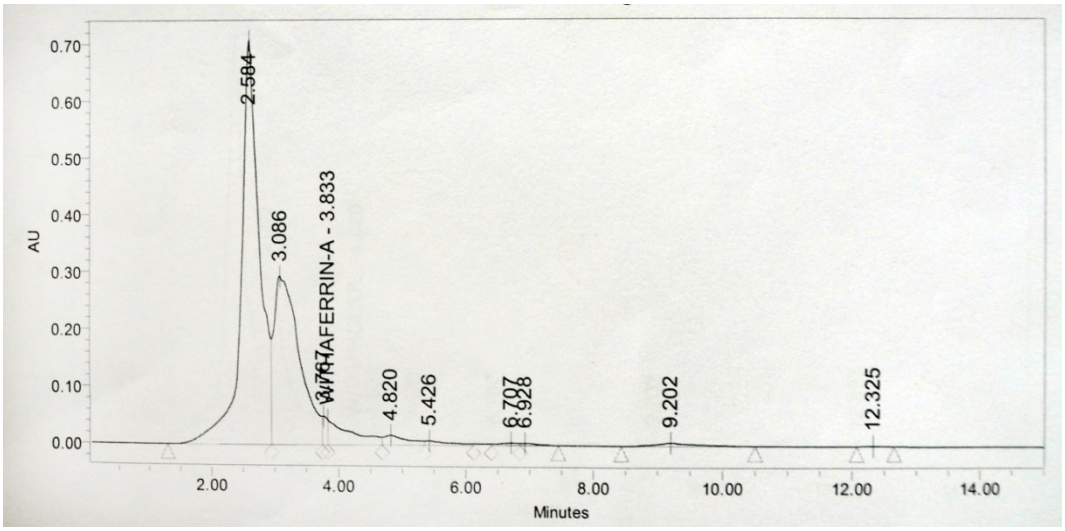


9a. Withaferin-A

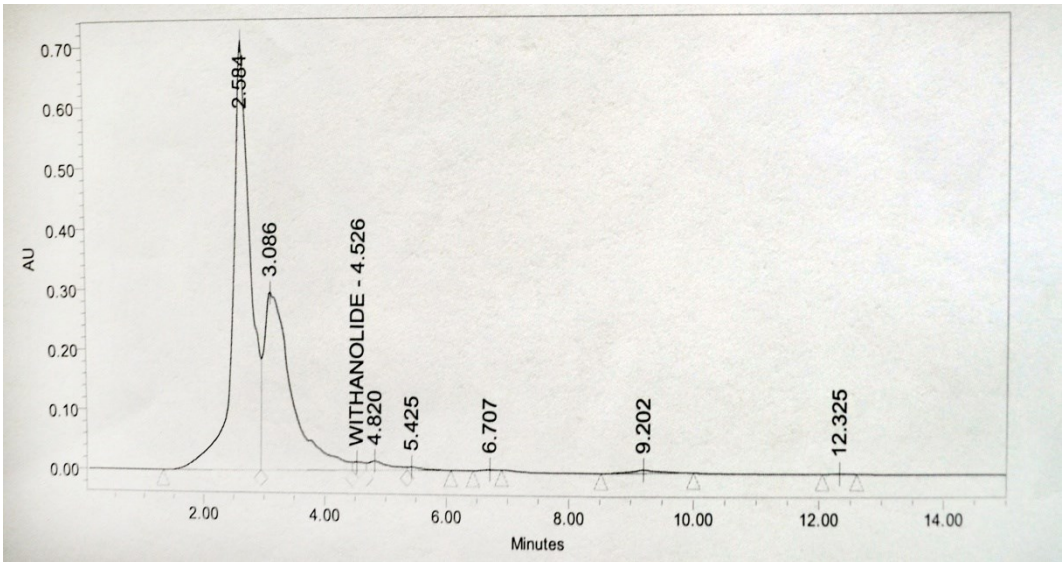


9b. Withanolide-A

Figure 9. (9a and 9b) HPLC analysis of withaferin-A and withanolide-A of hairy roots

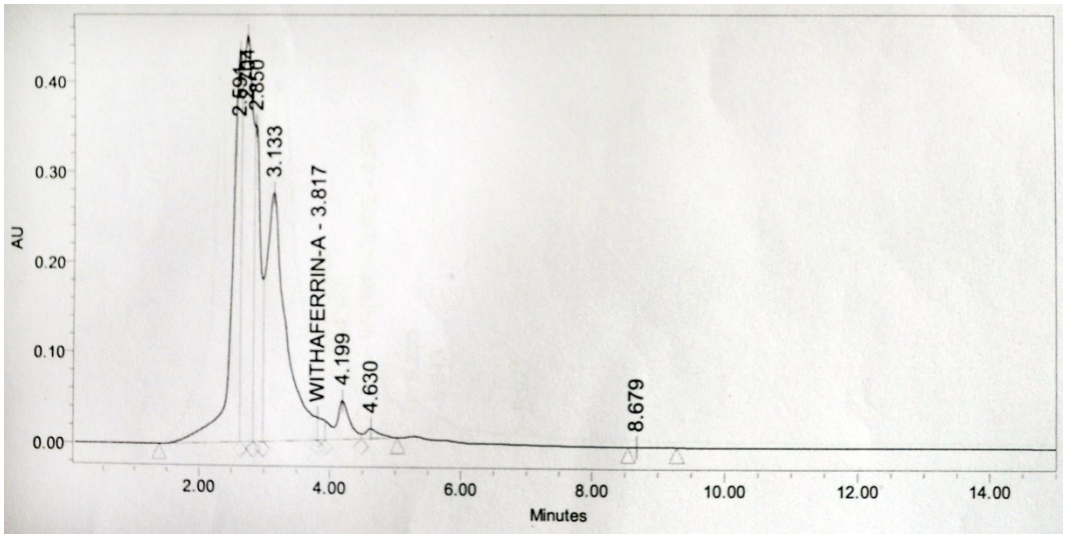


10a. Withaferin-A

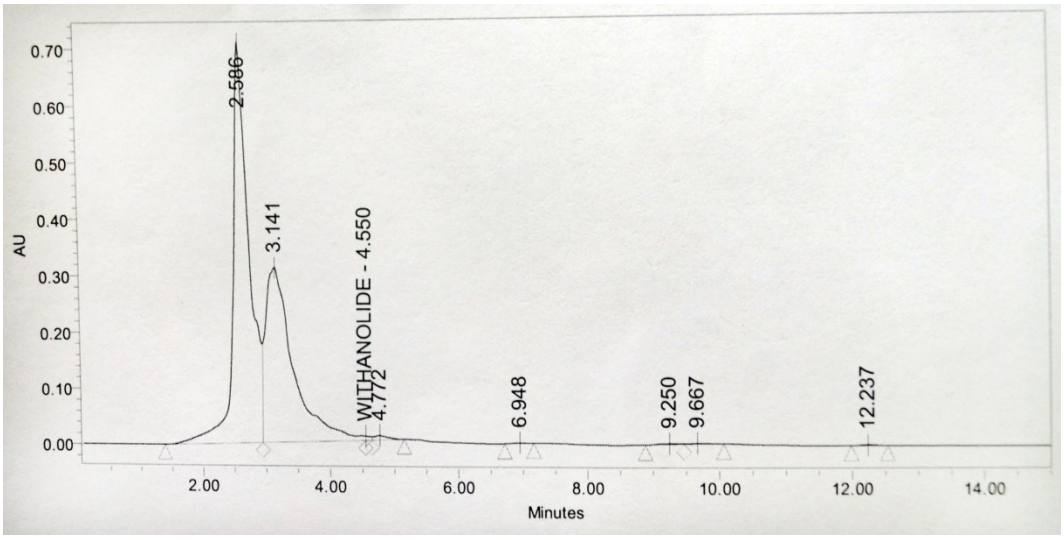


10b. Withanolide-A

Figure 10. (10a and 10b) HPLC analysis of withaferin-A and withanolide-A of normal root-shoot

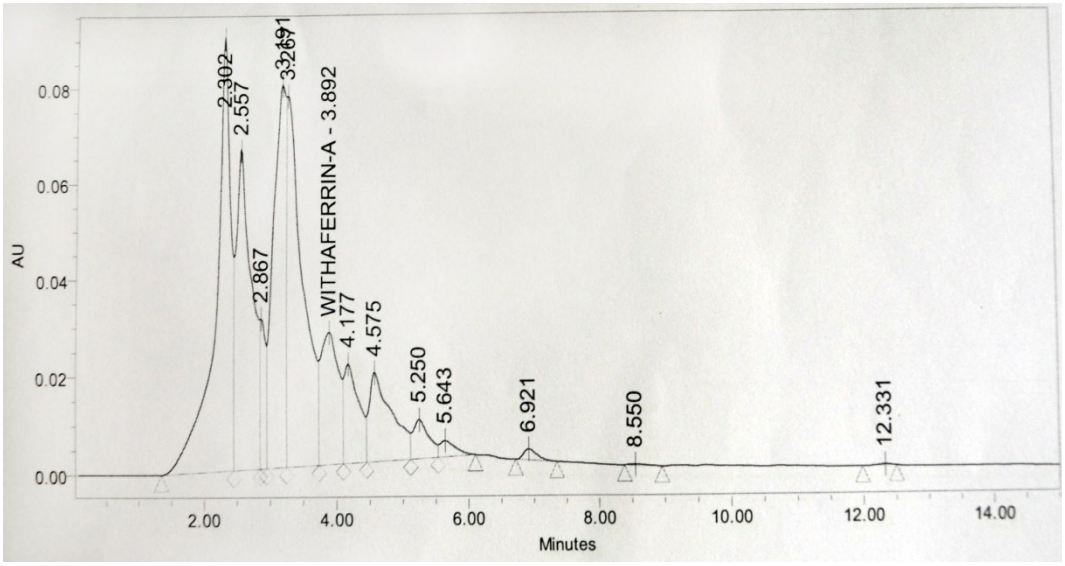


11a. Withaferin-A

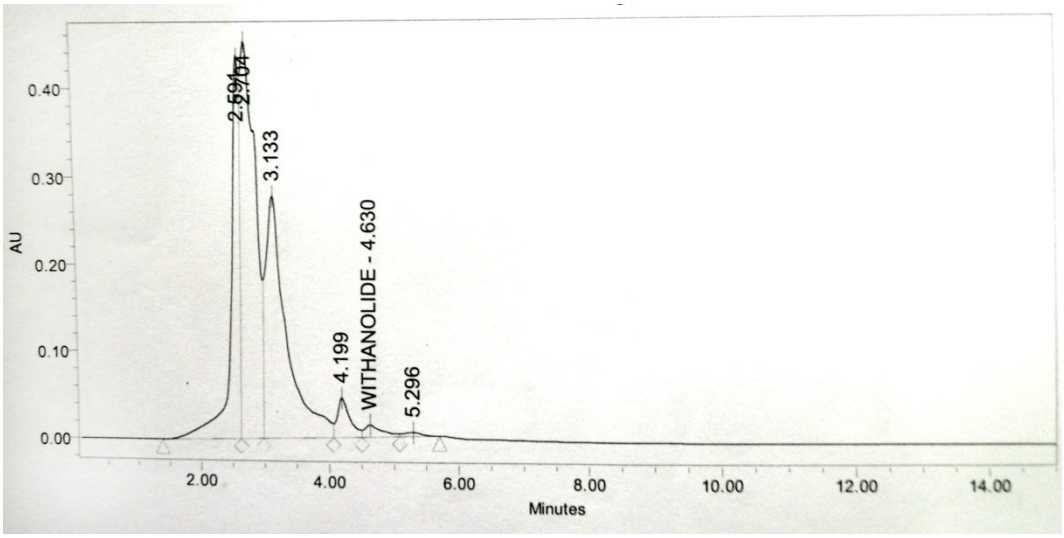


11b. Withanolide-A

Figure 11. (11a and 11b) HPLC analysis of withaferin-A and withanolide-A of normal root-leaf



12a. Withaferin-A



12b. Withanolide-A

Figure 12. (12a and 12b) HPLC analysis of withaferin-A and withanolide-A of field root

DISCUSSION

5. DISCUSSION

The main objective of this study was to establish hairy root cultures of *Withania somnifera* a highly demanded and commercially valuable medicinal plant for the production of pharmacologically important two withanolides, withaferin A and withanolide A. Development of a root culture is highly advantageous, as it is an alternative method for clonal propagation and germplasm conservation (Chaturvedi *et al.,* 1981). During the study, the virulent *Agrobacterium rhizogenes* strain, appropriate explant, proper procedure for elimination of bacteria after co-cultivation were determined which will be useful to the similar studies on other species of *Withania* and other members of Solanaceae family.

Different wild strains of *A. rhizogenes* - A4, LBA 9402, and MTCC 532 were evaluated for induction of transformed hairy roots in *W. somnifera* and different strains varied in their virulence for induction of hairy roots. *A. rhizogenes* strain A4 was the best for this species. The high virulence of this strain was analyzed in a wide range of host and found out that naturally occurring strains were superior to engineered strains in effecting plant transformation (Porter, 1991). The ability of an *A. rhizogenes* strain to transform a given plant species is strain dependent (Porter 1991; Maldonado–Mendoza *et al.* 1993; Zehra *et al.,* 1999). The type of explant is an important factor responsible for the rapid induction of hairy root in *Withania somnifera*. Cotyledons were better explants than hypocotyls and leaf in the present system. This observation is supported by the view that, the plant tissue infected is also a critical factor (Hu and Du, 2006).

The hairy roots produced were thin, soft and highly branched in the present system. This extensive branching, due to the presence of many meristems, accounted for higher growth rates of hairy roots in culture as this phenomenon was common for members of Solanaceae (Flores and Filner, 1985). The root growth was typically plagiotropic growth in plant growth regulator free medium and extensive lateral branching was observed. Plagiotropism of hairy roots was

characteristic as the result of *A. rhizogenes* mediated transformation (Gelvin, 1990).

The continuous rapid growth and high lateral root branching of the hairy roots on plant growth regulator-free basal medium clearly indicated the effect of integration and expression of *rol* genes into the genome of hairy roots which was confirmed through PCR amplification and Southern Blot hybridization. Metabolic status of hairy roots probably affected due to the altered hormone metabolism, transport properties which generally happen in transformation with the *rol* genes as discussed by Rhodes *et al*., 1997.

During the present study hairy roots growth in shake flask culture prolonged maximum up to 25 days which is contradictory to the observation made by Sivanandhan *et al.,* 2013, were growth prolonged to 30 days.

Exogenous addition of elicitors of biotic and abiotic origin in culture was considered to be one of the most promising strategies for the increased production of secondary metabolites (Radman *et al*., 2003). Production of secondary metabolites in plant tissue, cell cultures and adventitious root cultures could be enhanced through elicitation in many plants. Similarly, different elicitor application can be done in the present system developed as done in the plant by Sivanandhan *et al*., 2012. The hairy roots of *W. somnifera* established herein constitute an ideal system for the synthesis of withanolides in germ-free conditions and in the absence of expensive phytohormones in the culture system as advocated by Kumar *et al.*, 2005.

In the present study, an extensive investigation, was done on normal root culture besides to keep as a control for genetic transformation and production of withanolides. Production potential of compounds had been compared with leaf derived and shoot derived roots. Among the auxins tried IBA 0.2mg/l was optimum for the induction of normal root than IAA. The best auxin was determined as IBA for normal root induction in the investigation conducted by

Praveen and murthy, 2010. Whereas Wadegaonkar *et al.,* 2006 reported that combination of IAA and IBA was effective in the induction of adventitious roots from leaf explants of *W. somnifera*. Specific phytohormones supplemented exogenously can trigger the process of differentiation and induction pathways. An attempt was done to harvest roots by repeated culture of a single shoot. In one way, the method adopted is favourable as it reduced the labour and useful to those species that have scanty shoots under *in vitro* condition. On the other side, roots could not be harvested after 4^{th} subculture due to the callus formation at the cut end and roots ultimately became stunted. The exogenous supplementation of auxins in the medium for normal root cultures facilitated to accumulate more endogenous auxins which led to the callus formation at the root tips and hindered further elongation of the normal root cultures as suggested by Sudha *et al*. (2013). The roots derived from terminal shoots were found to be thick, and healthy compared to those derived from leaf explants. This favourable response might be due to the basipetal movement of auxin and its interaction with exogenous supplementation of auxin. The callus formation from the cut end of the terminal segment was pronounced and root formation was less. This callus formation was a hindering factor for the smooth initiation of roots.

For the qualitative and quantitative analysis thin layer chromatography and High performance liquid chromatography was carried out. In this study hairy roots, normal roots of shoot and leaf and field root samples were analysed and compared for withaferin-A and withanolide-A. The methanol extracts resulted in good resolution of spots. The spots were distinct and clear and maximum number of spots was obtained in the methanol to the other solvent fractions (Wasnik *et al.,* 2009). When the chromatograms of the various samples compared, it was found that a similar pattern of elution peaks occurred between 3.8- 4.2 mins. for withaferin-A and 4.2-4.8 mins. for standard withanolide-A. The area counts of the eluted peaks were also determined which helped to determine the concentration of the compounds in various samples.

Dalavayi *et al*. (2006) investigated the marker compound withaferin-A in different parts of plant (roots and leaves) and reported that the variation in retention time of peak may be due to the presence of other chemical constituents. Further analysis involving large scale purification of compound and structural studies of other major peaks identified should be done which will provide information on the biosynthetic pathway of the withanolides.

SUMMARY

6. SUMMARY

Today demand on medicinal plants is increasing tremendously and accordingly, the availability of many medicinal plants is decreasing. It is estimated that more than 90 % of the plant species used by the industry is collected from the wild and more than 70 % of the plant are from destructive harvesting and very few are in cultivation. It is a well-known fact that, many medicinal plant became rare and facing threat to the existence due to the indiscriminate and destructive collection. Especially, a number of root drug-yielding plants are facing threat and depletion in their habitats mainly due to indiscriminate collection and habitat destruction. Growing roots as isolated organ *in vitro* with faster growth potential and equal or better biosynthetic ability for active metabolites as that of field growing plants will have advantages.

Development of *in vitro* root culture will be an alternative technique for the large scale production of these secondary metabolites. The development of *Agrobacterium rhizogenes* mediated hairy root cultures offers a remarkable potential for commercial production of a number of low-volume and high value secondary metabolites. They are long-term aseptic root clones, genetically stable with growth rates comparable to those of the fastest-growing cell suspension cultures.

The roots of *Withania somnifera* contain a number of therapeutically applicable withanolides and the species has long gestation period (4 to 5 yrs) between planting and harvesting commercial cultivation. By considering these factors together with the increasing market demand of the drug, the present study focused to develop *Agrobacterium rhizogenes* mediated hairy root culture and assessed the growth kinetics and production of two biologically active metabolite, withanolide A and withaferin A. The major achievements and its applications are summarized below.

Establishment of aseptic seedlings was achieved in half strength MS medium supplemented with 0.5 mg/l GA_3 in Erlenmeyer flasks. By using GA_3, germination frequency could be enhanced marginally (93 %). Besides seedlings grown in GA_3 supplemented medium were long which could be useful to get more hypocotyl explants for hairy root induction. The maximum length (4.3 cm) of hypocotyls was obtained in half strength MS + 0.5 mg/l GA_3 medium. The aseptic seedlings had been utilized to harvest the appropriate explants cotyledons and hypocotyls for hairy root induction and cotyledonary nodes to initiate shoot cultures.

Aseptic shoot cultures were established from cotyledonary nodes of 6 weeks old seedlings in MS medium supplemented with 0.2 mg/l BAP and 0.05 mg/l IAA. Maximum shoot culture was obtained after 5-6 weeks. Terminal shoot cuttings and leaves, of these shoot cultures were used for normal and hairy root induction.

Best explants and auxin type and its concentration were optimized for normal root induction. Among the various explants tried, terminal shoot cuttings from shoot culture grown in half-strength MS solid medium with 0.2 mg/l IBA were more efficient (98 %) in inducing healthy normal roots than both terminal and basal leaf segments. Besides, roots induced on leaf explants were thin and produced less lateral roots. Half – strength MS medium favoured normal root biomass accumulation. The root biomass measured in terms of fw/dw increased during 20-25 days from both source, 3.4/0.3 g fw/dw from shoot derived root culture and leaf derived root culture, 2.7/0.22 g. For hairy root induction among different bacterial strains used A4 was found to be the virulent and cotyledon was the susceptible explant with highest frequency (82.3 %) of transformation. PCR analysis showed that the transformed root (hairy root) are able to express the *rol A* and *rol C* gene which is a clear indication of transformation of the explants of *W. somnifera* with Ri plasmid.

Total withanolide content in hairy root culture was more (22.3 mg/g dw of withaferin- A and 2.35 mg/g dw withanolide-A) than that of normal root culture (4.3 mg/g dw of withaferin- A and 1.65 mg/g dw). The best solvent systems for the qualitative detection of withanolides A and withaferin A by TLC was hexane:ethyl acetate:methanol in the ratio 7.5:2:0.5. The presence of withanolide A and withaferin A were confirmed when the Rf value coincided with the compound in both sample in the Co-TLC. The Rf-0.36 confirmed the presence of withaferin A and Rf-0.59 confirmed the presence of withanolide A.

Time course study indicated that total withanolide content was highest at the end of 3rd week of culture. Quantitative analysis of withaferin-A and withanolides-A in normal root culture of shoot and leaf derived root culture by HPLC showed that the concentration of both compounds analyzed were more in the former source. The analysis showed peak production of compounds, 0.086 mg/ml of withaferin-A and 1.65 mg/ml withanaloide-A, in shoot derived normal root and 0.055 mg/ml withaferin-A and 0.35 mg/ml withanolide-A in leaf derived normal root, during 3rd week of culture in both normal and hairy root culture (0.446 mg/ml of withaferin-A and 0.077 mg/ml of withanolide-A).

Since hairy root culture are biosynthetically and phytochmically close to the roots of the plant from which they are derived with respect to their secondary metabolites, the hairy roots of *W. somnifera* appear to be a potential source of total withanolides and biologically active compounds, withaferin A and withanolide A.

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7. REFERENCE

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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

APPENDIX II

Yeast Mannitol Broth

Components	g/l
$MgSO_4.7H_2O$	2.0
K_2HPO_4	0.5
NaCl	0.1
Mannitol	10.0
Yeast extract	0.4
Agar	7
pH	7.0

APPENDIX III

Reagents for Plasmid isolation using Alkaline Lysis

1. **Solution I (Lysis buffer I):**

50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0. Store at 0 °C

a. 10 ml 500 mM Glucose

b. 2 ml 500 mM EDTA pH 8.0

c. 2.5 ml 1M Tris pH 8.0

d. 85.5 ml H_2O

e. Autoclave and store at 4 °C

2. **Solution II (Lysis buffer II):**

Freshly prepared 0.2 N NaOH, 1 % SDS. Store at room temperature (RT)

3. **Isopropanol:**

Stored at -20 °C

4. **Solution III (Lysis buffer III):**

3 M KOAc, pH 6.0

a. 60 ml 5 M potassium acetate (49.07 g potassium acetate in 100 ml H_2O)

b. 11.5 ml glacial acetate

c. 28.5 ml H_2O

APPENDIX IV

Reagents for genomic DNA isolation

1. **Extraction buffer (2 X CTAB buffer)**

   2 % CTAB (Cetyl trimethyl ammonium bromide) w/v

   100 mM Tris (pH 8.0),

   20 mM EDTA (pH 8.0),

   1.4 M NaCl

   1.2 % PVP (Polyvinylpyrrolidone)

2. **CTAB solution (5 %)**

   5 % CTAB (w/v)

   0.7 M NaCl

3. CTAB Precipitation buffer

   1 % CTAB (w/v)

   50 mM Tris (pH 8.0)

   10 mM EDTA (pH 8.0)

4. High salt TE Buffer

   10 mM Tris (w/v)

   1 M NaCl (pH 8.0)

   0.1 mM EDTA (pH 8.0)

5. 10X TBE Buffer

   Tris Base

   Boric acid
   0.5 mM EDTA

APPENDIX V

Solutions used in PCR

1 Phosphate buffered saline (PBS): 13 Mm NaCl, 2 mM KC1, 10 mM Na_2HP0_4, 1 mM KH_2PO_4. The pH of the solution was adjusted to 7.4.

2 1% Triton X-100

APPENDIX VI

Solutions used for agarose gel electrophoresis

1. **50 X TAE** **(per liter)**

Tris base	242 0 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8 0)	100 ml

   Sterilized by autoclaving,

2. **DNA loading dye (6 X)** **(%)**

Xylene cyanol	0.25
Bromophenol blue	0.25
Glycerol	30.0

   Stored at 4 °C

3. **Ethidium Bromide (Stock)**

   10 mg/ml in distilled water. Used at a working concentration of 0.5 µg/ml. Store at 4 °C.

APPENDIX VII

Spraying Reagent Preparation

Anisaldehyde reagent

Freshly prepared 0.5 ml anisaldehyde in 10 ml glacial acetic acid, 85 ml methanol and 5 ml concentrated sulfuric acid and stored in amber bottles.

Liebermann- Burchard reagent

5 ml acetic anhydride and 5 ml concentrated sulphuric acid are added carefully to 50 ml absolute ethanol, under cool condition. The reagent must be freshly prepared before spraying.

ABSTRACT

Agrobacterium rhizogenes MEDIATED TRANSFORMATION OF ASHWAGANDHA (*Withania somnifera* (L.) DUNAL)

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Abstract of the thesis

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ABSTRACT

The present study describes aseptic seed germination, establishment of shoot and normal root culture, *Agrobacterium rhizogenes* mediated transformation for hairy root culture, confirmation of transformation by PCR method and detection of *rol* A and *rol* B genes, phytochemcial assays of root cultures and roots derived from mature field grown plants for qualitative and quantitative analysis of total withanolide content, withaferin A and withanolide A of *Withania somnifera* (L.) Dunal. Half- strength MS medium with 0.5 mg/l GA_3 were best to achieve maximum (93 %) seedlings rapidly. Multiple shoot culture was obtained by culturing the cotyledonary nodal explants in MS solid medium with 0.2 mg/l BAP and 0.05 mg/l IAA. Terminal shoot cuttings from shoot culture grown onto half- strength MS solid medium with 0.2 mg/l IBA were more efficient (98 % with 4-5 roots) in inducing healthy normal roots than both terminal and basal leaf segments. Hairy root culture was initiated by infecting cotyledons, hypocotyls leaf and internode explants with different wild type strains, A4, LBA 9402, and MTCC 532. The cotyledons infected with *A. rhizogenes* strain A4 induced hairy roots directly from the infected sites at a high frequency (90 %, 11.5 ± 0.2) than hypocotyl explants (12 %, 0.5 ± 0.05) which was best. The molecular evidence of *rol* A and *rol* C gene integration was confirmed by PCR amplification. Growth of the hairy root was measured by determining fresh weight and dry weight during time-course study in shake flask cultures. The maximum root biomass of hairy root culture (3.4 g) was noticed at 23rd day was more compared to normal root culture (2.6 g). Total withanolide content was more in transformed root culture (0.198 g) than normal root culture (0.103 g). The TLC analysis showed the compounds with same Rf values (0.36) and (0.59) as that of authentic samples of withaferin A and withanolide A respectively and it was confirmed again by Co-TLC. The HPLC analysis showed a more concentration of withaferin-A (22.3 mg/g dw) and withanolide A (2.35 mg/g dw) in hairy root culture than normal root culture (4.3 mg/g dw of withaferin-A and 1.65 mg/g dw of withanaloide-A).