Comparative Study On Expression Of Important Pathway Genes Among Different Phenophases Of The *In Vivo* And Cultured Root Tissues Of “Indian Ginseng”, *Withania Somnifera* In Concert With The Quantification Of Its Major Withanolide Accumulation.

Shobana Nancy, C

(12PBT013)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641 043

In Partial Fulfilment of the Requirement for the Degree of Master of Science in Biotechnology

March, 2014
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Signature of Head of the Department

Signature of Supervisor
ACKNOWLEDGEMENT
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“My salvation and honour depend on God”

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INTRODUCTION
1.0 INTRODUCTION

Primitive people have used plants to cure a variety of human ailments. Traditional medicines derived from medicinal plants are used by about 60% of the world’s population. A number of medicinal plants, traditionally used for over 1000 years named rasayana are present in herbal preparations of Indian traditional health care systems (Modak et al., 2007). Plants have an almost maximum ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. Most of these are secondary metabolites, of which 12,000 plant-derived agents have been isolated in the recent past (Samy and Gopalakrishnakone, 2008).

*Withania somnifera* (Solanaceae), commonly known, as ‘Ashwagandha’ is a subtropical undershrub used as an Ayurvedic herb. In *Withania* genus there are three species found in India namely, *W. somnifera, W. coagulans* and *W. obtusifolia*. It is distributed in tropical and subtropical region like Rajasthan, Madhya Pradesh, Punjab, Himachal Pradesh, Jammu and Kashmir, Western Himalayas and Tamil Nadu, in Tamil Nadu it is distributed in Trichy, Perambalur, Thanjavur, Ariyalur, Lalgudi and Kadalur (Kumar et al., 2011). *W. somnifera* is preferred for adjuvant use in the management of various psychosomatic conditions and it improves tissue vitality, physical and mental endurance, and neuromuscular strength. It is used as a sedative in the treatment of insanity, experimental data prominently label this plant as antistress, anticonvulsant, and tranquilizer (Mamidi and Thakar, 2011).

Several bio-actives, including withanolide, glycosides (also known as sitoindosides) and withanolide aglycones, are considered to be responsible for the medicinal properties of *W. somnifera* (Auddy et al., 2008). The major chemical components of *Withania* species are known to be the withanolides, which represent steroidal lactones characterized by a C28 basic skeleton with a 9 C atoms side chain and a 6-membered lactone ring (Furmanowa et al., 2001).

Withaferin A, withanolide A, and withanone are the major withanolides present in *W. somnifera* (Sabir et al., 2012). Various chemotypes of *Withania* accumulating more than 40 withanolides and several sitoindosides in aerial parts, roots and berries have been reported. Studies suggest that precursor molecules for withanolide biosynthesis are
isoprenoids. These isoprenoids are synthesized via classical cytosolic mevalonate (MVA) pathway and plastid localized 2-Cmethyl- D-erythritol-4-phosphate (MEP) pathway leading to biosynthesis of 24-methylene cholesterol (C30 terpenoid) (Gupta et al., 2013).

From biochemical, molecular and pathway engineering perspectives, it is imperative to characterize key pathway genes and to understand their regulatory role in the synthesis of bioactive metabolites. Major enzymes involved in biosynthetic pathway are Farnesyl diphosphate synthase, Arginine decarboxylase, chitinase, squalene epoxidase, Cycloartenol synthase, Glucosyl transferase (Gupta et al., 2010). Though extensive studies have been carried out on the pharmacological importance and metabolic profiling of W. somnifera constituents very little information is available on biosynthetic pathways of these bioactive steroidal molecules (Gupta et al., 2011).

Polymerase chain reaction (PCR)-based assays can target either DNA (the genome) or RNA (the transcriptome). Targeting the genome generates robust data that are informative and, most importantly, generally applicable. Reverse-transcription polymerase chain reaction remains the most sensitive technique for the detection of often-rare mRNA targets, and its application in a realtime setting has become the most popular method of quantitating steady-state mRNA levels (Bustin and Nolan, 2004). The secondary metabolites include various kinds of terpenes, such as mono-, sesqui-, di- and triterpenes. Overproduction of phytosterols and triterpenes by metabolic engineering might be an attractive strategy to produce a better quality of pharmacologically active medicinal plants (Lee et al., 2004).

Withania somnifera has been extensively studied in terms of its chemical profile which encompasses extraction, isolation and identification of various withanolides from different parts of the plant. These primarily abound in leaves and roots of the shrub which are the main tissues approved for remedial use in the traditional systems of medicine (Dhar et al., 2013). Marker compound means chemical constituents within a medicinal plant that can be used to verify its potency or identity. Phytochemical evaluation is one of the tools for the quality assessment, which includes preliminary phytochemical screening, chemo profiling and marker compound analysis using modern analytical techniques (Kshirsagar et al., 2008). Active constituents can be analyzed using several techniques
such as colorimetric, titrimetric, gravimetric, spectrometric and chromatographic techniques.

Recent methods are analysis by HPLC and HPTLC. High Performance Thin Layer Chromatography is one of the modern sophisticated technique that can be used for wide diverse applications. It is a simple and powerful tool for high-resolution chromatography and trace quantitative analysis is made possible. It is most widely used for quick and easy determination of quality, authenticity and purity of the crude drugs and market formulations (Mamatha, 2011). Moreover, different combination of solvent system of varying polarity has been used for the optimization of solvent system in high performance thin layer chromatography (HPTLC) methods (Patel et al., 2012).

The present study is initiated with the following objectives:

- To study and compare the expression pattern of key pathway genes involved in withanolide biosynthesis in *in vivo* and *in vitro* roots of *Withania somnifera*.
- To quantify and develop HPTLC finger prints for *in vivo* roots in comparison with the *in vitro* roots.
REVIEW OF LITERATURE
2.0 REVIEW OF LITERATURE

Herbal medicines being natural are preferred over synthetic remedies by a major section of the world. Medicinal plants are a major source of compounds of therapeutic value, and contain different phytochemical compounds resulting in numerous pharmacological activities. Since times immemorial, ancient Indians, Egyptians, Chinese and others have employed a variety of plants and plant products for curing all kinds of ailments. Approximately, 25000 plant based formulations are available in the indigenous medical texts (Gupta et al., 2004). The World Health Organization (WHO) has listed 21,000 plants, which are used for medicinal purposes around the world. Among these 2500 species are in India, out of which 150 species are used commercially on a fairly large scale. India is the largest producer of medicinal herbs and is called as botanical garden of the world (Seth et al., 2004). W. somnifera is increasingly becoming a popular adaptogenic herb and is available throughout the Western world as a dietary supplement. The herb has been identified by National Medicinal Plant Board of India as one of the thirty-two selected priority medicinal plants, which are in great demand in the domestic and international markets (Prajapati et al., 2003)

This chapter deals with the review of earlier work carried out in various aspects like growth, phytochemistry, pharmacological properties, gene expression studies and quantification of major withanolides related to Withania somnifera.

2.1 Withania somnifera: Indian Ginseng

2.1.1 Morphology

2.1.2 Photochemistry

2.1.3 Therapeutic uses of Withania somnifera roots

2.2 In vitro propagation of Withania Somnifera

2.3 Withanolide Biosynthesis Pathway

2.4 Key regulatory genes involved in withanolide biosynthesis

2.5 Quantification using High Performance Thin Layer Chromatography
2.1 *Withania somnifera*: Indian Ginseng

Solanaceae is one of the largest families in the plant kingdom and comprises about 85 genera and more than 3000 species (Subbaraju *et al.*, 2006). *Withania somnifera* which belongs to the family Solanaceae is commonly known as “Indian Winter cherry” or “Indian Ginseng”. It is used in the treatment of variety of physiological disorders and constitutes an important ingredient of more than 100 herbal formulations in Ayurveda, Siddha and Unani systems of medicine (Dhar *et al.*, 2013). It is one of the most important herb of Ayurveda (the traditional system of medicine in India) used for millennia as a Rasayana for its wide ranging health benefits (Singh *et al.*, 2011).

Numerous studies indicated that ashwagandha possesses antioxidant, antitumor, antistress, anti-inflammatory, immunomodulatory, hematopoetic, anti-ageing, anxiolytic, antidepresive rejuvenating properties and also influences various neurotransmitter receptors in the central nervous system (Sharma *et al.*, 2011). Pharmacological investigation suggests its safe and better utility than P. ginseng (Korean drug Ginseng) notably in view of “Ginseng abuse syndrome” of the latter (Grandhi *et al.*, 1994). Research on animal cell cultures has shown that the herb decreases the levels of the nuclear factor kappaB, suppresses the intercellular tumor necrosis factor, and potentiates apoptotic signalling in cancerous cell lines (Singh *et al.*, 2010).

2.1.1 Morphology

*Withania* species are perennial shrub with branched and non branched tap root system. The stem is herbaceous, erect and hairy; leaves are alternate, simple and the fruit is a berry. The use of morphological and leaf-epidermal features has been found to be greater interest in taxonomy. The use of leaf-epidermal features (epidermal cell, stomata and trichome) in systematics has become too distinctive and have been used as a great taxonomic tool of family, genus and species (Kumar *et al.*, 2010).
**Taxonomical Classification**

Kingdom : Plantae, Plants  
Subkingdom : Tracheobionta, Vascular plant  
Super division: Spermatophyta, Seeds plants  
Division : Angiosperma  
Class : Dicotyledons  
Order : Tubiflorae  
Family : Solanaceae  
Genus : *Withania*  
Species : *somnifera* Dunal

(Gupta and Rana, 2007)

**2.1.2 Phytochemistry**

Chemical analysis of Ashwagandha show its main constituents to be alkaloids and steroidal lactones (Singh *et al.*, 2010). The plant comprises withasomnine, somniferin and steroidal lactones like withanolides, withaferin, withanosides etc. (Kulkarni *et al.*, 2000). *In vitro* and *in vivo* molecular pharmacological investigations have elucidated association
of these activities of the herb with its specific secondary metabolites known as withanolides a class of phytosteroids named after *W. somnifera* (Sangwan *et al.*, 2008).

Withaferin A, the first member of this group, was isolated from *Withania somnifera*. Several properties of withaferin A have been reported: antiangiogenesis through NF-κB inhibition, cytoskeletal architecture alteration by covalently binding annexin II and apoptosis induction through the protein kinase C pathway in leishmanial cells (AbouZid *et al.*, 2010). Withanolide A is one of the most promising phytopharmaceuticals because of its recently reported impressive pharmacological properties: (1) induction of neuriteregeneration and synaptic reconstruction is important in dealingneurological disorders particularly Alzheimer’s and Parkinson’s diseases and (2) strong inhibition of carcinogenesis (Sangwan *et al.*, 2008). The withanolides are a group of naturally occurring C28- steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring (Mirjalili *et al.*, 2009).

**Fig 2.2 Major Withanolides of Withania somnifera**

(Sangwan *et al.*, 2008)
2.1.3 Therapeutic uses of Withania somnifera root:

The root of this plant is not only a major source of several alkaloids viz. tropine, pseudotropine & somniferine but also important steroids like withaferin A and withanolides (Pati et al., 2008). The root of Ashwagandha is regarded as tonic, aphrodisiac, narcotic, diuretic, anthelmintic, astringent, thermogenic and stimulant. The root smells like horse (“ashwa”), that is why it is called Ashwagandha (on consuming it gives the power of a horse) (Singh et al., 2011).

In view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention. Ashwagandha roots are a constituent of over 200 formulations in Ayruvedha, Siddha and Unani medicine, which are used in the treatment of various physiological disorders (Singh et al., 1998). The decoction of the root boiled with milk and ghee is recommended for curing sterility in women (Mirjalili et al., 2009). The root in combination with other drugs is prescribed for snake venom as well as in scorpion sting. It also helps in leucorrhoea, boils, pimples, flatulent colic, worms and piles (Misra, 2004). Root of Withania somnifera used for the treatment of asthma, bronchitis, edema, leucoderma, anorexia, consumption, asthenia, anemia, exhaustion, aging, insomnia, ADD/ADHD, neurasthenia, infertility, impotence, repeated miscarriage, paralysis, memory loss, multiple sclerosis, immune- dysfunction, carcinoma, rheumatism, arthritis, lumbag (Sharma et al., 2011).

The major active compounds of the roots are reported to be withanolides, glycosides and many different alkaloids. To date, up to 19 withanolide derivatives have been isolated from Withania roots (Harikrishnan et al., 2008). 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 (Senthil et al., 2011).
2.2 In vitro Propagation of *Withania somnifera*

Higher plants produce a great variety of secondary metabolites, which have been used as pharmaceuticals, nutraceuticals and food additives. Recent advances in the techniques and applications of plant cell and organ cultures have made it possible to produce these valuable secondary metabolites in large scale. Moreover, plant cell culture systems provide various ways to boost yields of desired metabolites, conveniently and cost effectively (Rao and Ravishankar, 2002). Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations. There has been significant progress in the use of tissue culture and genetic transformation to alter pathways for the biosynthesis of target metabolites. It also permits the production of pathogen-free material (Debnath *et al*., 2006).

This important medicinal plant has now become rare since it is not available in wild condition, the observation has been corroborated by earlier reports (Sivanesan 2007). Also, it is worth mentioning that withanolide A, a commercially important chemical, constitute a very small part of the plants *in vivo*. However, there are reports regarding scope of bio-generation of this an important chemical *in vitro* (Sangwan *et al*., 2007). Protocols for *in vitro* plant production via direct and indirect morphogenesis have many potential applications to any species particularly that of tremendous economic use and medicinal importance such as *Withania somnifera*. There are reports on *in vitro* culture of *Withania somnifera* using different explants (Chakraborty *et al*., 2013). There is also a report on the production of withanolide A in cell-suspension cultures of *W. somnifera* (Nagella and Murthy 2010).

Complementing biotechnology, attempts have been made in the recent years to induce Ri and Ti based genetic transformations in *W. somnifera* (Sharada *et al*., 2008). Banerjee *et al* (1994) reported that withaferin A production at different stages of growth in “hairy root” cultures obtained following infection with *Agrobacterium rhizogenes*. Hairy root cultures offer many advantages among which we can highlight the high and continuous yields of a wide range of metabolites and a high growth potential (Bensaddek *et al*., 2008).
The treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures (Hussain et al., 2012). It has been reported that acetyl salicylic acid stimulated accumulation of both withaferin A and withanolide A at higher concentration in hairy root cultures and also increasing concentrations of triadimefon, a fungicide increased withaferin A in dose dependent manner (Doma et al., 2012). Adventitious roots induced by *in vitro* methods showed high rate of proliferation and active secondary metabolism (Murthy et al., 2008). The bioreactor culture system is more advance than the traditional tissue culture system because the culture conditions in the bioreactor can be optimized by on-line manipulation of temperature, pH, concentration of oxygen, carbon dioxide and nutrients in the medium (Sivakumar, 2006). Airlift bioreactors (ALR) using low density beads with immobilized cells or enzymes are currently under research for a variety of applications in bioprocess engineering, and they have several advantages over alternate bioreactor designs. Airlift bioreactors combine high loading of solid particles and good mass transfer which are inherent for three-phase fluidized beds (Sajc et al., 2000).

Since the roots contain a number of therapeutically applicable withanolides, mass cultivation of roots *in vitro* will be an effective technique for the large scale production of these secondary metabolites (Wasnik et al., 2009). Agroclimatic factors are reported to play significant role in synthesis and accumulation of secondary metabolites and therefore it is rather difficult to optimize the maximum amount of chemical components under field grown conditions (Ananya and Kumar 2011).

### 2.3 Withanolide Biosynthesis Pathway

The extensive utility of *W. somnifera* in traditional system of medicine and recent interest generated in the bioactive properties of this plant has prompted many workers to elucidate the withanolide biosynthetic pathway (Razdan et al., 2012). Though various studies have been carried out to establish metabolic profiles and pharmacological activities of *Withania*, very limited information is available on biosynthetic pathways of withanolides, from this important medicinal plant. Recently, biochemical and molecular
studies have been initiated which led to characterize few genes/enzymes from this important plant (Gupta et al., 2013).

Putatively, withanolides (C-30) are synthesized via both mevalonate (MVA) and non mevalonate (DOXP) pathways through cyclization of 2,3-oxidosqualene to cycloartenol, wherein 24-methylene cholesterol is the first branching point towards the biosynthesis of various withanosteroids. Production of withanolides includes a series of desaturation, hydroxylation, epoxidations, cyclization, chain elongation, and glycosylation steps (Dhar et al., 2013).

Major enzymes involved in biosynthetic pathway are Farnesyl diphosphate synthase, Arginine decarboxylase, chitinase, squalene epoxidase, Cycloartenol synthase, Glucosyl transferase (Gupta et al., 2010). Recent evidence indicates that isoprenoid biosynthesis may take place differentially in different chemotypes (Chaurasiya et al., 2009) leading to biosynthesis of different kinds of withanolides and their content. (Sabir et al., 2012) have compared the production of major withanolides (withaferin A, withanolide A, and withanone) in in vitro tissues such as multiple shoots, rhizogenic roots, callus tissues, as well as field grown shoot and root tissues with the expression level of the pathway genes. Currently, we know very little about how metabolic pathways arise. A better understanding of the origin and nature of the genes and enzymes that comprise natural product pathways will enable us to probe the mechanisms underpinning the generation of metabolic diversity (Qi et al., 2006).
Fig 2.3 Withanolide biosynthesis pathway

MVA Pathway (Cytosol)
- Acetyl COA
  - HMG COA
    - HMG-CoA Reductase (HMGR)
    - Mevalonate
  - IPP
  - DMAPP
  - GPP
    - IPPS
    - Farnesyl Pyrophosphate
      - Squalene
        - 2-3 Epoxy squalene
          - Cycloartenol
            - SMT 1
            - 24-Methylene cycloartenol
              - Δ8,4 Sterol
                - WITHANOSIDE

MEP Pathway (Plastid)
- Pyruvate
  - GA-3P
    - DOXP
      - DXR
      - MEP
      - DMAPP
      - IPP
      - SESQUITERPENES

WITHANOLIDES
2.4 Key regulatory genes involved in withanolide biosynthesis

3-Hydroxy-3-methylglutary coenzyme A reductase (HMGR)

In plants, MVA is the general precursor of various identified isoprenoids, such as sterols, plant growth regulators, and terpenoids. 3-Hydroxy-3-methylglutary coenzyme A reductase (HMGR), located in the endoplasmic reticulum, catalyzes the conversion of HMG-CoA to MVA (Wu et al., 2012). The plant 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which catalyzes the conversion of 3-hydroxy- methylglutaryl-CoA (HMG-CoA) to mevalonate, has been considered as the first step in the MVA pathway in plants (Jiang et al., 2006). Although HMGR is encoded by a single gene in higher animals, archaea, and eubacteria, it is usually encoded by multiple genes in plants. This implies that plant HMGR genes have arisen by gene duplication and subsequent sequence divergence (Ma et al., 2011). Accumulating research results suggested that plant HMGRs are encoded by a gene family members and regulated by light, growth regulators, wounding and treatment with pathogen or elicitors (Diarra et al., 2013).

Farnesyl diphosphate synthase (FPPS)

Farnesyl diphosphate (FPP), which is synthesized by catalytic action of the enzyme farnesyl diphosphate synthase (FPPS), serves as a substrate for first committed reaction of several branched pathways leading to synthesis of compounds that are essential for plant growth and development as well as of pharmaceutical interest (Gupta et al., 2011). FPPS catalyses the sequential head-to-tail condensation of two molecules of IPP with one molecule of DMAPP to form the sesquiterpenoid precursor, FPP (Ma et al., 2011). It has been shown that overexpression of ginseng farnesyl diphosphate synthase in Centella asiatica hairy roots enhanced phytosterol and triterpene biosynthesis (Kim et al., 2010). FPPS is situated at a central branch point of the isoprenoid biosynthetic pathway and consequently has a key role in both eukaryotic and prokaryotic metabolism (Homann et al., 1996).
Squalene epoxidase (SE)

This enzyme is a noncytochrome-P450 type monooxygenase, takes part in a process to form a hydroxyl group that is characteristic of sterols and triterpenols, and functions as a rate-limiting step in the sterol biosynthesis in upstream of the P450-type oxygenases in the pathway (Uchida et al., 2007). Squalene epoxidase (SE; EC 1.14.99.7) is a mono-oxygenase that catalyzes the conversion of squalene to 2,3-oxidosqualene. The first gene encoding SE was isolated from a terbinafine-resistant *Saccharomyces cerevisiae* mutant. Squalene epoxidases are essential for the synthesis of cholesterol in mammals and ergosterol in fungi and, thus, comprise useful and medically important targets that can be used to lower cholesterol levels or to inhibit the growth of pathogenic fungi (Ruckenstuhl et al., 2006). (Gupta et al., 2013) made the first attempt of cloning and characterization of this gene from *W. somnifera*.

In recent years the SE-encoding genes of many other organisms were isolated and characterized, including those of pathogenic fungi, plants, mice, rats, and humans. Inhibitors used against SQE have been reported to inhibit the sterol formation in fungi, and also silencing of one of the three isoforms of SQE in *Panax ginseng* have led to decreased phytosterol formation indicating that SQE plays pivotal role in the regulation of phytosterol biosynthesis (Razdan et al., 2012).

Squalene synthase (SS)

Squalene synthase has been reported to play an important regulatory role in the phytosterol biosynthetic pathway. It was first reported by (Lee et al., 2004) that SS gene is a regulatory gene for triterpene biosynthesis and that the overproduction of squalene in transgenic plants stimulates the transcription of downstream genes involved in both phytosterol and triterpene biosynthesis. Moreover, overexpressing SQS in *Bupleurum falcatum* resulted in enhanced production of both phytosterol, saikosaponins and increased the mRNA accumulation of downstream genes including squalene epoxidase and cycloartenol synthase. Plant squalene synthase genes (SQSs) have been characterized
from Nicotiana tabacum, Glycyrrhiza glabra, Panax ginseng, and also some other plants (Bhat et al., 2012).

**Glucosyl transferase (GT)**

In higher plants, secondary metabolites are often converted to their glycoconjugates, which are then accumulated and compartmentalized in vacuoles. These glucosylation reactions are catalyzed by glucosyltransferases (GTases) (Taguchi et al., 2001). In general, glycosylation is the last step in the biosynthesis of secondary metabolites (Sun et al., 2013). A vast variety of glycosyltransferase genes have been identified thus far, which are currently classified on the basis of their phylogenetics into 70 families. So far, glycosyltransferases involved in plant secondary metabolism (i.e. plant secondary product glycosyltransferases (PSPGs) have all been grouped into glucosyltransferase family (Noguchi et al., 2007).

Transcriptional analysis using microarray comprising a large set of genes, including 109 secondary product glycosyltransferases suggest the role of glycosylation in defense response of Arabidopsis thaliana (Madina et al., 2007). GTs that use UDP-activated sugars as donors and various types of small molecules as acceptors are called UDP-glycosyltransferases (UGTs) and represent family 1GTs. Such UGTs are present commonly in plants and animals, but have been reported in a few cases only in microorganisms. In higher plants, UGT catalyzed glycosylation constitutes a prominent terminal modification in the biosynthesis of secondary metabolites and generates diverse natural glycosides (Sharma et al., 2007).

**Cycloartenol synthase (CAS)**

It has been well documented that CAS plays essential roles in the plant cell viability, and in the regulation of triterpenoid biosynthesis (Diarra et al., 2013). The differences in the biosynthesis of sterols between higher plants and yeast/mammals are generally accepted to begin at the cyclization step of 2,3-oxidosqualene, a common precursor. Phytosterols, such as campesterol and sitosterol, are biosynthesized via
cycloartenol and catalyzed by cycloartenol synthase (CAS) in higher plants (Ohyama et al., 2008).

**Isopentanyl Diphosphate (IPP)**

Isopentenyl diphosphate (IPP) is the central intermediate in the biosynthesis of isoprenoids, the most ancient and diverse class of natural products. Two distinct routes of IPP biosynthesis occur in nature: the mevalonate pathway and the recently discovered deoxyxylulose 5-phosphate (DXP) pathway (Lange et al., 2000). It was found that inhibition of the synthesis of plastidic isopentenyl diphosphate by use of fosmidomycin resulted in reduced seed dormancy in tomato and loss of carotenoid accumulation (Lindgren et al., 2003). It has also been reported that by controlling the conversion of IPP to dimethylallyl diphosphate, IPP isomerase will have an important role in regulating the biosynthesis of the major types of isoprenoid products formed (Phillips et al., 2008).

**1-Deoxy-D-xylulose 5-phosphate (DOXP)**

Isoprenoids are synthesized by the non mevalonate pathway (the 1-deoxy-D-xylulose 5-phosphate [DOXP] pathway, also called the MEP pathway) in a number of bacterial species, inside the plastids of algae and higher plants (Wiesner et al., 2002). In the DOXP pathway the C5 isoprene unit, isopentenyl diphosphate (IPP), is formed via DOXP from pyruvate and glyceraldehyde-3-phosphate rather than MVA (Cutler and Krochko, 1999). Moreover, plants overexpressing a gene coding for DOXP synthase gave rise to increased levels of carotenoids and ABA (Lindgren et al., 2003).

**4.6 Quantification using High Performance Thin Layer Chromatography (HPTLC)**

In recent years several analytical techniques have been established for the qualitative and quantitative analysis. The metabolic constituents, particularly secondary metabolites differ with the variety of *W. somnifera*, tissue type and sometimes with growth conditions. Such variations often lead to inconsistent therapeutic and health
promoting properties of various commercial *Withania* preparations (Chatterjee *et al.*, 2010). By using chromatographic fingerprints, the authentication and identification of herbal medicines can be accurately conducted even if the amount and/or concentration of the chemically characteristic constituents is not exactly the same for different samples of drug. Hence it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic component of the herbal drug (Chothani *et al.*, 2012).

Active constituents can be analyzed by several methods such as colorimetric, titrimetric, gravimetric, spectrometric and chromatographic techniques. Recent methods are analysis by HPLC and HPTLC (Mamatha, 2011). HPLC fingerprint analysis has now become a powerful tool for the quality control of raw plant materials. In comparison with HPLC, the greatest advantage of HPTLC procedure is that it does not require extensive clean up procedures of crude plant extracts even for quantitative analysis (Unnikrishnan *et al.*, 2008).

HPTLC is a valuable tool for reliable identification. It can provide chromatographic fingerprints that can be visualized and stored as electronic images (Mariswamy *et al.*, 2011). HPTLC is an inexpensive method for separation, qualitative identification, or semi-quantitative analysis of samples and it can be used to solve many qualitative and quantitative analytical problems in a wide range of fields, including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology and environmental analysis (Patel *et al.*, 2012). The advantages of HPTLC over other analytical methods are accurate sample application and in-situ scanning which facilitate reliability, rapidity and accuracy of analysis. It also allows simultaneous estimation of several samples utilising only a small quantity of a mobile phase, hence minimising the analysis time and cost (Ahamad *et al.*, 2014).
MATERIALS AND METHODS
3.0 MATERIALS AND METHODS

Comparative study on expression of important pathway genes among different phenophases of the *in vivo* and cultured root tissues of “Indian ginseng”, *Withania somnifera* in concert with the quantification of its major withanolide accumulation. The materials used and experimental procedures employed in the present study are described under the following headings.

3.1 Materials:

3.1.1 Plant materials

3.1.2 Chemicals

3.2 Methods:

3.2.1 Raising *Withania somnifera* plantlets in the field and sampling

3.2.2 *In vitro* culture studies

3.2.2.a Media preparation and sterilization

3.2.2.b *In vitro* propagation of *Withania somnifera*

3.2.2.c *In vitro* adventitious root induction

3.2.2.d Mass production of roots in suspension

3.2.3 Gene Expression Studies

3.2.3.a RNA Extraction

3.2.3.b RNA quality and quantity determination

3.2.3.c cDNA Synthesis and Quantification

3.2.3.d Quantitative real time PCR

3.2.4 HPTLC analysis of secondary metabolites

3.2.4.a Extraction of Secondary Metabolites

3.2.4.b Quantification of major withanolides
3.1 Materials

3.1.1 Plant Materials

The roots from one month old plantlet to the old matured roots of 5 month old plantlet grown from Jawahar 20 seeds sown in the field were harvested accordingly to their phenophases at monthly intervals and the adventitious root tissues grown in suspension were harvested from at 30D, 45D and 60 D of in vitro culture of *Withania somnifera* were taken for the study.

3.1.2 Chemicals

HIMEDIA and FISHER chemicals were used for this study unless otherwise mentioned. Nuclease and protease free water was used for the entire work. Reagents used for RNA isolation were purchased from Bangalore GeNei. cDNA synthesis and PCR studies were carried out using kits from Invitrogen Superscript Vilo and Sigma Aldrich.

3.2 Methods

3.2.1 Raising *Withania somnifera* plantlets in the field and sampling

The Jawahar 20 seeds of *Withania somnifera* were sown in the fields of Avinashilingam University in the 1st week of August 2013. The field was watered in regular intervals and the plants were maintained free of pest. For the study, the whole plant was removed along with the soil to maintain its soil conditions, the root tissue was cleaned with sterile water and cut into pieces. The sample was used immediately for isolating RNA due to its unstable nature and the remaining samples were dried and powdered. The powdered sample was used for the quantitative analysis. The same was continued at each growth stage of the plant till the matured wrinkled stage.

3.2.2 In vitro culture studies

Plant cell culture systems provide various ways to enhance the yield of desired metabolites. The roots contain a number of therapeutically important withanolides, mass cultivation of roots in vitro will be an effective technique for the large scale production of these secondary metabolites.
3.2.2.a Media preparation and sterilization

Full strength of Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) were used for all the plant tissue culture experiments.

The macro, micronutrients, vitamins and myo-inositol were taken from the stock solutions according to the requirement. Sucrose (30 g/l) was added and mixed well. Growth hormones 4.92µM IBA + 1.42µM IAA were added to it. The pH of the media was adjusted to 5.8. Solidifying or gelling agent (agar, 0.8%) was added to the media and steamed to melt the agar. It was then dispensed in clean culture bottles (30 ml per bottle) and autoclaved at 15 lbs pressure at 121°C for 20 minutes.

3.2.2.b In vitro propagation of Withania somnifera

The seeds of Withania somnifera were washed in tap water for about 10 minutes to remove the dust particles. After repeated wash, the seeds were cleaned twice with distilled water and soaked overnight for imbibitions. Water was decanted and the seeds were cleansed in 5% Tween20 for 5 minutes, followed by a wash in sterile distilled water. Seeds were then placed in 0.1% mercuric chloride under sterile conditions for 2-3 minutes with occasional swirling. Then finally the seeds were washed thrice with sterile distilled water and inoculated in half MS solid basal medium supplemented with 2% sucrose for germination. The inoculated bottles were cultured in dark at 25°C. Shoots from in vitro germinated seedlings were cultured in basal MS media and were maintained under 16 hours photoperiod at 25˚ C.

3.2.2.b In vitro adventitious root induction

For root induction studies, the leaf explants grown in vitro on MS medium were excised and trimmed into pieces of about 1cm² and were inoculated on MS semi solid medium (0.8% agar) supplemented with 3% sucrose along with 4.92µM IBA + 1.42µM IAA which has been already standardized. The pH of the medium was adjusted to 5.6 ± 0.2 before sterilization. The inoculated explants were cultured at 25°C and observed regularly. A photoperiod of 16 / 8 hr light was maintained throughout the culture period.
3.2.2.c Mass production of roots in suspension

The root tips and branches from *in vitro* induced adventitious roots were cultured in liquid MS basal media (suspension) in an air lift bioreactor for mass cultivation of roots. The bioreactor was provided with proper aeration supply and temperature was maintained at 22°C. Fresh media was supplemented every 15 days and cultured until one month.

3.2.3 Gene Expression studies

Major enzymes involved in biosynthetic pathway for the production of the secondary metabolite are taken to compare their expression level at each growth stage of the plant both in *in vivo* and *in vitro* grown root tissues.

3.2.3.a RNA Extraction

The fresh root tissue (100mg) was homogenized in a sterile mortar and pestle in 1ml TRIZOL solution and left at room temperature for about 3 minutes. The homogenized mixture was transferred carefully into an eppendorf and centrifuged at 12,000rpm for 20 minutes at 4°C. The supernatant was transferred into a new tube, to the supernatant 0.2ml of chloroform was added and mixed gently by tilting the tube up and down for about 10 times. The tube was incubated at room temperature for 2 to 3 minutes and centrifuged at 12000rpm for 15 minutes at 4°C. To the supernatant added 0.5ml of isopropyl alcohol. The tube was incubated at room temperature for 10 minutes and the centrifugation step was repeated. The pellet obtained was washed with 50µl of 70% ethanol and centrifuged at 8000rpm for 5 minutes at 4°C. The ethanol was discarded and the washing step was repeated. The pellets were air dried , not more than 10 minutes. The RNA obtained was dissolved in nuclease and protease free water. The resuspended RNA was stored in -20°C and used for cDNA synthesis.

3.2.3.b RNA Quality and Quantity Determination

The quality of the extracted RNA was checked using 1.3% formaldehyde agarose gel with λ DNA as a control. 3µl of the extracted DNase treated RNA along with the RNA loading dye was heated at 65°C in a water bath for 5 minutes and immediately
quenched in ice for 5 minutes. The heat inactivated RNA was loaded on to the wells and subjected to electrophoresis at 25V. The RNA profile was documented using Alpha digidoc system. The RNA was quantified using Biospec Nano (Shimadzu). 2μl of the RNA was used for quantification and the amount of RNA /μg was calculated.

3.2.3.c cDNA Synthesis and Quantification

RNA was normalized to 2 μg and used as a template for cDNA synthesis, where reverse transcription was carried out using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The thermal cycler conditions were used as follows: 25°C for 10 minutes, 42°C for 60 minutes and 85°C for 5 minutes.

The quality was checked using 1.3% agarose gel with a DNA ladder. The cDNA was diluted to 1:9 ratio for qRT-PCR quantification. The quantity of cDNA was checked using Biospec Nano(Shimadzu).

3.2.3.d Quantitative real time PCR

Real-time quantitative PCR was performed using 100 ng of cDNA in a 10 μl reaction volume using Luminoct SYBR® Green Master Mix (Sigma Aldrich) to analyse the expression of pathway genes involved in withanolide biosynthesis such as HMGR, CAS, SE, FPPS, and GT using gene specific primers. The Master Mix was prepared containing SyBR green, left and right primers, template (100ng), nuclease free water based on the manufacturers instruction.

Specific primers were previously designed in our laboratory using software primer3 online tool (Table 1). GAPDH gene was used as control to ensure that equal amount of cDNA was used in all the reactions. The master mix was carefully added into the 96 well plate without any bubbles. The thermal cycler conditions were used as follows: 5 minutes at 95°C, followed 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 5 minutes. The fluorescent product was detected at the last step of each cycle.
3.2.4 HPTLC analysis of secondary metabolites

3.2.4.a Extraction of secondary metabolite for HPTLC analysis

For quantitative HPTLC analysis, 1 g of the dried tissue was weighed, mixed with 1 ml of ammonia and incubated for 20 minutes at room temperature. 50 ml methanol was added and kept in sonicator for 20 minutes. The sonicated sample was then kept in a shaker for 2 hrs at 150 – 200 rpm. Filtered using Wattmann Filter paper 1 and the flow through was stored. The above step was repeated thrice and all the flow through was pooled. The pooled extracts was concentrated using Flash Evaporator (Equitron) and stored. Extractive value was calculated by standard procedure. Dissolved the extract in 5ml HPLC grade methanol and used for HPTLC analysis.

\[
\text{Extractive Value} = \text{Final Weight (FW)} - \text{Initial Weight (IW)}
\]

3.2.4.b Quantification of major withanolide accumulation

The root extracts were spotted to 10cm x 10cm precoated silica gel aluminium plate 60F254 (E.MERCK,Germany) plates as 8mm bands, under a stream of nitrogen gas, by means of a CAMAG Linomat V semiautomatic sample applicator fitted with a 100µl CAMAG HPTLC syringe. a mixture of Toluene: Ethyl Acetate: Formic acid were used in a ratio of 5: 5: 1 as the mobile phase. The chamber was presaturated with mobile phase for about 30 min at room temperature (25ºC±2).The developed plates were dried using a dryer. The banding patterns were visualized at 254nm, 366nm and white light using CAMAG TLC visualizer and the Rf values were calculated . The plate was derivatized using anisaldehyde, sulphuric acid and dried the plates at 110ºC for a minute. Densitometric scanning was performed using Camag TLC scannerIII at an absorbance of 530nm for withanolide A and 223nm for withaferin A after derivatization. Concentrations of the metabolite chromatographed were determined from the intensity of diffusely reflected light and evaluated by comparing the peak areas with linear regression (Jirge et al, 2011).
Table 1. Primer Sequence of pathway genes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Amplicon size</th>
</tr>
</thead>
</table>
| Ws GT       | Forward primer– 5' GTTTTCCTTCTTGCGAGTG 3'  
   Reverse Primer – 5' AGGTCCCAGTCCCTTTTTCAT 3' | 183          |
| Ws HMGR     | Forward primer – 5' TGCTGCAATATCGTCTCTG 3'  
   Reverse Primer – 5' CCGTCACTGATAGCCTCCAT 3' | 105          |
| Ws FPPS     | Forward primer – 5' TCGGGGGCTATCTGTTATTG 3'  
   Reverse primer – 5' CTCGGACGTGTATGGGAGTT 3' | 165          |
| Ws SE       | Forward primer – 5'- AGGGACCTGAGGAGCAAC-3'  
   Reverse primer – 5'-GGCTGATCCCATCACCAATCT-3' | 146          |
| Ws CAS      | Forward primer – 5' GCCTGGGCTTGATTATTGCTC 3'  
   Reverse Primer – 5' CACCCACCATCACTGTCTG 3' | 117          |
| GAPDH       | Forward primer – 5' CTCCATCACAGCCACTCAGA 3'  
   Reverse Primer – 5' GGTAGCACTTTCCCAACAGC 3' | 129          |
RESULTS AND DISCUSSION
4.0 RESULTS AND DISCUSSION

The present study was carried out to compare the expression profile of important pathway genes among different phenophases of the *in vivo* and *in vitro* root tissues of “Indian ginseng”, *Withania somnifera* in concert with the quantification of its major withanolide accumulation. The results procured during the course of the study are presented and discussed.

4.1 Raising *Withania somnifera* plantlets in the field and sampling

4.2 Mass production of *In vitro* adventitious roots in Suspension

4.3 Gene Expression studies

   4.3.1 RNA Extraction

   4.3.2 Gene Expression profile of *in vivo* root tissues

   4.3.3 Key pathway genes

   4.3.4 Expression of Glucosyl transferase (GT) in *in vitro* root tissue in comparison with the *in vivo* roots

4.4 Withanolide A accumulation in *in vivo* and *in vitro* root tissues

4.5 Withaferin A accumulation in *in vivo* and *in vitro* root tissues

4.6 Conclusion
4.1 Raising *Withania somnifera* plantlets in the field and sampling

The Jawahar 20 seeds of *Withania somnifera* were sown in the fields of Avinashilingam University in the 1\textsuperscript{st} week of August. The field was watered in regular intervals and the plants were maintained free of pest. The seeds started sprouting in a day’s time, in the first month the whole plant measured about 7cm in height, the root harvested in the first month was very thin and fragile. By 60 days (2\textsuperscript{nd} month) it was the flowering stage and the root started branching in this stage. At the 3\textsuperscript{rd} month berries were formed and it turned from green to yellow in the 4\textsuperscript{th} month. The 5\textsuperscript{th} month was the matured wrinkled stage, also said to be the withering stage. At each month interval the morphology of the root differed with the addition of branches. The thickness of the root increased at every month and at the 5\textsuperscript{th} month the root branches had a deep and tight anchoring to the soil.

For the study, the whole plant was removed along with the soil to maintain its soil conditions, the root tissue was cleaned with sterile water and cut into pieces. The sample was used immediately for isolating RNA due to its unstable nature and the remaining samples were dried and powdered. The powdered sample was used for the quantitative analysis. The same was continued at each growth stage of the plant till the matured wrinkled stage.
4.2 Mass production of *In vitro* adventitious roots in Suspension

Root serves as an anchor for a plant to keep it in place and most importantly they are the lifeline of a plant. They also produce certain important secondary metabolites containing therapeutic value. Fresh and healthy leaves from the cultured plantlets of *W. somnifera* were used as explants for root induction. The plants grown for one-two months on full strength MS basal media were selected for the induction. These plants were maintained in MS basal medium for at least two generations in order to avoid any residual hormonal effect. From the preliminary studies, MS media supplemented with 4.92µM IBA + 1.42µM IAA with 3% sucrose concentration in light was found to be the suitable media for highest percentage of root induction which has been standardized in our laboratory. Leaf explants were inoculated in the standardized media, after 12 days the
roots were initiated from the cut ends of the leaves Fig.2 (a). Initiated roots were maintained in the same media for 30 days. After 30 days, the adventitious roots tip (1g) from above combination were cultured in MS suspension medium in a bioreactor where the optimum conditions were maintained for the mass production of the adventitous roots Fig 2(b).

Plate 4.2 *In vitro* adventitious root mass production

(a) Root induction from leaf explants

(b) Multiplication Stage

(c) Mass production of roots in Bioreactor
4.3 Gene Expression studies

4.3.1 RNA Extraction

The whole RNA from 100mg of the root tissues were extracted using Trizol method. The quality of the extracted RNA was checked using 1.3% formaldehyde agarose gel with λ DNA as a control. The heat inactivated RNA was loaded on to the wells and subjected to electrophoresis at 25V. The RNA profile was documented using Alpha digidoc system. The RNA appeared to show intact bands in all stages of the field grown and in vitro root tissues. The extracted RNA was quantified using Biospec Nano (Shimadzu).

Plate 4.3 RNA of in vivo and in vitro root tissues

(a) *In vitro* root tissue

Lane 1 - 30D IV R  
Lane 2 - 45D IV R  
Lane 3 - 60D IV R

(b) *In vivo* root tissue

Lane 1 - 1M FG R  
Lane 2 - 2M FG R  
Lane 3 - 3M FG R  
Lane 4 - 4M FG R  
Lane 5 - 4M FG R
4.3.2 Gene Expression profile of in vivo root tissues

The first-strand complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The cDNA was used for the quantitative expression analysis of key pathway genes, such as HMGR, CAS, SS, SE, FPPS, and GT using gene specific primers. Specific primers were previously designed in our laboratory using software primer3 online tool. The primers for GAPDH gene were used as control to ensure that equal amount of cDNA was used in all the reactions. The results showed amplification pattern of these genes varied at each growth stage of the plant.

4.3.3 Key pathway genes

4.3.3.a Hydroxymethylglutaryl Co-A reductase (HMGR)

In plants, Mevalonic acid is the general precursor of various identified isoprenoids, such as sterols, plant growth regulators, and terpenoids. 3-Hydroxy-3-methylglutary coenzyme A reductase (HMGR), located in the endoplasmic reticulum, catalyzes the conversion of HMG-CoA to MVA and this has been considered as the first step in the MVA pathway (Wu et al., 2012).

Fig 4.2 Expression of HMGR in in vivo root tissue
The expression pattern of this gene studied in the *in vivo* root tissues has shown a steady rise in the expression level at each growth stage of the plant. Previous studies have shown that HMGR is an elicitor responsive gene against methyl jasmonate and salicylic acid which leads to positive regulation of this gene (Cao *et al.*, 2011).

### 4.3.3.b Farnesyl diphosphate synthase (FPPS)

Farnesylpyrophosphate synthase (FPPS) is a key enzyme in isoprenoid biosynthesis. FPPS catalyses the sequential head-to-tail condensation of two molecules of IPP with one molecule of DMAPP to form the sesquiterpenoid precursor, FPP. It has been shown that overexpression of *ginseng* farnesyl diphosphate synthase in *Centella asiatica* hairy roots enhanced phytosterol and triterpene biosynthesis (Kim *et al.*, 2010).

![Fig 4.3 Expression of FPPS in *in vivo* root tissue](image)

In the expression profile of FPPS there is a gradual up regulation upto 4th month old root tissue and at the 5th month the expression level started declining. The results indicate that the gene was highly expressed at the 4th month. Elicitor studies have also been carried out with this gene in *W.somnifera* and the results suggests that expression of
this gene is differentially regulated recognizing each effectors signal individually and is tightly regulated to the kind of the defense signals applied (Gupta et al., 2011).

4.3.3.c Squalene epoxidase (SE)

This enzyme is a noncytochrome-P450 type monooxygenase, takes part in a process to form a hydroxyl group that is characteristic of sterols and triterpenols, and functions as a rate-limiting step in the sterol biosynthesis in upstream of the P450-type oxygenases in the pathway. The biosynthesis of withanolide pathway up to squalene (catalyses by SS) is an anaerobic process. SE catalyses first step of oxygenation in this pathway and steps after squalene epoxidation are shared by sterol/brassinosteroids biosynthetic pathway (Sangwan et al., 2008). Similar to FPPS gene expression pattern SE also shows sequence increase in expression level at each growth stage and after the 4th month the expression level decreases. The highest expression is seen at the 4th month root tissue which correlates with the expression of FPPS gene.

**Fig 4.4 Expression of SE in in vivo root tissue**
4.3.3.d Cycloartenol synthase (CAS)

There are substantial evidence that CAS plays essential roles in the plant cell viability, and in the regulation of triterpenoid biosynthesis (Diarra et al., 2013). Phytosterols, such as campesterol and sitosterol, are biosynthesized via cycloartenol and catalyzed by cycloartenol synthase (CAS) in higher plants. This gene shows an irregular pattern of up regulation and down regulation in the expression profile at each growth stage and it is contrary to the expression of other key pathway genes that were analyzed. Until now very few studies have been carried out with this particular gene in plants, Corey et al.,(1993) reported the cloning and characterization of CASI, an Arabidopsis thaliana gene encoding cycloartenol synthase and the studies suggest that method used to clone this gene should be generally applicable to genes responsible for secondary metabolite biosynthesis.

Fig 4.5 Expression of CAS in *in vivo* root tissue

![Graph showing relative gene expression of CAS in *in vivo* root tissue](image-url)
4.3.3.e Glucosyl transferase (GT)

Glycosyltransferase (GT) constitute a superfamily of enzymes that catalyze conjugation of carbohydrate moieties to oligo/polysaccharides, proteins, lipids, terpenoids, flavanoids, alkaloids and other small molecules. The secondary metabolites in higher plants are often converted to their glycoconjugates in the presence of the catalyst glucosyltransferases (GTases), which are then accumulated and compartmentalized in vacuoles (Taguchi et al., 2001). Along with the other pathway genes GT also showed a steady increase in the expression profile at each growth stage and the highest expression was seen in the 5th month root tissue.

Fig 4.6 Expression of GT in *in vivo* root tissue
4.3.4 Expression of Glucosyl transferase (GT) in \textit{in vitro} root tissue in comparison with the \textit{in vivo} roots

The expression pattern of the glucosyl transferase gene in the \textit{in vitro} root tissues is in analogue with that of the expression pattern of the field grown root tissue. 30, 45 and 60 days old \textit{in vitro} root tissues were compared with the 1 to 5 month old \textit{in vivo} root tissues, the expression levels of the GT gene showed a significant increase in the expression pattern. The highest expression was seen at the 5\textsuperscript{th} month in the field grown root that correlates with the \textit{in vitro} root where the highest expression was shown by the 60 days old root tissue.

Fig 4.7 Expression of GT in \textit{in vitro} root tissue

![Graph showing relative gene expression of GT in in vitro root tissue at 30D, 45D, and 60D]

- **GT**
4.4 Withanolide A accumulation in *in vivo* and *in vitro* root tissues

The *in vitro* and *in vivo* molecular pharmacological investigations have elucidated that Withanolide A is one of the most promising phytopharmaceuticals. Withanolide A content of the *in vitro* roots, as well as from field grown native roots of *W. somnifera* were quantified using withanolide A standard and subjected to HPTLC analysis. Total withanolide A content was quantitatively different, low in *in vitro* developed roots as compared to field grown roots. The higher accumulation in the field grown root tissue was seen in the 4th month (623 µg/g DW) which can be correlated with the *in vitro* roots, the withanolide A accumulation was highest in the 45th day old root tissue (380 µg/g DW). In both the *in vivo* and *in vitro* root tissues the accumulation decreases gradually after the 4th month and 45th day. The study report of (Sabir *et al.*, 2008) evidence that the *in vitro* tissue systems of *W. somnifera* have the potential for withanolide synthesis that could be explored for large-scale production of valuable bioactive withanolides. Large scale cost effective withanolide A can be synthesized under controlled conditions as *in vitro* roots contain a significant amount of withanolide A similar to the native plant.

Plate 4.4 Quantification of Withanolide A- Developed plate (366nm)
Plate 4.5 Quantification of Withanolide A – Derivatized plate (white light)

Withanolide A

Withanolide A Standard
R<sub>r</sub> - 0.48

Plate 4.6 Quantification of Withanolide A – Linear regression graph

Substance Withanolide A @ 234nm  Regression mode : Linear

\[ Y = 898.116 + 4.665^*X \quad r=0.99672 \quad sdv = 6.53\% \]
4.5 Withaferin A accumulation in *in vivo* and *in vitro* root tissues

Withaferin A, a highly oxygenated steroidal lactone, is the principal withanolide in *W. somnifera*. The accumulation of withaferin A in *in vitro* and cultured root tissues were quantified using HPTLC. In the field grown roots the increase in withaferin A accumulation was steady until the 4\textsuperscript{th} month and started declining at the 5\textsuperscript{th} month. High amount of withaferin A accumulated at the 4\textsuperscript{th} month showing (463.41µg/g DW). In the *in vitro* root tissues withaferin A was seen only in trace amount and the highest was at the 45\textsuperscript{th} day (0.98µg/gDW). Previous investigations also prove that withaferin A is much lesser in root when compared to shoots of *Withania somnifera* (Sabir *et al.*, 2012).
Plate 4.7 Quantification of Withaferin A – Developed plate (366nm)

Plate 4.8 Quantification of Withaferin A – Derivatized plate (white light)
Plate 4.9 Quantification of Withaferin A – Linear regression graph

Substance Withaferin A @ 223nm  Regression mode : Linear

\[ Y = 366.965 + 6.244X \quad r=0.99945\quad sdv =2.91\%

Fig 4.8 Quantification of Withaferin A
4.6 Conclusion

The expression of the pathway genes is tightly linked to the pattern of accumulation of the withanolides. The expression level of gene in *in vivo* roots in comparison with the *in vitro* roots exhibited a direct co-relation with the maximum withanolide content. The root-contained withanolide A is de novo synthesized within roots from primary isoprenogenic precursors (Sangwan *et al.*, 2008). The results show that withanolide A was accumulated more in the roots of both *in vivo* and *in vitro* tissues. Literature supports that the biogenesis of withaferin A, a major constituent of the leaves, was however found to be tightly linked to shoots/ green tissue (Dhar *et al.*, 2013). Since the *in vitro* roots were induced from leaf explants only a trace amount of Withaferin A was observed in the root tissues. The present study adds to the record proving that withanolides are synthesized in different parts of the plant (through operation of the complete metabolic pathway) rather than imported.
SUMMARY AND CONCLUSION
5.0 SUMMARY AND CONCLUSION

The present study entitled “Comparative study on expression of important pathway genes among different phenophases of the in vivo and cultured root tissues of “Indian ginseng”, Withania somnifera in concert with the quantification of its major withanolide accumulation” was carried out to study the interrelation between the gene expression and accumulation of withanolides.

Withania somnifera (Jawahar 20) seeds were sown in fields of Avinashilingam University during the month of August (2013). The roots from one month old plantlet to the old matured roots from 5 month old plantlet were taken for the study.

For the in vitro adventitious roots, the leaf explants of W. somnifera was inoculated in MS media supplemented with 4.92µM IBA + 1.42µM IAA with 3% sucrose concentration. After 12 days the roots were initiated from the cut ends of the leaves. The adventitious roots tip from above combination were cultured in MS suspension medium in a bioreactor where the optimum conditions were maintained for the mass production. The root tissues grown in suspension were harvested at 30th day, 45th day and 60th day to carry out the study.

The whole RNA from the in vivo and in vitro root tissues was extracted using Trizol method. The extracted RNA was quantified using Biospec Nano (Shimadzu). The first-strand complementary DNA (cDNA) was synthesized using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The cDNA was used for the quantitative expression analysis of the key pathway genes.

The genes involved in the withanolide biosynthesis such as HMGR, CAS, SS, SE, FPPS, and GT were analyzed using gene specific primers. The primers for GAPDH gene was used as control. The results showed amplification pattern of these genes varied at each growth stage of the plant both in vivo and in vitro. All genes were highly expressed at the yellow berry stage of the plant and at the wrinkled berry stage the expression level started decreasing. In the in vitro root tissue the expression was maximum at the 60th day and can be correlated with the yellow berry stage in the field grown plants.
The major withanolides such as withaferin A and withanolide A were quantified using HPTLC. The withanolide A accumulation was seen high in the 4\textsuperscript{th} month root extract in the \textit{in vivo} plantlet and started declining from the 5\textsuperscript{th} month. A similar pattern was seen in the \textit{in vitro} root extract which showed the maximum accumulation at the 45\textsuperscript{th} day. Withaferin A was seen maximum in the 4 month old root extract in \textit{in vivo} and since the roots were induced from leaf explants only a trace amount was seen in the \textit{in vitro} root extract.

As the gene expression in both \textit{in vivo} and \textit{in vitro} root tissues starts declining after a certain stage, it can be further studied by using elicitors to find out the variation in expression pattern after the treatment in correlation with the withanolide accumulation. Inhibitors can also be used to identify the specific gene that plays a key role in the production of withanolides in the biosynthetic pathway. Characterization of other genes that interpret the complete biosynthetic pathway through high throughput metabolic profiling and sequencing will provide a better knowledge in understanding the withanolide biosynthesis in \textit{W.somnifera}. 
BIBLIOGRAPHY
6.0 BIBLIOGRAPHY


## APPENDIX-I

### COMPOSITION OF MS MEDIUM

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition (mg/L)</th>
<th>Stock Solution (W/V) (g)</th>
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<td><strong>MS Macro I (10 X)</strong></td>
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<tr>
<td>KNO$_3$</td>
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