ESTABLISHMENT OF *IN-VITRO* CULTURES FOR SUSTAINABLE PRODUCTION OF *WITHANIA SOMNIFERA* L. SECONDARY METABOLITES

By

MUHAMMAD ADIL

DEPARTMENT OF BIOTECHNOLOGY
QUAID-I-AZAM UNIVERSITY
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BY

MUHAMMAD ADIL

Supervised by

Dr. BILAL HAIDER ABBASI

Department of Biotechnology

Quaid-I-Azam University,

Islamabad, Pakistan

2017

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No part of this theis has been submitted anywhere else for any other degree. This thesis is submitted to the **Department of Biotechnology** in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Field of **Biotechnology** Department of **Biotechnology** University of: **Quaid-i-Azam.**

Student Name: Muhammad Adil. **Signature** ____________

a). **Dr. Ashiq Rabbani** ____________
Principal Scientific Officer
Plant Genetic Resources Program
Institute of Argi-Biotechnology & Genetic Resources (IABGR)
NARC, Park Road, Islamabad

b.) **Dr. Azra Yasmin** ____________
Associate Professor & Chairperson,
Environmental Science Programme
Fatima Jinnah University, Rawalpindi.

c). **Dr. Bilal Haider Abbasi** ____________
Supervisor

Dr. Muhammad Naeem ____________
Chairman

CERTIFICATE

This thesis submitted by Muhammad Adil is accepted by the Department of Biotechnology, Quaid-i-Azam University Islamabad as satisfying the thesis requirement for the degree of Doctor of Philosophy in Biotechnology.

Supervisor ....
Dr. Bilal Haider Abbasi

External Examiner..
Dr. Azra Yasmin
Professor
Department of Environmental Sciences
Fatima Jinnah University Rawalpindi

External Examiner..
Dr. Ashiq Rabbani
Principal Scientific Officer
Plant Genetic Resources Institute
NARC, Islamabad

Chairman..
Dr. Muhammad Naeem

Dated: 02/March/17

DECLARATION

The whole of the experimental work included in this thesis was carried out by me in the Plant cell culture Laboratory, Department of Biotechnology, Quaid-i-Azam University, Islamabad, Pakistan and in Plant cell culture Laboratory, University of Copenhagen, Denmark. The findings and conclusions are of my own investigation with discussion of my supervisor Dr. Bilal Haider Abbasi. No part of this work has been presented for any other degree.

MUHAMMAD ADIL

I Dedicate This

Thesis To

My Parents,

My wife & other Family members!

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List of Abbreviations

µM	Micromoler
2,4-D	2, 4 dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BAP	6-Benzyladenine
BTBB	Balloon Type Bubble Bioreactors
CIF	Callus induction frequency
cm	Centimeter
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DW	Dry weight
FW	Fresh weight
GA_3	Gibberellic acid
Gli	Glitz
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
IBA	Indole butyric acid
MDA	Malondialdehyde
MeJa	Methyl jasmonate
Mela	Melatonin
mg/g DW	Milligram/gram-Dry Weight
mg/L	Milligram/liter
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
PCA	Principle component analysis
PGRs	Plant growth regulators
POD	Peroxidase
RSA	Radical scavenging activity
SA	Salicylic acid
SH	Schenck and Hidebrandt
SOD	Supper oxide dismutase
TDZ	Thidiazuron
TFC	Total flavonoid content
TPC	Total phenolic content
WS	*Withania somnifera* L.

SUMMARY

Withania somnifera L. commonly known as Indian ginseng, has been used as a multipurpose medicinal plant and is a rich reservoir of pharmaceutically active triterpenoids that are steroidal lactones and known as withanolides. It belongs to genus *Withania*, a genus with recognized medicinal potentials against different ailments. Previously, it has been reported that *W. somnifera* also contains medicinally important phenolic and flavonoid compounds. These compounds have established antioxidant activities in body and are considered potent antioxidant than vitamin C and E. Little is known about the bioavailability of withanolides, while its synergistic combination with phenolic and flavonoids could further boost the therapeutic potentials of *W. somnifera* end products. In general, field grown plant material has been used for the commercial formulations of medicines. Different environmental conditions, pollutants and fungi, bacteria, viruses and insects are the factors that affect the quality of end products which may lead to heavy loss in yield and alteration in medicinal content of the plant. Bioprocess considerations are important alternatives to whole plant systems, in order to enhance secondary metabolites of interest. The present work was attempted to enhance secondary metabolites by establishment of adventitious roots, cell suspension and callus cultures of *W. somnifera*. The step-wise conclusions are presented as following.

In the first experiment, different light regimes were employed with varying concentration of melatonin, either alone or in combination with auxins (IBA/IAA) for growth and development of adventitious roots in *W. somnifera*. It was observed that 600 µM melatonin treatment alone favored 58% of roots induction from leaf explant cultures, incubated under continuous dark conditions. However, adequate root growth (number and length of roots) was observed under 16 h light/8 h dark at 600 µM melatonin. However, 78% roots induction was observed in cultures containing individual concentration of IBA (4.9 µM), followed by IAA (11.25 µM). The induction frequency reached to its maximum value of 80% in cultures containing melatonin (500 µM) in combination with IBA (4.9 µM). Growth of Adventitious root was significantly affected in cultures augmented with varying concentration of melatonin, single or in combination. The interactive effect of light and melatonin was found stimulating for adventitious root growth. Beside these melatonin significantly reduced light induce oxidative stress in adventitious roots and enhanced higher secondary metabolites content. Further, the DPPH free radical scavenging activity was maximum in melatonin treated roots. This

experimented suggested that melatonin mimics IAA activity in root growth and regulates well in 16-h light/8-h dark, thereby giving protection to plant system against light stress.

After 4 weeks of adventitious root initiation, from the best root induction medium, the roots were aseptically separated from the leaf explants and sub-cultured in MS liquid medium containing melatonin either alone or in combination with IAA/IBA. The cultures were maintained on orbital shaker under continuous agitation at 120-rpm; 16-hrs photoperiod at 25±2°C. Among the phytohormones treatments, maximum fresh weight (FW: 44 g/l) and dry weight (DW: 3.37 g/l) were observed in 6-weeks old cultures containing IBA (4.9 µM) in combination with melatonin (Mela: 8.6 µM). Further growth kinetics study was carried for intervals of 7-days during 6-weeks of cultures and maximum biomass accumulation was observed in 35-days old cultures. Decrease in biomass accumulation was observed when cultures were elicited with MeJa and SA. To overcome this, the addition of Mela in cultures proved supportive for biomass and growth curve dependent phenolic and flavonoid accumulation. Among the elicitors, MeJa (100 µM) treatment resulted maximum (35 mg/g DW) total phenolic and total flavonoids content (14 mg/g DW) in adventitious roots cultured in MS medium containing Mela in combination with IBA. Furthermore, a positive correlation of total phenolic content and antioxidant was observed in most cultures. This work demonstrated the stimulatory effect of Mela for biomass, phenolics and flavonoids accumulation in adventitious roots cultures elicited with MeJa.

In third experiment, we cultured leaf explant from *in vivo* seed derived plantlets of *W. somnifera*. For callus induction MS medium was fortified with varying concentrations of TDZ/BAP alone and in combinations with NAA (0.1/0.5 mg/l). We observed maximum callus induction frequency (78.3%), fresh weight (3.5 g/explant) and dry weight (0.29 g/explant) on MS medium containing TDZ (0.5 mg/l) in combination with NAA (0.5 mg/l). Further, leaf explant were cultured on optimized medium and exposed to monochromatic lights of 16-hr/8-hr cycle. The callus induction frequency on respective media reached to maximum (84%) in red light treated conditions. In comparison to control treatments callus biomass accumulation remained maximum under red light treatment, while maximum total phenolics and flavonoids content were observed in violet light treated callus cultures. The biomass dependent antioxidant activities were observed in callus cultures. Different light colors treatments evoked differential anti-oxidative enzymes responses in callus cultures and maximum protease, and SOD and POD activities were observed in green and violet light treated callus cultures, respectively. We

detected chlorogenic acid, moupinamide, withanomide Q, withaoside IV or X, withaferin A and withanoside V in HPLC chromatograms. Whereas, we were able to quantify chlorogenic acid, moupinamide and withaferine in significant amount and showed variations in amount with the given treatments. This study provided the basis for experiment to use combinations of red and violet light treatments to get optimum biomass with increased secondary metabolites content in *in-vitro* cultures of *W. somnifera*.

In fourth experiment, cell suspension cultures were established and secondary metabolites were elicited in these cultures. To obtain friable calli suitable for establishment of suspension cultures, zygotic embryos derived callus were initiated on different medium varying in composition of macro, micro, other organic additives and plant growth regulators. Callus obtained on different medium types showed significant variations in callus texture, biomass and secondary metabolites production. Desirable friable callus with loosely packed cells was obtained on Gli media and 35-days old callus was sub-cultured on respective medium for growth kinetics study. The obtained friable callus was transferred to Gli liquid medium and kept continuously in dark on shaker incubator for a period of 54 days. A 35-fold increase in biomass accumulation was observed in 48-days old cell culture. Maximum biomass (17.96 g/culture) and maximum phenolic content (10.74 mg/g DW) were observed in 48-days and 54-days old cell cultures, respectively. The established cell cultures were elicited with SA and MeJa which showed a significant decrease in biomass accumulation. The MeJa (20 µM) and SA (100 µM) treated cell suspension cultures produced higher phenolic (35.36 mg/g DW) and flavonoids (3.2 mg/g DW) content, respectively. Maximum (60%) DPPH free radical scavenging activity was observed in SA treated cell cultures, while maximum content of withaferin A, withanoside IV, moupinamide and chlorogenic acid were observed in MeJa treated cell cultures.

1. GENERAL INTRODUCTION

Withania somnifera L. (WS), also known as Indian ginseng or winter cherry, is an important medicinal plant, belongs to the genus *Withania* of Solanaceae or night shade family. This specie is a perennial shrub grows in drier regions up to 30-150 cm height with star shaped branching and covered in fine hairs with characteristic long tuberous roots. It has 48 (2n) chromosomes and distinguishing petiolate alternate leaves, greenish bisexual solitary flowers and globose orange-red fruits covered in large calyx. It has characteristic gamosepalous calyx and campanulate corolla each of five lobes.

Locally it grows wild in sandy loam to light red soil and dry climate, ranging from Northern areas of Pakistan to Karachi. Globally, it grows abundantly in India, Bangladesh, Sri Lanka, part of northern Africa and eastern Mediterranean area (Nasir et al. 1972). The name *Withania*, derived according to international code of Botanical Nomenclature from surname of Henry Witham, a paleo-botanist of early 19th century and the specific name somnifera means sleep-bearing due it`s sedative property. In traditional medicinal knowledge it is known as Ashwagandha (Asgund; Hindi) and Koti lal (Pashto) because of its horse smell and red berries respectively (Asthana and Raina 1988; Tripathi and Kimar 1996).

1.1. *Withania*: an important genus

The genus *Withania* is an important genus of solanaceae family that comprises of 23 species, widely distributed across the North Africa, Turkey, Canary Islands, Afghanistan, Egypt, India and Pakistan. Within the family Solanaceae, *Withania* belongs to subfamily Solanoideae, tribe Physaleae and sub-tribe Withaninae (Fig 1.1) (Olmstead et al. 2008). Among the worldwide list of *Withania* species, *W. somnifera* and *W. coagulans* are considered the most important due to their therapeutic potentials. A third species, *W. obtusifolia* Tackh was also reported from South India but according to Sundari et al. (1999) it is only a cytotype (2n = 24) of *W. somnifera* (2n = 48) and considered endemic to Jordan. Whereas *W. aristata* is endemic to Canary Islands and used as therapeutic remedy by the local community (Darias et al. 2001; Martin-Herrera et al. 2008).

The medicinal utilization of this genus is characterized by its use as herbal medicine either alone or in combination with other medicinal plants. The species of this genus are known for its withanolides content that gives the promising therapeutically value to their traditional end-products. Among the

species of this genus, *W. somnifera* and *W. coagulans* are well studied and have been in practice by local practitioners since 4000 years. Where *W. aristata*, commonly known as “orobal” or “saquido” used in folk medicine of Canary Island for antispasmodic, for rheumatic problems, eye problems and otitis, as well as for insomnia, constipation, and urinary pathologies (Martin-Herrera et al. 2008). *W. obustifolia* is called as Jordanian specie and considered closely related to *W. somnifera* and eight withanolides have been reported from this specie. There are several other species of this genus reported from Somalia and other parts of the world (Thulin 2002). But in Indo-Pak subcontinent *W. somnifera* and *W. coagulans* are extensively used in traditional medicinal recipes. *W. coagulans* is source of coagulating enzymes and used to make cheese from milk and recommended for dyspepsia, flatulent colic and other intestinal infections, whereas its smoke is inhaled to relive toothache. While, *W. somnifera* is a very valuable and popular in Ayurveda and Unani medicines, which is even compared with ginseng in view of the various therapeutic activities attributed to it. It is considered as anti-inflammatory, anti-tumor, anti-stress, antioxidant, mind-boosting, immune-enhancing, and rejuvenating. Historically its root has also been noted to have sex-enhancing properties.

Ashwagandha (WS) is widely used, prioritized Ayurvedic herb having annual demand of 7000 tons/yr but its actual production is 1500 tons/yr. Once it’s over harvesting from wild leaded a critical pressure on its natural reservoir and considerable efforts were made to conserve it for coming generations. It is included in the list of 32 prioritized plants as it is used in more than 200 Ayurvedic formulations as principle ingredient. The demand of WS in herbal market was estimated to be 9127.5 tons per annum in the year of 2004-2005 based on the trend, the current demand would be around 12500 tons. The good quality roots of Ashwagandha has selling price of 100-150 Rs/Kg, while additional profit return could be made by selling seeds (40-100 Rs/Kg) and leaves. According to Datta et al. (2010) the farm gate price for its roots in India is US$ 1.5 per kg.

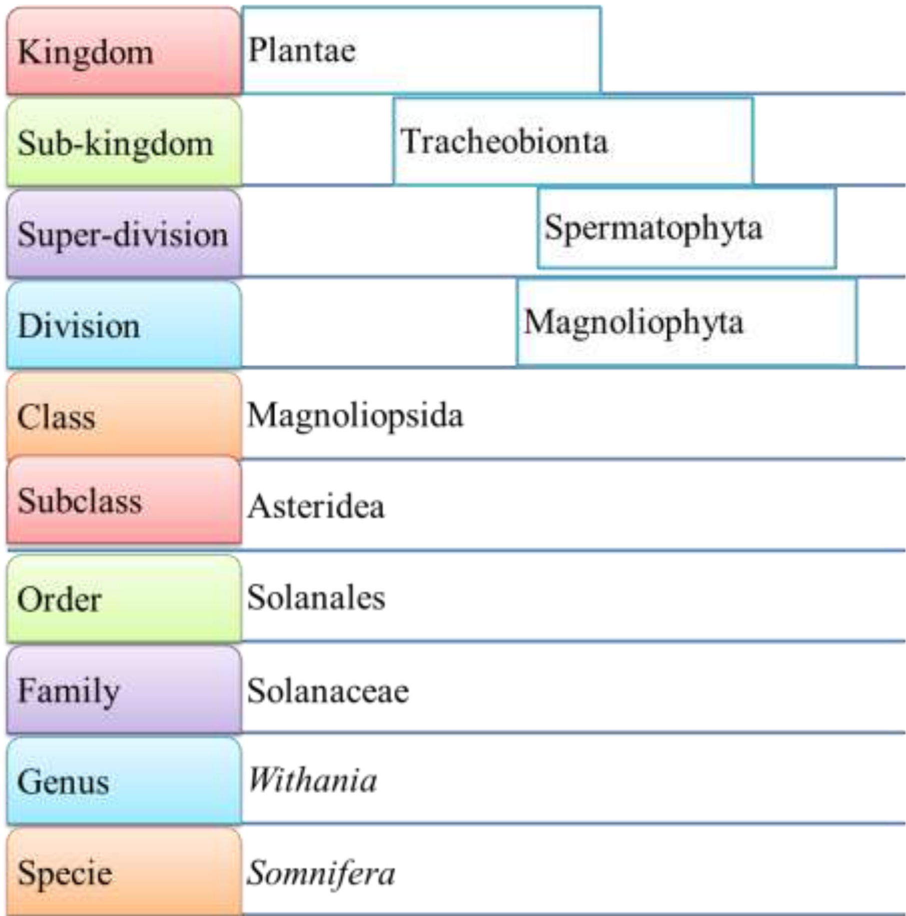

Figure 1.1: Taxonomic position of *Withania somnifera* L.

1.2. Medicinal uses of *Withania somnifera*

W. somnifera is recognized as medicinal herb throughout the world and particularly practiced in Ayurveda and Unani systems of traditional medicine for about 4000 years ago. In Ayurveda it is known as rasyanas, as it is being considered as lifesaving herb for all types of ailments. It is the best rejuvenative herb that maintain the proper tissue growth, specifically bones and muscles. While it also enhances the functions of reproductive system and adrenal glands. Its leaves are bitter in taste and recommended for fever as it has shown anti-malarial properties in recent studies. *W. somnifera* extract have shown promising results in curing the opthalmitis and have been recommended as cataracto-static agent (Thiagarajan et al. 2003). While a paste of its roots and bruised leaves are used as tincture to treat ulcer and painful swellings (Parajpati et al. 2003). Its leaves are also used as an anti-inflammatory, anti-proliferative and anti-helminthic medicine (Jabbar et al. 2006; Jayaprakasam et al. 2003; Jayaprakasam

and Nasir 2003). Ayurvedic practitioners have used its roots for centuries to treat health disorders as diverse as tumor and arthritis. There are numerous published reports on its pharmacological and chemical properties. The pharmacological properties of *W. somnifera* are summarized in the Table 1.1.

Table 1.1: Reported and tested pharmacological activities of *Withania somnifera* L.

General	Specific Pharmacological activities	References
Reproductive disorders	Testicular development	Abdul-Mugied et al. 2001
	Arousing sexual desire	Chauhan et al. 2014
	Spermatogenesis	Ahmad et al. 2010; Ambiye et al. 2013
	Abortifacient	Noumi and Djeumen 2007
	Menstruation	Yadav et al. 2005
Neurodegenerative disorders	Neuronal regeneration	Kuboyama et al. 2005; Tohda et al. 2005
	Anti-Anxiolytic	Battacharya et al. 2000; Gupta et al. 2008
	Anti-Arthritis	Rasool and Vararlakshmi 2007 & 2000
	Anti-Parkinson	Ahmad et al. 2005; De Rose et al. 2015
	Improvement of mental and brain aging	Wadhwa et al. 2016
	Neuroprotective	Ahmad et al. 2016; Manchanda et al. 2016
	Anti-Alzheimer	Shivamurthy et al. 2016
Cardiovascular disorders	Hypoglycemic	Gorelick et al. 2015
	Hypocholesteraemic	Saravanan and Ignacimuthu 2015
	Enhance myeloperoxidase activity and Calcium level	Khali et al. 2015; Mohanty et al. 2004
	Cardio-protective	Rao and Najam 2015
Gastro intestinal disorders	Cure inflammatory bowel disease	Pawar et al. 2011
	Anti-ulcer	Bhatnagar et al. 2005
	Cure Diarrhea	Alam et al. 2012
Anti-cancer	Breast cancer	Biswal et al. 2013
	Colon Cancer	Muralikrishnan et al. 2010
	Neuroblastoma	Kataria et al. 2013
	Prostate cancer	Roy et al. 2013
	Anti-oxidant	Visavadiya and Narasimacharya 2007
	Anti-leukemic	Turrini et al. 2016
Anti-microbial	Salmonella typhimurium	Owaise et al. 2005

General	Specific Pharmacological activities	References
	Murine aspergillus	
	E. coli	Aroa et al. 2004
	Staphylococcus aurous	Sundaram et al. 2001
	Neisseria gonorrhea	Kambiz and Afolayan 2008
Immunomodulatory		Davis and Kuttan 2000; Rasool and Varalakshmi 2006
Rejuvenative		Sing et al.1982; Kumar et al. 2005
Skin disorders	Prevent Squamous cell carcinoma	Samadi et al. 2010
	UVB protective	Mathur et al. 2004

Currently *Withania somnifera* root extract is used as a dietary supplement throughout the world including United States to boost energy and body performance. Beside all these it has a profound effect on nervous system and recommended to calm mind, relieve weakness and nervous exhaustion and promote healthy sleep. Nowadays it is also used in cosmetic industry for curing the skin rankles, protection from sun light, curing hair fall and dandruffs. In a traditional formulation Ashwagandha is mixed with dried ginger and lemon for skin toning, while in 2003 a patent was filed that claimed skin whitening properties of its alcoholic extract. Traditionally it has also been recommended for breast enlargement when used in butter together with the fruit of *Scindapsus officinalis* Schott., the root of *Saussurea lappa* Clarke. and the rhizomes of *Acorcus calamus* (Patkar 2008). There are some famous ayurvadic preparation of *W. somnifera*, Powder (Churna), Decoction (Kwatha), Medicated wine (Arishta), Medicated Ghee (Chrita) and Medicated Oil (Narayana taila). Further it`s *in-vitro* and in-vivo pharmacological studies are summarized in table 1.2.

Table 1.2: *In-vitro* and *in-vivo* pharmacological activities of *Withania somnifera* L. plant parts.

Plant part used	Extract type	Pharmacological activity	Study type	References
Roots	Aqueous suspension	Immunomodulatory	*In-vivo* and *in-vitro*	Rasool and Varalakshmi 2006a
Roots	Aqueous suspension	Suppressive, analgesic and antipyretic	*In-vivo* and *in-vitro*	Rasool and Varalakshmi 2006b

Plant part used	Extract type	Pharmacological activity	Study type	References
Roots, Stem and Leaves	Ethanol extract	Anticancer activity	*In-vitro*	Yadav et al. 2010
Roots and Leaves	Methanol, hexane and diethyl ether extracts	Antibacterial activity	*In-vitro*	Arora et al. 2004
Roots	Aqueous extract	Neuroprotective	*In-vitro*	Udayakumar et al. 2010b
Roots	Aqueous extract	Chondroprotective	*In-vivo* and *in-vitro*	Sumantran et al. 2008
Hairy roots	Ethanol extract	Rejuvenative	*In-vitro*	Kumar et al. 2005
Whole plant	*Withanai* Tablets	Cardio-protective	*In-vivo*	Hamza et al. 2008
Roots	Ethyl acetate	Anti-tumor	*In-vivo*	Christina et al. 2004
Roots	Hydrochloric acid extract	Anti-angiogenesis and Anti-proliferative	*In-vitro*	Mathur et al. 2006
Roots	Ethanol extract	antiglycating agent	*In-vitro*	Babu et al. 2007
Roots	Aqueous extract	Anti-inflammatory	*In-vivo*	Pawar et al. 2011
Roots	Methanol: Chloroform (3:1) extract	Neuroprotective	*In-vitro*	Kurapati et al. 2013
Leaves	Methanol	Anti-proliferative and anti-oxidant activity	*In-vitro*	Kaur et al. 2004
Roots	Ethanol	Immunomodulatory	*In-vivo*	Mikolai et al. 2009
Roots	Ethanol	Inhibition of pro-inflammatory molecules formation	*In-vivo*	Singh et al. 2007
Leaves	Ethanol	Anti-leishmanial	*In-vitro*	Chandrasekaran et al. 2013
Leaves	Ethanolic and aqueous Extracts	Antioxidant	*In-vitro*	Panchawat 2011
Leaves and Roots	Ethanol	Hypoglycemic and hypolippidemic	*In-vivo*	Udayakumar et al. 2009a
Roots	Aqueous suspension	Nephroprotective	*In-vivo*	Jeyanthi and Subramanian 2009
Roots	Aqueous-ethanol extracts	immunomodulatory	*In-vivo*	Kushwaha et al. 2012
Whole plant	Aqueous suspension	Hepatoprotective and Antioxidant	*In-vivo*	Sabina et al. 2013

Plant part used	Extract type	Pharmacological activity	Study type	References
Roots	Methanol extract	Neuronal regeneration	*In-vitro*	Tohda et al. 2000
Roots	Aqueous extract	Anti-microbial	*In-vitro*	Mehrota et al. 2011
Stem bark	Methanol and aqueous extracts	Anti-plasmodium	*In-vitro*	Kirira et al. 2006
Leaves	Methanol	Anti-proliferative	*In-vitro*	Senthil et al. 2007
Roots	Methanol	NF-κB transactivation	*In-vitro*	Iuvone et al. 2003
Roots	Powder Ethanolic	Anti-arthritic	*In-vivo*	Gupta et al. 2013; Begum et al 1988
Roots	Alcoholic extract	Hepatoprotective	*In-vivo*	Mansour and Hafez 2012
Leaves	Aqueous alcoholic extract	Immuno-regulatory	*In-vivo*	Khan et al. 2009
Fruits	Protein extract	Anti-cancer	*In-vitro*	Oza et al. 2011; Oza et al. 2009
Roots	Petroleum ether	Antioxidant	*In-vitro*	Shahriar et al. 2013
Roots	Methyl alcohol	Neuroprotective	*In-vivo*	Kasture et al. 2009
Roots	Powder	Hypocholesteremic and antioxidant	*In-vivo*	Visavadiya and Narasimhacharya 2007
Roots	Aqueous Chloroform	Anti-cancerous	*In-vivo*	Mathur et al. 2006; Mathur et al. 2004
Aerial parts	hexane, dichloromethane, ethyl acetate and methanol	Antimicrobial	*In-vitro*	Mwitari et al. 2013
Roots and Leaves	Aqueous alcoholic extract	Antitumor	*In-vitro*	Malik et al. 2009
Leaves	Lectin like protein	Antifungal	*In-vitro*	Ghosh 2009
Roots	Powder	Improves semen quality	*In-vivo*	Mahdi et al. 2011
Roots	Aqueous extract	Improve insulin sensitivity (anti-diabetic)	*In-vivo*	Anwer et al. 2008
Roots	Ethanol extract	Ameliorate metformin-induced hypothyroidism	*In-vivo*	Jatwa and Kar 2009
Roots	Ethanol extract	Anti-metastatic	*In-vitro*	Yang et al. 2013

1.3. *Withania:* Medicinal phytochemistry

The genus *Withania* is known for its group of compounds known as withanolides and most of the pharmacological activities are linked to it. For phytochemical investigation *W. frutescens*, *W. somnifera*, *W. coagulanse*, *W. aristata* and *W. adpressa* have extensively been studied (Abdeljebbar et al. 2009; Chatterjee et al. 2010) and have reported several types of withanolides. Additionally, *Withania* is also an active source of medicinally important flavonoids, phenolics, alkaloids and terpenoids (Kumar et al. 2015; Udayakumar et al. 2009b; Udayakumar et al. 2010a; Uddin et al. 2012).

Withanolides or withanosteriods, group of compounds containing C_{28}-steroidal lactone ring and varies according to its functional groups, but this definition is no more acceptable as recent studies have reported compounds which are not lactone in nature. Withanolides are highly oxygenated phytochemicals, and the oxidation at various sites of skeleton is responsible for the structural variations in different classes of withanolides (Choudhary and Yousuf 2013; Kulkarni and Dhir 2008). So far several hundreds of withanolides have been reported in literature, but withaferin A have got far most attention and several patents have been filed since than its discovery. Withaferin A have proven to be potent anticancer drug that have shown promising results against all kind of cancer cell lines. Beside this natural kind of withanolides, several semisynthetic derivatives have been reported with increased anti-proliferative activity (Zhang et al. 2014).

1.3.1. Phytochemistry of *Withania somnifera*

Phytochemical investigation of *W. somnifera* has shown a variety of biologically active compounds of medicinal importance (Fig 1.2 and Table 1.3). The metabolic constituents, particularly secondary metabolites differ with the variety of *W. somnifera*, tissue type and sometimes with growth conditions (Abraham et al. 1968). The chemistry of this plant have shown the presence of several chemical groups of diverse compositions such as steroidal lactones, alkaloids, flavonoids, tannins, etc. (Dhanani et al. 2013; Fernando et al. 2013; Kapoor 2000). At present, more than 12 alkaloids, 40 withanolides, and several sitoindosides (withanolide containing sugar moiety at C_{27}) have been identified from fruits, leaves, roots and stem of *W. somnifera* (Mirjalili et al. 2009). The major constituent of this plant, withanolides, are mainly synthesized through isoprenoids biosynthesis pathway and found abundantly in leaves. Where isoprenoids bio-synthesis occurs through classical cytosolic mevalonate (MVA) and plastid localized 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways that leads to 24-methlene

cholesterol (C30 terpenoid) and thought to be the central molecule for various withanolides biosynthesis (Gupta et al. 2013; Sangwan et al. 2008).

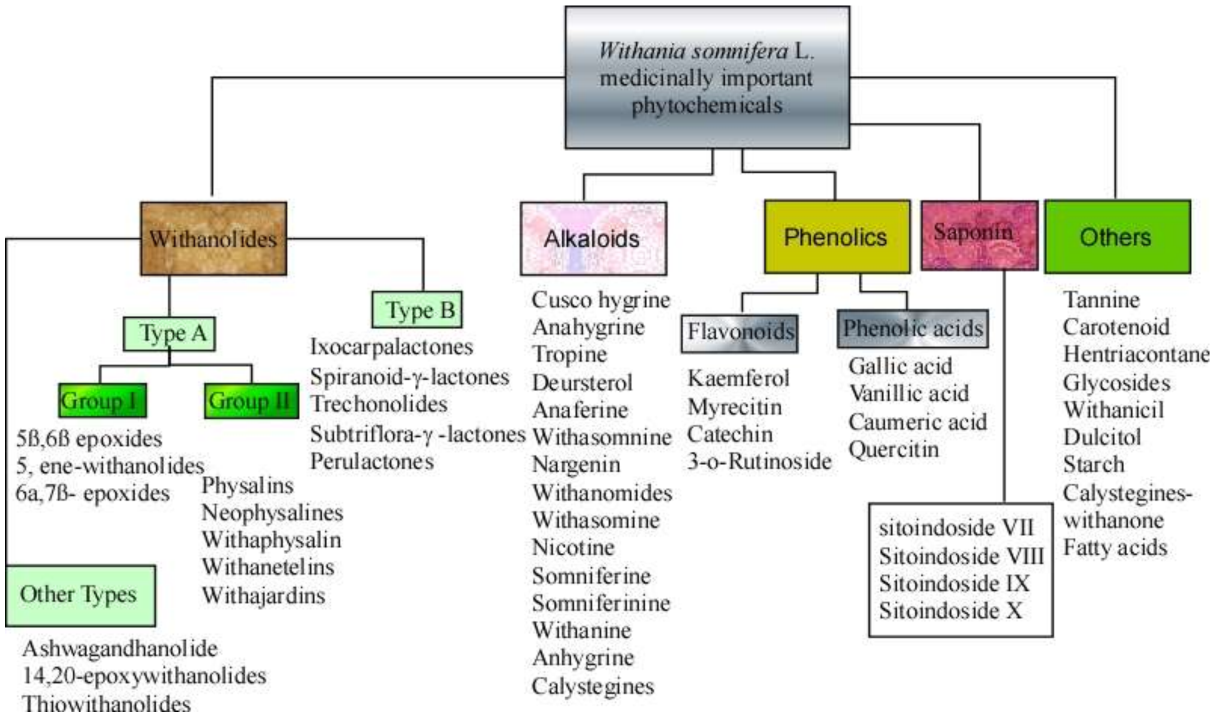

Figure 1.2: Medicinally important phytochemicals of *Withania somnifera* L. (adopted from: Chen et al. 2011).

Kandil et al. (1994) reported flavonol glycoside and phenolics from *W. somnifera* and confirmed the presence of 6, 8-Dihydroxykampferol 3-rutinoside along with quercetin, 3-*O*-rutinoside and 3-rutinoside-7-glycoside. A comprehensive review on its phytochemistry have elaborated the presence of alkaloids, amino acids, steroids, volatile oil, starch, reducing sugars, glycosides, hentriacontane, dulcitol and withaniol in roots (Uddin et al. 2012). Basic alkaloids include cuscohygrine, anahygrine, tropine, pseudotropine, anaferine, isopelletierine, withananine, withananinine, pseudo-withanine, somnine, somniferine, somniferinine, Neutral alkaloids include 3-tropyltigloate and an unidentified alkaloid (Gupta and Rana 2007; Kapoor 2000). Other alkaloids include withanine, withasomnine, and visamine (Khare et al. 2007). The green berries contain amino acids, a proteolytic enzyme, condensed tannins, and flavonoids. Beside these compounds it has a high proportion of free amino acids which include proline, valine, tyrosine, alanine, glycine, hydroxyproline, aspartic acid, glutamic acid, cystine and

cysteine (Dhalla et al. 1961). Alam et al. (2011) reported eight polyphenols (gallic acid, syringic, benzoic, p-coumaric and vanillic acids as well as catechin, kaempferol and naringenin) in methanolic extracts of *W. somnifera* and reported higher level of catechin in these extracts. The phytochemicals present in *W. somnifera* are described in table 1.3.

Table 1.3: Chemical structures and biological activities of phytochemicals identified in *Withania somnifera* L.

Compound name	Structure	Biological activity	Reference
Cusco-hygrine		Psychoactive	Elsakka et al. 1990
Anahygrine		Anti-inflammatory	Santhi et al. 2011
Tropine		Hallucinogenic	Grynkiewicz and Gadzikowska 2008; Pigatto et al. 2015
Doursterol		Analgesic	Misra et al. 2008; Tanakara et al. 1933; Nigro et al. 1982
Ashwagandhin olides		Cardio-tonic	Das et al. 1964; Prasad 1993; Subbaraju et al 2006

Compound name	Structure	Biological activity	Reference
6,8-Dihydroxykampferol		Antioxidant	Kandil et al. 1994
Pseudotropine		Analgesic	Elsakka et al., 1990
Anaferine		Sedative Hypnotic	Elsakka et al. 1990; Singh et al. 2011
isopelletierine			Elsakka et al. 1990
withasomnine		CNS-Depressant Sedative Antimicrobial	Schröter et al. 1966; Sharma et al 2011
naringenin		Antioxidant	Alam et al. 2011; Ahmad et al. 2005

Compound name	Structure	Biological activity	Reference
Campesterol		Cardiovascular protective	Chatterjee et al. 2010
Stigmasterol		Anticancer	Misra et al. 2008
β-sitosterol		Hypocholesterolemic	Misra et al. 2008
Pheophytins a & b		Antitumor	Chatterjee et al. 2010
Withaphysanolide A		Cytotoxic	Ma et al. 2007

Compound name	Structure	Biological activity	Reference
Withaferin-A		Anti- angiogenic Anti-tumor Anti-cancer	Mohan et al. 2004; Sharada et al. 1996
Withanone		Anticancer Neuroprotective	Konar et al. 2011; Vaishnavi et al. 2012; Widodo et al. 2010
Withanolide-A		Immunomodulatory Neurotic regeneration	Kuboyama et al. 2005; Malik et al. 2007
27-Hydroxy withanone		Anti-cancer	Chaurasiya et al. 2008; Lal et al. 2006; Saddique et al. 2014
27-Hydroxy withanolide B		Anti-proliferative	Chaurasiya et al. 2008; Zhang et al. 2014
17-Hydroxy, 27-deoxy withaferin-A		Immunomodulatory	Kushwaha et al. 2012; Supriya 2009

Compound name	Structure	Biological activity	Reference
27-Deoxy withaferin-A		Anticancer	Siddique et al. 2014
12-Deoxy withastramonolide		Anticancer	Sivanandhan et al. 2014a; Rana et al. 2012
Withanoside-IV		Antidementia Neuronal regeneration	Kuboyama et al. 2006; Nakayama and Tohda 2007
Withanoside II		Inhibit tachyphylaxis	Matsuda et al. 2001
Withanoside-VI		Inhibit tachyphylaxis	Chatterjee et al. 2010; Trivedi et al 2016
Withanoside IX		Neuron regeneration	Matsuda et al. 2001; Nakayama and Tohda 2007; Singh et al. 2011 Zhao et al. 2001

Compound name	Structure	Biological activity	Reference
Physagulin D		Cyclooxygenase-2 inhibitory	Jayaprakasam and Nair 2003
1-Octanol		Antioxidant	Chatterjee et al. 2010
3,4,5 Tryhydroxy cinnamic acid		Anticancer Antioxidant	Chatterjee et al. 2010 Batra and Sharma 2013
Withanamides		Cure from Alzheimer disease Neuroprotective	Jayaprakasam et al. 2010; Vareed et al. 2014; Jayaprakasam and Nair 2007
Somniferine		Hypnotic	Mir et al. 2013; Mirjalili et al. 2009; Jumdar and Guha 1933

Compound name	Structure	Biological activity	Reference
Sitoindoside VII		Antistress	Singh et al. 2011; Bhattacharya et al. 1987; Mirjalili et al. 2009
sitoindoside IX		Antioxidant	Rastogi et al., 1998; Vimal et al., 2010
withanolide-D		Induce apoptosis	Chaurasiya et al. 2008; Lavie et al. 1968; Mondal et al. 2010
Withanolide-G		Immunosuppressive	Glotter et al. 1973; Huang et al. 2005; Roja et al. 1991; Vitali et al. 1996
Withasomine		Anti-inflammatory	Santhi and Swaminathan 2011

1.4. Importance of Phenolic and flavonoids in biological activities of *Withania*

The nutritional and pharmacological properties of medicinal plants are a result of synergistic interactions of many different phytochemicals. To date, very few have reported phenolic compounds in *W. somnifera* leaves, roots and fruits. While *Withania* have extensively been studied for its withanolides composition and several withanolides have been reported for effective therapeutic potential (Table 1.2). Dhanani et al. (2013) believed that the extract of *W. somnifera* is a complex mixture that also contain flavonoids and phenolics and attributed the antioxidant activity to these compounds as well. Similarly, Alam et al. (2011) reported phenolic and flavonoid compounds from leaf, root, stem and fruit extracts and strongly correlated DPPH free radical scavenging activity to these compounds. Few other studies have also reported these compounds in *W. somnifera* (Choudhary and Yousuf 2013; Fernando et al. 2013; Mehrotra et al. 2011; Sharma et al. 2012; Udayakumar et al. 2010). Alam et al. (2011) further investigated using HPLC and found five phenolics (Gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol and naringenin) compounds in *W. somnifera.*

Previously, Ferreira et al. (2010) reported the antimalarial activity of *Artemisia absenthium* due to the synergistic effect of flavonoids and phenolics with artemisinin. Similarly, extensive reviews have concluded the importance of these compounds in dietary sources and other plants (Ghasemzadeh and Ghasemzadeh 2011; Rice-Evans et al. 1997). Phenolic and flavonoids belongs to polyphenols, secondary metabolites of plants that gives taste, color and enhance the nutritional properties of food (Chenyier, 2005). These polyphenols are low molecular weight compounds that have diverse biological effects ranging from antioxidant activity to anticancer and cardio-protective potentials. Recent studies have shown higher antioxidant activity of these compounds than vitamin E and C. Farnando et al. (2013) attributed the antioxidant activity of *W. somnifera* leaves to its phenolic content.

1.5. Plant secondary metabolites production using *in-vitro* tissue culture technology

Plant secondary metabolites are bioactive compounds produced in plants beside the primary biosynthetic pathways and are considered as products of biochemical side tracks in plant cell. These side track compounds have profound effects on humans and animals as well for the plant itself like resistance, attraction of the pollinators and signaling etc. These compounds are in use of human traditional recipes since 6000 or in modern medicine as pure isolated compound for healing purposes. Today, only 10% of

all used medicinal plant species are cultivated, with by far the larger majority being obtained from wild collections. The collection of plants for medicinal purposes from wild have caused loss of genetic diversity and habitat destruction. Furthermore the quality and quantity of the active compounds in wild grown plants is compromised by the geographical and environmental conditions and also adulterated by the misidentified/closely related plant species. From this background *in-vitro* tissue culture technology have got considerable interest, especially strategies to enhance the end products yield. The manipulation of *in-vitro* tissue culture is well known and acceptable to scientist and industrialist.

There are numerous successful stories of plant tissue culture based enhanced production of secondary metabolites. Micro-propagation, cells, hairy roots and adventitious root cultures have successfully been used for the secondary metabolites production on commercial scale. Recently in our laboratory, we have successfully established cell suspension culture of *Artemisia absenthium*, regeneration system for medicinally consistent *Silybum marianum* and *Pipper nigrum*, and adventitious root culture for *Prunella vulgaris*. Similarly, others have established adventitious root culture systems of several valuable medicinal plants such as *Morinda citrifolia*, *Echinacea purpuria* and *angustifolia*, *Hypericum perforatum* using large scale (500–1000 L) bioreactors. Previously, adventitious root culture system of *Panax ginseng* using 10-ton scale bioreactors for the production of ginsenosides have successfully been reported (Paek et al. 2009).

1.5.1. *In-vitro* tissue cultures of *W. somnifera*

Members of the genus *Withania* produce a large number of secondary metabolites that show biological activities. The most interesting ones from pharmacological point of view are withanolide and flavonoids among many other metabolites. However, *in-vitro* cultures are developed to enhance these metabolites, particularly withanolide which is present in minute quantities in wild plants of *W. somnifera*. Table 1.3 represents the *in-vitro* cultures of *W. somnifera* for production of desired secondary metabolites.

Table 1.4: Use of *in-vitro* tissue culture approaches for enhanced *W. somnifera* L. secondary metabolites production.

Culture type	Culture media and Plant growth regulator used	Method used	Active ingredient detected	Amount	References
Callus	MS, 2,4-D	-	-	-	(Manickam et al. 2000)
Callus	MS, 2,4-D	-	-	ND	(Roja et al. 1991)
Callus	MS, 2,4-D±Kin	-	-	-	(Rani et al. 2003)
Callus	MS, 2,4-D±Kin	-	-	-	(Rani and Grover 1999)
Callus	B5/MS, NAA/BAP±IBA	-	Withanolide A Withanon Withaferine A Withanolide B Withanolide E	0.176 µg g^{-1} DW 0.044 µg g^{-1} DW ND ND ND	(Sharada et al. 2007)
Callus	MS, IBA±2,4-D		Phenolics	20 mg g^{-1}	(Singh et al. 2011)
Embryogenic callus	MS, IAA±IBA	Ca free media	Withaferine A	Higher than callus	(Swathia et al. 2013)
Shoot	MS, BAP±TDZ		Withaferine A		(Ray and Jha 2001)
Shoot	MS/NN, BAP, spermidine	Liquid media, MeJa/SA	Withanolide A Withanolide B Withaferin A Withanone	8.48 mg g^{-1} DW 15.47 mg g^{-1} DW 29.55 mg g^{-1} DW 23.44 mg g^{-1} DW	(Sivanandhan et al. 2012)
Regenerated plants	MS, Kin±IBA	-	Withanolide A Withanone	0.023 % 0.023 %	(Rana et al. 2012)

Culture type	Culture media and Plant growth regulator used	Method used	Active ingredient detected	Amount	References
			Withaferine A 12-deoxy-withanostramonolide Withanostramonolide 27-hydroxywithanone Withanoside	0.241 % 0.225 % 0.094 % ND 0.009 %	
Shoots			Withaferin A		(Ray and Jha 2001)
Galls	-	*Agrobacterium tumefaciens*	Withaferin A Withanolide D	0.07-01 %DW 0.085-0.025 % DW	(Ray and Jha 1999)
Hairy roots	-	*A. rhizogenes*	Withaferin A	0.44 % DW	(Bandyopadhyay et al. 2007)
Hairy roots	MS	*A. rhizogenes* Sucrose 5% ± Triadimeform	Withaferine A	3866 mg g^{-1} DW	(Doma et al. 2012)
Hairy roots	½ MS	*A. rhizogenes*	Withaferine A	72.3 mg g^{-1}	(Saravanakumar et al. 2012)
Hairy roots	MS	*A. rhizogenes strain R1601*, 40 g/L sucrose, liquid	withanolide A	157.4 µg g^{-1} DW	(Murthy et al. 2008)
Hairy roots	MS	*A. rhizogenes* 4% sucrose pH 6	Withanolide A	13.84 mg g^{-1} DW	(Praveen and Murthy 2012)
Hairy roots	MS	Salicylic acid	Withanolide A Withanone	132.44 mg g^{-1} DW 84.35 mg g^{-1} DW	(Sivanandhan et al. 2013)

Culture type	Culture media and Plant growth regulator used	Method used	Active ingredient detected	Amount	References
			Withaferine A	70.74 mg g^{-1} DW	
Adventitious roots	½ MS, IAA±IBA	Shake flask Bubble column bioreactor	Withanolide	3 mg g^{-1} 10 mg g^{-1}	(Wadegaonkar et al. 2006)
Adventitious roots	½ MS, IBA	$NH_4^{\pm}$: NO_3^-	Withanolide A	11.76 mg g^{-1}	(Murthy and Praveen 2012)
Adventitious roots	½ MS, IBA/IAA	Bioreactor	Withanolide A	0.0009%	(Wasnik et al. 2009)
Adventitious roots	MS, IBA ± NAA	Shake flask, Chitosan elicitation	Withanolide A Withanolide B Withaferin A Withanoside IV Withanoside V	323.85 mg g^{-1} 0.275 mg g^{-1} 4.347 mg g^{-1} 0.528 mg g^{-1} 0.450 mg g^{-1}	(Sivanandhan et al. 2012)
Cell suspension	¼ B5, 2,4-D±Zeatin± Sucrose	Shake flask± elicited with Salicin	Withaferine A	25 mg L^{-1}	(Ciddi 2006)
Cell suspension	MS, 50% sucrose	Shake flask *A. tumefaciens* $CuSO_4$ ± *V. dahaliae* extract (5%)	Withaferine A	36.57 mg L^{-1}	(Baldi et al. 2008)
Cell suspension	MS/N6/B5/NN, BAP±Kin	Shake flask Carbon source, pH	Withanolide A	2.5 mg g^{-1} DW	(Nagella and Murthy 2010)
Cell suspension	MS	Nitrogen source, Macro-elements	Withanolide A	3.96 mg g^{-1} DW	(Nagella and Murthy 2011)

Culture type	Culture media and Plant growth regulator used	Method used	Active ingredient detected	Amount	References
Cell suspension	MS,Picloram±Kin±glutamin±5%sucrose	Bioreactor Squalene ± Chitosan	Withanolide A Withanolide B Withaferine A Withanone 12-deoxy withastramonolide Withanoside IV Withanoside V	7606.75 mg 4823.05 mg 3732.81 mg 6538.65 mg 3176.63 mg 2623.21 mg 2861.18 mg	(Sivanandhan et al. 2014)
Cell suspension	MS, Glutamine, picloram ± Kin	Shake flask ± Sea weed extract	Withanolide A Withanolide B Withaferine A Withanone	5.04 mg g^{-1} 2.59 mg g^{-1} 2.36 mg g^{-1} 4.32 mg g^{-1}	(Sivanandhan et al. 2013)
Cell suspension	MS, 2,4-D ± Kin	Co-culturing ± *Piriformospora indica*	Withaferine A	2.04 times	(Ahlawat et al. 2016)
Transformed cells	MS	Shake flask ± Squalene synthase gene over expression	Withanolide A Withaferine A	0.16 mg g^{-1} 4.98 mg g^{-1}	(Grover et al. 2013)

1.5.2. Adventitious root culture

W. somnifera is a rich source of wide range of medicinally important secondary metabolites and its roots are extensively used for centuries in traditional medicines. Production of its important metabolites from naturally grown plant is not always satisfactory. It is often restricted to growth phase, season, nutrients availability, cultivation practices and post-harvest processing methods. Because of these constrains since past decades, adventitious root (AR) culture system have been extensively studied as an alternative source of secondary metabolites. Several studies have proved the importance of intact plant tissues culture over cell suspension culture in yield of secondary metabolites. Further adventitious roots culture are more acceptable to general public then hairy roots due to reluctance of public to genetically modified tags. The rapid growth rate of AR culture make it more attractive and feasible option for commercial scale production of secondary metabolites.

AR are post-embryonic roots that arises from unusual sites like, stem, old roots, leaves and callus. Anatomically these roots are different then lateral roots and arises from cells around cambium or parenchyma of phloem (De Klerk 1995; Ford et al. 2002; Smart et al. 2002). The induction of these roots is of key importance in AR cultures establishment and secondary metabolite production. Where auxins and explant type plays vital role and previously Indole acetic acid (IAA) and indole butyric acid (IBA) have successfully been used in the process (Table 1.4).

Murthy et al. (2008) describes four discrete steps involved in successful production of plant secondary metabolites using AR culture system. These stages are induction, proliferation, elicitation, large scale culturing and downstream recovery of secondary metabolites. Each step requires extensive consideration and selection of optimum parameters (PGRs, Media composition, environmental conditions etc.) to ensure enhance biomass and secondary metabolites production. There are several successful stories of secondary metabolite production in adventitious root culture at commercial scale such as *Morinda citrifolia*, *Echinacea* (*purpuria* and *angustifolia*), *Hypericum perforatum* and *Panax ginseng* (Baque et al. 2012). Where Baque et al. (2011) used leaf explant derived ARs of *M. citrifolia* in 500 L balloon type bubble column bioreactor and obtained higher level of anthraquinone, phenolics and flavonoids. Similarly, 20-1000 L BTBB have successfully bee reported for enhanced production caffiec acid derivatives using ARs of *E. purpurea.* Despite of immense importance of AR culture, *W. somnifera* still

a lot of considerations are needed to optimize the process for maximum biomass and secondary metabolite accumulations at commercial scale.

1.5.2.1. Induction of adventitious roots and role of melatonin

The initiation of ARs from explant is called induction and it is the unique property of plants that are formed at post-embryogenic stage of life. AR formation is a distinct form of organogenesis that involves the *de novo* initiation of a meristem as a result of several endogenous and exogenous (PGRs, light and temperature) signals. These signals are monitored through auxin-controlled signaling pathways that links other auxin responsive factors than those involved in lateral root formation (Gutierrez et al. 2009; Sorin et al. 2005; Sorin et al. 2006). During AR induction the role of classical auxins (IAA, IBA and NAA) and environmental factors such as wounding and light are well described by Blakesley (1994) but their role at molecular level is still not well understood. Different auxin types elicit differential rooting responses in *in-vitro* explants and this could be probably due to differences in binding capacity of these auxins to auxin receptor (T1N1). But Verstraeten et al. (2013) thinks differently and believes that several other factors such as transport, metabolism, conjugation and differential signal transduction mechanisms are involved in auxin induced AR induction. Beside these, several other phytohormones like gibberellic acid, abscisic acid, ethylene, alkamides and nitric oxides, polyamines and flavonoids are also reported to be involved in the AR induction and formation process (Pacurar et al. 2014). For ethylene it is proven that it increases the rate of cytokinin catabolism to increase the endogenous auxins level and thus lead to root organogenesis response (Verstraeten et al. 2013).

Beside the unleashed myth of AR induction mechanism, recently melatonin have also proven to be responsible for AR induction and formation in plants. Since the discovery of melatonin, it was believed to be a vertebrate hormone that regulates the sleeping cycle of animals (Kolář et al. 2003; Lerner et al. 1958). But its subsequent discovery in plants leaded the plant physiologist to unveil the possible rule of this animal hormone in plants. Its structural resemblance with IAA made it a candidate functional auxin (Pelagio-Flores et al. 2012). But the physiological role of melatonin in plants varies according to plant species, concentration and environmental factors i.e. light etc. Previously, it was proved as AR inducing regulator in *Hypericum perforatum* L. (Murch et al. 2001), *Prunus cerasus* L. (Sarropoulou et al. 2012), *Lupinus albus* (Arnao and Hernández-Ruiz 2007), *Brassica juncea* (Chen et al. 2009) and *Arabidopsis* (Pelagio-Flores et al. 2012). The mechanism of action of melatonin is still poorly understood and needs

further studies to explore its mechanism of action. While, (Wen et al. 2016) reported root promoting role of melatonin by regulating endogenous auxins and nitric oxide signaling in *Solanum lycopersicum* L. Contrarily, Kim et al. (Kim et al. 2016) reported unsound effect of melatonin in Maize coleoptile elongation, root growth and ACC synthase gene expression. Other than auxin like function, melatonin has several other physiological roles in plants i.e. fruit and flower development, shooting, increase oxidative stress resistance, and delayed senescence.

In animals the endogenous production of melatonin by pineal gland is effected with light and dark oscillation. The same phenomenon was studied by Kolář et al. (Kolář et al. 2003) in *Chenopodium rubrum* when exposed to light and dark cycles. But Manchester et al. (2000) reported contradictory results in *Aloe vera* and St. john`s wort. The biosynthetic pathway of melatonin is well ascribed and involves four sequential enzymes (TDC, T5H, SNAT, and N-aceylserotonin O-methyltransferase) to synthesis melatonin from tryptophan (Arnao and Hernández-Ruiz 2015).

1.5.3. Callus and cell suspension culture

Plant callus and cell suspension culture techniques were developed and established as an experiment necessity for solving basic plant biology questions like role of plant growth regulators, mechanism of tissue differentiation, plant cell response to pathogen and external stresses, metabolism and pathways elucidation. Apart from these studies callus culture have attained the promising industrial applicability in plant propagation, raising genetically modified plants, production of plant secondary metabolites and conservation of elite and endangered clones and species respectively. Callus is derived from Latin word, callum that means hard and in medical terms it is associated with hardening of dermal tissues. While in plants callus is referred to callose formation due to wounding. While in plant tissue culture, the term callus is used for the masses of disorganized cell. However, this definition is no longer accepted and confirmed that histologically callus cells resembles with the mother cells in explant (Atta et al. 2009). Later on Sugimoto et al. (Sugimoto et al. 2010) confirmed that the transcriptomic profile of the calli cells most closely resemble to that of root meristem.

Callus cells are totipotent and may give rise to whole plant or can be used to get the important secondary metabolites, produced in intact plant. Callus formation and its characteristics is an important controlling step for establishment of successful cell suspension cultures to get the desired applicability. Callus could

be formed from single differentiated cell through dedifferentiation (or simple cell division) process and may produce different kind of callus such as friable or compact callus or organogenic callus (rooty, shooty or embryogenic callus). Callus formation is a complex process that is still not well described at molecular level but it is largely accepted that exogenously application of auxins and cytokinin reprograms the differentiated cell to divide. Other plant hormones such as melatonin, brassinosteriods or abscisic acid have also proven to induce callus. Selection of PGR type and concentration, explant age and type are crucial to get the desired callus (friable or compact) of rapid growth rate and higher secondary metabolite content.

The first successful attempt for *in-vitro* production of withanolides and other secondary metabolites of *W. somnifera* was made from callus culture by Ciddi (Ciddi 2006). While earlier study of Roja et al. (Roja et al. 1991) reported withanolides production in *in-vitro* grown roots and claimed callus cultures were deficient in this regard. Similarly, Rani and Grover (Rani and Grover 1999) detected withanolides in trace amount in callus and cell culture of *W. somnifera*. Later on several other used callus but with different PGRs types then previously reported one, and observed higher withanolides content in callus culture (Table 1.4). Similarly Swathia et al. (2013) reported higher withanferine A content in embryogenic callus than undifferentiated calli. The production of secondary metabolites in callus culture still needs further considerations to get the desired higher content of secondary metabolites. But this system is constrained by batch to batch refreshment of the media for callus growth and make it more tedious option for large scale production. However, this dilemma is stunned by the use of cell suspension culture and several reports are available in literature since a decade ago. But still media composition, media strength, PGRs concentration, type and combination, carbon source and media pH are important factors to be carefully manipulated for enhanced production of secondary metabolites.

1.6. Effect of light quality on secondary metabolite production

Light is an important factor affecting growth, organogenesis and the formation of plant products including both primary and secondary metabolites (Shohael et al. 2006). The stimulatory effect of light on the formation of compounds, including flavonoid and anthocyanins has been shown in plants (Kreuzaler and Hahlbrock 1973; Tariq et al. 2014). In addition to the abovementioned effects of light on the production of plant secondary metabolites, light is also involved in regulating the secretion mechanism of secondary products (Liu et al. 2002). It is widely believed that the synthesis of secondary

metabolites in plants is part of the defense responses of plants to stress. The culmination of over a century of plant photobiology research shows that plants possess complicated photo-sensory networks that monitor and respond to a wide spectrum of ambient light energies.

There is no available report to study the effect of light quality on secondary metabolite production in the *in-vitro* cultures of *W. somnifera*. Lee et al. (2007) studied the *in-vitro* regenerated plantlets of *W. somnifera* under different spectral lights and concluded the profound effect of light quality on stomatal conductance, chlorophyll and carotenoid content. And concluded a profound growth under red and blue light mixture.

1.7. Anti-oxidant activity

1.7.1. *Antioxidants*

Compounds of synthetic or natural origin that stop or hold-up the oxidation of substrates (i.e. mostly lipids, but can also be a DNA molecule, carbohydrate or protein) even if the antioxidants are present in a considerably lesser amount comparatively oxidized substrate called anti-oxidants (Halliwell 1995). Antioxidants are used to maintain quality of food mainly by preventing lipid constituent ionic oxidative deterioration and also protect the human body from toxic effects of free radicals as well retard the progress of many chronic diseases (Gulcin et al. 2004). *W. somnifera* also contain higher amount of antioxidants increase the intrinsic properties of the end products. There are two types of antioxidants found in *Withania*, water soluble (Phenolic compounds, Vitamin C&E and folic acid) and lipid soluble (Carotenoids, etc.) antioxidants. The content of antioxidant in *Withania* varies with genotypes, plant part, environmental factors, and post-harvest treatments (Soengas Fernández et al. 2011). Restriction on the use of synthetic antioxidants due to their probable side-effects has increased the demand of natural antioxidants (Velioglu et al. 1998). Antioxidant activity of many phenolic compounds, including flavonoids, has attracted considerable attention and reported to be more powerful antioxidants than vitamins C, E and β-carotene which are largely in routine use. These natural antioxidants are also reported to decrease the risk of degenerative diseases and could have a protective effect against oxidative stress (Vinson et al. 1998). Antioxidants, such as vitamin A, vitamin C, vitamin E, carotenoids, polyphenolic compounds and flavonoids are found in plenty in vegetables and fruits and these

antioxidants control the free radical damage, reduce the risk of chronic diseases while atherosclerosis can be prevented by the consumption of dietary antioxidants from these sources (Barros et al. 2007).

1.7.2. Antioxidant Activity Determination Methods

Antioxidant properties have been studied in several plant species for the development of natural antioxidant formulations in the areas of food, medicine and cosmetics (Miliauskas et al. 2004). A number of *in-vitro* methods have been developed for estimation of antioxidant activity that is grouped to two main types:

1. Hydrogen ion transfer reactions
2. Electron transfer reactions (Huang et al. 2005).

This method`s diversity is due to the complexity of the analyzed substrates, often mixtures of dozens of compounds having different functional groups, polarity, and chemical behavior (Szabó et al. 2007)

1.8. Aims and objectives

The aim of the current work was to establish adventitious roots and cell suspension cultures of *W. somnifera* L. under different *in-vitro* conditions to enhance the secondary metabolites productions for sustainable use. Furthermore, attempts were made to correlate secondary metabolites production with antioxidant activity of *in-vitro* cultures in response to different treatments. The aim of the current study covers following objectives.

1. Establishments of adventitious roots culture and evaluation of melatonin role in adventitious root growth and secondary metabolites production.
2. Establishment of callus culture and evaluation of different light colors treatments on callus growth and secondary metabolism.
3. Establishment of cell suspension culture and evaluation of different media compositions for callus morphology and growth.
4. To evaluate the effect of different abiotic elicitors on biomass and secondary metabolites production.

2. INTERACTIVE EFFECTS OF MELATONIN AND LIGHT ON GROWTH PARAMETERS AND BIOCHEMICAL MARKERS IN ADVENTITIOUS ROOTS OF *WITHANIA SOMNIFERA* L.

2.1. ABSTRACT

Light plays a pertinent role in plant photo morphogenesis and it is believed to have an impact on the melatonin-induced physiological functions. In the current study, different light regimes were employed with varying levels of melatonin, either singly or in combination with auxins for the growth and development of adventitious roots in *Withania somnifera* L. It was observed that 600 µM melatonin favored maximum adventitious root induction frequency (58 %) in cultures incubated under continuous dark conditions. However, adequate root growth (number and length of roots) was observed under 16 h light/8 h dark at 600 µM melatonin. Nevertheless, the interactive effect of light and melatonin was found stimulating for profound production of commercially important secondary metabolites. Correlation among growth parameters and biochemical markers was also observed in the current report. Data on total phenolic content and total flavonoid content were found at higher coincidence with each other and with DPPH antioxidant activity. In conclusion, exogenously applied melatonin mimics IAA activity in root growth and regulates well in 16-h light/8-h dark, thereby giving protection to plant system against light stress.

2.2. INTRODUCTION

Withania somnifera L. (Indian ginseng) of family Solanaceae is a renowned medicinal herb with multiple pharmacological functions. It has been used in more than 100 indigenous medicinal recipes globally for over 3000 years. A very common problem associated with medicinal plant preparations is the extreme variability in the phytochemical content (Khan et al. 2015). This variability, in medicinal herbal products from wild plants, occurs due to contamination with biological and environmental pollutants, adulteration with misidentified species, quantitative and qualitative variation of bioactive compounds as well as unsustainable harvest (Abbasi et al. 2010; Khan et al. 2013). Application of plant *in-vitro* technology can circumvent these issues of variability in Withania end products.

However, the tissue culture of *W. somnifera* is still under the trials of optimization for better productivity (Mir et al. 2014; Murthy et al. 2014). One of the contemporary features of tissue culture, adventitious root formation is the result of a complex and distinctive process regulated by endogenous and exogenous factors (Ford et al. 2002; Sorin et al. 2005).

Melatonin, (N-acetyl-5-methoxytryptamine) is a naturally occurring indole amine initially discovered in vertebrates (Lerner et al. 1958) and has also been detected in several plant species (corn, rice, wheat, barley, and oats) after its discovery as phytohormone (Cao et al. 2006; Dubbels et al. 1995; Kola´r and Macha´ckova´ 2005; Tan et al. 2012). Manchester et al. (2000) reported higher melatonin concentration in black and white mustard seeds compared to the vertebrate's blood melatonin level. The comparatively much higher melatonin levels in plants are thought to be a compensatory response by plants because they, unlike animals, lack mobility as a means of coping with harsh environments. As the effect of melatonin on plant's physiology is being explored, there are now many reports which give an insight into its role in cell division and root development (Arnao and Herna´ndez-Ruiz 2007), regulating circadian rhythms and other photoperiod dependent processes (Herna´ndez-Ruiz et al. 2004; Kola´rˇ et al. 2003; Murch and Saxena 2002). Moreover, it has been employed as an alternative to IAA due to structural similarities (Pelagio-Flores et al. 2012). The endogenous level of melatonin is effected by photoperiod, and higher melatonin level have been observed in plants during dark (Wolf et al. 2001).

There is no report available which studies the impact of melatonin on leaf explant from *W. somnifera.* Furthermore, the synergistic effect of light and melatonin on adventitious root formation is also a less explored area. In this work we studied the effect of light and melatonin on adventitious root formation,

and the results were compared with those obtained from auxin-melatonin and auxin-induced root growth.

2.3. MATERIALS AND METHODS

2.3.1. Plant material and explants preparation

Seeds of *Withania somnifera* L. were obtained from National Agriculture Research Centre (NARC) Islamabad, Pakistan. Healthy and viable seeds were selected using float test method. These seeds were germinated in pots under growth room conditions. Leaf explants (*3.5 mm^2) were excised from 30 days old potted plantlets. Sterilization process was completed following the protocol of (Abbasi et al. 2010). Briefly, explants were treated with 70 % ethyl alcohol for 3 min, and then thoroughly washed stepwise with autoclaved distilled water followed by 0.1 % mercuric chloride treatment for 5 min and finally rinsed three times with autoclaved distilled water.

In-vitro conditions for adventitious roots growth and development. The surface sterilized leaf explants were inoculated on MS medium (Murashige and Skoog 1962) containing 3 % sucrose (w/v) and 0.8 % (w/v) agar in 100 ml conical flask supplemented with (4.9, 7.3, 9.8, and 19.6 µM) of IAA or IBA and incubated in plant growth chamber with a photoperiod of 16-h light/8-h darkness, light intensity of 100 µmol/m2/s, and temperature of 25 ± 2 C. In subsequent experiments different concentrations of melatonin (300, 400, 500 and 600 µM) alone or in combination with IBA (4.9 µM) were employed for induction and development of adventitious roots. For evaluation of effects of light regimes on regulation of melatonin during adventitious rooting, explants inoculated on MS media supplemented with IBA (4.9 µM) or melatonin (600 µM) or melatonin (600 µM) in combination with IBA (4.9 µM). The culture flasks were then kept in exposure to three different light treatments as (1) 24 h continuous light, (2) 24 h continuous darkness and (3) 16-h light/8-h darkness. For dark treatment flasks were thoroughly covered with aluminum foil. Data on adventitious roots were collected as percent root induction, number of roots per leaf explant, mean root length, number lateral roots and root fresh biomass respectively.

2.3.2. Analytical methods

For fresh weight (FW) determination, the fresh roots were harvested from 49 days old culture and gently pressed on filter paper to remove excess water, weighed and labelled according to the culture conditions. Subsequently, the plant materials were dried in oven at 35 C for 24 h and dry weight (DW) was recorded.

The dried roots were powdered and extracted in 80 % (v/v) methanol according to the protocol of Ali and Abbasi (2013). The methanolic extracts were stored at 4 ^{0}C for further analysis. For the estimation of total phenolic content (TPC), Folin-Ciocalteu (FC) reagent was used according to the method of Velioglu et al. (1998).

Total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method reported by Chang et al. (2002). For antioxidative enzyme activities, homogenate of *in-vitro* grown adventitious roots was extracted with ice-cold 0.5 M Tris–HCl (pH 6.8) buffer. The extracts were centrifuged at 10,000 rpm for 20 min at 4 ^{0}C and resulting supernatant was used for enzyme assays.

UV–visible spectrophotometer (Halo DR-20, UV–VIS spectrophotometer, Dynamica Ltd, Victoria, Australia) was used to determine absorption of extracts by the method of Khan et al. (2013).

Free radical scavenging potential was determined by the method of Abbasi et al. (2010) by using 2,2-diphenyl-1- picrylhydrazyl (DPPH) as free radical producer. The absorbance of the reaction mixture was recorded at 517 nm by spectrophotometer and 2 mg of butylated hydroxyanisole (BHA) per 4 ml of methanol was used for background correction as a decaying agent of DPPH to diminish the purple color of DPPH. The radical scavenging activity was calculated according to following formula and was expressed as % DPPH activity:

$$\%DPPH = 100 * (1 - \frac{AE}{AD})$$

Where A_E is absorbance of the solution, when extract (sample) was added at a particular level and A_D is the absorbance of the DPPH° solution with nothing added (Blank or negative control).

2.3.3. Data analysis

All experiments were repeated twice. Mean values of various treatments were subject to analysis of variance (ANOVA) and significant difference was separated using Duncan's Multiple Range Test

(DMRT). SPSS (Windows version 7.5.1, SPSS Inc., Chicago) was used to determine the significance at $P<0.05$.

2.4. RESULTS AND DISCUSSION

2.4.1. Effects of auxin on adventitious root growth

Indole acetic acid (IAA) and Indole butyric acid (IBA) were exploited in preliminary experiments under the control (16 h light/8 h dark) conditions. In these experiments, individual concentration (4.9 µM) of IBA produced maximum (20 ± 0.26) number of adventitious roots per leaf explant at higher (78.3 ± 0.392) rate of root induction and of maximum fresh biomass (1.94 ± 0.011) in lesser (17 days) incubation period. However, maximum (14 ± 0.096) lateral root number and lateral root length (4.5 ± 0.067) were observed in IAA (8.55 µM) treated leaf explant (Table 2.1). Murthy and Praveen (2012) induced direct adventitious rooting from leaf explant of *W. somnifera* on strength MS medium containing 0.5 mg l^{-1} IBA. Sivanandhan et al. (2012) reported adventitious root induction from leaf derived calli of *W. somnifera* on strength MS medium fortified with IBA in combination with NAA. The higher stability of IBA than IAA might be the possible reason for its potent adventitious roots production (Nordstro¨m et al. 1991). Similarly transportation, metabolism or uptake mechanism and conversion of IBA to IAA may also contribute to the superior adventitious root induction (Baraldi et al. 1995; Epstein and Ludwig-Mu¨ller 1993).

Table 2.1: Effects of different concentrations of IAA or IBA on adventitious root growth parameters from leaf explant in *Withania somnifera* L.

PGRs	Conc. (µM)	Days Required	%rooting	No. of roots/explant	Root length (cm)	lateral root number	Fresh biomass (g)
IAA	5.7	19±0.22	50.8±1.1	6.7±0.87	1.8±0.078	6.8±0.146	0.55±0.0139
	8.55	19±0.22	59±1.13	8.2±0.25	4.5±0.067	14±0.096	0.38±0.0023
	11.25	16±0.21	63.5±0.751	9±0.21	2.6±0.064	12±0.076	0.68±0.0086
	22.8	20±0.23	57.2±0.96	7.1±0.15	1.7±0.14	11±0.5	0.29±0.004
IBA	4.9	17±0.2	78.3±0.392	20±0.26	3.6±0.14	6.3±0.6	1.94±0.011
	7.35	17±0.2	69.1±0.177	13.45±0.58	3.7±0.054	9.9±0.83	1.73±0.056
	9.8	18±0.2	60.8±0.426	9.67±0.79	2.3±0.092	10±0.9	0.85±0.049
	19.6	17±0.23	63±0.212	15±0.75	3.6±0.18	7.6±0.1033	0.74±0.038

2.4.2. Effects of melatonin on adventitious root growth

In subsequent experiment, maximum root induction frequency (83.1 ± 0.123) was observed in leaf explant, treated with 4.9 µM IBA in combination with 500 µM melatonin and it was two times higher than melatonin treated leaf explant. However, it was statistically not different from control (Fig. 2.1). Previously, best adventitious rooting response has been observed on IBA in combination with melatonin in *Prunus cerasus* shoot tip explant (Sarropoulou et al. 2012), which is supporting our data. It shows that combination of auxin with melatonin is superior to melatonin-alone treatments in inducing healthy adventitious roots in leaf explant of *W. somnifera*. Adventitious roots obtained in melatonin augmented media were green in appearance and highly branched (Fig. 2.2c).

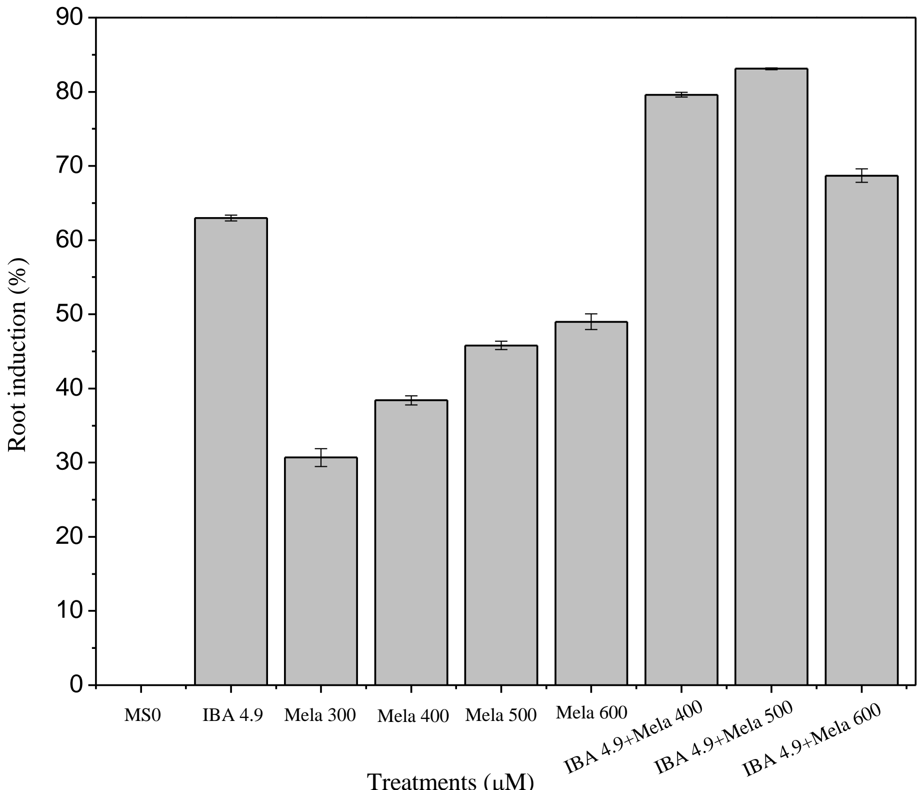

Figure 2.1: Effect of different concentrations of melatonin either alone or in combination with IBA on adventitious root induction frequency in leaf explant of *Withania somnifera* L. The values are the means of triplicates with ± S.E.M..

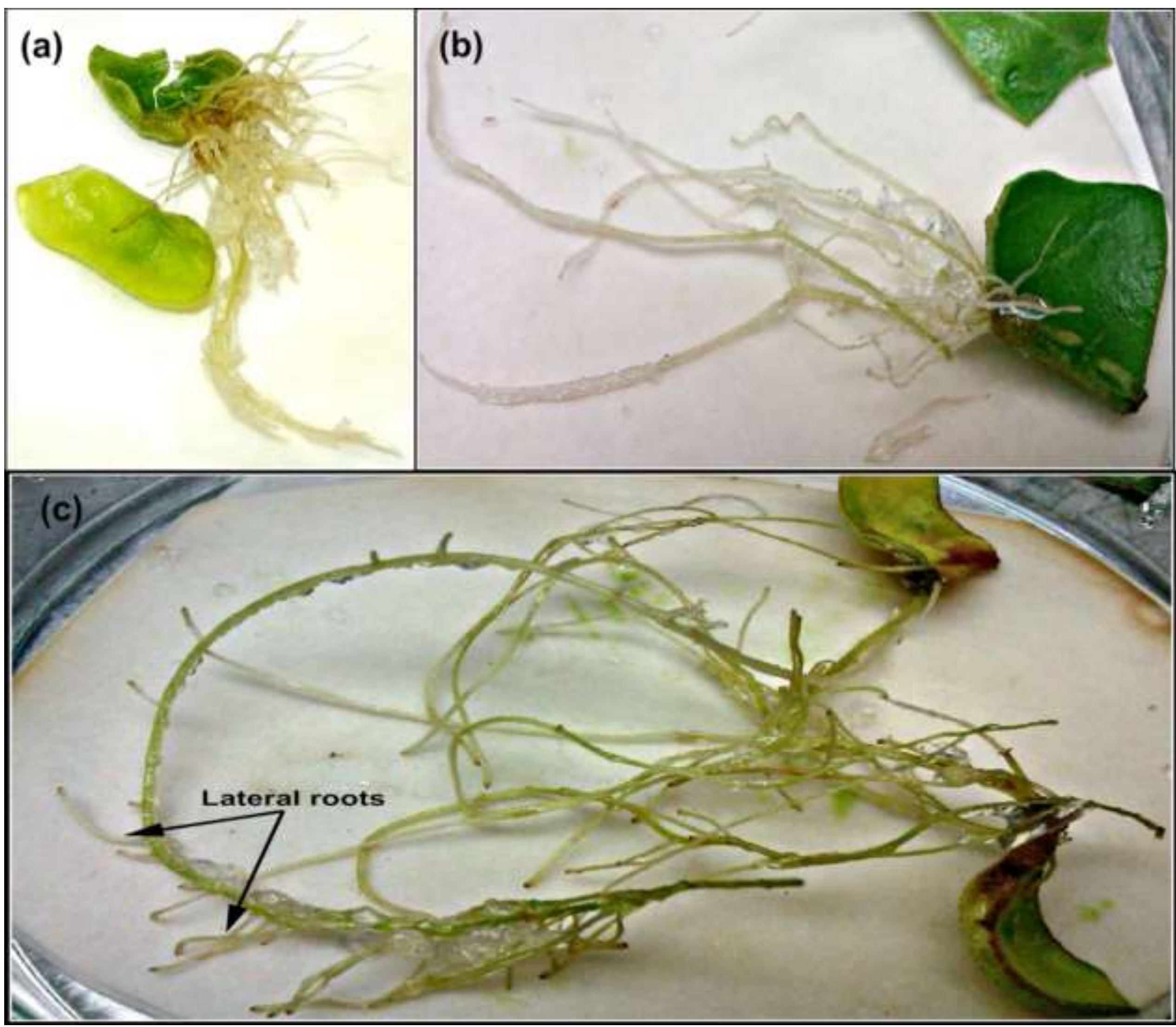

Figure 2.2: Adventitious roots **(a)** induction in leaf explant **(b)** elongated roots and **(c)** lateral root number.

Furthermore, the number of roots, lateral root length and root fresh biomass were significantly affected by different concentrations of melatonin/in combination with IBA treated leaf explants (Fig. 2.3). 600 µM of melatonin-alone produced maximum (7.3 ± 0.41) roots per explant in our study.

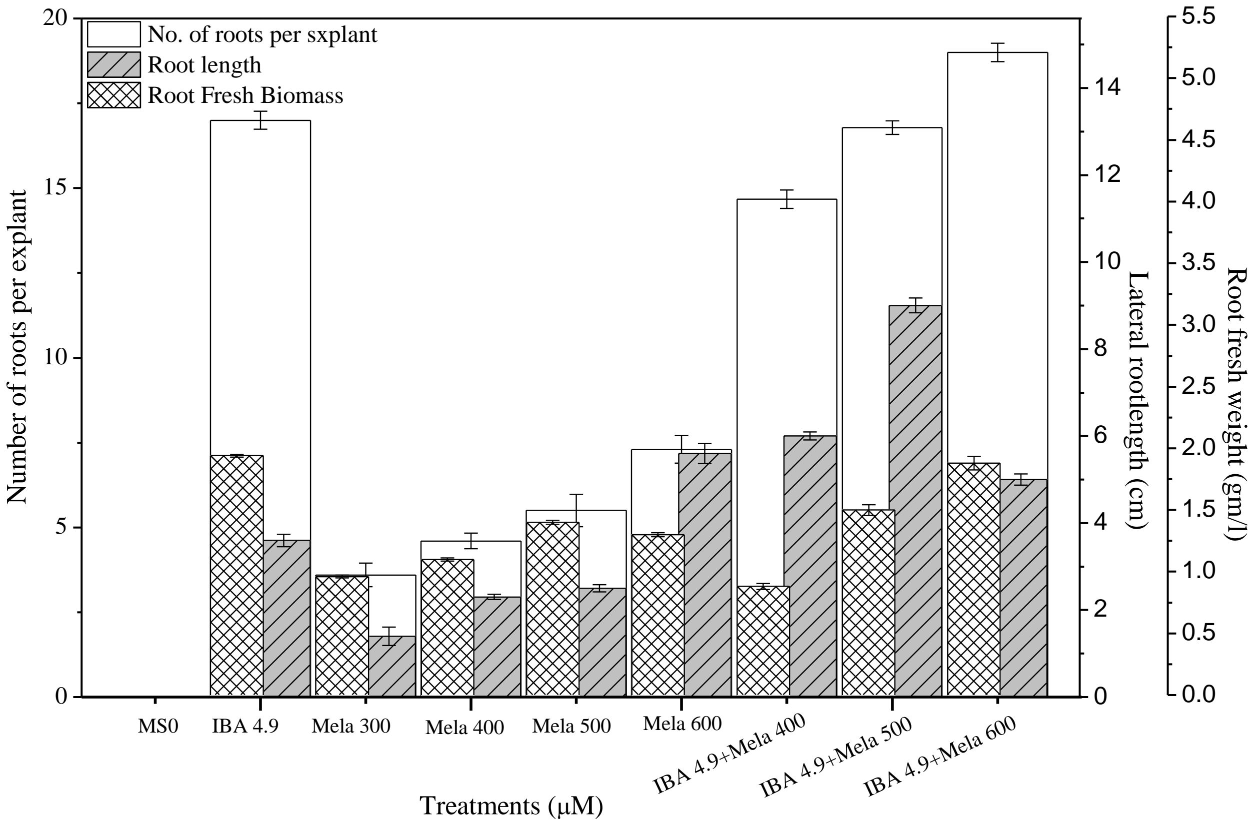

Figure 2.3: Effect of melatonin and its combination with IBA on adventitious root growth of *Withania somnifera* L. The values are means of triplicates with ± S.E.M.

Approximately, threefold increase in number of roots per explant was observed when melatonin 600 µM was augmented with IBA 4.9 µM. However, maximum (9 ± 0.17 cm) root length was observed on MS medium containing IBA in combination with 500 µM melatonin and this value was about three times greater than that of controlled treatment. Fresh biomass was comparatively higher (1.94 ± 0.01 g) at IBA (4.9 µM) augmented MS medium and followed by 1.88 ± 0.05 g on the MS medium containing 4.9 µM IBA along with 600 µM melatonin.

Previously, root promoting properties of melatonin were reported by Arnao and Herna´ndez-Ruiz (2007) and Sarropoullou et al. (2012) in *Lupinus albus* and sweet cherry, respectively. However, contrary to our findings, they also reported callogenesis in melatonin treated cuttings of sweet cherry; whereas, we observed callogenesis in a-naphthalene acetic acid (NAA) treated leaf explant and direct rooting from melatonin treated leaf explants. Probably, the adventitious rooting properties of melatonin might be due to its structural similarities with IAA or may increase the endogenous level of auxin in plants (Pelagio-Flores et al. 2012).

2.4.3. Effects of photoperiod on melatonin treated leaf explant

The present study shows that 16/8-h photoperiod, continuous dark and light conditions were sufficient to cause significant variations in melatonin-induced root growth (Fig. 2.4). In 24-h dark, early root initiation was observed followed by 16/8-h photoperiod. Inclusively, maximum root induction percentage was observed in 16/8-h photoperiod incubation conditions irrespective of the regulator used. Whereas leaf explant responded well to melatonin alone concentration in MS medium under 24-h dark conditions. Continuous light conditions were proven oppressive for root initiation on MS medium containing IBA or melatonin alone or in combination (Fig. 2.4a). In similar way, 16/8-h photoperiod favored maximum number of adventitious roots per explant when explant was cultured on MS medium containing IBA in combination with melatonin. Whereas only melatonin produced optimum number of roots per explant when kept in continuous dark (Fig. 4b). For root fresh biomass light was found more appropriate but when the duration of light was exceeded from 16-hr, a reduction in root fresh biomass was recorded (Fig. 2.4c). Light plays an important role in root morphogenesis and is believed to effect the function of melatonin in plants (Afreen et al. 2006; Kangasja¨rvi et al. 2012; Vollsnes et al. 2012). In our study, the roots induced in continuous dark conditions were slender in appearance and sluggish in growth. Importantly, the leaf explant incubated under continuous dark condition in medium containing melatonin remained green for several days that indicates its association with biosynthesis of chlorophyll (Arnao and Herna´ndez-Ruiz 2009).

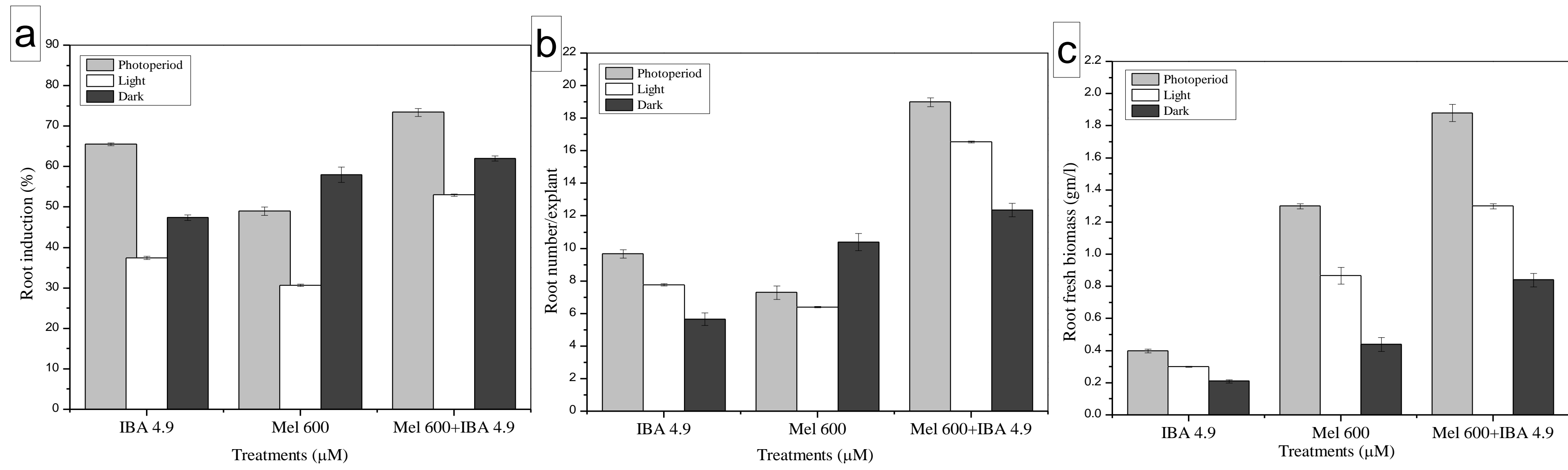

Figure 2.4: Effect of melatonin and indole-3-butyric acid (IBA) alone or in combination, coupled with 16/8h photoperiod or 24h dark or 24h light condition on percent adventitious root induction (a), number of roots per explant (b) and Fresh root biomass.

2.4.4. Antioxidative enzymes activities

During morphogenesis, the antioxidative enzymes (SOD and POD) play vital role in protecting the cells from deleterious effects of free radicals, produced during the normal physiological process (Suzuki and Mittler 2006). The biotic and abiotic stresses disrupt the metabolic balance of cells, resulting in accumulation of reactive oxygen species (ROS) and oxidative burst (Mittler et al. 2004). The total protein content, Protease, Superoxide Dismutase (SOD) and Peroxidase (POD) activities of adventitious roots varied significantly with the variations in light conditions during incubation (Fig. 2.5). Maximum (940 µg BSAE/mg FW) total protein content and protease activity (3.44 U/g FW) were observed in roots exposed to continuous lighting and on IBA alone (Fig. 2.5). With application of melatonin the total protein content and protease activity were reduced to 700 µg BSAE/mg FW and 2.36 U/g FW, respectively. The lowest total protein content and protease activity was observed in medium incorporated with melatonin at 24-h dark incubation conditions (Fig. 2.5).

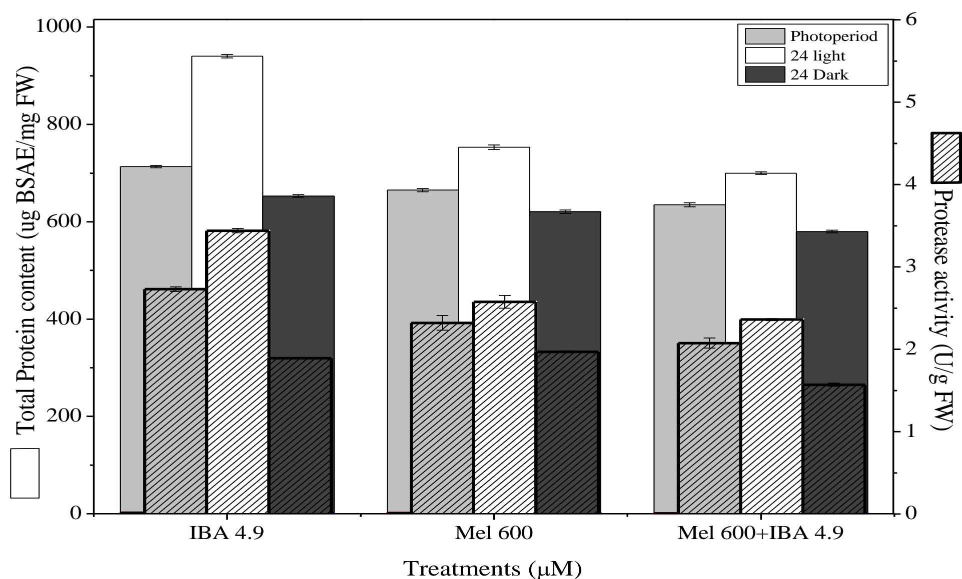

Figure 2.5: Effects of photoperiod in conjunction with melatonin alone or in combination with IBA on total protein content and protease activity of adventitious roots. The values shown represent the means of triplicate extractions ± S.E.M.

Continuous light exposure lead to higher total protein content and ultimately resulted in maximum SOD and POD enzyme activities. Maximum (0.22 nM/min/mg FW) SOD and POD (0.97 nM/min/mg FW) activities were observed in adventitious roots obtained at 4.9 µM IBA containing MS medium under the continuous 24-h light conditions (Fig. 2.6). Whereas in complete dark (24-h) incubation conditions resulted in lower enzymatic activities and these values were reduced further when melatonin was introduced to medium. Similar observations were made by Arnao and Herna´ndez-Ruiz (2014) and ascribed that melatonin alleviated light-induced stress in roots. Contrary to ours findings, Shi et al. (2015) reported that melatonin activates several antioxidants to reduce abiotic stress in Bermuda grass.

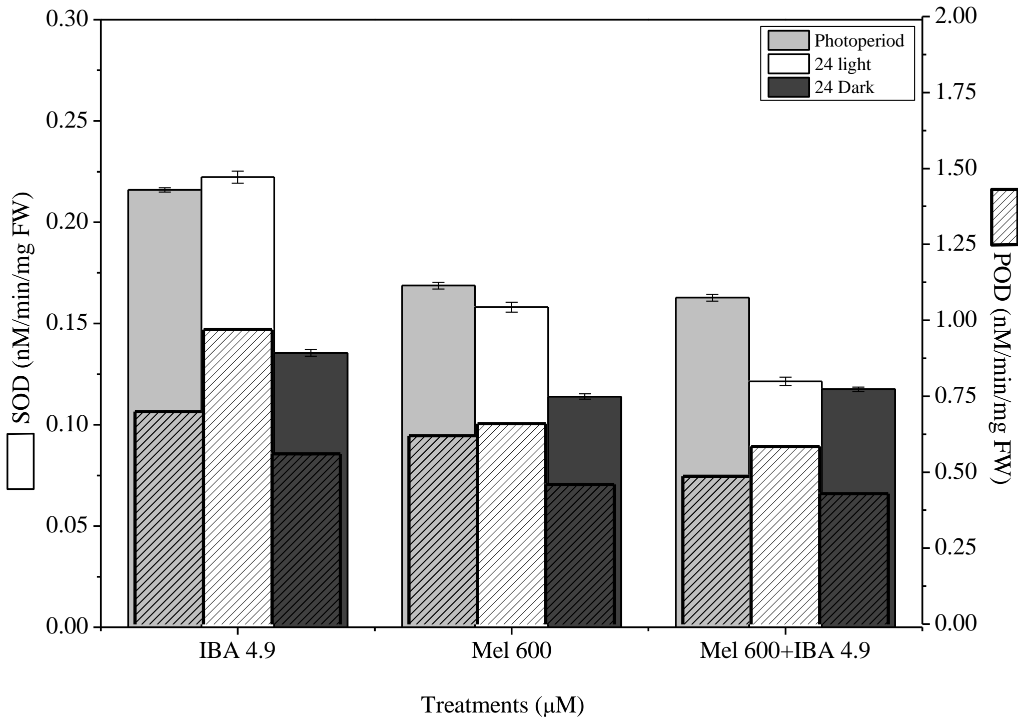

Figure 2.6: Antioxidative enzymes activities in adventitious roots of *W. somnifera* L. grown under different incubation conditions. The values shown represent the means of triplicate extractions ± S.E.M.

2.4.5. Secondary metabolites production and antioxidant activity

Secondary metabolism in roots could be linked to the stress produced during their incubation in illuminated conditions. Similar findings were made by Yokawa et al. (2011), where immediate and

strong burst of reactive oxygen species (ROS) was observed in plant cells. Plants produce secondary metabolites (phenolics and flavonoids) as a defense mechanism to scavenge ROS. Therefore, enhanced levels of these secondary metabolites were found in light grown roots. The content of these metabolites varied significantly according to the type of plant regulator exploited and was found strictly dependent on light/dark regimes (Fig. 2.7).

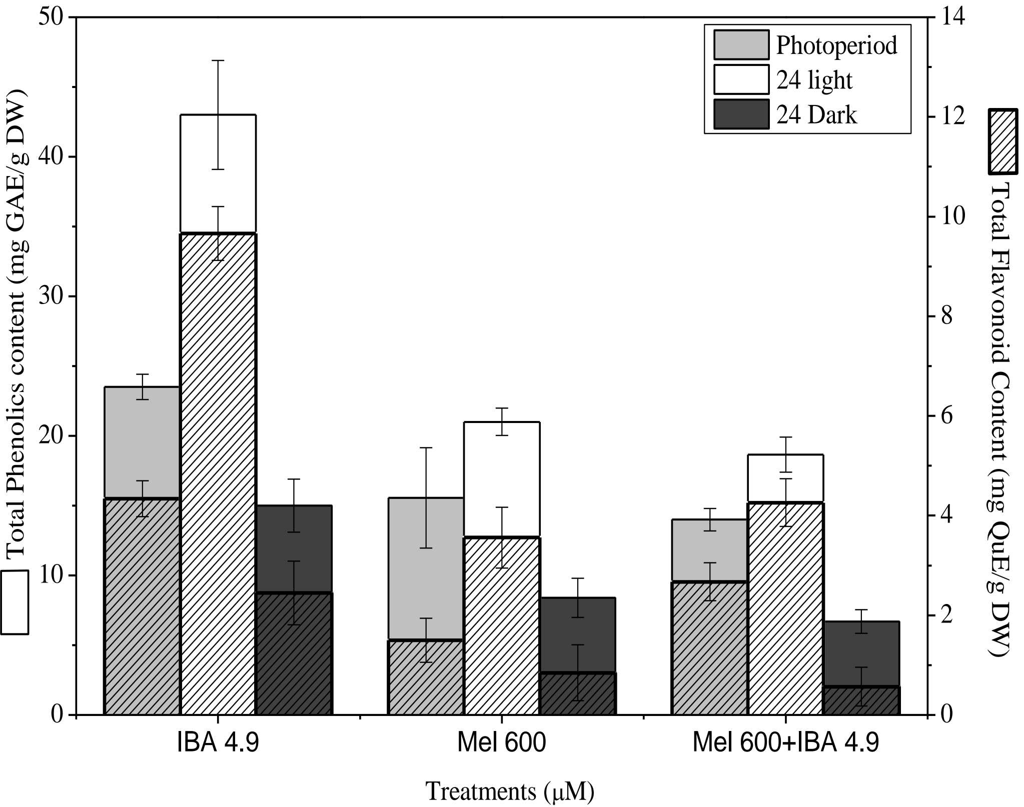

Figure 2.7: Total phenolics and Flavonoid content (mg/g DW) in adventitious roots grown under different photoperiod regimes. The values shown represent the means of triplicate extractions ± S.E.M.

Adventitious roots obtained on MS medium containing melatonin, contained lower levels of these metabolites. The decreased levels of secondary metabolites in roots strongly confers that melatonin alleviated light-induced stress in roots (Arnao and Herna´ndez-Ruiz 2014). Furthermore, the enhanced biosynthesis of these metabolites were correlated with DPPH-scavenging activity (Fig. 2.8). But roots

grown in continuous dark on medium combined with melatonin showed relatively higher scavenging activity and confers the potential of melatonin to act as an antioxidant compound (Bajwa et al. 2014; Tan 2015).

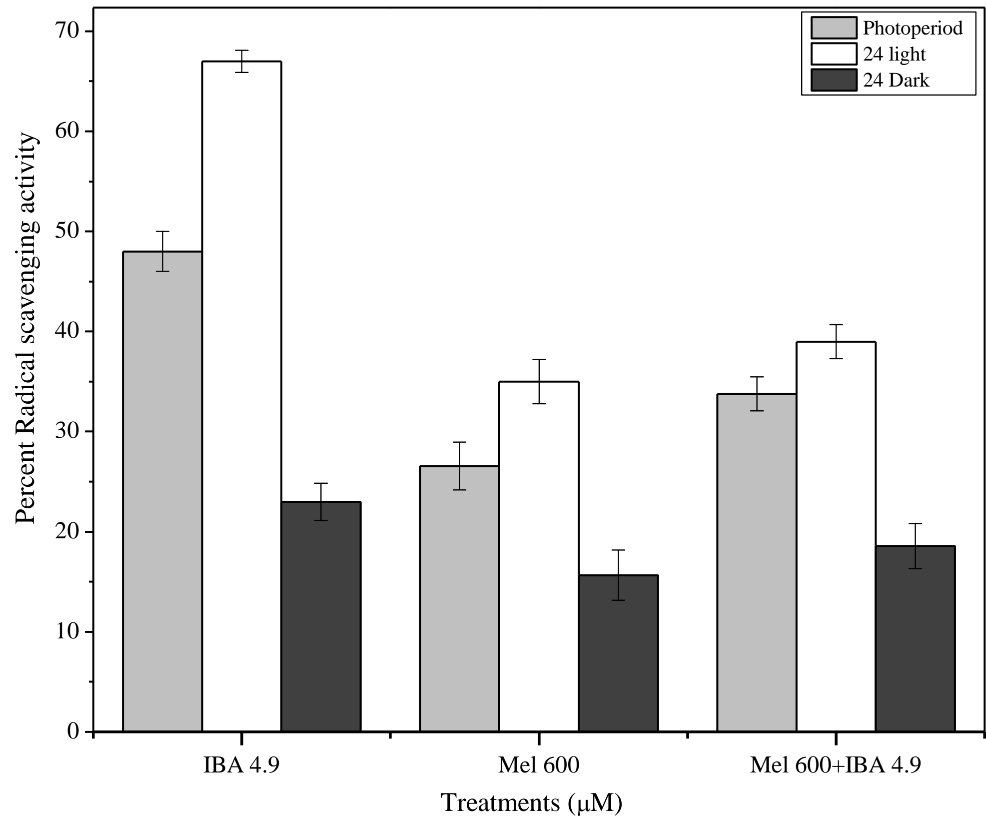

Figure 2.8: DPPH free radical scavenging activity in methanolic extracts of adventitious roots grown in-vitro under different photoperiod regimes. The values shown represent the means of triplicate extractions ± S.E.M.

2.5. CONCLUSIONS

The effect of day length and melatonin on *W. somnifera* leaf explant has been investigated. Results showed that melatonin in higher concentration favored maximum adventitious rooting in constant dark. However, the root growth parameters (number and length of roots) were adequate in 16 h light/8 h dark. Furthermore, antioxidant activities and free radical scavenging compound (Phenolics and Flavonoids) content was effected by both light regime and concentration of melatonin. The auxin like as well as protective function of melatonin has been elucidated in this study. The results obtained in this work directs at future research for large scale cultivation of adventitious roots and ultimately enhanced production of plant secondary metabolite

3. ELICITORS ENHANCED BIOMASS ACCUMULATION AND ANTIOXIDANT COMPOUNDS IN ADVENTITIOUS ROOT CULTURE OF *WITHANIA SOMNIFERA* L.

3.1. ABSTRACTs

Withania somnifera is a medicinally important plant widely used in the folk medicine against many serious ailments. In the present study, effects of important elicitors including Melatonin (Mela), Methyl Jasmonate (MeJa) and salicylic acid (SA) were estimated on growth kinetics, secondary metabolites accumulation and withanolides production in adventitious root suspension cultures of *W. somnifera*. We observed optimum biomass accumulation in adventitious roots cultivated on Murashige and Skoog (MS) medium supplemented with 6.4 µM Mela and 4.9 µM Indole Butyric Acid (IBA). Growth kinetics of root cultures was evaluated on weekly basis for a period 6 weeks (42 days). Maximum fresh and dry weight of 13 g L-1 and 0.15 g L-1 respectively were recorded for day 35 on media containing Mela/IBA.

Furthermore, maximum total phenolic content of 35.8 mg g-1 DW and highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of 75.3% were observed in roots elicited with MeJa 100 µM and 25 µM respectively in Mela/IBA containing medium. FT-IR and HPLC analysis revealed production of notable withanolides such as moupinamide, withaferin A and chlorogenic acid at considerable amounts in the roots elicited with MeJa (300 µ mol) raised in vitro at IBA/Mela containing media.

3.2. INTRODUCTION

The genus *Withania* comprises of 23 species, widely distributed in the drier parts of the tropical and subtropical zones; ranging from the Canary Islands, the Mediterranean region and northern Africa to Southwest Asia (Nambiar 1993; Hunziker 2001; Mirjalili et al. 2009). Among them two species, *W. somnifera* and *W. coagulans* are of significant importance in plant based pharmaceutical industries. The market demand of plant material for *W. somnifera* has increased tremendously from 7028 tons in 2002, to more than 10000 tons in the recent years (Shrivastava and Sahu 2013; Verma and Singh 2014). In India, it's per hectare production is about 0.78 tons that is not fulfilling the current market demand (Ashashri et al. 2015). Despite of its natural availability in Pakistan its demand is not in accordance to its supply, however it is imported (3.8 tons per year) from India and China.

Roots of *W. somnifera* are used in over 200 formulations in Ayurveda, Siddha and Unani medicine, which are used in the treatments of various physiological disorders (Asthana and Raina 1988; Mirjalili et al. 2009). The therapeutic potential of these roots is attributed to the presence of phenolics, flavonoids and steroidal lactones. Withanolides present in its roots, have proven to be effective in treatment against bronchial asthma, inflammation, cancer, and autoimmune diseases (Misico et al. 2011). In comparison to conventional cultivation procedures, plant cell cultures have emerged as a promising platform for the biosynthesis of valuable metabolites in limited time and space (Khan et al. 2013). Adventitious roots act as biosynthetic factories for production and accumulation of much valuable health promoting phytochemicals including phenolics, flavonoids, alkaloids etc. (Khan et al. 2015).

Plants accumulate secondary metabolites under different stresses like temperature, elicitors or signal molecules, irradiation of different intensities of UV or visible light, injury, nutrient deficiencies, pathogen attack, herbicide treatment etc. (Dixon and Paiva 1995; Dias et al. 2016). In vitro application of elicitors notably Methyl Jasmonate (MeJa) and Salicylic acid (SA) to enhance production of plant secondary metabolites has proven an effective strategy (Thanh et al. 2005; Murthy et al. 2014). Previously, root cultures of *W. somnifera* were evaluated for sustainable production of withanolides (Praveen and Murthy 2010; Sivanandhan et al. 2013&2014). However, production and enhancement of commercially important secondary metabolites is yet to be discovered.

Therefore, the aim of the current study was the establishment of an efficient and reproducible protocol for formation of adventitious roots in *W. somnifera*. Furthermore, evaluation of the essential components

of plant antioxidant system was elucidated to understand the phenomenon of elicitation for production of withanolides through root suspension cultures.

3.3. MATERIALS AND METHODS

3.3.1. Induction and elicitation of adventitious root culture for biomass accumulation

Adventitious roots (AR) were induced from leaf explant of in-vitro grown seed derived plantlets, as described previously (Adil et al. 2015). AR were excised from leaf explant, cut into inoculum size of about 9-12 cm and transferred to 250 ml flasks containing 50 ml MS (Murashige and Skoog 1962) liquid media; varying in concentration and combination of Indole-3-butyric acid (IBA; 4.9, 7.38 and 9.8 µM), Indole-3-acetic acid (IAA; 5.7, 8.55 and 11.25 µM) and Melatonin (Mela; 2.15, 4.3, 6.4 and 8.6 µM). Further gibberellic acid (GA_3; 4.33 µM) was used in combination with IBA (4.9 µM) or IAA (5.7 µM) respectively.

For elicitation, sterilized methyl jasmonate (MeJa; 25, 50 and 100 µM) and salicylic acid (SA; 100, 200 and 300 µM) were introduced to 21 days old adventitious roots suspension culture. Roots were harvested after 3 weeks from elicitation media. MS liquid media containing IBA, IAA, IBA/GA_3, IBA/Mela and Mela without elicitors were used as control. Triplicate flasks were used in all experiments. Before autoclave (121 ^{0}C for 20 min) pH of all media was adjusted to 5.7 and 30 g L^{-1} sucrose was added as carbon source. All cultures were incubated in a shaker incubator at 16 h photoperiod with light irradiance of ~ 40 µmol m^{-2} s^{-1}, 100 rpm and the temperature was maintained at 25 ± 2 ^{0}C.

Roots were harvested from the medium after 6 weeks cultivation period and biomass was estimated according to described protocol of Murthy and Paek (2016). Briefly, prior to estimation of fresh weight (FW; g) harvested roots were washed in water and were blotted on filter paper to remove excessive water. For dry weight (DW; g) determination roots were dried at 35 ^{0}C for 24 h in forced air unit. Root F/D (FW/DW) ratio and percent Dry Weight (% DW) were estimated according to formulas given below:

$$\frac{F}{D} = \frac{\text{Root Fresh Weight (g/L)}}{\text{Root Dry weight (g/L)}}$$

$$\% \text{ DW} = \left[\frac{\text{Root Dry weight (g/l)}}{\text{Root Fresh weight (g/l)}}\right] * 100$$

3.3.2. Total Phenolic and Flavonoid content estimation

The dried powdered root samples were extracted as described by Ali et al. (2013). Briefly, each finely powdered root sample (300 mg) was mixed with 15 ml of 80% (v/v) methanol. The mixture was thoroughly vortexed for 3 min followed by three times of 10 min sonication with a resting period of 30 min in between and finally centrifuged at 8,000 rpm for 10 min. The supernatants were collected and either immediately used for analysis or stored at 4 ^{0}C for further analysis.

For the estimation of total phenolic content (TPC), Folin-Ciocalteu (FC) reagent was used according to the valuable protocol of Velioglu et al. (1998). For this purpose, a reaction mixture of 200 µl was prepared in 96-well plate and absorbance was measured at 630 nm by using UV/VIS–DAD spectrophotometer (Halo DR-20, UV–Vis spectrophotometer, Dynamica Ltd., Victoria, Australia). The calibration curve (0-50 µg/ml, $R^2 = 0.986$) was plotted by using Gallic acid as standard. The TPC was expressed as Gallic acid equivalents (GAE)/g of dry weight (DW).

For determination of total flavonoid content (TFC), the aluminum chloride colorimetric method of Chang et al. (2002) was used. Absorbance of the reaction mixture was measured at 415 nm by using UV/VIS–DAD spectrophotometer. The calibration curve (0-40 µg/ml, $R^2 = 0.897$) was plotted by using quercetin as standard and TFC in the samples were expressed as quercetin equivalents (QE)/g DW.

3.3.3. Free Radical Scavenging Assay

Free radical scavenging potential was determined according to the protocol of Abbasi et al. (2010) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used as producer of free radicals. The absorbance of the reaction mixture was recorded at 517 nm by spectrophotometer. Two mg of butylated hydroxyanisole (BHA) per 4 ml of methanol was used for background correction as a decaying agent of DPPH° to diminish the purple color of DPPH°. The radical scavenging activity was calculated according to following formula and was expressed as % DPPH activity:

$$\%DPPH = 100 * \left(1 - \frac{AE}{AD}\right)$$

Where A_E is absorbance of the solution, when extract (sample) was added at a particular level and A_D is the absorbance of the DPPH° solution with nothing added (Blank or negative control).

3.3.4. FT-IR and HPLC analysis of root extract

The FT-IR analysis of methanol extract of root sample was recorded by bench-top TENSOR 27TM Fourier Transform Infra-Red (FTIR) spectrometer (Bruker, Germany) equipped with universal PIKE-MIRacleTM Single Reflectance ATR accessory (PIKE Technology, Germany). The FTIR-ATR spectra was recorded at room temperature (25 °C) yielding an IR spectrum over the range of wave number 4000-600 cm^{-1} with a speed of 10 scans per spectrum. All data were initially corrected for background spectrum and base line. The transmittance (%) mode was used for spectral measurements.

HPLC analysis for withanolides was performed with some modification to the reported method of Mundkinajeddu et al. (2014). We used Jasco HPLC system (LC-Net II), consisting of a quaternary pump (PU-2089), an auto sampler (AS-2059 Plus) and a PDA detector (MDA-2018). The compounds separation were achieved using 5 µm Luna C18 250 x 4.6 column and solvents [A = Water Ultra-Pure ± 0.05% TFA (pH. 2.6), B = MeOH HPLC grade]. Mobile phase was run using gradient elution at 0, 15, 20 and 23 min of 30, 80, 80 and 30% of B respectively. The flow rate was 0.7 ml/min and injection volume of 20 µl. The eluent was detected and analyzed at 230 nm. The chlorogenic acid, moupinamide and withaferine A were detected and quantified in root samples using their authentic standards obtained from sigma.

3.3.5. Statistical analysis

All experiments were carried out in triplicates. Mean values of various treatments were subjected to analysis of variance (ANOVA) and significant difference were separated using one-way ANOVA with Tukey's test using Statistix software (8.1 versions). For graphical presentation Origin lab (8.5) was used and error bars were represented as standard error (SE).

3.4. RESULTS AND DISCUSSION

3.4.1. Effects of auxins and elicitors on biomass accumulation in adventitious roots suspension culture

In preliminary experiments, varying levels of melatonin and auxins were tested in-vitro for formation and biomass accumulation of adventitious roots. More biomass accumulation (1.38 DW g L-1) was observed for melatonin (Mela) at 6.4 µM than IBA and IAA at 4.9 µM and 5.7 µM respectively (Table 3.1). Adventitious roots obtained on IBA/Mela containing media were conspicuously branched, while

on IBA alone media they were less branched, thicker and longer in length (Fig 3.1B&D). Adventitious roots obtained on Mela alone media were white in color, thin and less branched (Fig 3.1C).

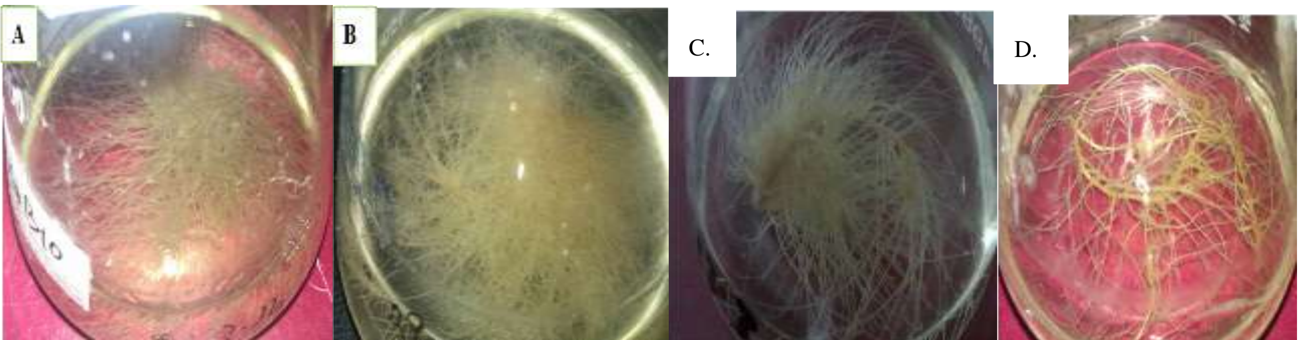

Figure 3.1: Biomass production of adventitious roots of *Withania Somnifera* after 6 weeks of culture period in MS liquid contain (A) IBA/GA3 (4.9/4.33 µM), (B) IBA/Melatonin (4.9/6.4 µM) (C) Melatonin (6.4 µM) alone and (D) IBA (4.9) alone.

Biomass accumulation was further enhanced (3.37 DW gL^{-1}) by addition of 6.4 µM Mela to the MS media containing 4.9 µM IBA (Table 3.1). Interestingly, higher level (18.6 mg g^{-1}) of total phenolic content (TPC) was observed in IBA/Mela (4.9/8.6 µM) containing medium, followed by IBA/GA_3, whereas maximum total flavonoid content (TFC) was observed in medium containing IBA (8.9 µM) (Table 3.1). Mela is considered as one of the most potent anti-oxidant and stress alleviator in plants (Shi et al.2015). Previously, in-vitro application of Mela resulted in better shoot proliferation with lower total phenolic content in *Panax quinquefolium* (Uchendu et al. 2011). This might be due to the different organogenic routes on the same growth conditions. Nonetheless, Chen et al. (2009) observed Mela-concentration dependent growth response in etiolated seedling of *Brassica juncea*.

Table 3.1: Effects of different auxins and melatonin (alone or in combinations) on adventitious root growth and secondary metabolite content of *Withania somnifera* after 6 week of culture.

Treatments (µM)		Fresh Weight (g L^{-1})	Dry Weight (g L^{-1})	F/D ratio	% DW	TPC (mg g^{-1} DW)	TFC (mg g^{-1} DW)
IBA	4.9	10 ± 1.3^{cde}	1.06 ± 0.13^{cd}	9.4	10.60	5.9 ± 0.11^{h}	7.42 ± 0.01^{b}
	7.38	6.4 ± 1.4^{de}	0.874 ± 0.18^{cde}	7.3	13.66	9.8 ± 0.09^{f}	4.06 ± 0.06^{g}
	9.8	4.9 ± 1.3^{de}	0.64 ± 0.08^{cde}	7.65	13.06	13.0 ± 0.13^{c}	$8.16 \pm 0.02^{a*}$
IAA	5.7	4.4 ± 0.13^{de}	0.362 ± 0.04^{de}	12.2	8.23	11.1 ± 0.40^{de}	7.06 ± 0.003^{c}
	8.55	4 ± 0.81^{de}	0.392 ± 0.02^{de}	10.2	9.81	12.8 ± 0.11^{c}	3.52 ± 0.14^{h}
	11.25	0.43 ± 0.12^{e}	0.064 ± 0.06^{e}	6.7	14.92	10.2 ± 0.32^{ef}	4.95 ± 0.09^{e}
IBA/GA3	4.9/4.33	28 ± 3.8^{b}	2.22 ± 0.11^{b}	12.6	7.93	15.2 ± 0.35^{b}	6.57 ± 0.05^{d}
IAA/GA3	5.7/4.33	13 ± 1.8^{bcde}	1.39 ± 0.23^{c}	9.4	10.69	7.5 ± 0.09^{g}	6.44 ± 0.03^{d}
IBA/Mela	4.9/8.6	$44 \pm 8.5^{a*}$	$3.37 \pm 0.04^{a*}$	13.1	7.66	$18.6 \pm 0.17^{a*}$	4.05 ± 0.02^{g}
IAA/Mela	5.7/8.6	22 ± 4.2^{bc}	1.32 ± 0.31^{c}	16.7	6.00	7.8 ± 0.13^{g}	4.59 ± 0.06^{f}
Melatonin	2.15	7.1 ± 0.51^{cde}	0.592 ± 0.02^{cde}	12.0	8.34	7.4 ± 0.30^{g}	2.57 ± 0.06^{i}
	4.3	8.8 ± 0.78^{cde}	0.754 ± 0.20^{cde}	11.7	8.57	9.4 ± 0.08^{f}	4.69 ± 0.07^{f}
	6.4	19 ± 3.5^{bcd}	1.38 ± 0.10^{c}	13.8	7.26	13.6 ± 0.09^{c}	2.55 ± 0.12^{i}
	8.6	16 ± 2.4^{bcde}	1.07 ± 0.29^{cd}	15.0	6.69	11.4 ± 0.29^{d}	2.38 ± 0.05^{j}

*Values are means ± SD of three replicates and values with in column followed by different letters are significantly different ($P<0.05$)

Concerning growth kinetics, maximum fresh weight and dry weight of 13 g L-1 and 0.15 g L-1, respectively, were observed on day 35 of culture in MS medium containing IBA/Mela (4.9/4.33 µM), followed by media containing IBA/GA3 (4.9/4.3 µM). Furthermore, lesser biomass accumulation with slower multiplication rate was observed on liquid MS medium augmented with 5.7 µM IAA (Fig 3.2).

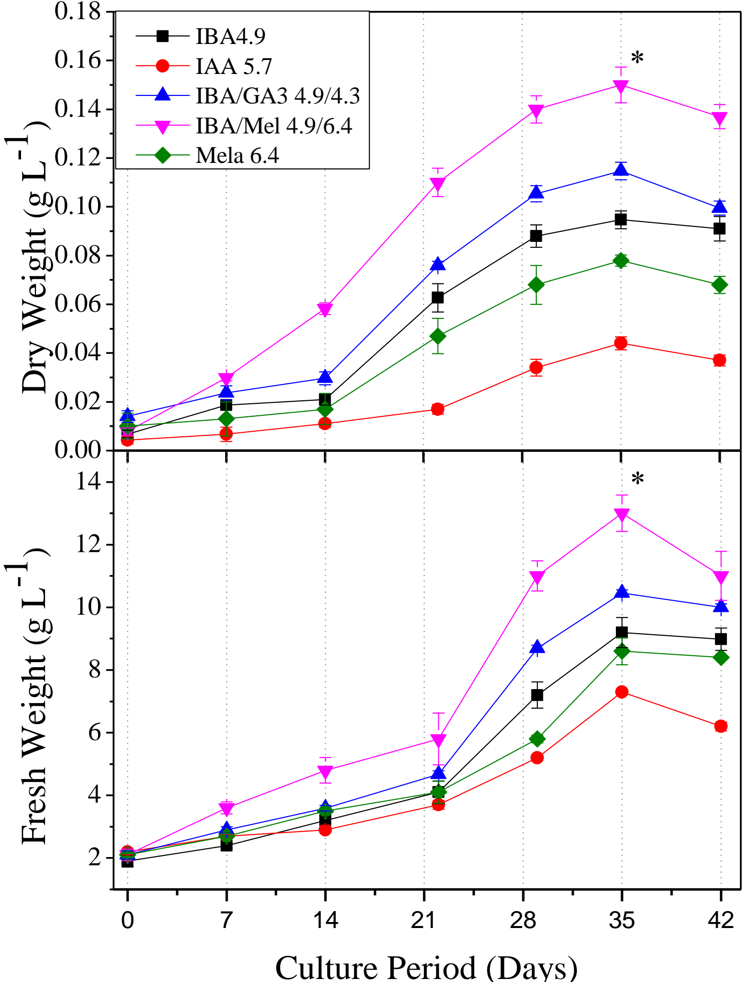

Figure 3.2: Growth kinetics of adventitious root submerged culture of *Withania somnifera* MS liquid medium supplemented with different PGRs (µM) alone or in combination. Data points are mean ± standard error of three replicates.

Elicitors (SA and MeJa) were introduced to the culture medium at day 21 while roots were harvested at day 42 for fresh and dry biomass (g L-1) determination. Within all the tested levels, biomass accumulation was decreased up to two folds and five folds when higher concentrations of SA and MeJa were used respectively. When MeJa was used in low concentration in IBA/Mela and Mela alone containing MS media, root fresh biomass 13.54 g L-1 and 8.99 g L-1, respectively were recorded at higher levels than their respective control treatments (elicitor's free media) (Fig 3.3A&B). In present study, lower levels of SA and higher levels of MeJa in media containing Mela produced more biomass of adventitious roots.

Contrary to our findings, See et al. (2011) reported no significant effect of MeJa on cell biomass of *M. malabathricum*. Whereas, Shabani et al. (2009) reported significant reduction in root growth of *G. glabra* after MeJa treatment, while SA did not affect root growth. In our study presence of melatonin facilitated roots growth in response to MeJa and SA treatments. We speculate that melatonin strongly supported the adventitious root growth by protecting the root tips and macromolecules from oxidative stress caused by the elicitors (Adil et al. 2015). Previously, it was reported that exogenous application of melatonin enhances seminal root length and root biomass of transgenic rice (Park and Back 2012). Similarly, Hernández-Ruiz et al. (2005) reported increased coleoptile growth of canary grass, wheat, barley and oat by exogenous application of melatonin.

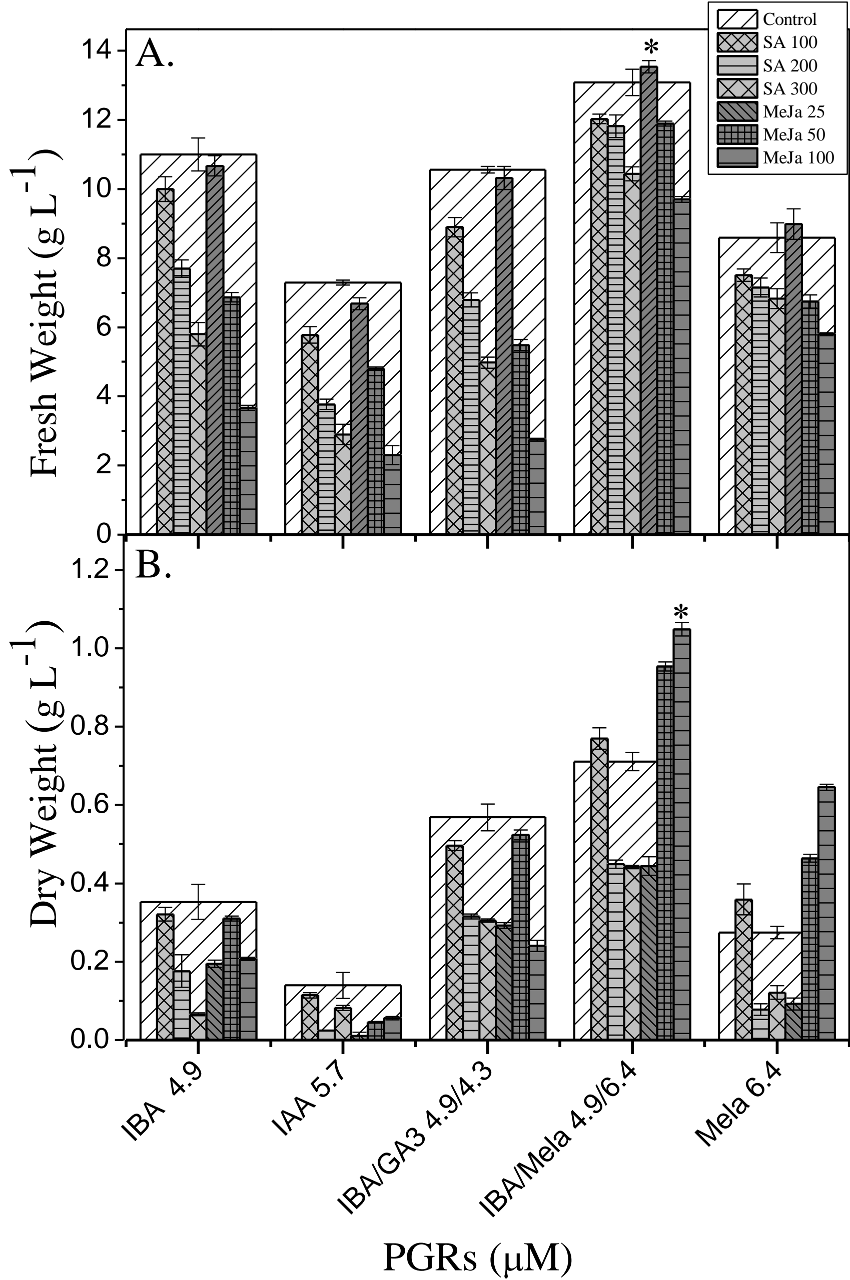

Figure 3.3: Effect of methyl jasmonate (25, 50 and 100 µM) and salicylic acid (100, 200 and 300 µM) on adventitious root growth *Withania somnifera*. These elicitors were added to 21-days-old cultures and harvested after 7 days for growth estimation. Values are mean ± standard error of three replicates. Values are significantly different ($P<0.05$).

3.4.2. Effects of elicitors on biochemical markers during adventitious roots suspension culture

Maximum levels (35.8 mg g-1 DW) of phenolics were observed when IBA/Mela medium was elicited with MeJa (100 µM), which was in parallel to the root biomass accumulation (Fig 3.4). Within the control treatments (without elicitors), total phenolic content (18.6 mg g-1 DW) was observed in roots grown on media containing IBA/Mela, which was four fold to the lowest level (4.9 mg g-1 DW) on medium containing only IBA. Comparatively, higher total phenolic content was observed in MeJa treated roots than SA.

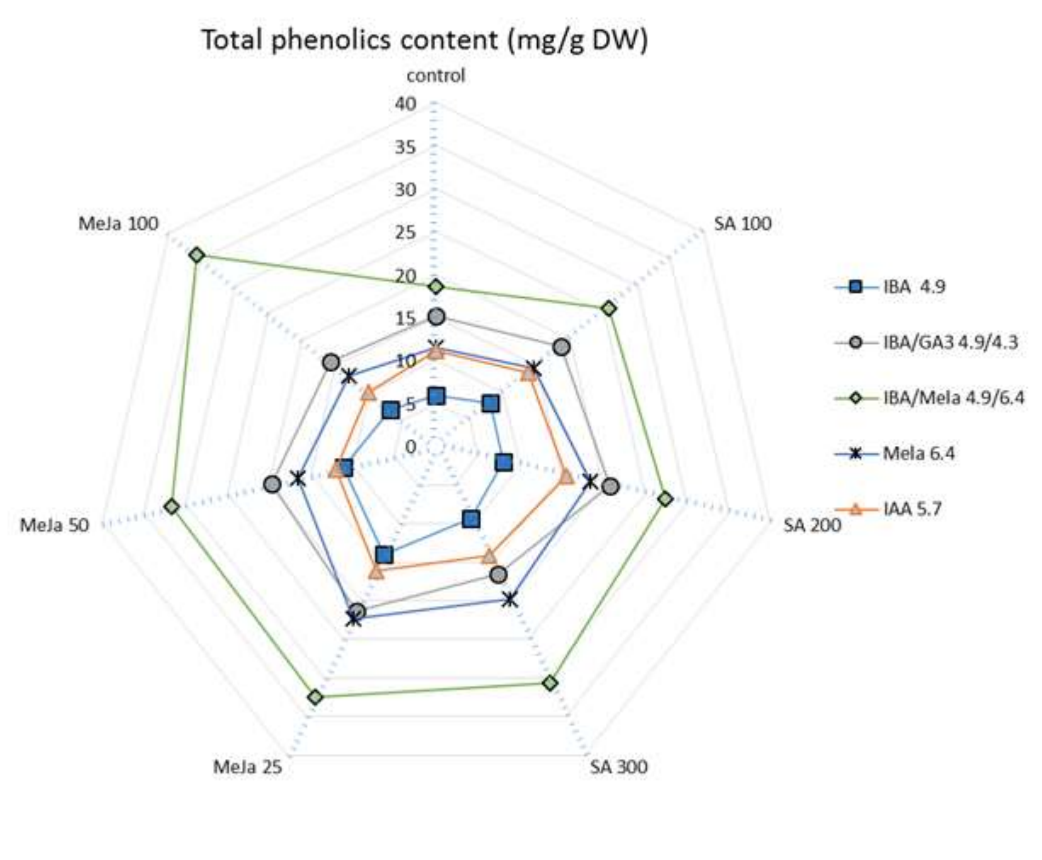

Figure 3.4: Total phenolic content (mg Gallic acid/g DW) in adventitious root culture of *Withania somnifera* on MS medium, elicited at day 21 of the culture with methyl jasmonate (25, 50 and 100 µM) and salicylic acid (100, 200 and 300 µM). Values are mean of three replicates.

It is well illustrated in literature that phytohormone composition effect secondary metabolites productivity during in vitro plant cell culture (Murthy et al. 2014). Lowest total flavonoid content (0.9 mg g-1 DW) was observed in roots obtained on media containing IBA/Mela that reached to its maximum value of 13.7 mg g-1 DW, when MeJa (50 µM) was introduced into the same media at day 21. Similarly, TFC content (5.4 mg g-1 DW) was observed in media incorporated with IBA/GA3; that reached to 10.7 when elicited with MeJa (50 µM), while less significant variation in TFC content was observed in IBA containing media (Fig 3.5). Overall, MeJa strongly increased the TPC and TFC than SA. In term of total phenolic and flavonoid yield (mg L-1), IBA/Mela containing media give higher yield followed by IBA/GA3 (Fig 3.6).

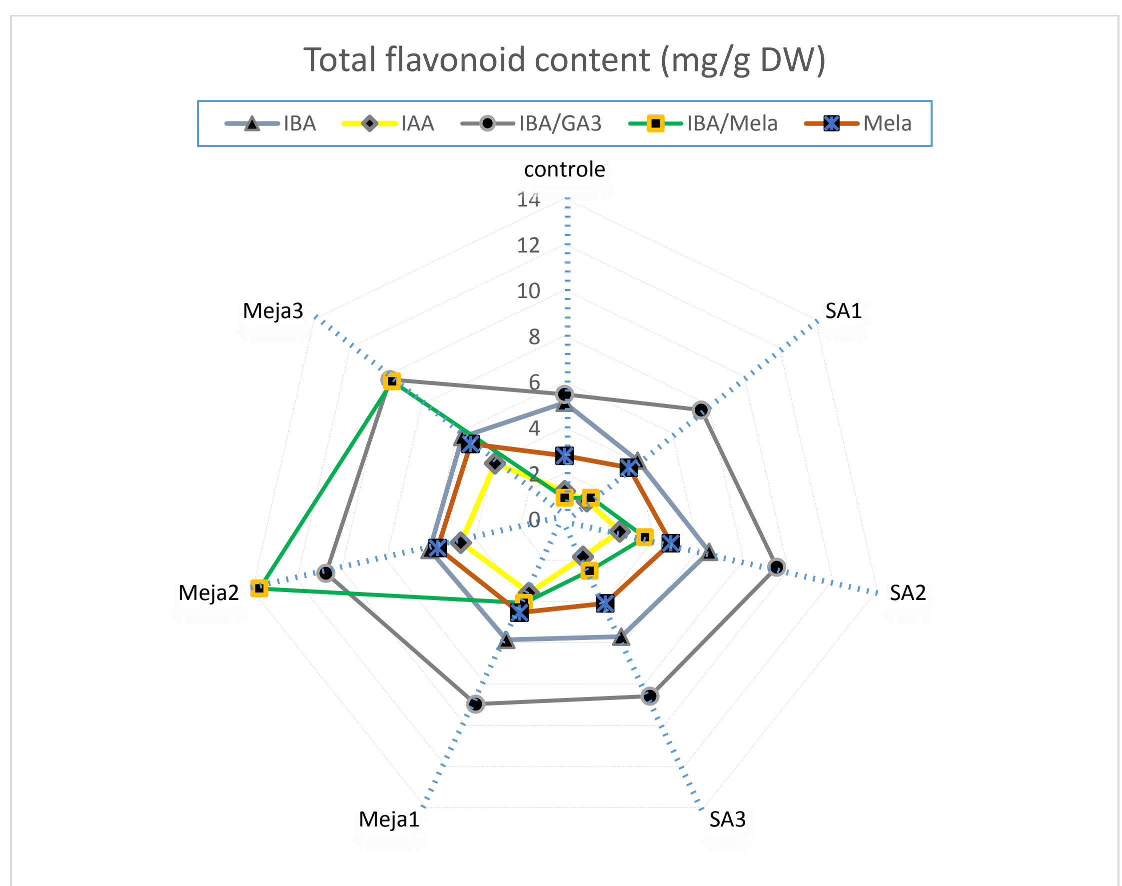

Figure 3.5: Total Flavonoid content (mg/g DW) in adventitious root culture of *Withania somnifera* on MS medium elicited at day 21 of the culture with different concentrations of salicylic acid and methyl jasmonate. Values are mean of three replicates ± standard error.

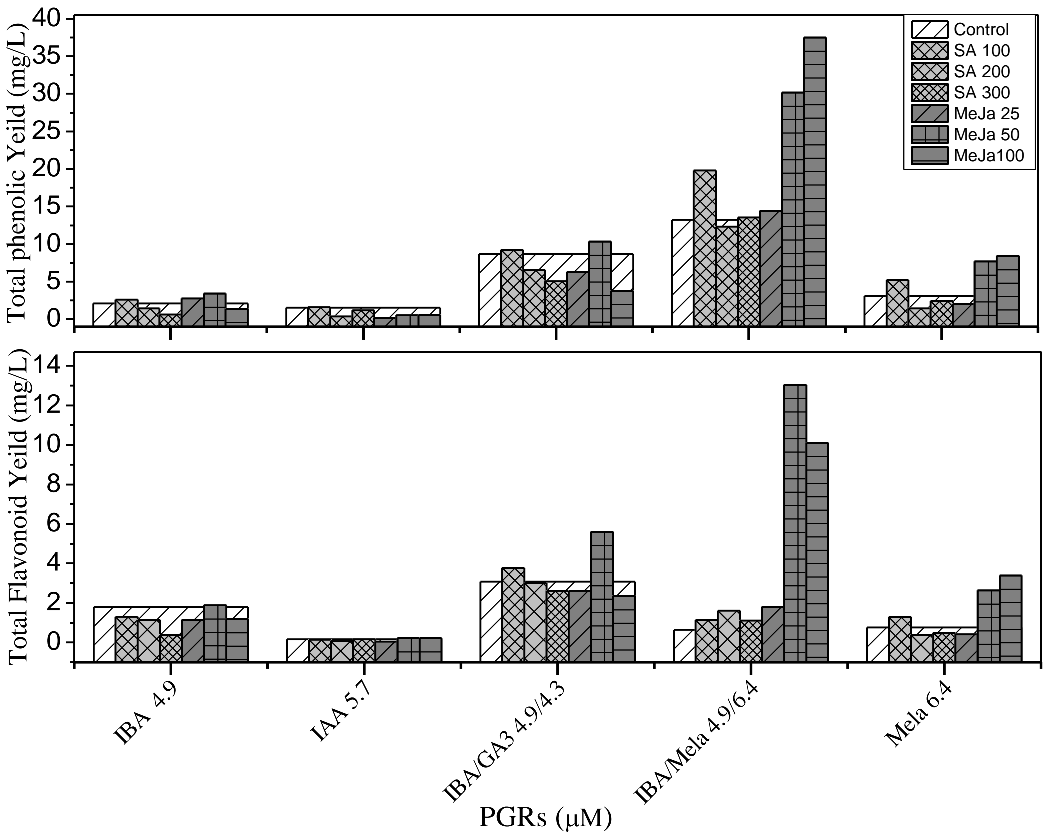

Figure 3.6: Changes in secondary metabolites yield (phenolic and flavonoid) of adventitious root of *Withania somnifera* during seven week of culture in shake flask. (A) Phenolic yield per litter of medium from dry weight. (B) Flavonoid yield per litter of medium from dry weight.

As shown in Fig 3.7, the DPPH free radical scavenging activity (DFRSA%) of the adventitious root samples revealed that roots treated with MeJa 50 µM, at day 21 on media containing IBA/Mela exhibited maximum activity (4 fold to control). When roots in medium incorporated with IBA and melatonin alone were elicited the DPPH free radical scavenging activity raised to 65 and 50 %, respectively.

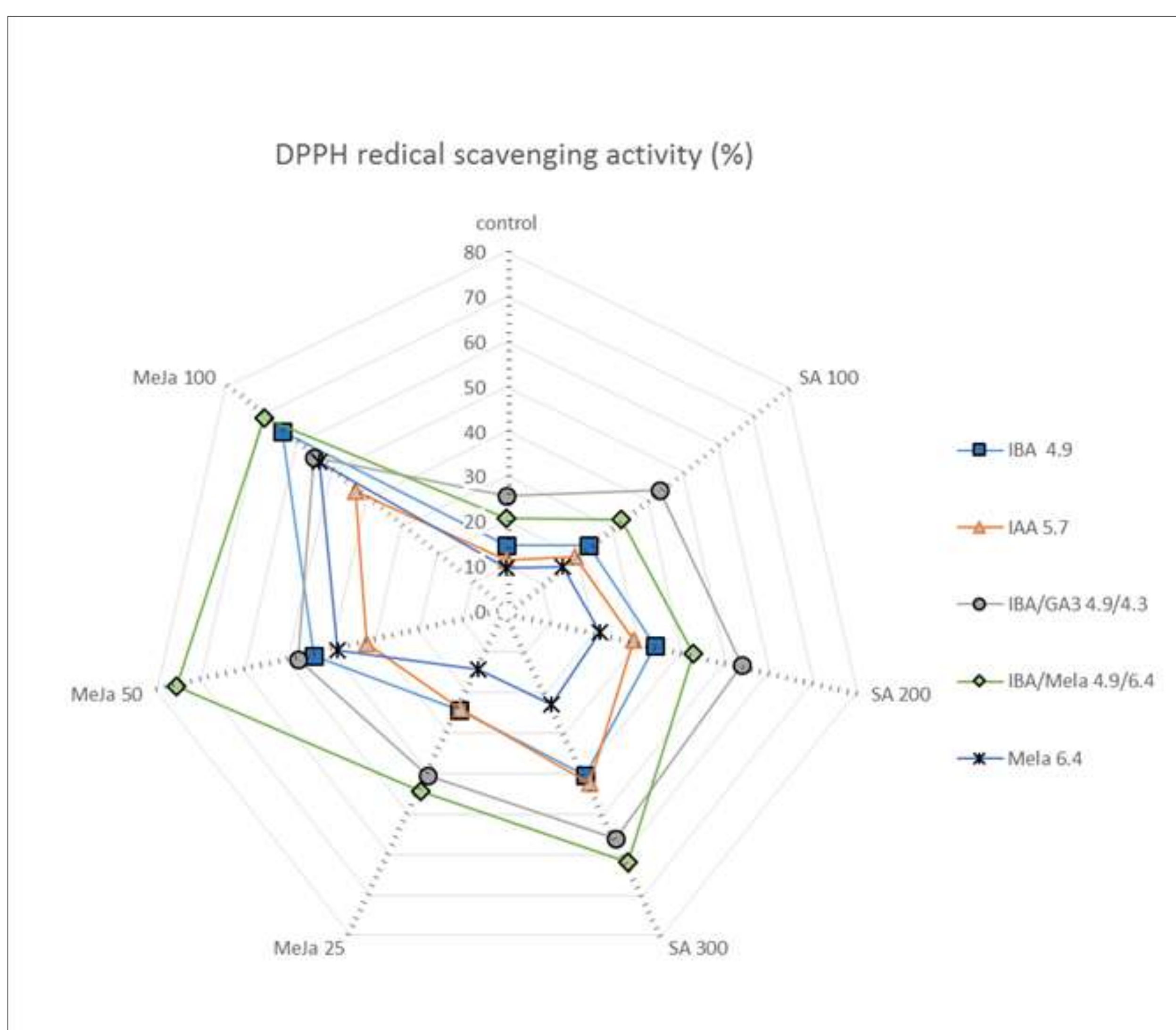

Figure 3.7: DPPH free radical scavenging activity (%) in adventitious root culture of *Withania somnifera* on MS liquid media augmented with different concentrations of salicylic acid and methyl jasmonate at day 21 of the culture. Values are mean of three replicates.

To find the relationship between free radical scavenging activity, total phenolic yield (TPY), total flavonoid yield (TFY) and dry weight (DW) Pierson`s correlation test was performed. The DPPH free radical scavenging activity were positively correlated with TPY (r = 0.55), and TFY (r = 0.64) with statistical significance of $p<0.05$ (Table 3.2).

Table 3.2: Pierson correlation for biochemical and growth markers after the harvest of adventitious root of *Withania somnifera*.

	DPPH	***Total Phenolic Yield***	***Total Flavonoid Yield***	***DW***
DPPH	1			
Total Phenolic Yield	0.55	1		
Total Flavonoid Yield	0.64	0.81	1	
DW	0.46	**0.91**	0.77	1

Bold value shows significant correlation at P<0.05

In addition, significantly high correlation (r = 0.81, p<0.05) was found between TPY and TFY. While with regression statistics, r = 0.65 and r^{square} = 0.43; taken as set, the predictors TPY, TFY and DW account for 43% of variance in DPPH free radical scavenging activity (Fig 3.8).

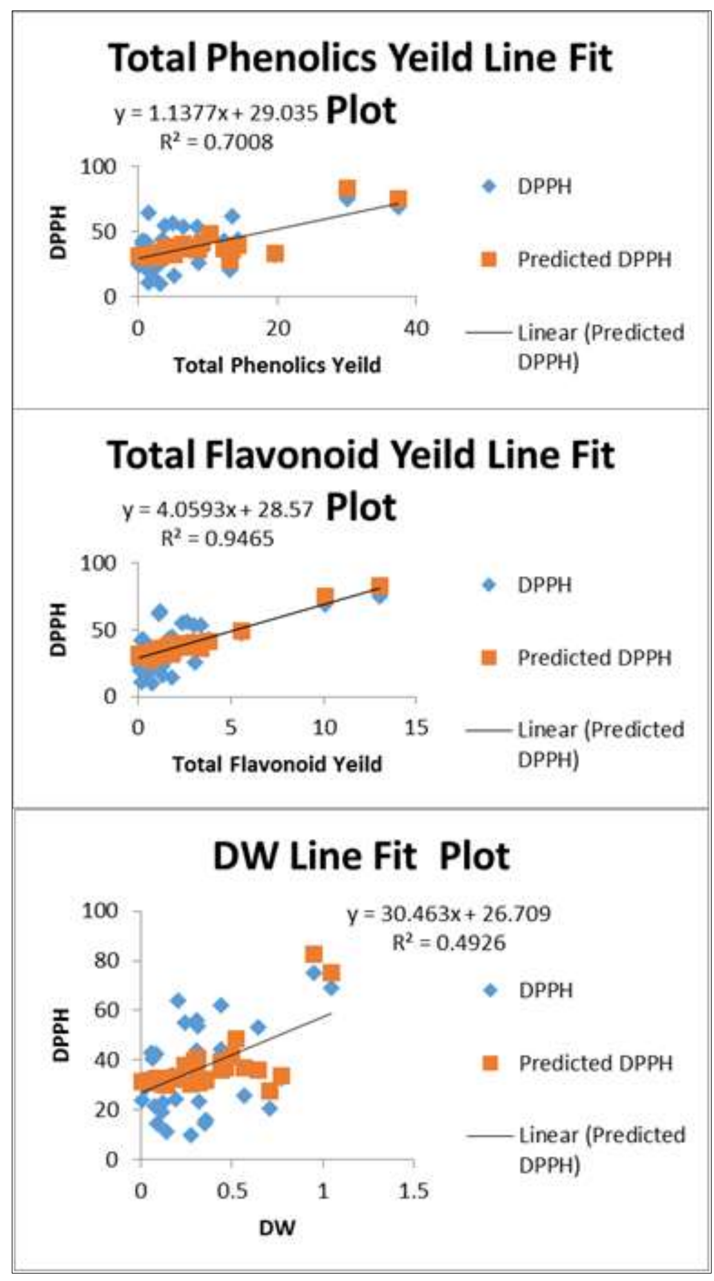

Figure 3.8: Relationship between Dry Weight (DW), Total phenolic and flavonoid yield with DPPH radical scavenging activity of *Withania somnifera*.

3.4.3. Fourier Transform Infrared (FT-IR) and HPLC analysis of root cultures

FT-IR analysis revealed presence of different functional groups in the methanolic extracts of the in vitro raised root cultures. On the basis of measurement of vibrations of the polar bonds, different functional

groups were identified including -OH, -CO, C=O, C---C aromatic ring and –CH in FT-IR (region 400 to 4000 cm^{-1}) fingerprint (Fig 3.9). The biochemical fingerprints were made from the vibrational features of methanolic extracts and the functional groups were assigned on the basis of previous available reports (Minhas et al. 2012). FT-IR spectroscopy has been exploited as an authentic tool for macro-fingerprints of vital compounds in different plant species (Liu et al. 2016).

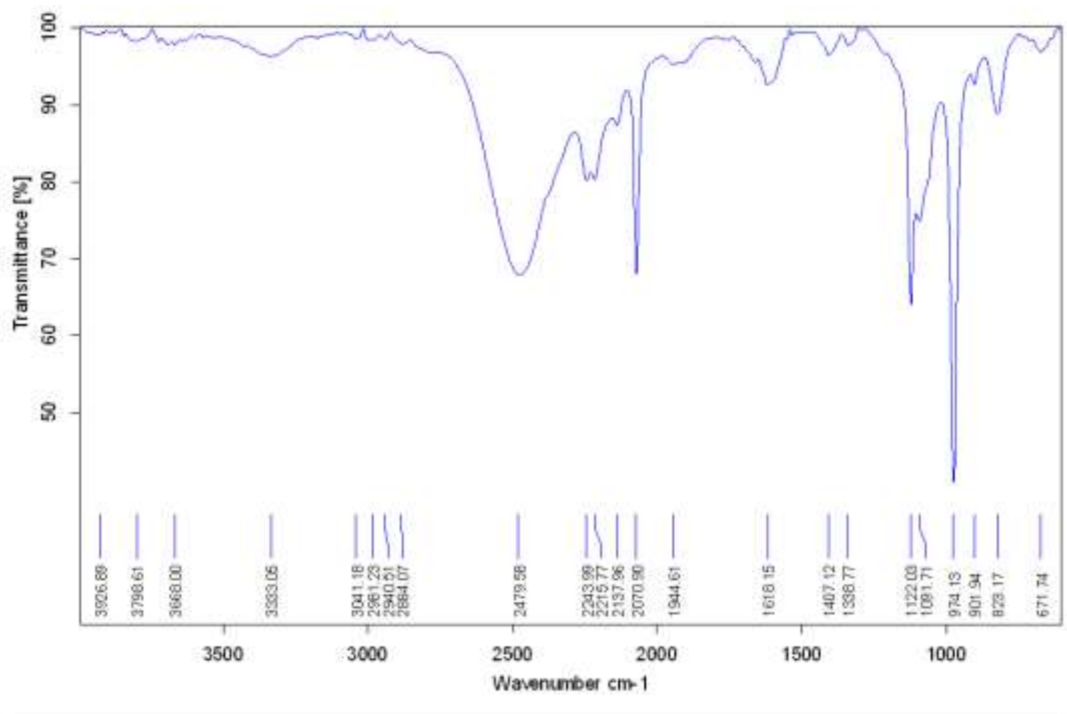

Figure 3.9: FT-IR spectra of methanol extract of adventitious roots of *Withania somnifera*.

Separation of *W. somnifera* methanol extracts of adventitious roots through HPLC showed the detection of different important bio-active compounds. Chlorogenic acid (RT=9.06 min), Moupinamide (RT=15.17 min), Withanomide Q (RT=15.54 min), Withanoside IV (RT=19.03 min), Withaferin A (RT=19.39 min), and Withanoside V (RT=23.05 min) were detected at varying levels in the different AR samples. The quantity of the detected compounds varied significantly with type of adventitious roots, phytohormones combinations and elicitor.

Chlorogenic acid was found at highest level (55.6 mg/g DW) in the roots cultivated on media containing IBA/Mela treated with MeJa 50 µ mol. However, in IBA/GA_3 containing media, SA (300 µ mol) resulted in considerable amount (37.2 mg/g DW) of chlorogenic acid (Fig 3.10). Previously chlorogenic acid has

been revealed to have significant physiological roles in formation of adventitious roots, cell wall building and de-novo organogenesis (Mondolot et al. 2006; Narukawa et al. 2009; Franklin and Dias 2011).

Notable withanolides such as Moupinamide and Withaferin A were detected at highest levels in the roots elicited with MeJa (50 μ mol) raised in-vitro at IBA/Mela containing media. Moupinamide content was found at reduced level in IBA alone containing media, However Withaferin A was found at higher level in response to SA (300 μ mol) during adventitious rooting on IBA/GA_3 media (Fig 3.10c). The enhanced production of withanolides in the elicited root cultures can be anticipated with the direct relation of these compounds with morphological differentiation during adventitious roots formation in present study whereby, elicitors at mild levels play an important role in the synthesis of withanolides (Sharada et al. 2007). Recently, Sabir et al. (2013) detected withanolide A in roots of field grown and in-vitro cultures. Moreover, Dhar et al. (2012) hypothesized the tissues specific biosynthesis of withanolides and observed comparatively higher concentration of these compounds in leaves then roots. Further he concluded that Withaferine A concentration varies strongly with the growth phases and peaked at fruiting stage that tending to decrease at fruit ripening stage. Withanolides are also known to act as growth regulators coinciding in their physiological functions with brassinosteroids (Sangwan et al. 2008).

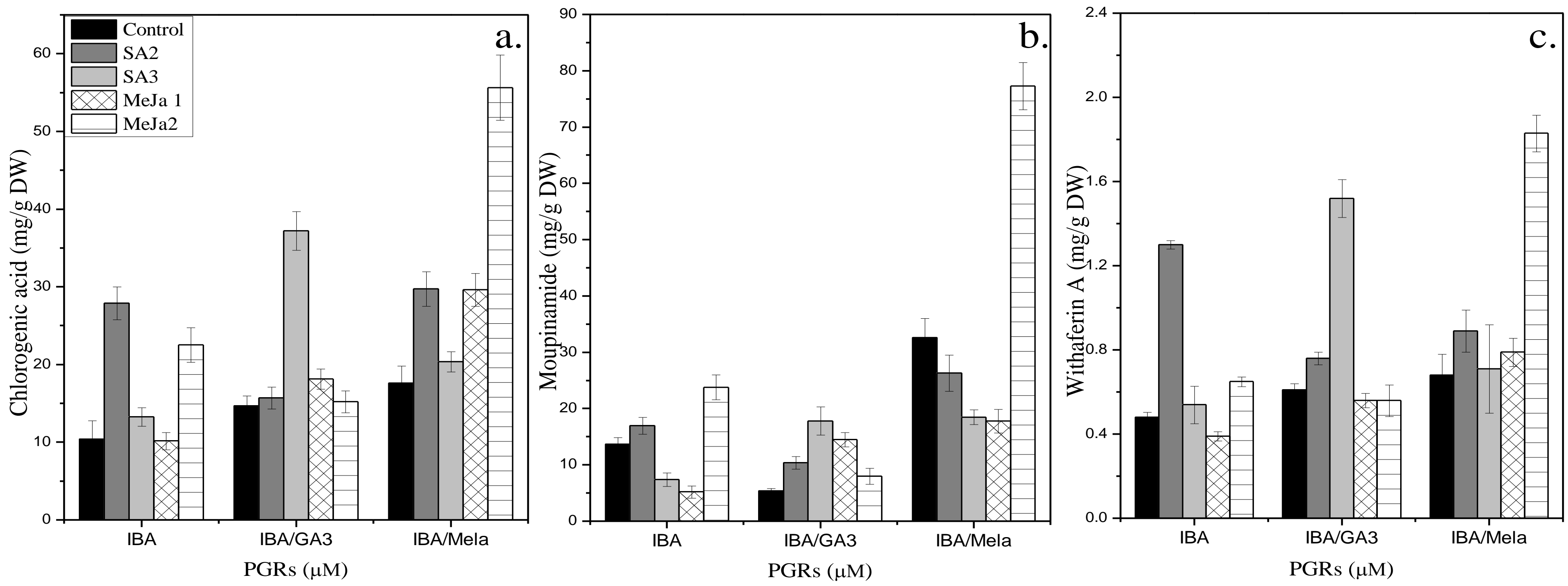

Figure 3.10: The effect of elicitors on Chlorogenic acid, Moupinamide and Withaferin A content (mg/g DW) in *Withania somnifera* adventitious roots after 21 days of suspension culture.

3.5. CONCLUSIONS

The results of present study indicated that the biomass and secondary metabolites accumulation were affected by phytohormone type and concentration, and by melatonin during shake flask culture of *W. somnifera* adventitious roots. Moreover, data indicated that for adventitious root biomass production, melatonin in combination with IBA is more effective than others and growth remained modest even abiotic elicitors were used to switch the pathways for secondary metabolites production. These findings can be used for large scale commercial production of *W. somnifera* adventitious roots and it will be helpful to fulfil the market demand for this species.

4. RED LIGHT CONTROLLED CALLUS MORPHOGENETIC PATTERNS AND SECONDARY METABOLITES PRODUCTION IN *WITHANIA SOMNIFERA* L.

4.1. ABSTRACT

Withania somnifera is an important medicinal plant with increasing market demand around the globe is studied for *in-vitro* callus induction, biomass accumulation, secondary metabolite content, stress level and anti-oxidant enzymes content at tailored wavelengths of light. *In-vivo* grown leaf explants were cultured on Murashige and Skoog (MS) media containing Thidiazuron (TDZ), α-naphthalene acetic (NAA) acid and Benzyl aminopurine (BAP). The optimum MS medium containing 0.5 mg L^{-1} of TDZ and 0.5 mg L^{-1} NAA was cultured with leaf explant and incubated under different wavelengths of lights. Among the different wavelengths, red light was proved optimum for maximum biomass accumulation during seven weeks of culture, while total phenolic and flavonoid content were maximum under violet light than others. Compared to other wavelengths, red light grown callus extract showed significantly higher content of chlorogenic acid, maupinamide and withaferin A. Higher malondialdehyde (MDA) and protease activity were observed at blue and violet light treatments, respectively. Antioxidant enzymes, superoxide dismutase (SOD) and per-oxidase (POD) were maximum at violet light treatment while remained minimum at red light. Whereas, maximum total protein content was observed at normal white light fluorescence. This represents the sensitivity of leaf explant derived calli to the different light colors. We observed maximum antioxidant activities in red light treated calli followed by blue and violet light treatments. This study concludes that red light treatment was optimum for maximum biomass accumulation and anti-oxidant activity in calli of *W. somnifera*.

4.2. INTRODUCTION

Withania (*W.*) *somnifera* is an important medicinal plant commonly known as winter cherry, Indian ginseng or poison gooseberry, belongs to Solanaceae or nightshade family. Traditionally, it has been used for more than 3,000 years against various ailments, as an essential constituent of over 200 traditional medicinal formulations (Kaileh et al. 2007). The modern clinical studies have proven *Withania`s* pharmaceutical products have lifesaving potentials including immunoregulatory, anti-cancer, anti-arthritic and recovery from neurodegenerative disorders (Mirjalili et al. 2009; Singh et al. 2011; Bano et al. 2015). The higher market demand for *W. somnifera* has caused a tremendous burden on its natural reservoirs (Supe et al. 2006). Due to habitat destruction, over exploitation and illegal collection of this plant, it is at the verge of eminent danger of extinction in Pakistan. Considering its paramount medicinal significance novel means and ways should be implemented on the conservation and sustainable utilization of this important plant. In comparison to conventional cultivation procedures, plant cell cultures have emerged as a promising platform for the biosynthesis of valuable metabolites in limited time and space.

Light controls plants growth and development mainly in two ways: photosynthesis and photo-morphogenesis. Plant responds differentially to respective wavelengths or colors of light through photoreceptors called phytochromes, cryptochromes and phytotropin (Casal 2000). In plant tissue culture three aspects of photo-environment mostly influence *in-vitro* growth and morphogenesis; these are: wavelength, flux density and photoperiod. As a number of *in-vitro* studies have mentioned light having significant effects on callus growth and morphogenesis, inhibition of axillary shoot proliferation and induction of specific enzyme activity which are concerned with production of important secondary products (Afshari, Angoshtari et al. 2011). By now three kind of photoreceptors known as phytochrome (red and far red detector), cryptochrome (blue and UV-B detector) and phototropin (blue and UV-A detector) are thought to be involved in plant development (Ascencio-Cabral et al., 2008, Gutiérrez-Pulido et al. 2008). There are some reports on the involvement of light on phytohormone synthesis, for example it has been shown that CK biosynthesis is stimulated to occur in red light, but is prevented when far red is used (Afshari, Angoshtari et al. 2011) It is presumed that monochromatic light regimes may help to optimize and control growth by evoking the photo-oxidative changes in plants that may lead to the increased content of phytochemicals (Brazaitytė et al. 2016).

However, there is still a lack of information regarding the monochromatic light conditions for growth and secondary metabolites content of different plant species. Thus, we conducted this study to improve the alternative and renewable method of callus culture for sustainable production of *W. somnifera* biomass with higher secondary metabolites content. Nevertheless, the secondary metabolites production pathways are significantly affected by environmental factors and light quality/quantity have proven to be an effective elicitor for enhanced secondary metabolite content (Jimenez-Garcia et al. 2013; Tariq et al. 2014; Ahmad et al. 2016). Therefore, we performed this study to determine the impacts of different wavelengths of light on callus growth and secondary metabolites accumulation in *W. somnifera* with the aim to establish callus culture for increase biomass and secondary metabolite content. Further, the callus cultures were exposed to several light colors and were studied to ascertain the optimum light wavelengths to increase the callus growth and secondary metabolites content of *W. somnifera*. Moreover, the biochemical markers like total protein content, protease activity, per-oxidase activity, super oxide dismutase activity and malondialdehyde content along with HPLC based quantification of withanolides were carried out to determine the plant secondary metabolism during callus formation in response to different wavelengths of light.

4.3. MATERIALS AND METHODS

4.3.1. Leaf explant preparation and optimization of callus induction

Leaf explants were excised from 7 weeks old growth room potted plants of *Withania somnifera*. Explants (leaf sections~2.5 cm) were excised from intact plantlets. During sterilization, the explants were thoroughly rinsed with ethanol (70%) for 5 minutes followed by rinsing for 3 minutes in 0.1 % (w/v) mercuric chloride solution ($HgCl_2$). Finally, the explants were washed three times with sterile distilled water and were dried on sterile blotting paper. The sterilized explants were cultured for callus initiation on Murashige and Skoog (MS) (1962) medium containing 3% sucrose and 0.8% agar in 100 ml conical flasks supplemented with combined or single concentration of thidiazuron : TDZ (0.1, 0.5 and 1 mg L^{-1}), benzyladenine : BAP (0.5 and 0.1 mg L^{-1}) and α-naphthalene acetic acid : NAA (0.1, 0.5, 1.0 and 1.5 mg L^{-1}). The pH of the media was adjusted to 5.8±0.2 prior to autoclave at 121 0C for 30 min. Three replicates of each treatment were maintained at 16/8-hour light/dark photoperiod under cool-white light (~100 μmol/m^2/sec) and 25±2 ^{0}C room temperature. Data on the growth patterns during callus

organogenesis was recorded after 5 weeks of culture as callus induction frequency (CIF; %) and biomass formation (Fresh weight and dry weight; g explant^{-1}).

After 5 weeks of culture on respective media callus induction frequency (CIF) was recorded according to the following formula:

$$CIF=\left\{\frac{\text{Number of calus producing explant}}{\text{Total number of explants}}\right\}*100$$

4.3.2. Effect of monochromatic lights on callus growth

For different monochromatic light treatments the ascribed method of Tariq et al. (2014) was used. Briefly, for violet (350-400 nm), blue (380-560 nm), green (480-670 nm), yellow (530-780 nm) and red (610-715 nm) illuminations the tubes of Philips (32 W), Keliang (220 V; 50 Hz), Litex (40 W), Philips (36 W) and Binxiang (25 W) private limited were used respectively. The MS media having 0.5 mg/l of both TDZ and NAA, placed under the cool white fluorescent light (16:8 hr light and dark) was used as control and experiment was performed in triplicate. After five weeks of treatments CIF, callus fresh weight (g/explant) and dry weight (g/explant) were recorded as mean ± standard error.

4.3.3. Estimation of total phenolics and flavonoids content

Callus from each treatment was collected and washed with distilled water and dried at 60 ^{0}C for 48 h. The dried callus were finely powdered and 300 mg of powdered callus were extracted in 1500 µl of 80% methanol. The weight was recorded as total extractable amount of callus (mg/300mg DW). The obtained extract was re-suspended in 1 ml of pure methanol and was used immediately for biochemical screening or stored at 4 ^{0}C. For total phenolics content (TPC) estimation the ascribed method of Ali et al., (2013) was used and expressed as mg Gallic acid equivalents (GAE)/g DW. Total flavonoid content (TFC) was calorimetrically determined according to the method of Wu et al. (2006) and was expressed as mg quercetin equivalents (QAE)/g DW.

4.3.4. Anti-oxidant potential

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity (DFRSA), total antioxidant capacity (TAC) and total reducing power (TRP) were used for estimation of the anti-oxidant potential

of *W. somnifera* callus. For DPPH free radical scavenging activity, the ascribed protocol of Abbasi et al. (2016) was used. The DPPH free radical scavenging activity was calculated according to following formula and was expressed as % DPPH activity:

$$\%DPPH=100*(1-\frac{AE}{AD})$$

Where A_E is absorbance of the solution, when extract (sample) was added at a particular level and A_D is the absorbance of the DPPH° solution with nothing added (Blank or negative control).

The method of Jafrie et al. (2016) was used for estimation of TAC and TRP. Briefly, 0.1 ml of callus extract was mixed with 1 ml of reaction mixture containing sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammoniummolybdate (4 mM) and incubated for 90 min in a water bath at 95 ^{0}C. After cooling (to room temperature) the antioxidant potential of the sample was measured at 697 nm by use of a spectrophotometer and was expressed as mg ascorbic acid equivalent. While, 0.1 ml of methanol plus reagent solution was used as blank. Whereas, for TRP assay 0.2 ml of callus extract was mixed with 0.5 ml phosphate buffer (2 mM; pH=6.6) followed by addition of 0.5 ml potassium fericynide [$K_3Fe(CN)_6$] and incubated for 20 min in water bath at 50 ^{0}C. After cooling, 10% of tri-chloroacetic acid was added and centrifuged at 3000 rpm for 10 min to isolate 0.5 ml of upper the layer. The isolated upper layer was mixed with an equal volume of distilled water followed by addition of 100 µl of 0.1% of ferric chloride ($FeCl_3$). The absorbance of reaction mixture was measured at 700 nm where 200 µl of methanol was used as control and reducing power was expressed as mg ascorbic acid equivalent.

4.3.5 Determination of plant cell defensive enzymes activities

Fresh callus was harvested after 35 days from each of the tested light treatments and processed for protein extraction using the method of Nayyar and Gupta (2006). For total protein content (TPC) estimation the method of Lowry et al. (1951) was used with some modifications. Briefly, a reaction mixture of 1 ml was prepared by taking 200 µl of each sample and mixed with reagent A (2 g Na-K tartrate x 4 H2O, 100 g Na2CO3, 500 ml 1N NaOH) in a separate test tube. The tubes were then incubated at 50 ^{0}C in water bath and cooled back to room temperature. To this mixture 20 µl of reagent B (2 g Na-K tartrate x 4 H2O, 1g CuSO4 .5 H2O and 10 ml of 1N NOH) was added, mixed and incubated for 10 min in 50 ^{0}C water bath. Then 600 µl of reagent C (Folin-Ciocalteau reagent) was added to each tube and incubated for 10 min in 50 ^{0}C water bath. Finally the reaction mixture was cooled again and

centrifuged for measuring the absorbance at 650 nm using spectrophotometer. Bovine serum albumin (BSA) was used as standard and total protein content was expressed as mg BSA equivalent. Enzymes were extracted from fresh callus according to the previously ascribed method of Ali et al. (2015). The super oxide dismutase (SOD) activity was estimated according to the protocol of Giannopolities and Ries (1977). Where the reaction mixture was prepared by sequentially mixing of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 mM p-nitro blue tetrazolium chloride (NBT), 2 mM riboflavin, 0.1 mM EDTA and 60 µl of enzyme extract. The previously ascribed protocol of Chisari et al. (2007, Fang and Kao 2000) was followed for peroxidase activity (POD). Briefly, a reaction mixture of 27.5 mM H_2O_2, 100 mM Guaiacol, 50 mM K-phosphate buffer of pH 7 and 20 µl of samples was prepared.

4.3.6. Determination of protease activity and MDA content/stress level

Callus protease activity was determined by the Lowry-Folin method as ascribed by McDonald and Chen (1965). Briefly, 100 mg of powdered callus was incubated in 4ml of 1% casein in citrate phosphate buffer; pH 7.0 for one hour at 30°C, followed by addition of 5ml trichloroacetic acid (5%) to precipitate the residual protein and then centrifuged at 13000 g for 15 min. The obtained 5 ml supernatant was mixed with 5 ml of reaction mixture (2% sodium carbonate, 2.7% sodium potassium tartrate and 1% copper sulphate) followed by addition of 1 N NaOH and incubated for 10 min. Finally FC reagent was added to the reaction mixture and absorbance was measured at 660 nm after 60 min of incubation. The previously ascribed thiobarbituric acid (TBA) based method of Bailly et al. (1996) was used for Malondialdehyde (MDA) content estimation and MDA content was expressed as µM/g FW.

4.3.7. HPLC based secondary metabolite analysis

HPLC analysis for withanolides was performed with some modification to the ascribed method of Mundkinajeddu et al. (2014). The Jasco HPLC system (LC-Net II), consisting of a quaternary pump (PU-2089), an autosampler (AS-2059 Plus) and a PDA detector (MDA-2018) was used for putative detection of withanolides. The compounds separation were achieved using 5 µm Luna C18 250 x 4.6 column and Solvents (A = Water Ultra-Pure ± 0.05% TFA (pH. 2.6), B = MeOH HPLC grade). Mobile phase was run using gradient elution at 0, 15, 20 and 23 min of 30, 80, 80 and 30% of solvent-B respectively. The flow rate was 0.7 ml/min and injection volume was 20 µl. The eluent was detected

and analyzed at 230 nm. For background correction standards of chlorogenic acid (100 mg/l), Moupinamide (25 mg/l) and withaferin A (146 µMbis) were passed through HPLC to assign respective peaks. While for other withanolide compounds reference extract called "Georgios shoot 2 in MS" was used.

4.3.8. Statistical and PCA analysis

Mean values of three replicates from the treatments were subjected to analysis of variance (ANOVA) and significant difference were separated using one-way ANOVA with Tukey's test using Statistix software (8.1 versions). For graphical presentation Origin lab (8.5) was used and error bars represent standard error (SE). For PCA analysis we used R (version 3.3) and selected 6 metabolites data from HPLC text file to build a 6-dimensional data set. All the samples were classified into one of 7 groups: violet, blue, green, yellow, red and white colors.

4.4. RESULTS AND DISCUSSION

4.4.1. Callus induction and growth

The efficacy of callus induction from leaf explants of *Withania somnifera* was screened out for optimum PGRs type and concentration after 5 weeks of culture (Table 4.1). Callus was initiated on MS medium containing TDZ, BAP and NAA either alone or in combinations. While BAP alone failed to induce callus in leaf explants. The explant started to turn brown after one week of culture. Maximum callus induction frequency and fresh weight (g explant^{-1}) of 78.3% and 3.8 g, respectively were observed on MS medium containing TDZ (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}). The minimum callus induction frequency of 38.4% was observed on MS medium containing NAA (0.5 mg L^{-1}) alone, which confers the hypothesis of plant cell growth needs proper ratio of cytokinin and auxin. Callus fresh and dry weight remained minimum (P<0.05) on medium containing NAA alone; whereas an increase in growth was observed when NAA was used in combination with TDZ or BAP. It was observed that with increase of NAA concentration callus induction was optimum but adventitious root formation overwhelmed callus growth.

At all the tested PGRs treatments compact green calli with upper top snowy layer of growing cells were observed (Fig 4.1A). Contrarily, Rani et al. (2003) reported maximum callus induction from cotyledanary leaf explant of *W. somnifera* on MS medium containing 2, 4-D and Kin. This could be due to the differences in the explant type and its origin that responded differently. In agreement with current findings, Ali et al. (2014) reported callus induction in leaf explant of *Artemisia absinthiam* on MS medium containing TDZ and NAA.

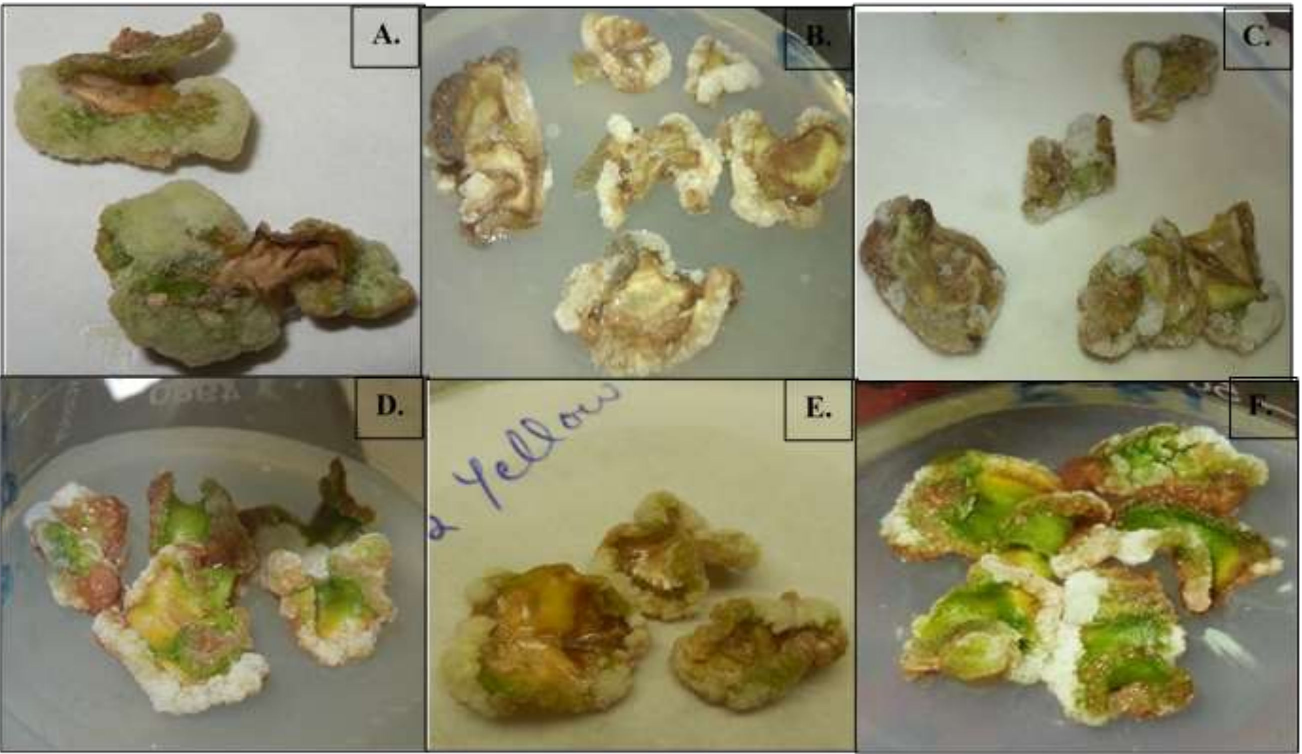

Figure 4.1: The leaf derived callus morphology of *Withania somnifera* effected by light quality (A.) White light, (B.) Violet light, (C.) Blue light, (D.) Green light, (E.) Yellow light and (F.) Red light.

Table 4.1: The implication of different plant growth regulators (PGRs) for optimum callus induction and callus growth of *Withania somnifera* L.

PGRs (mg L^{-1})					
TDZ	**BAP**	**NAA**	**CIF** (%)	**FW** (g explant^{-1})	**DW** (g explant^{-1})
1	0	0	49.3 ± 2.84^{EF}	0.46 ± 0.61^{CDE}	0.05 ± 0.007^{H}
0	0	0.5	38.4 ± 1.2^{H}	0.16 ± 0.13^{DE}	0.04 ± 0.005^{I}
0	0.5	0	-^{I}	-^{E}	-^{I}
0.1	0	0.1	55.6 ± 1.76^{D}	1.68 ± 0.21^{B}	0.11 ± 0.003^{E}
0.1	0	0.5	62.67 ± 1.45^{BC}	1.6 ± 0.26^{B}	0.18 ± 0.0015^{C}
0.1	0	1.0	66.3 ± 2.60^{BC}	1.8 ± 0.23^{B}	0.17 ± 0.002^{C}
0.1	0	1.5	50.3 ± 1.76^{EF}	0.78 ± 0.28^{C}	0.08 ± 0.008^{F}
0.5	0	0.5	78.3 ± 1.35^{A*}	3.5 ± 1.02^{A*}	0.29 ± 0.0019^{A*}
0	0.1	0.5	43.67 ± 1.9^{GH}	0.97 ± 0.09^{C}	0.105 ± 0.007^{E}
0	0.1	1.5	61.3 ± 0.89^{C}	0.83 ± 0.046^{C}	0.062 ± 0.0037^{G}
0	0.1	2.0	46.68 ± 2.85^{FG}	0.68 ± 0.078^{CD}	0.14 ± 0.005^{D}
0	0.5	0.5	67.89 ± 1.22^{B}	0.94 ± 0.05^{C}	0.21 ± 0.001^{B}

*Means are significantly different at $P<0.05$, data sets with different alphabets are significantly different from each other.

4.4.2. Effect of monochromatic light on callus induction and growth

The leaf explant cultured on optimized MS medium varied significantly in callus induction frequency, fresh weight and dry weight, when incubated under different monochromatic light at 25±2 ^{0}C (Fig 2).

The callus induction frequency (CIF) was observed to be 84% under red, 79% under normal (white), 65.26% under green, while only 55.6% under blue, 49.33% under yellow and 41.6% under violet light treatments. The callus induction frequency among white and red light treatments were not significantly different at *P*<0.05. The maximum fresh weight and dry weight of 4.4 g explant^{-1} and 0.2 g explant^{-1}, respectively under red-light treatment was significant (*P<0.05*) higher than other treatments (Fig 4.2).

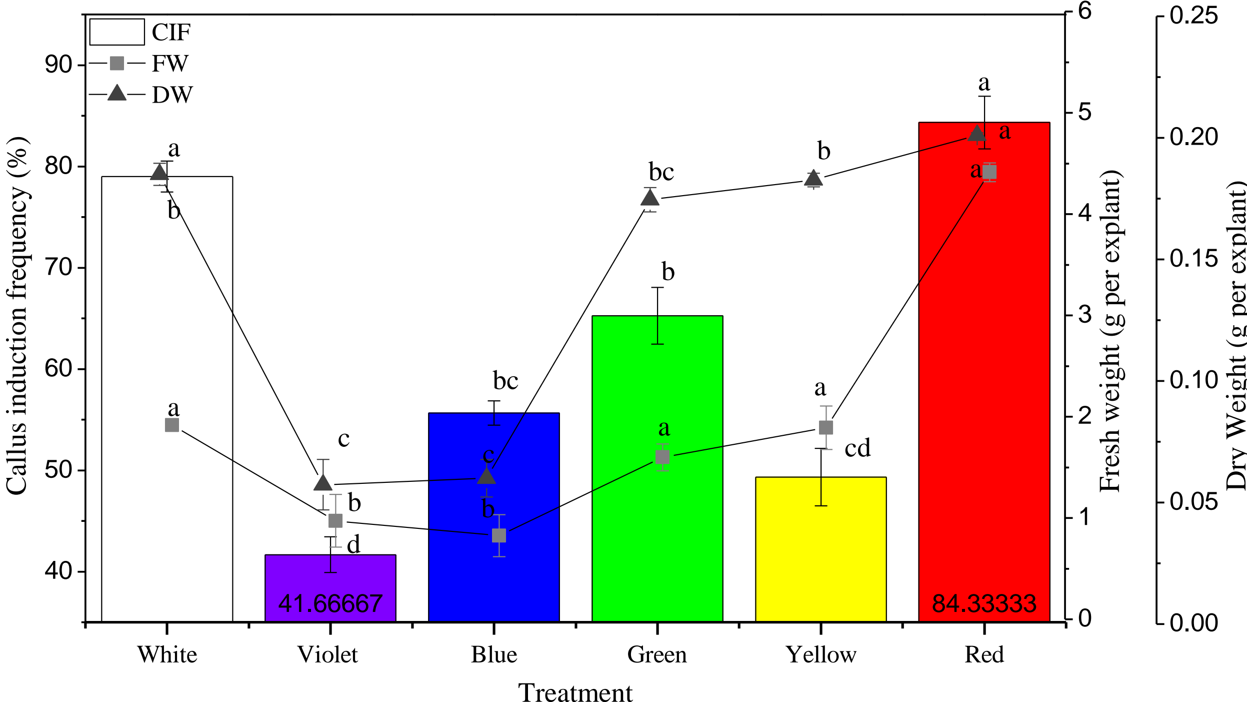

Figure 4.2: Effect of different light colours on callus induction and growth from leaf explant of *Withania somnifera*. Data columns and points with error bars are mean of three replicates and alphabets indicates significance level at P value <0.05.

The calli under red-light treatments were greener and compact in appearance with higher fresh weight and dry weight, and with fewer symptoms of necrosis. This could be related to activation of phytochromes system by the red-light to promote callus induction and growth. The green color of the callus corresponds to chlorophyll content and it is suggested that red light promotes its synthesis in *Ceratodon purpureus* (Lamparter et al. 1997). Similarly, Kim et al. (2004) found higher biomass

accumulation in *Chrysanthemum* plant under red light treatment as compared to blue. Comparatively, to the callus obtained under red and control light the callus under violet light was more necrotic and white in color with minimum fresh weight and dry weight (Fig 4.1F). The possible explanation for higher biomass accumulation in callus under red light might be due to the fact that endogenous cytokinin synthesis is enhanced in callus when exposed to red light (George et al. 2008). Ours findings were contradictory to the findings of Ahmad et al. (2016) where they observed white light treatment was supportive to callus growth of *Stevia rebaudiana*. However, Guo et al. (2007) found increase in callus biomass of *Saussurea medusa* when exposed to red light irradiation.

4.4.3. Callus crude extract amount, total phenolics and flavonoids content

Callus obtained from leaf explant of *W. somnifera* under different treatments of light showed significant variations in total extractable amount, phenolic and flavonoid contents (Fig 4.3). Maximum extract of 5.4 mg/300 mg DW was observed in callus ($P<0.05$), cultured under red-light for 7 weeks, this was followed by 4.6 mg (white) and 4.45 mg (yellow). While minimum amount of extract was noticed for callus obtained under green-light treatment. The extract value corresponds to the amount of organic substances produced under respective treatment and may represent the extent of gene expression involved in biosynthesis of these substances. While, total phenolic and flavonoid content were maximum (42 and 2.5 mg g^{-1} DW, respectively) under the violet-light as compared to the other spectra`s of light. While, Guo et al. (2007) reported maximum phenolics and flavonoids content in blue light treated callus of *Saussurea medusa*. The red-light treated callus stands second with values of 39.6 mg g^{-1} DW and 2.08 mg g^{-1} DW for phenolic and flavonoid, respectively, at $P<0.05$ (Fig 4.3). The possible reason for the significant variation in extractable amount, phenol and flavonoid content could be differential evoking response of signal transudation in cells. Significantly, lower phenolic (18.8 mg g^{-1} DW) and flavonoid (0.57 mg g^{-1}DW) were observed in callus treated with white-light. These compounds play an important role in stress alleviation in plants and the variation in their content corresponds to the degree of stress caused by different monochromatic light.

The effect of light quality on callus secondary metabolite content of *W. somnifera* is not available in the literature, while it is well studied for other plant species. Like, Wang et al. (2001) reported enhanced secondary metabolites production in red light treated hairy roots of *Artemisia annua*. While there are some different reports in literature that might be due to the varying responses of different plant species

to different light colors (Tariq et al. 2014; Ahmad et al. 2016). However, Shohael et al (2006) reported highest eleutherosides content in red-light treated somatic embryo culture of *Eleutherococcus senticosus*.

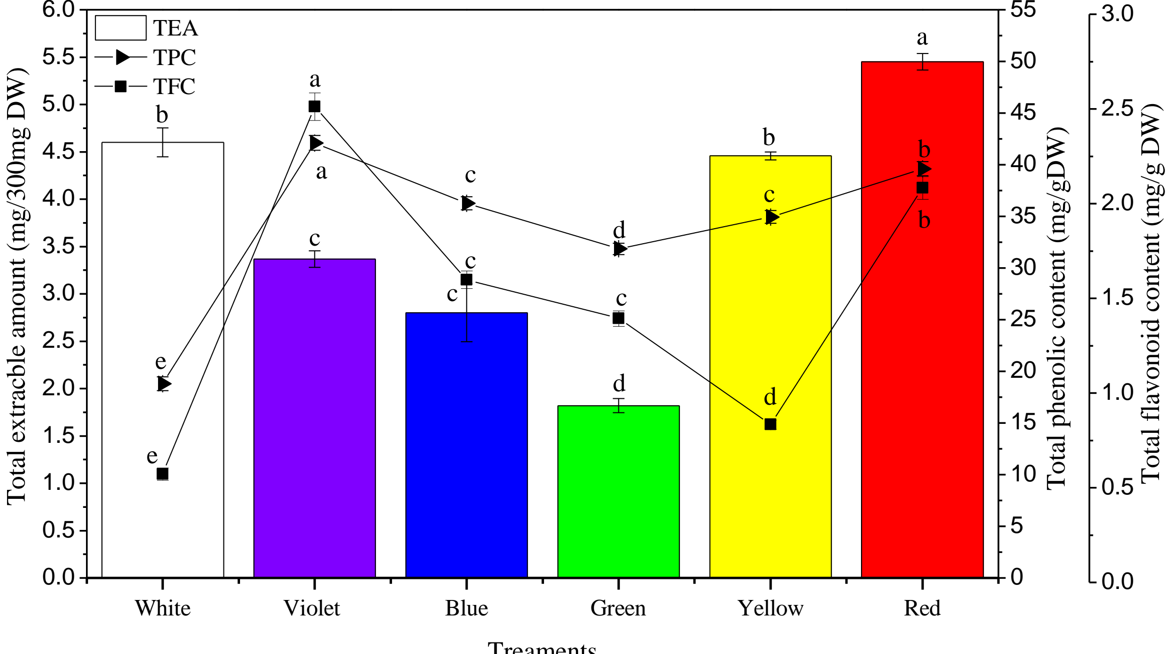

Figure 4.3: Effect of light quality on callus extractable amount, total phenolics and flavonoids content of *Withania somnifera*. Data columns and points with error bars are mean of three replicates and alphabets indicates significance at P value <0.05.

4.4.4. Callus Anti-oxidant activity

Similar to total phenolic and flavonoid content in callus extract, we observed significant variation in anti-oxidant activities as well. The antioxidant potential varied according to the type of free radical produced in reaction mixture and probably it might be due to the affinity of different molecules (in extract) to the type of radicals (Folta et al. 2001). Interestingly, significantly ($P<0.05$) maximum DFRSA (64%), TAC (4.5 mg AAE g^{-1} DW) and TRP (2.6 mg AAE g^{-1} DW) were observed in red light treated calli, compared to the other treatments (Fig. 3.4). Minimum DFRSA (21.6%), TAC (2.26 mg

AAE g^{-1} DW) and TRP (1.8 mg AAE g^{-1} DW) were found in calli treated with white-light. There were less significant (P value less than 0.05) variation in antioxidant activity of calli treated with blue, green and yellow lights. The variation in antioxidant activity were strongly correlated to TPC and TFC of the callus and can be well explant from flavonoid and phenolic content prospective.

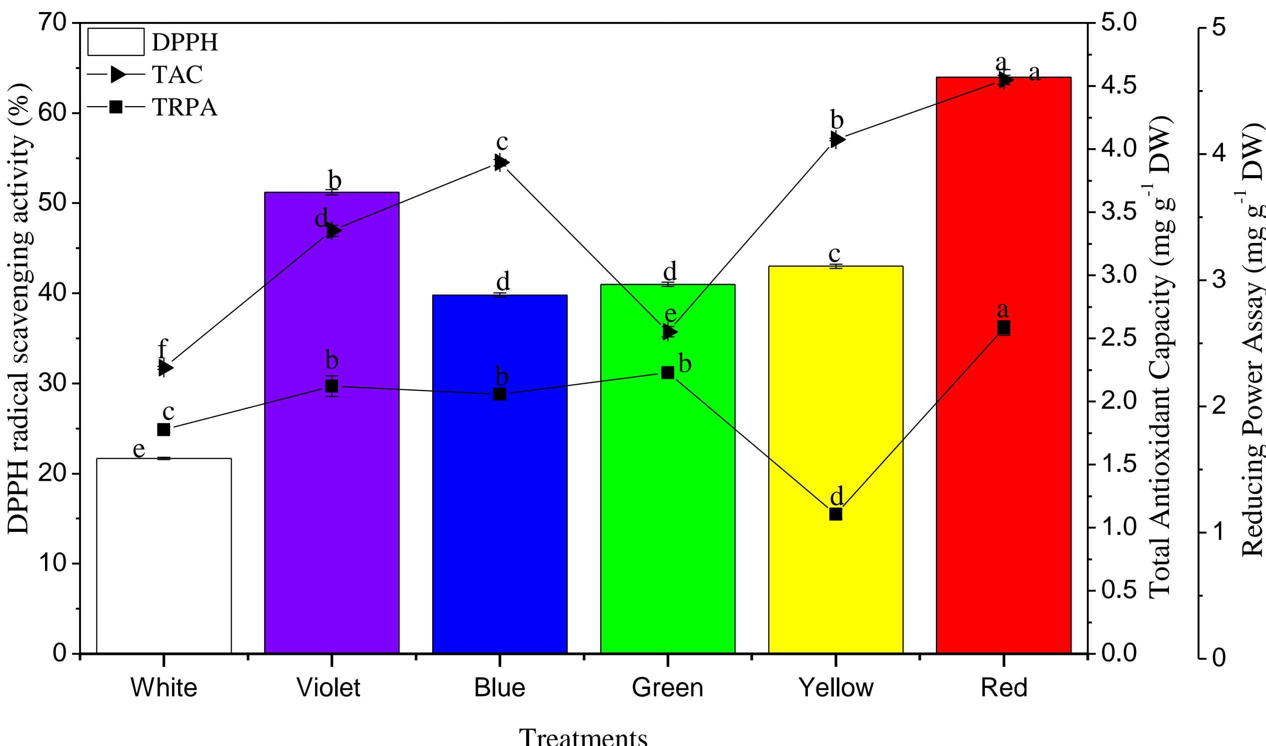

Figure 4.4: Light quality induced changes in DPPH free radical scavenging activity, total antioxidant potential and reducing power assay of callus cultures of *Withania somnifera*. Data columns and points with error bars are mean of three replicates and alphabets in indicates significance at P value < 0.05.

4.4.5. Total protein content, protease activity and MDA content

The effect of different light spectra on callus total protein content, protease activity and MDA content after 7 weeks of culture is shown in Figure 4.5. Maximum total protein content of 376.6 µg BSAE mg^{-1} FW was observed in callus treated with normal white light fluorescence while at the same treatment the

protease activity (6.1 U g^{-1} FW) and MDA (0.47 µM g^{-1} FW) content were minimum with respect to blue and violet light treated callus (Fig. 4.5). Red light stands second in total protein content and protease activity, while minimum total protein (172.6 µg BSAE mg^{-1} FW) content was observed in callus treated with blue light. Hypothetically, decrease in growth in blue light treatments ultimately resulted in lower total protein content in callus (Folta et al. 2001; Gibon et al. 2009).

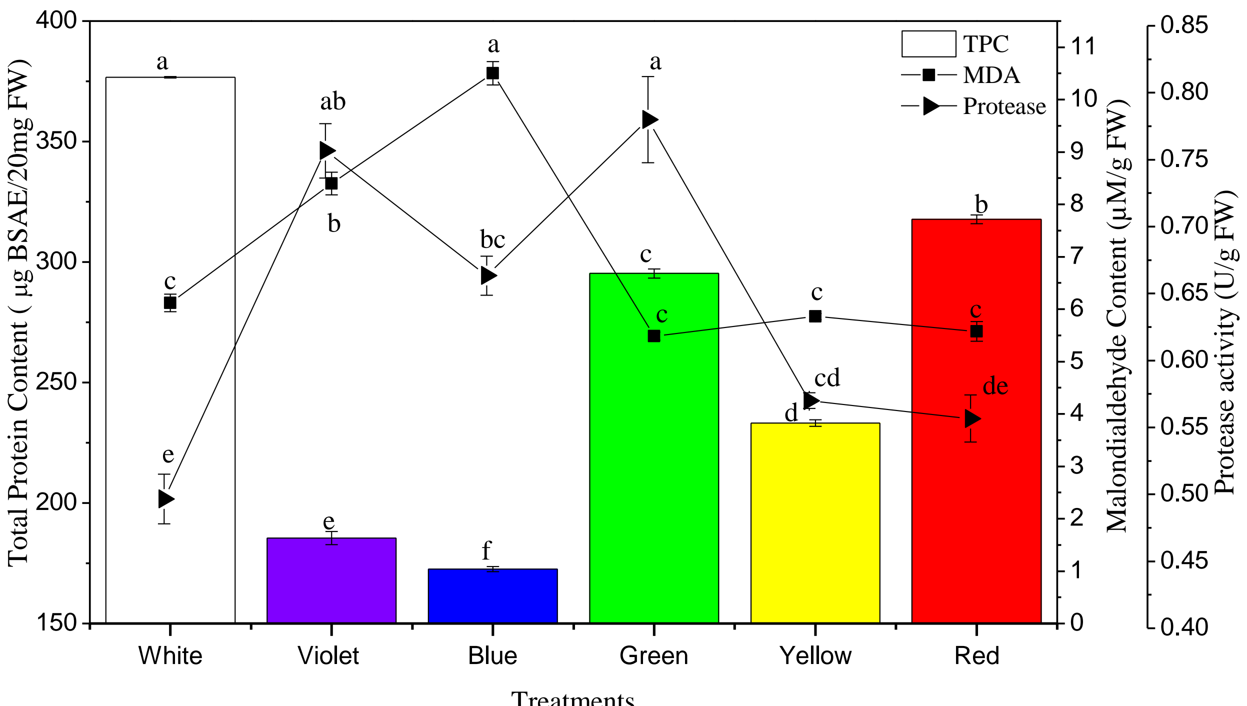

Figure 4.5: Differential expression of proteins, MDA and proteases under different light colours in callus cultures of *Withania somnifera*. Data columns and points with error bars are mean of three replicates and alphabets indicates significance at P value < 0.05.

The variation in protein content could be due to the variation in the expression pattern of genes regulated through phytochromes (phy A to phy E) and cryptochrome by sensing different light spectra (Quail 2002). The maximum total protein content in white light treatment (16/8 hr) might be due to the dark and light cycle that lead to accumulate higher level of transcription factor called phytochrome interacting factors (PIFs), which in turn regulates gene expression for different physiological responses. Previous

study has proved that phytochromes has protein kinase activity, that phosphorylate the proteins and phosphorylated form of proteins has a profound effect on its activity and interaction with other proteins (Dewir et al. 2006). Higher MDA content in violet and blue light treated calli suggesting that this light induces damage to cell membrane due to oxidative stress and ultimately hamper the callus growth.

4.4.6. SOD and POD activity

The sequence of metabolic pattern that occurs during callus growth under different spectral light treatments involves the activation of specific enzymes to cope with the stress. As the first line of defense SOD catalyze the conversion of O_2^- (produced in cell due to high light energy stress) into H_2O_2 and O_2 (41). Further the H_2O_2 is decomposed by POD by oxidation of co-substrate such as phenols or other antioxidant compounds (Meloni et al. 2003). In this report, we observed maximum SOD (0.15 nM/min/mg FW) and POD (0.29 nM/min/mg FW) activity in violet-light treated callus that was followed by blue light (0.09 and 0.2; respectively), whereas these activities were comparable to white and red light treated calli (Fig. 4.6).

The maximum SOD and POD activity in violet light treatment could be attributed to plant antioxidant system for elimination of ROS to attain higher callus growth. The decline in the SOD and POD level in calli treated with red and white lights might be due to the lesser degree of oxidative damage induced by these treatments. Contrarily, Shohael et al. (2006) reported higher SOD activity in red light treated somatic embryos of *E. senticosus*. Keeping in view MDA and protease activity (Fig. 4.5) was higher in violet light treatment; accordingly, the SOD and POD activity were maximum under the same culture conditions.

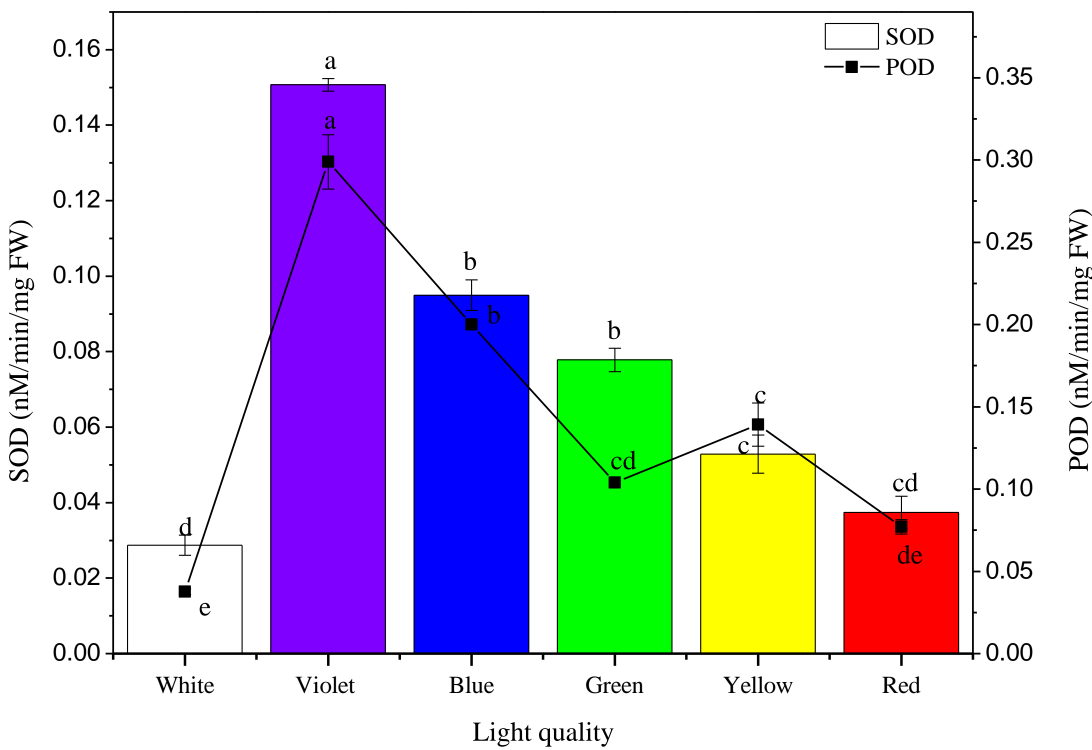

Figure 4.6: Activity of anti-oxidative enzymes, Peroxidase (POD) and superoxide dismutase (SOD) activities in callus cultures during different light treatments on optimized MS media, the white light treated callus used as control. Values are mean ± SE of three replicates and alphabets shows significance at P value < 0.05.

4.4.7. HPLC fingerprinting and PCA analysis

The metabolite profile of these *in-vitro* grown calli were analyzed and compared with standard compounds of withanolides (withanomide Q, withaoside IV or X, withaferin A and withanoside V), moupinamide and chlorogenic acid. Significant variation in peak area were observed in calli grown under different light treatments. Chlorogenic acid and withanolides content varied significantly in callus cultures with respect to light wavelengths. Red light treated callus yielded significantly higher (11.5 mg/g DW) chlorogenic acid, moupinamide (41.5 mg/g DW) and withaferin A (24.28 mg/g DW).

However, violet light treated callus stand second in chlorogenic acid and moupinamide contents. White light treated callus contained the lower level of these medicinally important secondary metabolites.

Figure 8 displays a PCA score plot of *W. somnifera* callus obtained under different light treatments. PCA 1and PCA 2 explained total 74.4% and 91.3% of variations, respectively on the basis of the specific metabolites. Callus samples from blue and Violet light treatments were clustered together closely, indicating the similar metabolites compositions. Similarly, white light and green light treated were clustered together but with distinct fingerprints then others. While red light and yellow light treated calli were distinct and far away from each other on the plot. This data suggests that the selected 6 metabolites in the extract were sufficient to separate the callus samples on the basis of light treatments.

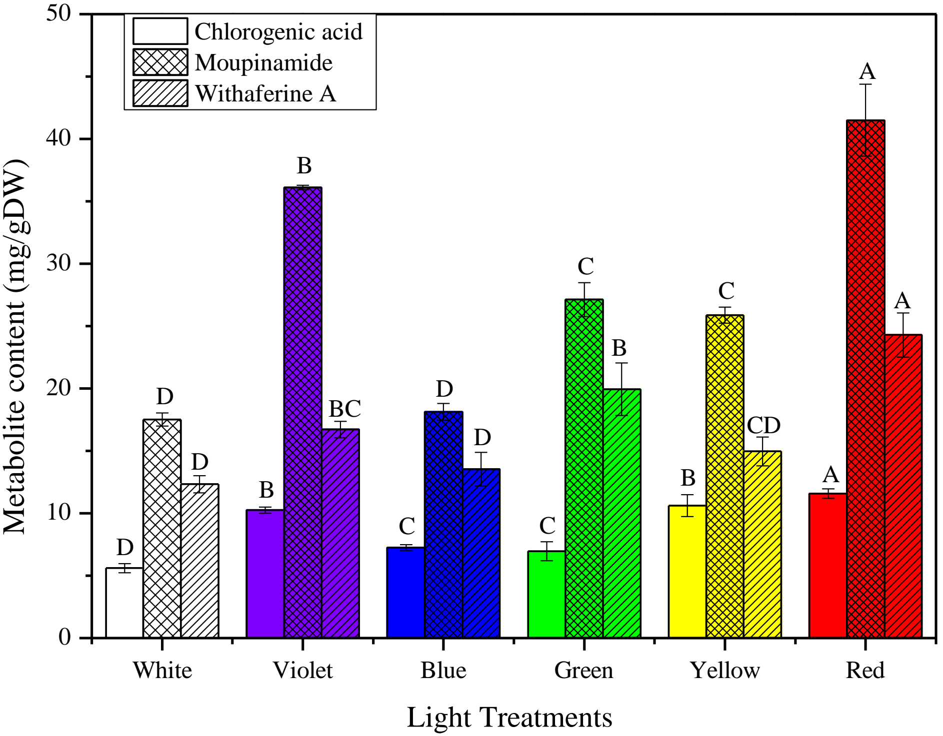

Figure 4.7: Effect of light colours on chlorogenic acid, moupinamide and withaferin A contents (mg/g DW) in callus cultures of *Withania somnifera*. Values are mean ± SE of three replicates and alphabets shows significance at P value < 0.05.

The concentrations of various secondary plant products strongly depend on the growth conditions and it is obvious that in vitro stress conditions have a strong impact on the metabolic pathways responsible

for the accumulation of the related natural products (Khan et al. 2015b). In corroboration to data of biochemical markers, it is evidenced that withanolides production in calluses raised under red light was synchronized by the profound expression of other phytochemicals like total phenolic and flavonoid content (Fig 4.7). Since withanolides biosynthesis involves the conversion of phenylalanine to cinnamic acid by phenyl ammonia lyase (PAL) enzyme (Khan et al. 2015b). Therefore it might be hypothesized that PAL enzyme has been triggered during callus growth under red light for signaling the phenylpropanaoid pathway to circumvent the *in-vitro* oxidative stress by production of higher level of withanolides for acquiring callus organogenesis in leaf explants.

In the Figure 4.8, a PCA score plot of *W. somnifera* callus was plotted on the basis of obtained HPLC chromatograms (Fig. 4.8) to differentiate the effect of light colors on callus HPLC finger prints. PCA 1and PCA 2 explained total 74.4% and 91.3% of variations, respectively on the basis of the specified metabolites. Callus samples from blue and Violet light treatments were clustered together closely, indicating the similar metabolites compositions. Similarly, white light and green light treated were clustered together but with distinct fingerprints then others. While red light and yellow light treated calli were distinct and far away from each other on the plot. This data suggests that the selected 6 metabolites in the extract were sufficient to separate the callus samples on the basis of light treatments.

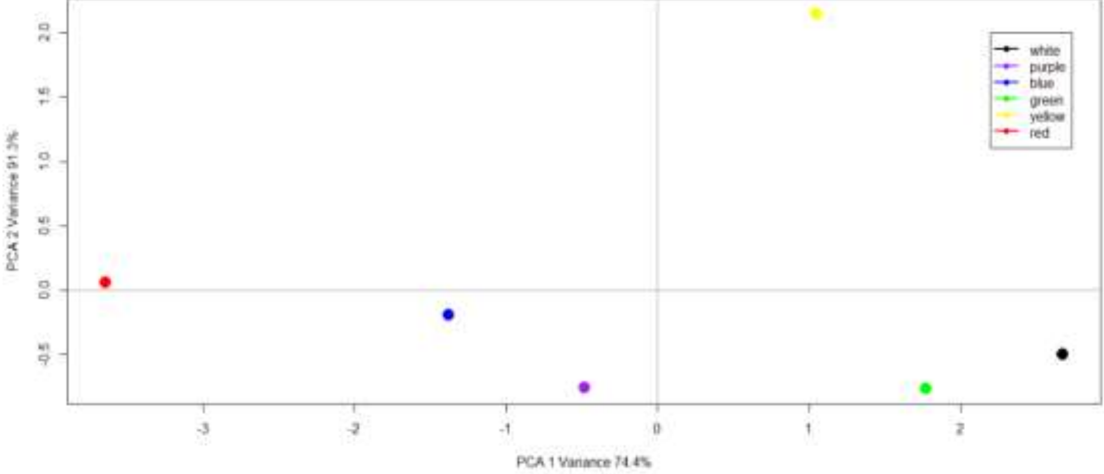

Figure 4.8: Principal component analysis (PCA) analysis plot with principal components 1 and 2 of callus extracts obtained after different light treatments.

4.5. CONCLUSIONS

We concluded that red light treatment is supportive for maximum biomass accumulation but with slight reduction in phenolic and flavonoid content, less photo-oxidative stress with reduced SOD and POD activity and higher antioxidant activities in *W. somnifera* callus culture. On the other hand, violet light is useful for maximum phenolic, flavonoid, chlorogenic acid and withanolides production. This study also shows that differences in SOD, POD, Protease activity and MDA content in callus culture of *W. somnifera* could be linked with the differential oxidative stress response in the cell to light quality for alleviating the stress. From PCA analysis the effect of respective light colors on metabolites variation could be clearly pictured. Future research will refine the use of optimum light quality for higher biomass as well as for enhanced secondary metabolites production and must focus on the metabolic pathways that are modulated during the process

5. EFFECT OF MEDIA TYPE ON CALLUS MORPHOLOGY AND ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *WITHANIA SOMNIFERA* FOR ENHANCED PRODUCTION OF SECONDARY METABOLITES

5.1. ABSTRACT

An *in-vitro* cell suspension culture of *Withania somnifera* L. was established for commercially important secondary metabolites production. Cultures were initiated from zygotic embryos, cultured on different media types; varying in composition of macro, micro, other organic additives and plant growth regulators. Friable callus with rapid multiplication rate was obtained on Glitz (Gli) media and 35 days old callus was sub-cultured on the respective media for growth kinetics study. Maximum (15 g) fresh weight of callus was observed on day 56 of culture with shorter lag phase on Gli media. For cell suspension culture obtained callus on Gli medium was introduced to Gli liquid (40 ml) medium in blue cap bottles and kept in shaker incubator for 54 days. Biomass accumulation and secondary metabolites content were studied in 6 days old cell cultures for 54 days. Moreover, the established cell cultures were elicited with methyl jasmonate (MeJa; 5, 10 and 20 µM) and salicylic acid (SA; 50, 100 and 200 µM) and growth parameters and secondary metabolites accumulations were studied using calorimetric methods and high performance liquid chromatography (HPLC). Maximum levels of total phenolic content 35.36 mg GAE/g DW (Control: 10.73 mg GAE/g DW), total flavonoid content 3.2 mg QE/g DW (control: 0.92 mg QE/g DW) and DPPH free radical scavenging activity 60.48 % (control: 35.5 %) were detected in elicited cell suspension cultures. Among the withanolide content, maximum content for withanoside IV and withaferine A were detected in MeJa treated cell cultures. The results suggest that media composition can be used to improve biomass and secondary metabolites production in cell suspension culture of *W. somnifera* L.

5.2. INTRODUCTION

The optimum growth of plant cell and tissue is crucial determinant for successful establishment of *in-vitro* cultures. The appropriate *in-vitro* tissues growth requires specific nutrients according to the explant type and plant species used for culture initiation (Saad and Elshahed 2012). The systematic experimentations and trails of years resulted plant tissue culture medium for successful plant tissue culture experiments (Trigiano and Gray 1999; Saad and Elshahed 2012). It is obvious from literature that an ideal plant tissue culture medium comprises of macronutrients (< 0.5 mM L^{-1}), micronutrients (< 0.5 mM L^{-1}), amino acids, nitrogen supplements, carbon source, vitamins, plant growth regulators and gelling agent (Murashige and Skoog 1962; De Fossard 1976). The variations in composition of medium significantly affect plant cell and tissues growth and friable callus is always considered desirable for establishment of cell suspension culture.

Withania somnifera (L.) (Solanaceae) is highly reputed medicinal plant, extensively used in Ayurvedic recipes and commonly known as Ashwagandha/winter cherry/Indian ginseng (Bano et al. 2015). It is used by the herbalist as decoctions (Kwatha), infusions, ointments (Chrita; medicated ghee or Narayana taila), powder (Churna), and syrups (Arishta; medicated wine). It occurs globally and has several therapeutic properties e.g. antioxidant, antimicrobial, antifungal (Alam et al. 2012), adaptogenic (Bhattacharya and Muruganandam 2003), cardioprotective (Mohanty et al. 2004), anticancer (Mohan et al. 2004), neuroprotective (Ahmad et al. 2005), immunomodulatory (Davis and Kuttan 2000), hepatoprotective (Sabina et al. 2013) and anti-inflammatory (Bhattacharya et al. 1997). These therapeutic activities are attributed mainly due to the presence of steroidal lactones i.e., withanolides and polyphenols present in *W. somnifera* (Mirjalili et al. 2009).

In India cultivable land is declining day by day due to various developmental activities while Pakistan is already importing medicinal plants raw material from India. There are no possibilities of substituting food crops with medicinal and aromatic plants in fertile land, due to increasing food requirements. Furthermore, Because of low germination of seeds and excessive exploitation, it has become an endangered plant (Chaturvedi et al. 2007). In this case, the only option left is to use plant cell organ and tissue culture technology to cultivate *W. somnifera* L. in large scale bioreactors. In most of the cases it needs proper optimization of each and every step like media composition, plant growth regulators and use of specific elicitors to enhance the secondary metabolites production. Pharmaceutical companies

and local drug manufacturers depend on the raw material supply, but metabolic inconsistency in the raw material compromises there end products efficacy (Mir et al. 2014a, Kumar et al. 2012). Plant tissue culture technology offers the only reliable and efficient alternative for obtaining the consistent plant material for effective drug formulations (Skrzypczak-Pietraszek et al. 2014). There are several available reports that attempted to establish cell and organs cultures of *W. somnifera*. But still its commercial scale production is hindered by lack of exclusive information for establishment of commercial scale production unit to meet its market demand.

Previously, the production of withanolide D, withaferin A and withanone have been reported in cell suspension, hairy root and shoot cultures of *W. somnifera* (Roja and Heble 1991; Banerjee et al. 1994; Ray et al. 1996; Vitali et al. 1996; Furmanowa et al. 2001; Ray and Jha 2001; Sangwan et al. 2007; Murthy et al. 2008). Comparatively low yield of secondary metabolites were found in cell cultures (Baldi et al. 2008a; Sabir et al. 2011). Nagella and Murthy (2010, 2011) attempted to stimulate withaferin A production in cell suspension culture by using plant growth regulators (PGRs), macro, micro compositions, carbon sources, inoculum mass, media strength and type. The highest production of withanolide A content was recorded in the medium with 2.09 KNO_3 (4.36 mg g^{-1} DW) and the greatest biomass [147.81 g l^{-1} fresh weight (FW) and 14.02 g l^{-1} (dry weight (DW)] in the medium containing a 0.59 concentration of NH_4NO_3.

In present study, we studied the effect of different media composition on zygotic embryos derived callus morphology and growth of *W. somnifera* L. Further, we studied the effect of elicitors (SA and MeJa) on biomass accumulation and secondary metabolites production in cell suspension culture of *W. somnifera* L.

5.3. MATERIALS AND METHODS

5.3.1. Seed sterilization, zygotic embryos isolation and culture conditions

Seeds were isolated from fruits of *W. somnifera* L. and surface sterilized according to ascribed protocol of van der Valk et al. (1992) with some modification. Briefly, isolated seeds were sterilized in NaOCl solution (2%, w/v) containing Tween 20 (10 drops per 100 ml) for 24 hr, rinsed in sterilized distilled water (dH_2O), followed by a dip in ethanol (70%, w/v) for 20 sec and then rinsed with sterilized distilled water (dH_2O). Seeds were further sterilized with 5% H_2O_2 (v/v) for 10 min and rinsed again with distilled

water (dH_2O) in air-laminar flow cabinet. The sterilized seeds were de-coated under stereo microscope, zygotic embryos were isolated aseptically and embryos without apparent injury were cultured horizontally on media varying in composition (Table 5.1) in petri-plates. There were three replicates, each treatment consist of five embryos and were kept in continuous dark at 25 ± 1 ^{0}C in growth room.

Treatment media are detailed in Table 5.1 and were prepared according to standard protocols (Litvay et al. 1985; Schenk and Hildebrandt 1972; Murashige and Skooge 1962; Hargreaves et al. 2009). The 2,4-D and sucrose concentration (3%) remained the same in all for media types while gelrite (3 g L^{-1}), phytagel (1.8 g L^{-1}) and agarose (0.8%) were used as gelling agents in Glitz, SOM and SH/MS media respectively.

Table 5.1: Media composition (mg/l) for callus initiation from zygotic embryo of *Withania somnifera*.

Media components	Gli	SOM	MS	SH
Majors				
KNO_3	950	11685	1900	2500
$MgSO_4.7H_2O$	925	925	180.54	195
KH_2PO_4	170	425	170	
$CaCl_2.2H_2O$	11	1100	332.02	151
NH4NO$_3$	825	1380	1650	
$NH_4H_2PO_4$				300
Minors				
$MnSO_4.4H_2O$	21	16.9	16.9	10
H_3BO_3	31	6.2	6.2	5
$ZnSO_4.7H_2O$	43	8.6	8.6	1
KI	4.15	0.83	0.83	1
$CuSO_4.5H_2O$	0.5	0.025	0.025	0.2
$Na_2MoO_4.H_2O$	1.25	0.25	0.25	0.1
$CoCl_2.6H_2O$	0.125	0.025	0.025	0.1
Iron Source				
$FeSO_4.7H_2O$	30	27.8	36.70 (Iron EDTA)	19.8

Media components	Gli	SOM	MS	SH
$Na_2EDTA.2H_2O$	40	37.3		
Vitamins				
Thiamine HCl	5	1	0.1	5
Nicotinic acid	5	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5	0.5
Amino acids				
L-Glutamine	500	2250		
Casein hydrolysate	1000	2500		
Inositol	100	5000	100	1000
Glycine		2	2.0	
Sucrose	30,000	30000	30,000	30,000
Agarose	3000 (gelrite)	1800 (phytagel)	0.8%	0.8%
2,4-D	1	1	1	1
BAP	0.5			
Media pH	5.7	5.7	5.7	5.7

5.3.2. Callus induction and growth kinetics

To study the effect of media composition on callus induction, isolated zygotic embryos were incubated on different media (Gli, SOM, SH and MS) and callus biomass accumulation was estimated after 6-weeks of culture in triplicates. For growth kinetics study of callus, 0.5 g of callus inoculum were cultured on the respective media and biomass accumulation was estimated with in intervals of 7 days for 56 days period.

5.3.3. Cell suspension culture growth kinetics and elicitation

To establish cell suspension culture, 25 days old friable and proliferating callus on Gli media was selected as inoculum source for cell culture. 30 g of fresh callus was added to 70 ml liquid Gli media and stirred for 10 sec in blender at 120 rpm and was used as stock for cell culture in 250 ml blue cap bottles. The blended callus in liquid medium were poured into volumetric cylinder and were allowed to

settle down the suspended small cells aggregates. The supernatant liquid media was poured off and remaining cells aggregates of ~ 0.5 g fresh weight were introduced in triplicates to blue cap bottles containing 40 ml of fresh Gli liquid media. Observations and data of growth kinetics were performed with an interval of 6-days for 54 days of period. All the experiment were performed in triplicates and cultures were incubated in 24-hr dark conditions at 25 ± 1 ^{0}C.

For elicitation of cell culture, salicylic acid (SA) and methyl jasmonate (MeJa) were used in different concentrations; SA (50, 100 and 200 µM) and MeJa (5, 10 and 20 µM). These elicitors were added to the culture media on day 21 and cells were harvested after 48 days for biomass and secondary metabolites estimation. Fresh and dry weights were recorded in triplicates as g per culture.

5.3.4. Analytical methods

For cells and callus fresh weight and dry weight estimation the described method of Ali and Abbasi (2013) was used. The obtained dry callus and cells were extracted according to the modified method of Ali et al. (2013). Briefly, dried sample (300 mg) was mixed with 70 % methanol (2 ml). The mixture was mixed thoroughly by vertexing, followed by sonication for 30 min and then centrifugation for supernatant isolation. The procedure was repeated thrice to ensure complete extraction and isolated supernatant was used for analysis or stored at 4 ^{0}C for future use.

For total phenolics (TPC) and total flavonoid content (TFC) determination described protocols of velioglu et al. (1998) and Chang et al. (2002), respectively were used. The calibration curve (0–50lg/ml, R^2 =0.968) was plotted by using Gallic acid as standard and the TPC was expressed as mg Gallic acid equivalents (GAE)/g of dry weight. While, for the total flavonoids content, calibration curve (0–40lg/ml, R^2 = 0.998) was plotted by using quercetin as standard and was expressed as mg quercetin equivalents (QE)/g of dry weight.

Antioxidant activity was determined by DPPH free radical scavenging assay (FRSA) according to described method of Abbasi et al. (2010). Where, 2 mg butylated hydroxyanisole (BHA) in 4 ml methanol with 0.5 ml of DPPH solution was used as positive control for background correction and percent antioxidant activity in samples were calculated according to following formula:

% DPPH free radical scavenging activity = 100 x (1-AE/AD)

Where AE is absorbance of the solution when an extract was added at a particular concentration and AD is the absorbance of the DPPH solution with nothing added.

5.3.5. HPLC based fingerprinting

Chromatographic analysis was carried out on Jasco HPLC system (LC-Net II), consisting of a quaternary pump (PU-2089), an auto-sampler (AS-2059 Plus) and a PDA detector (MDA-2018). Separation of crude methanolic extract was performed on 5 µm Luna C18 (250 x 4.6) column and solvents (A = Water Ultra-Pure ± 0.05% TFA (pH. 2.6), B = MeOH; HPLC grade) at 30 ^{0}C. Mobile phase was run using gradient elution at 0, 15, 20 and 23 min of 30, 80, 80 and 30% of solvent-B respectively. The peaks for specific compounds in samples were assigned by diode array detector at 230 nm. For quantification and assigning of respective peaks external standards of chlorogenic acid (100 mg/l), Moupinamide (25 mg/l) and withaferin A (146 µMbis) were used. For quantification, series of working solutions of standards were passed through HPLC and peak responses were plotted against injected mass. On the basis of obtained plot, a calibration curve was created and obtained equation were used for quantification of respective compounds in our samples. Furthermore, compounds peaks were identified by comparing the retention times of unknown peaks with the peaks of reference standards in each chromatographic method.

5.3.6. Statistical analysis

Mean values of three replicates from the treatments were subjected to analysis of variance (ANOVA) and significant difference were separated using one-way ANOVA with Tukey's test using Statistix software (8.1 versions). For graphical presentation Origin lab (8.5) was used and error bars represent standard error (SE).

5.4. RESULTS AND DISCUSSION

5.4.1. Effect of media composition on callus growth and secondary metabolites content

The optimum nutrient concentration is a critical determinant in controlling the cells/ organ growth and secondary metabolism (Rao and Ravishankar, 2002). In the present study, the response time of explant (zygotic embryo) and callogenesis varied depending on media composition. Indeed, the tested media varied in compositions of $CaCl_2.2H_2O$, KNO_3, minor elements ($MnSO_4.4H_2O$, H_3BO_3, $ZnSO_4.7H_2O$ and KI), amino acids (L-Glutamine and Casein hydrolysate), BAP and gelling agents (Table 5.1). Out of the

tested medias, Glitz (Gli) media was supportive for maximum callus induction from zygotic embryos of *W. somnifera* L. and resulted significantly ($P<0.05$) maximum biomass accumulation compared to other tested media types (Table 5.2). Previously, Hargreaves et al. (2011) used modified Litvay medium (Glitz) (Litvay et al. 1985) for somatic embryogenesis in zygotic embryos of Christmas trees. Callus of compact and creamy texture with minimum biomass accumulation was observed in MS media. While callus on SH media was white and friable in appearance with slower growth rate (Fig. 5.1). Contrary to maximum biomass accumulation on Gli media, maximum total phenolic content was observed in callus obtained on MS media. While maximum flavonoids content was recorded for callus grown on SOM media and was followed by callus grown on MS media (Table 2). Similarly, MS medium was proved to be supportive for secondary metabolite production from *Artemisia absinthium in-vitro* cultures (Ali and Abbasi 2013; Ali et al. 2016). Previously, Kim et al. (2004) used immature zygotic embryo of *Catharanthus roseus* and reported embryogenic callus for successful regeneration of plants on MS media.

These differences in response of mature zygotic embryos to callus induction and proliferation on different media composition suggest that the amount of calcium chloride, nitrogen source, minor elements and vitamins were crucial for rapid cell growth and division. It has extensively been described in literature that calcium ion acts as transducer of hormonal and environmental signals to the responsive elements of cell metabolism (Evans et al. 1991). Furthermore $Ca^{\pm}$ modulated plant physiological responses to stress conditions, like salinity, chilling or anoxia (Jaleel et al. 2007).

Table 5.2: Effect of media composition on callus biomass and secondary metabolite content. Data were collected after 6 weeks of culture. Values are mean ± SD of three replicates.

Media	Callus morphology	FW (g/culture)	DW (g/culture)	% water content	TPC (mg/g DW)	TFC (mg/g DW)
MS	Creamy-compact	3.11 ± 0.12	1.7 ± 0.02	45.66	9.15 ± 0.05	0.44 ± 0.015
SH	White-fragile	3.77±0.05	0.7 ± 0.002	82.03	4.54 ± 0.10	0.216 ± 0.003
Gli	Green-fragile	14.36±0.17	3.44 ± 0.05	76.05	6.32 ± 0.17	0.154 ± 0.007
SOM	Green-compact	4.38 ± 0.05	0.97 ± 0.04	77.91	2.44 ± 0.08	0.63 ± 0.006

In addition, $Ca^{\pm}$ has been found to be involved in strengthening the cell wall and plant tissues and most importantly it increases the selective uptake of other essential elements from the media as well (Ozaki et al. 2005). Beside the nutrient concentration variation inclusion of BAP into the Gli media might also be the reason for rapid cell division and growth, as hypothesized in cytokinin to auxin ratio hypothesis. Callus obtained on the respective media were refreshed after 6 weeks and were studied for growth kinetics of 56 days to compare the growth pattern of callus on respective media (Fig. 5.2). Maximum callus fresh weight with shorter lag phase and green color of loosely packed cells were obtained on Gli media at day 56 of the culture (Fig. 5.1a). A prolonged lag phase of 21 days was observed for calli grown on SH, MS and SOM media. Callus grown on SH and SOM media retained their white and green colors respectively throughout the sub-culturing periods (Fig. 5.1b&d). While after 42 days of culture, calli on MS media turned brown of crispy texture from creamy green of compact texture. While on SOM and MS media the fresh biomass was reached to its maximum at day 49 of the culture and showed a decline afterward. Whereas on SH media growth curve tended to decline after day 42 of the culture (Fig. 5.2).

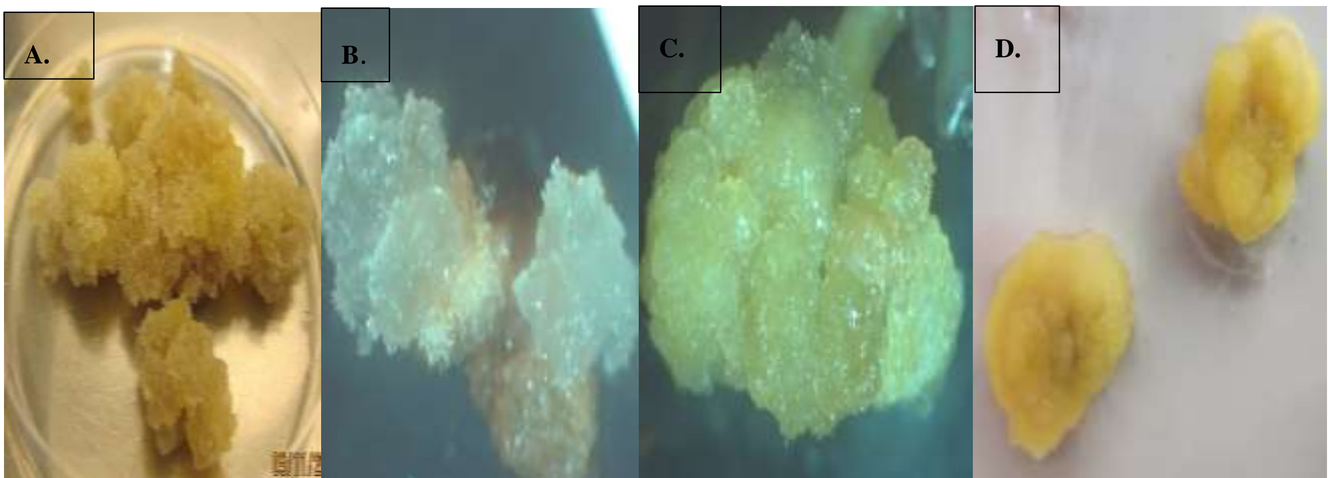

Figure 5.1: The zygotic embryo derived callus morphology effected by media composition (A.) Gli media, (B.) SH media, (C.) MS media and (D.) SOM media.

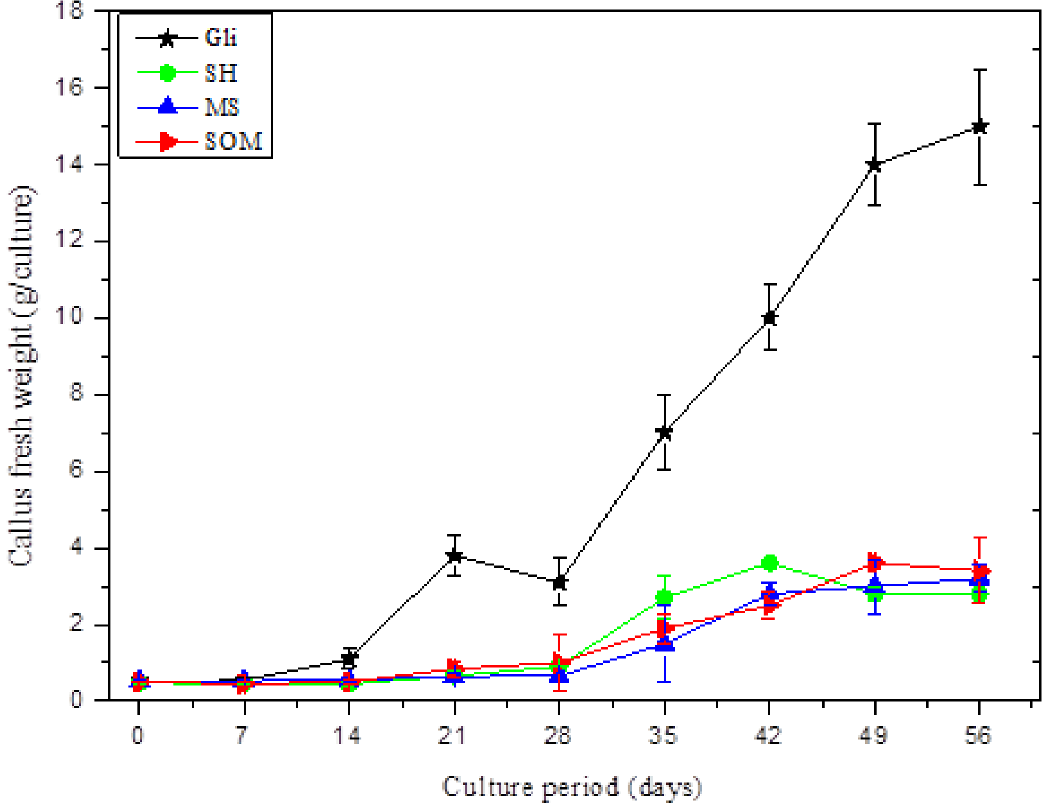

Figure 5.2: Callus growth curve (FW; g/culture) on different media compositions. Values are mean ± standard error.

Friability of callus tissue is highly desirable when establishing cell suspension culture (Akaneme and Eneobong 2008). It has been reported that higher concentration of Ca in media inhibits the uptake of other essential elements like Mg, Mn, Zn, etc. and this could be the possible explanation of retarded growth of callus on MS and SOM media (Muhammed et al. 1987; Schimansky et al. 1981). However, Ozaki et al. (2005) found less significant effect of Ca concentration on uptake of the other essential elements and concluded the elements ionic radius based competitive binding affinity to the receptors on roots. Beside this, Łojewski et al. (2014) observed that media organic constituents also play important role in accumulation of growth promoting essential elements in plant tissues. Norstog and Smith (1963) discovered enhanced plant cell growth when media was augmented with glutamine and alanine. Myo-inositol is believed to play a role in cell division because of its end products, ascorbic acid and pectin in the cell that incorporates into phosphoinositides and phosphatidyl-inositol (Kadhimi et al. 2014).

5.4.2. Establishment of cell suspension culture and growth kinetics

It is crucial to know the growth phases of cell culture to find the suitable day of elicitation for attaining the maximum biomass as well as secondary metabolites production. The friable green callus with loosely packed cells, obtained on Gli media was selected as source of inoculum for cell suspension culture establishment and blue cap bottles of 250 ml size were used to study growth behavior of cells in agitated liquid media for 54 days. Biomass accumulation of the cell culture of *W. somnifera* L. displayed a relatively speedy growth curve of just 6 days of lag period and followed by a prolonged log phase of 42 days on Gli liquid medium (Fig. 5.3 & 5.4). Doubling in fresh weight and dry weight was observed on day 12 of culture. However, maximum fresh weight and dry weight at day 48 were 17.9 g/culture and 5.07 g/culture, respectively. Furthermore cell suspension culture were creamy white at log phase and brown in color at death phase of culture (Fig. 5.3, D&E). The obtained packed cell volume of the cell suspension culture was about 10.3 ml in 100 ml liquid medium (Fig. 5.3, F). Previously, Sivanandhan et al. (2014) reported maximum biomass accumulation at day 28 of the cell culture in MS medium containing combination of picloram, Kin, L-glutamine and sucrose.

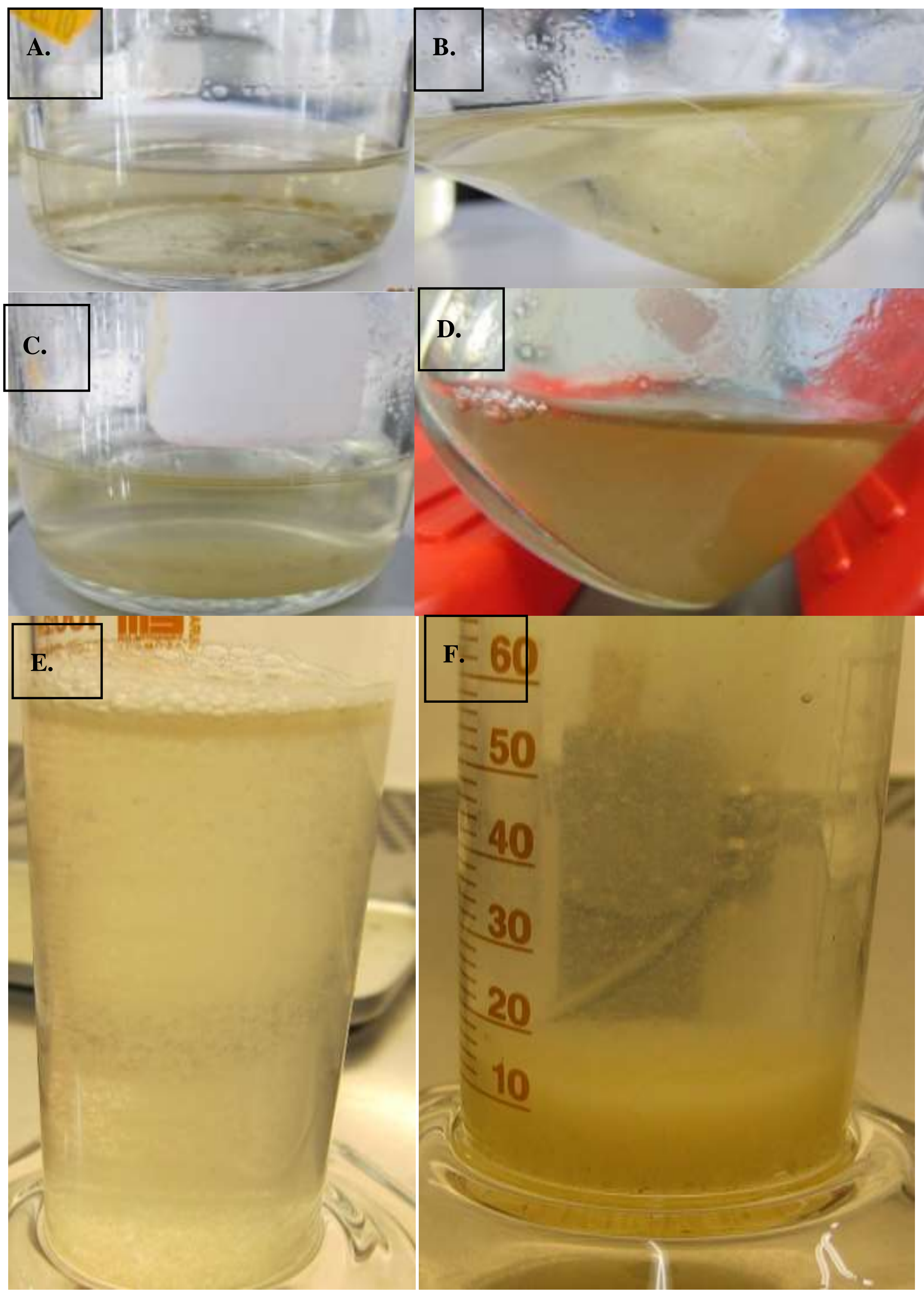

Figure 5.3: Cell suspension cultures of *Withania somnifera* L. during **(A.)** Lag phase, **(B.)** Log phase, **(C.)** Stationary phase, **(D.)** Death phase. While **(E.)** Suspended cells and **(F.)** Compact cell volume.

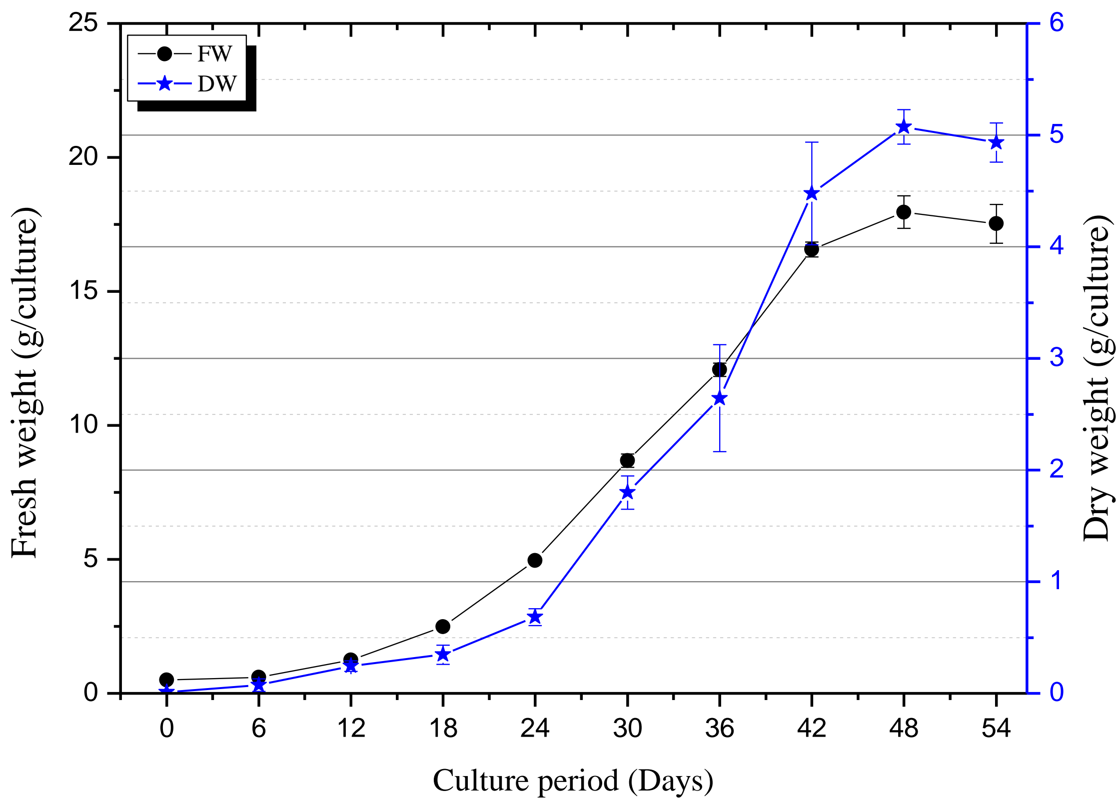

Figure 5.4: Growth kinetics of cells suspension culture of *Withania somnifera* L. on Gli media. Values are mean ± standard error of three replicates.

5.4.3. Total phenolic and flavonoid content in cell suspension culture

It was previously confirmed that secondary metabolite production occurs after the cells entering to exponential phase of the growth (Ouyang et al. 2005). In ours study, accumulation of total phenolic and flavonoids in cell suspension culture showed a growth dependent pattern on Gli liquid media (Fig. 5.5). On day 30th of the cell culture total phenolic and flavonoid content were 3 fold and 2 fold respectively, while cell dry weight was 9 fold with respect to dry weight at day zero of the culture. Maximum, 10.6 mg GAE/g DW and 1.05 mg QE/g DW total phenolic and flavonoid contents, respectively were reached on day 48 of the cell suspension culture and afterward a decline in these values was observed.

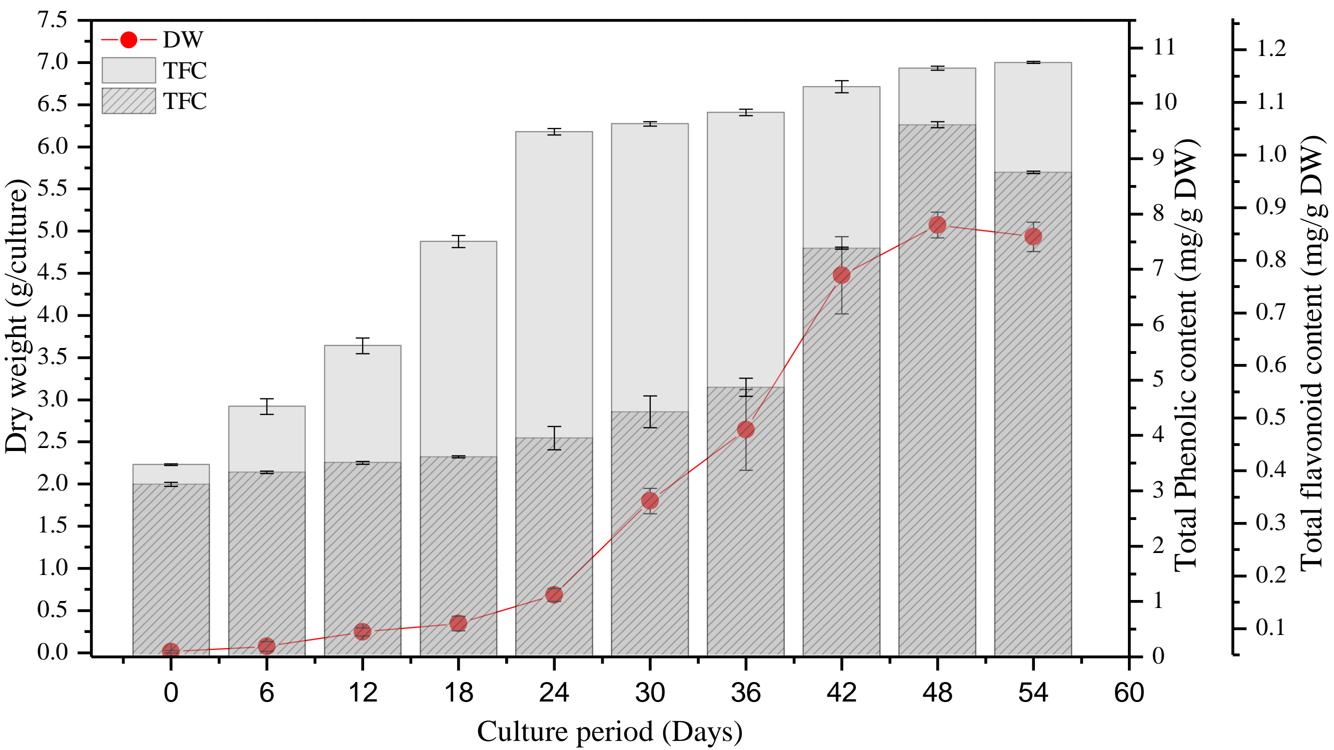

Figure 5.5: Total phenolic and total flavonoid contents with respect to cell culture growth curve (DW). Values are mean ± standard error.

5.4.4. Effect of elicitors on cells growth in cell suspension culture

Optimization of culture age is one of the critical factors to improve secondary metabolite productions in *in-vitro* cultures Hence, from growth kinetic study we incorporated methyl jasmonate (MeJa) and salicylic acids (SA) to the established cell suspension culture of *W. somnifera* L. at day 21 (exponential phase) of the culture. Previously, Sivanandhan et al. (2012) found 30 days old roots culture the optimum age for elicitation. However, Sakunphueak et al. (2010) recommended 21 days old culture the optimum age for elicitation. In ours preliminary investigations we selected 21 days old cell culture as the optimum age for elicitation experiment and observed a significant ($P<0.05$) variations in cell biomass accumulation after elicitation (Fig. 5.6). The harvested cells fresh weight of *W. somnifera* L. varied from 18 g/culture (control) to 10.8 g/culture (~2-fold decrease) and 4.65 g/culture (~ 4-fold decrease) when

MeJa (20 µM) and SA (200 µM) respectively were used as elicitors. Similarly, decrease in cell dry weight was observed and it varied from 5.04 g/culture (control) to 1.74 g and 0.89 g when elicited with MeJa 20 and SA 200 respectively (Fig. 5.6). So, here we concluded that SA was more effective in cell growth reduction. The resulting cells after 54 days of culture were dark brown in color when treated with SA. For comparison, statistically significant (*P<0.05*) reduction in biomass accumulation was observed in SA treated cell culture as compared to control and MeJa treated cells.

Rao et al. (1997) reported that exogenous application of SA, enhanced the lipid peroxidation and caused oxidative damages to *Arabidopsis thaliana* that ultimately caused reduction in biomass accumulation and similar observations were made by in tobacco and *Taxus chinensis* cultures (Yu et al. 2002). Similarly, Xu et al. (2015) found significant reduction in cells growth of *Vitis vinifera* L. when elicited with higher concentrations of elicitors. However, Ali et al. (2015) reported 3-fold increase in MeJa treated cells dry biomass with shorter log phase of *Artemisia absinthium* L. cell culture.

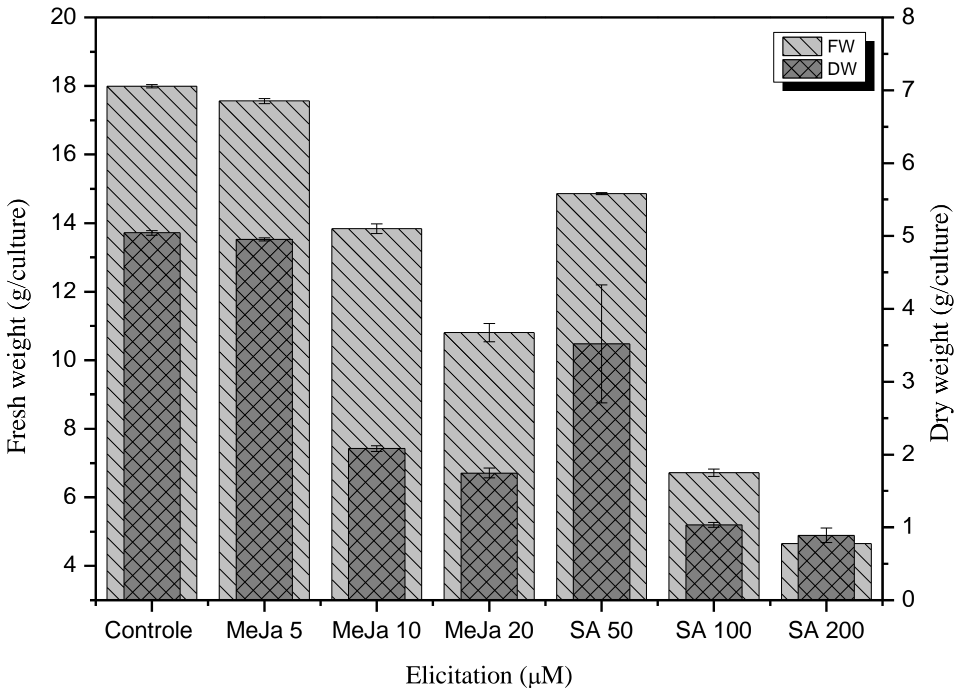

Figure 5.6: Effect of elicitors, methyl jasmonate (MeJa; 5, 10 and 20 µM) and salicylic acid (SA; 50, 100 and 200 µM) on biomass accumulation in cell suspension culture of *Withania somnifera* L. after 48 days of culture period. Values are mean ± standard error.

5.4.5. Effect of elicitors on total phenolic and total flavonoid content

Plant cell culture serves as a toolbox for production of useful secondary metabolites and could be manipulated for maximum production by employing suitable elicitor at suitable concentration. Where elicitors are signaling molecules which mediates the plant defense pathways that leads plants to produce various protecting compounds such as phenolics, flavonoids and other low molecular weight metabolites (Tan et al. 2004; Ali et al.2006). Previously, SA and MeJa have successfully been used as extracellular engineering tools for enhanced production of valuable secondary metabolites in plant cell cultures. In the present study we applied elicitors (MeJa and SA) in cell suspension culture of *W. somnifera* L. and observed different stimulatory responses for secondary metabolites production, depending on elicitor's type and concentration. Thus, in SA (200 µM) treated cell suspension culture an increase (3.5-fold) in total flavonoid content was observed. While, with respect to control the total phenolic content reached to its maximum value of 35.36 mg GAE/g DW when MeJa (20 µM) was used as elicitor (Fig. 5.7). In conclusion SA elicited the phenolic compounds production while in MeJa treated cultures total flavonoid production was maximum. This difference could be due to the differential modes of actions of these elicitors in plant cells metabolic pathways. Previously, SA acid treatment activated PAL enzymes in callus culture of *S. miltiorrhiza* that increased the production of salvianolic acid B and caffeic acid (Dong et al., 2010). While, Kenmotsu et al. (2013) observed transcriptional activation of farnesyl diphosphate synthase in MeJa treated cell culture of *Aquilaria microcarpa*. So from this study we recommend to use the combinations of these elicitors for enhanced production of these compounds.

On the other hand we compared the DPPH free radical scavenging activity in cell culture of *W. somnifera* L. with its total phenolic and flavonoid contents. It was found that DPPH free radical scavenging activity was dependent on total flavonoid content and independent on cell biomass. Highest antioxidant activity (60.5%) and maximum flavonoid content (3.2 mg QE/g DW) were recorded in cell culture treated with SA (200 µM) (Fig. 5.7). A decline in antioxidant activity was observed with further increase of SA concentration. Among MeJa treated cell culture extracts contributed to the DPPH free radical scavenging activity in range of 42 to 55%. Significantly lower antioxidant activity (35%) was observed in control treatment.

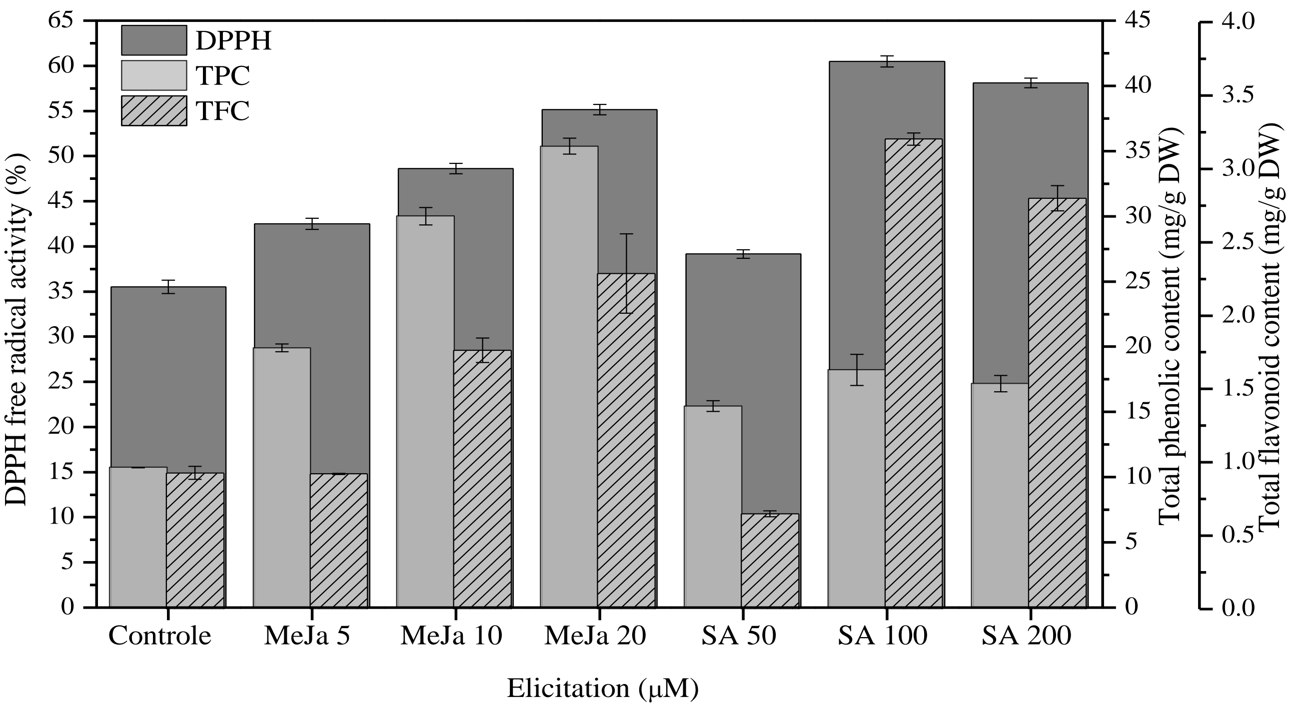

Figure 5.7: Effect of methyl jasmonate (MeJa) and salicylic acid (SA) on secondary metabolites contents in cell suspension culture of *Withania somnifera* L. Values are mean ± standard error.

5.4.6. Effect of elicitors on chlorogenic acid and withanolide content

Among different tested concentrations of MeJa and SA, 20 μM MeJa addition into growth media resulted an enhanced production of chlorogenic acid (24.78 mg/gDW; 6-fold), moupinamide (18.78 mg/g DW; 4-fold), withanoside IV (11.46 mg/g DW; 2.3-fold) and withaferine A (11.19 mg/g Dw; 2-fold) when compared with control (Fig 8). This could be due to the Go´mez et al (Gómez et al. 2010) hypothesis that agrees with altered allocation of resources in plants to buffer the stress by increasing plant cell growth and survival. We observed less significant (*P<0.05*) difference among control and cells treated with 50 μM SA. While an increase was observed when SA concentration was doubled and beyond this decrease in content of detected compounds were observe. The results suggested that the cells treated with elicitors had different abilities to produce secondary metabolites. Hence, cell treated with MeJa (20 μM) resulted maximum integrated peak area, followed by MeJa (10 μM) and SA (100

μM) (Fig 8). The increased metabolites may indicate that transcript levels of genes involved in the secondary biosynthesis has been induced by MeJa (Gadzovska et al. 2007).

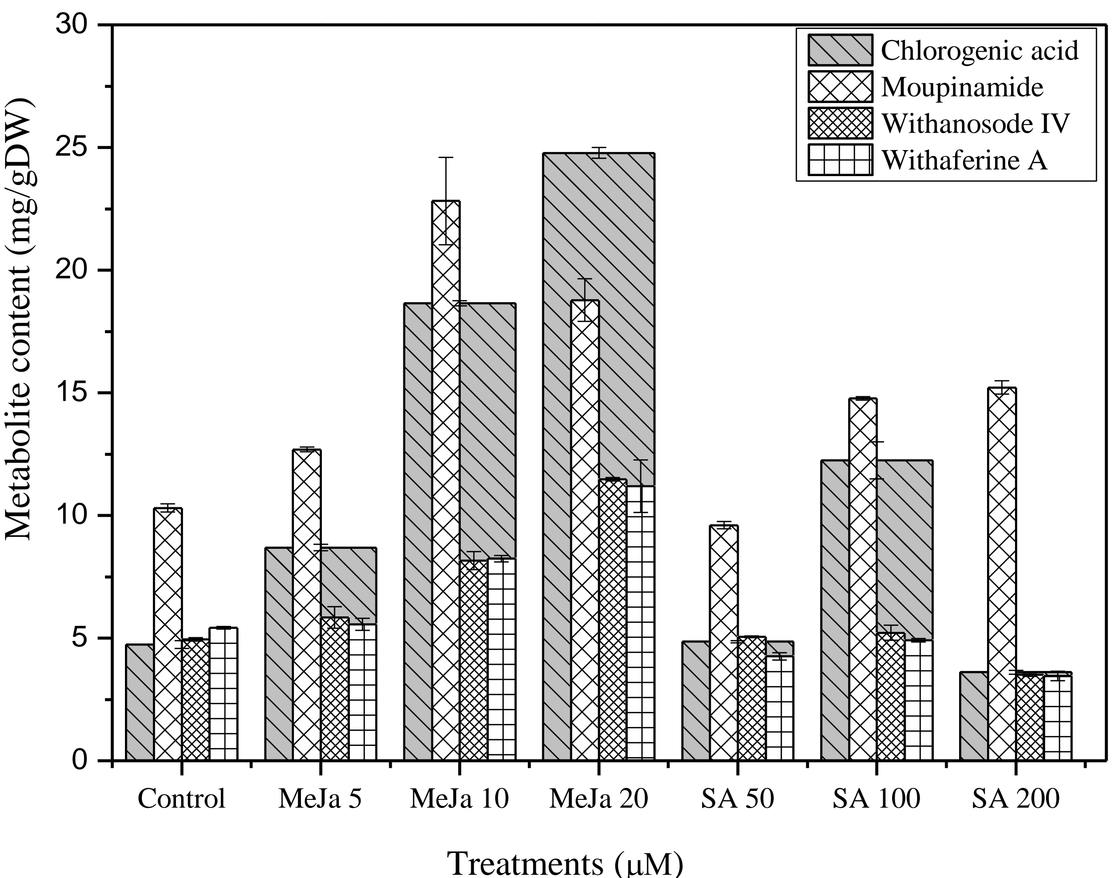

Figure 5.8: Effect of methyl jasmonate (MeJa) and salicylic acid (SA) with respect to control on chlorogenic acid, moupinamide, withanoside IV and withaferin A contents (mg/g DW) in cell suspension cultures of *Withania somnifera*. Values are mean ± SE of three replicates.

MeJA and SA have been long known to contribute to defense against plant pathogens. Our results hits to the possibility of these hormones to play a pivotal role in reprogramming plant development. Sa´nchez-Sampedro et al. (Sánchez-Sampedro et al. 2005) reported that addition of MeJa to *S. marianum* cell suspension cultures rapidly induced the activity of chalcone synthase (i.e., an enzyme in silymarin biosynthesis). Our results are in accordance with previous reported study on hairy root culture of *Withania somnifera*, where MeJA elicited the production of withanolide A, withanone, and withaferin

A (Sivanandhan et al. 2013). A study revealed that MeJA is better than SA in stimulation of total phenolic compounds production in banana (Edouard Ncho et al. 2016). Similarly, Manivannan et al (Manivannan et al. 2016) reported that MeJa mediated-elicitation significantly increased the production of total phenolics, total flavonoids and acacetin content in cell suspension culture of *Scrophularia kakudensis*. The possible explanation to this could be the induction of endogenous ROS in plant cells upon MeJa treatment that leads to the activation of cascade of antioxidant metabolism and results the enhanced production of secondary metabolites (Lamb and Dixon 1997; Zhang and Xing 2008).

5.5. CONCLUSIONS

Thus, it was concluded that the optimum multiplication rate of friable callus induced from zygotic embryos of *W. somnifera* L. can be achieved on Glitz (Gli) media under 24-hr dark conditions. During the present study SOM, SH and MS medium were not supportive for attaining maximum biomass accumulation in 56 days of culture period. While MS medium was proved to be affective for secondary metabolite production. Further it was found that maximum biomass accumulation in cell suspension was achieved on day 48 of the culture and secondary metabolites accumulation showed the same pattern. The use of elicitors at day 21 of culture, evoked secondary metabolite production response and SA was proved to be supportive for flavonoids production. While, MeJa treated cells favored the phenolic compounds production. The antioxidant activity of *W. somnifera* L. cultures showed a depending pattern on flavonoid content and maximum antioxidant activity was observed in SA (100 µM) treated cell cultures. Hence, the results suggest to further exploit the Gli media for enhancement of secondary metabolites in cell suspension culture of *W. somnifera* L. to meet the pharmaceutical industries demand.

6. CONCLUSIONS AND FUTURE PROSPECTS

6.1. Conclusions

In the present study we established adventitious roots, callus and cell suspension cultures for increased biomass and secondary metabolites production of *Withania somnifera* L. *W. somnifera* L. is known for its therapeutic properties and known as Indian ginseng due its rejuvenative properties. Due its higher commercial demand, metabolic inconsistency, lack of uniform cultivation practice and availability of little arable land, we adopted these alternative approaches to harvest metabolically consistent plant material. The adventitious roots culture system developed in this study assured increase in biomass and secondary metabolites and could be scaled up for commercial scale production. Moreover, in this study we used different monochromatic lights and observed red light treatment was supportive for biomass accumulation in callus culture of *W. somnifera* L. Cell suspension culture system was established to bypass the tedious repeated sub culturing steps of callus culture and we found the modified Glitz media was supportive for cell growth. The metabolites content were confirmed on chromatographic and FT-IR fingerprints. Following conclusions can be made from our results:

- ❖ First experiment was used in-vivo seed derived leaf explant and cultured on MS media containing melatonin and auxins (IAA and IBA) either alone or in combination and in conjunction with different light regimes. The combination of IBA with melatonin resulted maximum root induction from leaf explant with maximum roots per explant, roots fresh weight and root length. Further, 16-h light and 8-h dark proved supportive for adventitious root induction and growth. It was also observed that melatonin treatments significantly lowered light induced oxidative stresses in adventitious roots.
- ❖ In second experiment, adventitious root induced from leaf explant were successfully multiplied in liquid media. The results showed maximum biomass accumulation in MS liquid media containing IBA in combination with melatonin; however total flavonoid content were maximum in IBA alone treated cultures. Moreover, maximum fresh weight and dry weight were achieved in 35 days old adventitious root cultures treated with combination of IBA and melatonin. It was observed that in elicitors treated cultures melatonin positively supported the biomass accumulation and secondary metabolites

yield. A linear relation of total phenolic content and flavonoid content with DPPH free radical scavenging activity was observed in adventitious root cultures. It was concluded that adventitious roots culture produced in melatonin enriched media have positive correlation with biomass and secondary metabolite yield.

- ❖ In third experiment, the callus cultures obtained from in-vivo grown leaf explant on MS media containing TDZ and NAA showed optimum proliferation as compared to other tested plant regulators. Further, obtained calli on optimized media conditions were treated with different monochromatic lights. Comparable, biomass accumulation were observed in different light colors treatments and red light treatment was supportive for callus biomass; however maximum total phenolic and flavonoids content were achieved in violet light treated callus cultures. The presence of withanolides in callus cultures were putatively confirmed on high-performance liquid chromatography (HPLC). It was deducted that monochromatic light treatments differentially evoked cells defensive enzymes and also effected the callus metabolites profile.
- ❖ In fourth experiment, improved zygotic embryos derived callus growth and cell suspension culture were observed on modified media composition. Increase in macro nutrients in media resulted retarded callus growth of compact texture; while increase in micronutrients and addition of organic additives favored friable callus of higher biomass with shorter lag phase and prolonged log phase. Cell suspension culture was established and maximum biomass and secondary metabolites content were achieved in 48-days old cell culture. The use of elicitor further improved secondary metabolite production with retarded cell growth. Further the withanolide composition was confirmed by high performance liquid chromatography finger printings.

6.2. Future prospects

- ❖ The lab-scale protocols optimized for adventitious roots and cell cultures in this project should be scaled up to bioreactor level, in order to enhance the production of secondary metabolites in bulk and to investigate further the other inducible metabolites of medicinal interest. Furthermore, this work has the potential to be used in conjunction with other

biochemical engineering techniques to get value added plant materials of enhanced therapeutic potential.

- ❖ The optimized adventitious roots and cell cultures can be used further to investigate the effect of biotic elicitors, alone or in combinations with abiotic elicitors, monochromatic lights treatments and *in-vitro* culture conditions (pH, temperature, etc.) on biomass and secondary metabolite production. Furthermore, it can be used for bioconversion of compounds to valuable secondary metabolites of medicinal or nutritional interest. It can also be extended to use precursor feeding approach to enhance the secondary metabolites yield in cultures.
- ❖ The melatonin inclusion approach in this study, which increased the biomass accumulation and minimized the elicitors induced biomass reduction in adventitious roots culture should be extended to cell suspension keep prolonged cells viability.
- ❖ Other approaches like carbon source and cell line selection can be made to improve secondary metabolites production. Furthermore, the suggested media composition in this study should be exploited further to enhance secondary metabolites yield.

The *in-vitro* cultures obtained through these protocols, which contains different secondary metabolites, should be tested for anticancer, neuroprotective, anti-stress and rejuvenative properties, since *W. somnifera* L. has been reported for these properties. Furthermore, different fractions of its culture`s extracts should be made to investigate the formation of novel compounds due to different *in-vitro* treatments.

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Submitted articles

Adil M, Abbasi BH and Khan MA, Elicitors enhanced biomass accumulation and antioxidant compounds in adventitious root culture of *Withania somnifera* L. Physiology and Molecular Biology of Plants

Adil M and Abbasi BH, Red light controlled callus morphogenetic patterns and secondary metabolites production in *Withania somnifera* L. Photochemistry and Photobiology: B.

Adil M and Abbasi BH, Effect of media type on callus morphology and establishment of cell suspension culture of *Withania somnifera* for enhanced production of secondary metabolites. Plant Cell, Tissue and Organ Culture (PCTOC)

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DOI 10.1007/s11240-015-0844-x

ORIGINAL ARTICLE

Interactive effects of melatonin and light on growth parameters and biochemical markers in adventitious roots of *Withania somnifera* L.

Muhammad Adil[1] · **Bilal Haider Abbasi**[1] · **Tariq Khan**[1]

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Abstract Light plays a pertinent role in plant photomorphogenesis and it is believed to have an impact on the melatonin-induced physiological functions. In the current study, different light regimes were employed with varying levels of melatonin, either singly or in combination with auxins for the growth and development of adventitious roots in *Withania somnifera* L. It was observed that 600 μM melatonin favored maximum adventitious root induction frequency (58 %) in cultures incubated under continuous dark conditions. However, adequate root growth (number and length of roots) was observed under 16 h light/8 h dark at 600 μM melatonin. Nevertheless, the interactive effect of light and melatonin was found stimulating for profound production of commercially important secondary metabolites. Correlation among growth parameters and biochemical markers was also observed in the current report. Data on total phenolic content and total flavonoid content were found at higher coincidence with each other and with DPPH antioxidant activity. In conclusion, exogenously applied melatonin mimics IAA activity in root growth and regulates well in 16-h light/8-h dark, thereby giving protection to plant system against light stress.

Keywords Light · Melatonin · Adventitious root · Auxins · Antioxidant activity · Phenolics

✉ Bilal Haider Abbasi
bhabbasi@qau.edu.pk

[1] Department of Biotechnology, Quaid-i-Azam University, Islamabad 45320, Pakistan

Introduction

Withania somnifera L. (Indian ginseng) of family *Solanaceae* is a renowned medicinal herb with multiple pharmacological functions. It has been used in more than 100 indigenous medicinal recipes globally for over 3000 years. A very common problem associated with medicinal plant preparations is the extreme variability in the phytochemical content (Khan et al. 2015). This variability, in medicinal herbal products from wild plants, occurs due to contamination with biological and environmental pollutants, adulteration with misidentified species, quantitative and qualitative variation of bioactive compounds as well as unsustainable harvest (Abbasi et al. 2010; Khan et al. 2013). Application of plant in vitro technology can circumvent these issues of variability in *Withania* end products. However, the tissue culture of *W. somnifera* is still under the trials of optimization for better productivity (Mir et al. 2014; Murthy et al. 2014).

One of the contemporary features of tissue culture, adventitious root formation is the result of a complex and distinctive process regulated by endogenous and exogenous factors (Ford et al. 2002; Sorin et al. 2005).

Melatonin, (*N*-acetyl-5-methoxytryptamine) is a naturally occurring indole amine initially discovered in vertebrates (Lerner et al. 1958) and has also been detected in several plant species (corn, rice, wheat, barley, and oats) after its discovery as phytohormone (Cao et al. 2006; Dubbels et al. 1995; Kolár and Machácková 2005; Tan et al. 2012). Manchester et al. (2000) reported higher melatonin concentration in black and white mustard seeds compared to the vertebrate's blood melatonin level. The comparatively much higher melatonin levels in plants are thought to be a compensatory response by plants because they, unlike animals, lack mobility as a means of coping

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Muhammad Adil <adilbiotech@gmail.com>

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