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Effect of different plant growth regulators on biomass accumulation of *Withania somnifera*

By

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21PBC007

II M.Sc. BIOCHEMISTRY

Department of Biochemistry, Biotechnology and Bioinformatics

A thesis Submitted to Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore - 641 043.

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

MAY 2023

CERTIFICATE

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KAVIYA.M

CONTENTS

CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF PLATES

LIST OF PLATES

INTRODUCTION

1.0 INTRODUCTION

Plants not only play a vital role for food and fuel, but also it can used as a medicine for most of the health ailments and its treatment, due to the presence of various secondary metabolites present in them. From last few decades, phytoconstituents such as alkaloids, tannins and flavonoids present in the plants are used as medicine due to their minimal toxicity. *Withania somnifera* (Linn) Dunal belonging to the family solanaceae is used as a popular medicinal plant throughout the world (Ezez *et al*., 2023). According to the World Health Organization (WHO), it is calculated that 80% of the global population depends on herbal medicine for curing diseases and disorders (Kaur *et al.,* 2021). Any part of the plants which has the secondary metabolites that can be used for the treatment of diseases are called medicinal plants. Medicines derived from plants are adiministered either oral or it can be applied directly onto the skin or inhaled (Ullah *et al.*, 2014).

Plant propagation, production of secondary metabolites and conservation of plant resource have improved a lot in recent years through plant tissue culture techniques. This technique provides sustainable opportunities to solve problems in medicinal plant culture and conservation biology (Akin *et al*., 2020). In plant tissue culture a single explant, can be multiplied into thousands of plants under controlled *in-vitro c*ondition. Plant tissue culture is a type of *in- vitro* culture that involves aseptic culture of single cell, organs, or whole plant under controlled nutrition and environment. Meristem tip culture of banana plants that was devoid from banana bunchy top virus and brome mosaic virus were produced through plant tissue culture technique (Oseni *et al*., 2018). Plant tissue culture could also provide a means of disease-free healthy clones for extraction of pure drugs compounds (Mir *et al.,* 2014).

Withania somnifera (Linn) Dunal commonly known as Ashwagandha is an important adoptogenic herb in which its roots, seeds and leaves are used in preparations of Ayurveda and Unani medicines. This plant is widely distributed throughout the dry and subtropical parts of India (Meena *et al.,* 2020). *Withania somnifera* roots are used for more than over 200 formulations in Ayurveda, Siddha, and Unani medicines used in treatment (Girme *et al*., 2020). *Withania somnifera* has also been used for over 3000 years in indigenous medicine. Several studies collectively reveal the metabolic insight of more than 200 primary and secondary metabolites of *Withania somnifera* (Pandey *et al.,* 2017).

Secondary metabolites that are present in the medicinal plants that act as medicine which were synthesized *in-vitro* through plant tissue culture technique. In recent years, use of specialized techniques such as cell immobilization, elicitation, metabolic engineering and *insitu* product removal have increased the production of secondary metabolites through plant tissue culture (Pant *et al*., 2014). *In-vitro* secondary metabolite production is based on two separate phases, (1) mass production and (2) secondary metabolites (Scarpa *et al.,* 2021). Withaferin-A is one of the the major secondary metabolite present in withanolide group of *Withania somnifera. Withania somnifera* has immense anti-inflammatory and anti-tumour activity (Bhasin *et al.,* 2019). Withanolides present in *Withania somnifera* act as hormone precursor and helps to change the human physiological hormone (sex hormones) as required (Kaur *et al.,* 2018). Elicitation is also one of the effective methods used in tissue culture to improve secondary metabolite in cell and organ culture (Sivanandhan *et al.,* 2014).

Plant hormones regulate the physiological processes in plants including growth, time of flowering, flowers development and sex determination including senescence of leaves and fruits (Kukreti *et al.,* 2013). The available literature on *Withania somnifera* suggests that the plant responds differently to different culture conditions and Plant Growth Regulators (PGRs). In the majority of studies, cytokinins like 6-benzylaminopurine (BAP) and kinetin (KIN) have been used for optimum shoot proliferation in *Withania somnifera* under *in-vitro* condition (Kaur *et al.,* 2021).

The *in- vitro* suspension culture are used in many ways to improve secondary metabolite production. Secondary metabolites production is increased through selection of high producing PGRs, precursor feeding, elicitation and metabolic engineering, transferring root cultures, micropropagation and bioreactor cultures (Parameswari *et al.,* 2017). Large scale production through plant tissue culture using bioreactors will provide a means of transferring large volume of plants onto the market at lower prices (Autade *et al*., 2016). The main problems for cultivation of *Withania somnifera* in India is that the crop is raised through direct seed propagation along with climatic change and there is tremendous variation on root growth, root morphology, root yield and active ingredients (Patel *et al*., 2013). Biotechnologically, there is hardly few information available on the cell suspension culture of *Withania somnifera* (Sabir *et al*., 2011).

To date few reports are available on the influence of Plant Growth Regulators on accumulation of secondary metabolites in *in-vitro* suspension culture of shoots of *Withania somnifera.*

Based on the above observation, the present study was designed with objective

- To standardize suspension culture condition for effective production of *Withania somnifera* (Linn) Dunal shoots *in-vitro.*

REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

Plant tissue culture provides an attractive alternative for the production of pharmaceutically important secondary metabolites (Namdeo *et al*., 2021). Shoot multiplication through *in-vitro* plant regeneration of *Withania somnifera* will help to develop large scale propagation and conservation for its pharamaceutical compounds (Ara and Choudhary, 2014).

The literature related to the work **"Effect of different plant growth regulators on biomass accumulation of *Withania somnifera*"** was surveyed extensively and is presented in this chapter

2.1. *Withania somnifera* (Linn) Dunal

2.2. Medicinal uses of *Withania somnifera* (Linn) Dunal

2.3. Secondary metabolites of *Withania somnifera* (Linn) Dunal

2.4. Pharmacological properties of *Withania somnifera* (Linn) Dunal

2.5. *In-vitro* studies on *Withania somnifera* (Linn) Dunal

2.6. Bioreactors

2.1. *Withania somnifera* (Linn) Dunal

Withania somnifera also known as Indian ginseng or Indian winter cherry (Munir *et al.,* 2022). It is medicinal plant widely used in traditional medicine for more than 3000 years (Santhanu *et al*., 2021). *Withania somnifera* is otherwise called as Ashwagandha. Ashwagandha are used in the treatment of sleep disorder, reduce anxiety and hypo-thyroidism, helps to reduce the stress and enhance muscle strength (Mikulska *et al*., 2023). "Ashwa" means horse and "gandha" is defined as smell in Sanskrit. *somnifera* in latin is defined as the sleep inducing. This medicinal herb has the sedative property. This herb helps to improve the health and immunity of a person. *Withania somnifera* has anti-bacterial activity, anti-diabetic activity, anti-inflammatory activity and anti-cancer activity (Santhanu *et al.,* 2021). *Withania somnifera* belongs to the solanaceae family (Patnaik *et al.,* 2015)

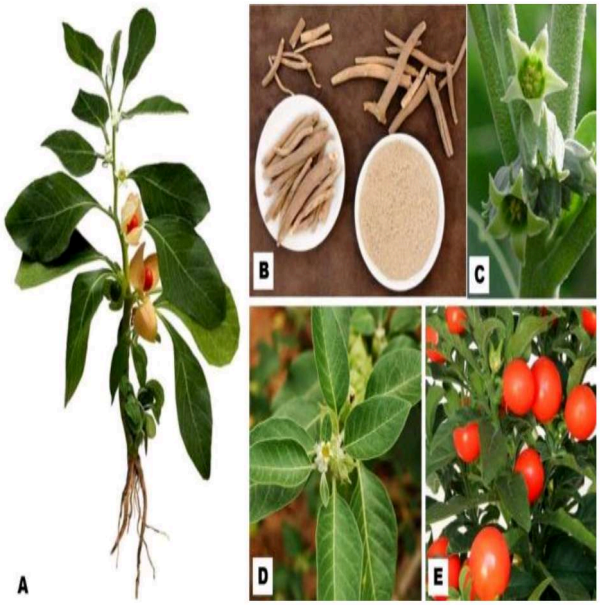


Figure 2.1. *Withania somnifera* (Linn) Dunal plant (Sengupta *et al*., 2018)

A-Whole plant, B- Root, C- flower, D- leaves, E- fruits

2.1.2 Taxonomical classification

Kingdom : plantae,plants

Subkingdom : Tracheobionta, vascular plants

Super division : Spermatophyta, Seed plants

Division : Angiosperma

Class : Dicotyledons

Order : Tubiflorae

Family : solanaceae

Genus : Withania

Species : somnifera Dunal (Narinderpal *et al.,* 2013)

2.1.2 Distribution

It also grows in the Pakistan, Afghanistan, Morroco, Jordan, Australia and Spain (Polumackanycz *et al., 2023*). This shrub is commonly distributed in the Mediterranean region and spread throughout the Northern Africa to Southwest Asia. In India it is cultivated in the sub-tropical regions of Gujarat, Rajasthan, Punja, Uttar Pradesh, Maharashtra and Madhya Pradesh (Santhanu *et al., 2021*).

2.1.3. Morphological characteristics

Withania somnifera is an evergreen, tormentors and small woody shrub and this shrub grows to a height 30-150cm. The plant is covered with wooly pubescence ranging from white to brown (Santhanu *et al.,* 2021). It is a perennial herb and have an odour of horse's urine. The stem of this plant appears in brownish dark colour and it is erect in nature (Gaurav *et al.,* 2015). This shrub has an oval shaped leaves and have margins arranged in alternative manner. The leaves are having length 10cm and wide 2.5- 5 cm (Santhanu *et al.,* 2021). The margins present in the leaves of this plant are slightly waved. Leaves are ovate or oblong in shape. This shrub has a long woody tuberous root that are grayish in colour (Gaurav *et al.,* 2015). Flowers are greenish

or lurid yellow in colour (Narinderpal *et al.,* 2013). Flowers are small and height is 1cm long and few flowers occur in umbellate cyme pattern (Saidulu *et al.,* 2014). Seeds are yellow in colour, flat in shape, small in size and reniform in nature (Meena *et al.,* 2020). The ripe fruit is orange-red in colour and has milk coagulating properties (Umadevi *et al.,* 2012). The corolla of this shrub is 5 lobed and 5-8mm long and colour ranges from light yellow to yellow green. The fruit is a hairless berry with round in shape and enveloped by large calyx. The fruit have 5-8mm across and occurs in red colour when ripped. The flowering of this plant occurs from October to June and fruiting occurs from October to July (Gaurav *et al.,* 2015). Roots are stout, fleshy, bearing fiber-like secondary branches arising from the main root having a strong odour and bitter, acrid taste (Paul *et al*., 2021)

2.1.4. Vernacular names (Santhanu *et al.,* 2021)

Languages	**Plant names**
Bengali	Ashvagandha
English	winter cherry
Gujrati	Ghodakun,Ghoda,Asoda and asan
Hindi	Punir,Asgandh
Kannada	Aswagandhi,pannaeru,Viremaddlinagadde,Kiremallinagida
Konkani	Fatarfoda
Malayalam	Ammukuram
Marati	Asgund, Ashvagandha
Punjabi	Asgand,Isgand
Rajasthani	Chirpotan
Sanskrit	Ashwagandha
Telugu	Pulivendram,panneru-gadda,pannaeru

2.2 Medicinal values of *Withania somnifera* (Linn) Dunal *:*

This plant helps to cure the sleeping disorder insomnia and helps in the rejuvenation of nervous system. It reduces the blood pressure and the stress caused by ulcer. It is used in the treatment of arthritis. It increases the red blood count and melanin content in the hair (Umadevi

*et al.,*2015). It is used in the treatment of neurodegenerative condition like Parkinson's and Alzheimer's disease (Rad *et al.,*2021). In Ayurvedic medicine, it is used as analgesic to stimulate sexual impulse and increase sperm count in men and it is used in pregnancy for breast development in women (Kumar *et al.,*2015). *Withania somnifera* is used to calm the mind and used in treatment of joint diseases. The herb help to prevent the weakness, chronic fatigue, dehydration, impotency, convalescence, bone weakness and muscle tension as a traditional medicine (John, 2014).

2.2.1. Leaves

The leaves of the plant are used for rheumatism and hemorrhoids treatments and is also used as an ointment (Ezez *et al.,* 2023). It is used as an internal agent for fever and externally it is used to cure wounds. It is also used in the treatment of ophthalmitis, tumours, anthrax pustules, syphilitic sores and erysipelas as an external agent (Sharma *et al.,* 2011). The leave juice of Withania *somnifera* is useful in the treatment of conjunctivitis (Umadevi *et al.,* 2012). It contains 12 variants of withanolides and 5 unidentified alkaloids (Santhanu *et al.,* 2021). The leaves also contain many free amino acids, chlorogenic acid, glucose, condensed tannins and flavonoids (Jana *et al*., 2018). Insect repellent properties was found to be present in the leaves of this plant (saleem *et al.,* 2020). In Ayurveda, the juice of this leaves is used as an ear drops in ear discharge and leave paste is applied on enlarged cervical glands or swelling of other glands as it reduces oedema and pain (Meher *et al.,* 2016). The bitter leaves of the plant have characteristic odor, used as an anti-helmantic and infusion is given in fever (Dhar *et al.,* 2015)

2.2.2. Roots

The roots of this plant are been used as diuretic, narcotic, anti-helminthic, thermogenic, stimulant and aphrodisiac (Krutika *et al*., 2016). The paste form of *Withania somnifera* roots are applied in the human body to reduce the inflammation of the joints. It is applied locally in the specific region of the body to treat ulcers, carbuncles and painful swellings. The combination of this root with other drug is used to treat the snake bite and scorpion sting. It is also used in the treatment of piles, pimples, leucorrhoea and boils (Singh *et al.,* 2011). Decoction of the root is used to cure cold and chills and is used to strengthen the uterus after miscarriage. The combination of root decoction and bark is used to treat asthma (Krutika *et al.,* 2016). Dried roots

are used as tonic for hiccup, cold, female disorders, ulcers and as a sedative in care of senile debility (Umadevi *et al.,* 2012). In Ayurveda, the root paste of *Withania somnifera* is applied on enlarged cervical glands or swelling of other glands to reduce pain and oedema and is used oil massage in vata diseases and weakness (Meher *et al.,* 2016)

2.2.3. Fruits and Seeds

The green berries of *Withania somnifera* plant contain condensed tannins, proteolytic enzymes, flavonoids and amino acids (Shanthanu *et al.,* 2021). Fruits contain high amount of amino acids that include proline, alanine, tyrosine, valine, cystine, cysteine, glutamic acid, glycine and hydroxy proline (Jana *et al.,* 2018). The berries are used to coagulate the milk in cheese making and act as a substitute for rennet. In Ayurveda, these fruits are used to treat memory loss (Sharma *et al.,* 2011). The seeds of this plant have anti-helminthic properties and is used to remove silver acnes from cornea. The seeds boost up the sperm count and help in the testicular development (Hussain *et al.,*2023). Fruits are used as a blood purifier and helps in the growth of the infant (Bhasin *et al.,* 2019).

2.3 Secondary metabolites of *Withania somnifera* (Linn) Dunal

The chemically active compounds that are produced in plants in response to stress is known as secondary metabolites. They include alkaloids, flavonoids, terpenoids, saponins, tannins, steroids and glycosides (Visweswari *et al.,* 2013). The major secondary metabolite that are present in the different parts of *Withania somnifera* are C-28 steroidal lactone triterpenoids named as withanolides. The class of withanolides include withanolide A, Withaferin-A, Withanone and withanolide D (Dutta *et al*., 2019).

2.3.1. Alkaloids

They are heterogenous group of natural substances with nitrogen as basic group and possess pharmacological properties. They have anti-inflammatory and antipsychotic activity (Badyal *et al*., 2020). Alkaloids are used in the treatment of tumours, diarrhoea and nocturnal leg cramps. They are also used in the treatment of palpitation and psychiatric disorders. Studies reveal that alkaloids extracted from extract of *Withania somnifera* roots helps in the smooth muscle concentration in intestinal, uterine and trachea (visweswari *et al.,* 2013). The alkaloids

that are present in the *Withania somnifera* are somniferinine, somninine, withanine, withananine, nicotine and tropeltigloate (Hussain *et al.,* 2023). The other alkaloids are pseudo-withanine, pseudo-tropine, choline, 3-a-gloyloxytropane, isopelletierine, cuscohygrine and anaferine (John 2014).

2.3.2. Flavonoids

These are large group of naturally occurring phenolic compounds present in fruits and vegetables to provide colours for them. Flavonoids are sometimes referred to as phytophenols. These phytophenols help to reduce the risk of coronary heart diseases and factors responsible for cancer (Badyal *et al*., 2020). They also help in the reduction of osteoporosis and show improvement in the blood cholesterol levels (Visweswari *et al*., 2013). The important flavonoids present in *Withania somnifera L. (Dunal)* are quercetin and kaempfrol (Hussain *et al.,* 2023).

2.3.3 Steroids

The organic compound that contains four cyclohexane rings are known as steroids. The main steroidal lactone that are present in *Withania somnifera* are withanolides. They are responsible for reducing cholesterol and stress levels, enhance learning ability, improves memory power and helps to treat the tumour cells in cancer (visweswari *et al.,* 2013). The class of steroidal lactones that are present in *Withania somnifera* are Withaferin A, withanolides A-Y, Withasomniferin-A, withanone, withasomidienone and withasomniferols A-C (John 2014). Steroids that are present in *Withania somnifera* are diosgenin, cholesterol, stigmasterol, β-sitosesterol (Hussain *et al.,* 2023).

2.3.4 Saponins

They are heterogenous group of natural products present in medicinal plants. Terpenoids and steroidal saponins are two types of saponins. Saponins that are present in *Withania somnifera* have been used as detergents, molluscicides and pesticides. It is used as foaming agent in industries (Visweswari *et al* 2013). The important saponins containing an additional acyl group are sitoindoside VII and VIII (Ragini *et al.,* 2021)

2.3.4. Glycosides

The molecule in which the sugar is bound to a non- carbohydrate group is known as the glycosides. They are small organic molecule. They act as diuretic and have sedative effect on heart and muscle when taken in small quantities. They can be used in the treatment of severe dry coughs (Visweswari *et al.,* 2013).

2.3.5. Tannins

They are the member of the polyphenol family and help the plant to resist against the infection. Tannins present in *Withania somnifera* inhibit the growth of microbes and fungi. They are used in healing of wound and mucous membrane. They show anti-inflammatory and analgesic activities (Visweswari *et al*., 2013).

2.3.6. Withanolides

Withanolides are immunity boosters and has anti-viral properties against COVID-19 (Khanal *et al*., 2020). Withaferin A present in the leaves is the most important of the withanolide that contribute to anti-tumour and antibiotic activities (Supe *et al*., 2011). Modulation of an Withanolides and their derivatives including Withanolide A, Withanosides, Withaferin-A and withanolidedenosomin are used in treating neurodegenerative disorders through neuroinflammatory modulation and stimulating neurite outgrowth and regeneration (Abdelwahed *et al,*.2023). Root contains steroidal lactone withaferin A, 17-hydroxy-27-deoxywithaferin A and withanolide glycosides *viz.* withanosides I-VII, withanolides A, B and D as major phytoconstituents (Tandon *et al*., 2020)

2.4 Pharmacological properties of *Withania somnifera (*Linn) Dunal

Withania somnifera possess antistress, antimicrobial, immunomodulatory, antitumour, anti-inflammatory, antioxidant, neuroprotective and hematopoetic activities because of the secondary metabolites present in them (Tripathi *et al.,* 2018). Withanolide is the main biochemical constituent present in *Withania somnifera.* Withanolides that help in anti-tumour activities are Withaferin-A and withalongolide A. Both withaferin A and withalongolide A block the heat shock protein which cause cancer cell apoptosis (Abdelwahed *et al*., 2023).

2.4.1. Anti-aging activity

A study was conducted on *Withania somnifera* in a double blind clinical trial to prove its anti-aging properties. A group of 101 healthy males at the age of 50-59 years were used as the subject for the study (Singh *et al.,* 2010). The subjects were administered with the 3 grams of herb for one year. The result revealed that subjects showed improvement in hair melanin, haemoglobin and red blood cell count (Narinderpal *et al*., 2013).

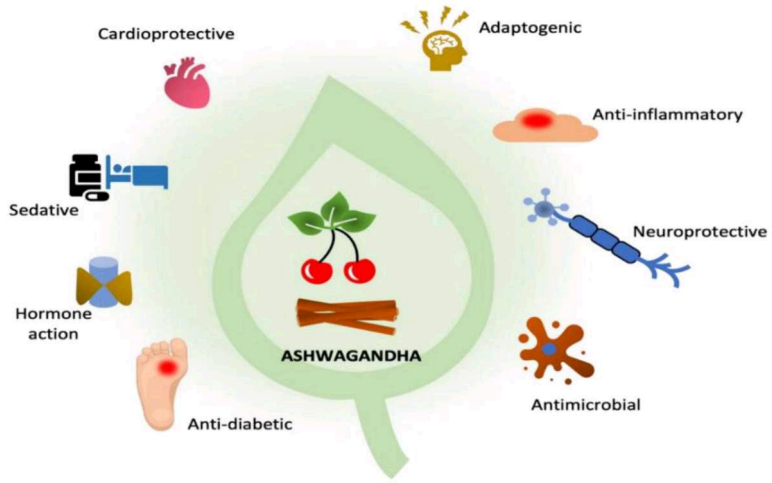


Figure 2.4: Health benefits of *Withania somnifera* (Mikulska *et al*., 2023)

2.4.2. Anti-microbial activity

The root of *Withania somnifera* contain monomeric glycoprotein that inhibit the growth of bacterium such as *Clvibacter michigan-ensis subsp.*when aqueous leaf extract were given orally it showed bacteriostatic effect against *S .typhimurium* as exhibited by the anti-biotic chloramphenicol (Saleem *et al.,* 2020). The bacteria that are found high susceptible to *Withania somnifera* are *Corynebacterium bacillus, Streptococcus and Staphylococcus* species (John, 2014). An experimental study on mice was conducted to prove the anti-microbial activity. The aqueous fruit extract was given orally to mice against *Salmonella i*nfection and the result revealed that mice survival rate was increased by decreasing the bacterial load in the vital organs

of the mice (Thakur *et al.,* 2021). *In-vitro* grown *Withania somnifera* exhibit anti-bacterial activity against *Salmonella typhimurium* (Hussain *et al.,* 2023). The leaf extract of *Withania somnifera* exhibited inhibition of *Enterococcus spp.* and MRSA against antibiotics like erythromycin and ampicillin (Bisht *et al.,* 2014)

2.4.3. Anti-cancer Activity

Root extract of *Withania somnifera* possess good anti-cancer activities. A study was conducted on mice for the treatment of skin cancer. In this study the root extract used to reduce the skin lesion on mice (Thakur *et al.,* 2021). The secondary metabolite Withaferin A is responsible for the anti-cancer activity and it inhibits the tumour cells of human carcinoma of nasopharynx (Jain *et al.,*2012). Research on animals prove that the *Withania somnifera* reduces the levels of nuclear factor kappa B and enhances the signals of apoptosis in cancer cell lines (Jana *et al.,* 2018).

2.4.4. Anti-inflammatory activity

A study revealed that the anti-inflammatory activity shown by *Withania somnifera* is similar to the activity of hydrocortisone at 5 mg/kg dosage (Kumar *et al.,* 2015). The secondary metabolite, Withaferin A and 3-b-hydroxy-2,3-dihydrowithanolide present in the *Withania somnifera* is responsible for the anti-inflammatory acivity (Mandal *et al.,*2017). Methanolic fractions of the extract showed high anti- inflammatory activity than the drug hydrocortisone. The root powder of *Withania somnifera* were given orally to decrease the glycosaminoglycan content which was higher than that of the drugs hydrocortisone and phenylbutazone (Halder *et al.,* 2015). *Withania somnifera* can inhibit the cyclooxygenase enzyme which act as the biological mediators of anaphylactic and inflammatory reactions (John, 2014)

2.4.5. Antioxidant activity

The extract of *Withania somnifera* reduces the lipid peroxidation that are caused by copper in aging of wister rats (John, 2014). A study was done on rat by giving *Withania somnifera* in ayurveda herbal formulation and the result revealed that the formulation enhanced the superoxide dismutase activity in the pancreas of diabetic rats (Kumar *et al.,* 2022). One experimental study reported that the glycowithanolides were given once for 21 days and the

increase in free radical scavenging enzymes was observed in relation to dose (Mandal *et al.,* 2017).

2.4.6. Anti-fungal activity

The monomeric glycoprotein was isolated from the root of *Withania somnifera* showed inhibition of growth of fungi such as *Aspergillus flavus, Fusarium verticilloides* and *Fusarium* oxysporum (Saleem *et al.,* 2020). Methanolic extract of leaf possess anti-fungal activity against *Dreschlera turica*, *Aspergillus niger* and *Aspergillus fumigates.* Hexane extract of root of *Withania somnifera* showed antifungal activity against *Fusarium oxysporum* and *Alternaria brassica* (Khanchandani *et al.,* 2019). Study showed that the aqueous extract of leaves of *Withania somnifera* inhibit the mycelia growth and spore formation of some phytopathogenic fungi such as *Alternaria brassicae, A.solani, Botyris fabae, F.oxysporum* and *Phytophthora infestans* (Nefzi *et al*., 2016).

2.4.7. Anti-arthritic activity

Anti-arthritic activity was proved in *Withania somnifera* by the study on rat model of adjuvant induced arthritis. *Withania somnifera* root powder was given to rats and result obtained showed the less disintegration of cartilage by observing the amount of bone collagen in the joints. The plant is used to maintain the stability of collagen fibres by inhibiting the collagenase (Hussasin *et al.,* 2023). The plant is used as an analgesic that calms the nervous system and help to relieve from stress (Mandal *et al.,* 2017).

2.4.8. Hepatoprotective activity

Mohanty *et al.,* 2008 studied the hepatoprotective activity of *Withania somnifera* root powder. The extract of root help to maintain the level of lipid peroxidation by providing the hepatoprotective effect. Verma *et al.,* 2009 examined the hepatoprotective effect in the aqueous root extract of *Withania somnifera* on the hepatic cell of *Clarias batrachus*. The result revealed that the root extract consists of flavonoids and neurotransmitters responsible for the hyperactivity of the endomembrane leading to excretion of molecules through endocytosis (Jain *et al.,* 2012). Alcoholic extract of the leaves of *Withania somnifera* exhibit hepatoprotective activity by inhibiting the CCl_4 induced alterations in transaminase activity (Meher *et al.,* 2016).

Histopathological studies reveal that the alcoholic extract of *Withania somnifera* inhibits ochratoxin A which cause liver inflammation and suppresses macrophage chemotaxis (Gaurav *et al.,* 2023)

2.4.9. Anticonvulsant activity

This activity was present in the dried powder, decoction and alcoholic extract of *Withania somnifera* by acting against phenobarbitone and electroshock. Alcoholic extract show more activity than the dried powder and decoction (Meher *et al.,* 2016).

2.4.10. Antidiabetic activity

Withania somnifera root powder, when given orally for 30 days-maintained blood glucose stability much better than the oral hypoglycemic drug daonil in diabetic patient. A study was conducted on rat with alloxan induced Diabetes Mellitus to prove anti diabetic activity of the plant. *Withania somnifera* root and leaf extract were given to the rats. The result showed decrease in the blood glucose, urine sugar, glucose-6-phosphatase and the amount of tissue glycogen and increase in the insulin levels (Hussain *et al.,* 2023). A study was conducted to prove the antidiabetic effect of root and leaf extract of *Withania somnifera* on streptozotocin induced diabetic rats. Result showed that leaf and root extract possess anti-diabetic activity in streptozotocin induced diabetic rats. The activity was found higher in root extract compared to leaf extracts (Sarangi *et al*., 2013).

2.4.11. Cardioprotective activity

In the present world of young generation cardiovascular diseases like myocardial infarction and myocardial ischemia-reperfusion are the major cause of death (John, 2014). *Withania somnifera* is prescribed as ethnomedicine for cardiovascular diseases (Javadian *et al.,* 2017). This plant help to increase the heart rate, relaxation and contraction of heart and inhibit the lipid peroxidation as similar to the function exhibited by the vitamin E, a cardioprotective antioxidant (Ojha *et al.,* 2009). A strong cardioprotective activity was shown by the hydro-alcoholic extract of *Withania somnifera* by the experiment done on the animal model of myocardial necrosis induced by isoprenaline in wistar albino rats (John, 2014).

2.4.12. Neuroprotective activity

In the majority of the central nervous system related disorder, *Withania somnifera* has been used as a therapeutic agent. Studies conducted on animal model prove the neuroprotective activity by overcoming the oxidative damage and excitotoxicity because of the secondary metabolites present in them (Ahmad *et al.,* 2016). The root extract of the plant help to induce the growth of axon and dendrite (Durg *et al*., 2015). Animal study was conducted on rats to investigate the glutamatergic, brain cholinergic and gamma aminobutyric acid induced allergic receptors by the administration of *Withania somnifera* root extract (sitoindosides VII-X). The results obtained showed increase in the acetylcholinesterase activity in lateral septum and decrease activity in the diagonal band of lateral septum and globus pallidus (Saleem *et al.,* 2020). A study on animal model proved that the root extract of *Withania somnifera* increase the brain antioxidant enzymes levels and total protein. When animals are exposed to lead nitrate the increase in total protein protects the brain (Kumar *et al.,*2015).

Anti-Parkinsonian activity

Clinical trial was done by the researchers to prove anti parkinsonian activity of *Withania somnifera*. The result revealed that the plant helps to cure the catalepsy, 6-hydroxydopamine elicited toxic manifestation and tardive dyskinesia and paved a path for the new therapeutic approach to treat Parkinson disease (Jana *et al.,*2018). A study revealed that the root extract of *Withania somnifera* 100mg/kg body weight was found to be protective in 1-methyl 4- phenyl 1,2,3,6-tetrahydropyridine induced catalepsy (Singh *et al.,* 2017). A study was conducted on fruit flies to prove the anti-Parkinsonian activity. Result revealed that methanol extract of *Withania somnifera* root counteracts the deficits associated with Parkinson's disease (Mikulska *et al*., 2023)

Anti-Alzheimer's activity

A study was conducted by the scientists of National Brain Research Centre, New delhi on animal model of mice to prove the anti-Alzheimeric activity. The root extract of *Withania somnifera* were given to the mice. The result obtained revealed that the extract was helpful in curing the memory loss of the mice. Based on the investigation root extract was used in the treatment of Alzheimer disease (Jana *et al.,*2018). Withaferin- A isolated from *Withania*

somnifera is used to treat the cerebral functional deficits including amnesia in elderly patient. Oral administration of withanolide IV may ameliorate neuronal dysfunction as it is metabolized into sominone and sominone can directly cross the blood brain barrier (Singh *et al*., 2017). Studies conducted on human nerve cells, *Withania somnifera* has been shown to neutralize the toxic effects of β-amyloid an implication in neurocognitive impairment during HIV infection (Mikulska *et al.,* 2023)

2.4.13 Immunomodulatory activity

Withania somnifera provides best immune response towards the diseases when it is formulated as rasayana without any side effects. The extract of plant prevents the delayed type hypersensitivity reaction and enhance the phagocytic activity in animal study of mice (John, 2014). In an animal study it was found that extract of *Withania somnifera* given to cyclophosphamide treated animals increased the β-esterase positive cells in the bone marrow (Jana *et al*., 2018)

2.5. *In- vitro* studies on *Withania somnifera* (Linn) Dunal

In- vitro propagation through plant tissue culture techniques has proved itself as a reliable and promising tool for clonal propagation of healthy and disease-free plants throughout the year. In the case of *Withania somnifera,* Plant tissue culture technology could be of immense value as it facilitates in generating large-scale healthy, genetically uniform plants with defined chemical content for pre-clinical and translational studies (Kaur *et al.,* 2022)

2.5.1. Effect of Plant Growth Regulators on *Withania somnifera* (Linn) Dunal

A study revealed that the good development of callus type was observed in the MS media supplemented with BAP (2mg/l) for best regeneration, IBA (2mg/l) for root formation and BAP (1.5) + IAA (1.5mg/l) for multiple shoot formation, when MS supplemented with GA3 it was found the best media for seed germination (0.5mg/l) as well as shoot elongation (0.3mg/l) and MS +2, 4- D (0.5) + Kinetin (0.2mg/l) was media for maximum callus induction under invitro condition (Shelke *et al.,*2020). Another study revealed that nodal segments were cultured on MS solid medium supplemented with five different concentrations of Kin (0.25 to 2.0 mg/L). On comparing all the concentrations and combinations of Kin, MS+1.0 mgl-1 Kin and MS+0.5 mgl-

1 Kin mediums were found to be most effective for mass propagation and IAA at 0.5 mgl-1 was found to be applicable for rooting of shoots as it formed healthy and maximum number of roots (Shukla *et al*., 2010). A study revealed that of three cytokinin (BAP, KIN and 2-iP), BAP was found to be more efficient than other cytokinin with respect to initiation and subsequent proliferation of shoots. Multiple shoot buds were induced within 2–3 weeks of culture and the maximum frequency (95 %) was observed on MS medium supplemented with BA (2.5 μM) which induced (24.8 ± 2.33) shoots with a shoot length of (5.86 ± 0.52 cm) after 8 weeks of culture (Fatima *et al*., 2012).

2.5.2. Micropropagation technique

Micropropagation is the process of vegetative growth and multiplication from plants tissues or seeds in aseptic and favourable conditions on artificial growth media. Callus cultures were initiated from nodal segments on Murashige and Skoog medium supplemented with 2,4-D, BAP and KIN. The highest frequency (85%) of organogenic callus induction was observed in MS medium containing 1 mg L ha-1 BAP and 2 mg L ha-1 KIN (Chandana *et al.,* 2018).

2.5.3. Cell suspension culture

Plant cell suspension culture is a cost effective and simple biological process for the synthesis of plant derived secondary metabolites at large scales (Alcantara *et al.,* 2021). Cell suspension culture was established from the explant of callus culture of cotyledonary leaf, internode and root. Result revealed that the highest growth rate was observed in MS medium containing 2 mg/L 2,4, -D and 0.5mg/L KIN at fourth subculture. The cell suspension culture obtained from cotyledonary leaf callus showed the highest packed cell volume (38.93%) at fourth subculture in MS liquid medium with 2.0mg/L 2,4, D and 0.5mg/L KIN followed by internode (38.44%) and root (35.29%). The highest dry weight (15.55%) for cotyledonary leaf calli followed by internode calli (15.1g/L) and root calli (14.55g/L) was observed in the same MS medium (Bhoyar *et al*., 2015).

2.6. Bioreactors

The term bioreactor describes the largescale vessels used for plants, in literature plant cells are described as extremely sensitive for shear forces facilitating the use of low

shearbioreactors. Air lift bioreactor is an example of low shearbioreactors (Martin *et al*., 2005). Agitation based bioreactors are grouped into three main tank systems according to their agitation construction, mechanically agitated, pneumatically agitated and non-agitated bioreactor systems. Air lift bioreactors systems are very useful for masss propagation of various plant species because of their simple design causing less degradation of cells, tissues, shoots and organs (Kaya *et al.,* 2018).

MATERIALS AND METHODS

3.0.MATERIALS AND METHODS

Various materials and experimental procedures followed in this study **"Effect of different plant growth regulators on biomass accumulation of *Withania somnifera*"** are given under the following headings

3.1. Materials

3.1.1. Plant material

3.1.2. Chemicals

3.2. Methods

3.2.1. Media preparation

3.3. *In-vitro* propagation of *Withania somnifera* (Linn) Dunal

3.3.1. Germination of *Withania somnifera* (Linn) Dunal seeds (JAWAHAR 20)

3.3.2. Inoculation of the explants

3.3.3. *In- vitro* shoot proliferation

3.3.4. *In- vitro* suspension culture of *Withania somnifera* (Linn) Dunal

3.3.5. Mass production of *Withania somnifera* using bioreactor

3.1. MATERIALS

3.1.1. Plant material

Seeds of Jawahar-20 variety were collected from the University of Agricultural Sciences, Bangalore. Surface sterilized seeds of *Withania somnifera* JA-20 variety were germinated under *in- vitro* condition and maintained on half strength MS basal medium and germinated shoots were transferred on to MS medium and subculture at regular intervals. The shoots were maintained in plant growth regulators for shoot multiplication. Then the shoots were transferred to suspension medium followed by bioreactor.

3.1.2. Chemicals

Chemicals used for this study were purchased from HiMedia unless otherwise mentioned. Double distilled water was used for the entire work.70% alcohol and sodium hypochlorite were used as surface disinfectant.

3.2. METHODS

3.2.1. Media preparation

Full strength of MS medium (Murashige and skoog,1962) were used for all the plant tissue culture experiments with plant growth regulators. The composition of stock solution is presented in **Appendix 1.**

The macro, micronutrients, vitamins, myo-inositol and glycine were taken from the stock solution according to the requirement for growth of *Withania somniferaL. (Dunal)*. Sucrose (30g/L) was added and mixed well. The media was maintained at 5.7±0.1 pH. Solidifying or gelling agent (agar,8g/L) was added to the media and its steamed to melt the agar, then it was transferred into clean 250,350 ml of culture bottles (25 ml/bottle) and autoclaved at 15 lbs pressure at 121°C for 20 minutes. Growth hormones BAP and KIN were considered as hormonal media.

3.*3 In- vitro* propagation of *Withania somnifera* (Linn) Dunal

3.3.1 Germination of *Withania somnifera* (Linn) Dunal seeds (JAWAHAR-20)

The seeds were soaked overnight in tap water then washed twice with distilled water to remove dust particles. The seeds were washed in tap eater till the dust particles are completely removed. The seeds were soaked for overnight for imbibition process to take place. After overnight soaking the seeds were inoculated into the half strength MS medium under aseptic environment in laminar airflow chamber. Before inoculating, the distilled water in the seeds were decanted then the seeds were rinsed in 70% (v/v) alcohol for 3-5minutes, followed by a sterile water washing. The seeds were rinsed in 2.8% sodium hypochlorite for 60 seconds under sterile condition with swirling. Then the seeds were rinsed in sterile distilled water for three to four times to remove the excess sodium hypochlorite solution. The seeds were then inoculated into half strength MS solid basal medium supplemented with sucrose (30g/L). The inoculated culture bottles were incubated in dark until the seeds get germinated. After the appearance of first leaflet, the bottles were placed at photoperiod of 16/8 hours with 3000 lux to enhance the growth of the seedling.

3.2.2. Inoculation of the explants

The explants were inoculated under aseptic environment. The laminar airflow chamber was surface sterilized using 70% ethanol. The tools (forceps, scalpels, sterile cotton, sterile tissues and petriplates) that are used for inoculation must be autoclaved and kept in the laminar airflow chamber before UV. The laminar airflow chamber was sterilized by UV radiation for 20 minutes. Prior to inoculation hands should be sterilized with 70% alcohol. The forceps and scalpels were wiped with 70% alcohol and sterilized in the red hot flame, cooled and used for inoculation. The source of explant was the shoot obtained from the germinated seedlings and is been inoculated on to the MS basal medium. The inoculated cultures were incubated at 25±2°C. the growth was enhanced by providing the 16/8 h of photoperiod.

3.2.3. *In -vitro* shoot proliferation

The source of explant for shoot multiplication was obtained from the nodal region of two-month-old culture of MS basal medium. The nodal region was carefully excised under aseptic environment in laminar airflow chamber and 3-4 explants per bottle were inoculated onto MS basal medium supplemented with BAP (1mg/L). The explants were maintained under the photoperiod of 16/8 hours under the light intensity 2000 lux and a constant temperature of

25±2°C was maintained. Multiple shoots were obtained from the explants culture after a period of 30 days.

3.2.4 *In-vitro* suspension culture of *Withania somnifera* (Linn) Dunal

To establish shoot culture,30 days old grown explants obtained from the media of cytokinin (1mg/L BAP) used for the shoot proliferation were carefully excised and different mass of initial inoculums were subcultured in 30 ml of MS liquid media with different concentration and combination of cytokinin as mentioned in **Table 1.1** and allowed to grown with regular shaking in orbital shaker with 2000lux photo intensity and at temperature of 25±2°C. For every 7 days the media was changed and the weight of the plant material was calculated. After 30 days the explants were harvested from which the biomass and growth index was determined by measuring fresh weight of the explants. Media without hormone was used as the control.

Table1.1 MS basal media supplemented with different concentration and combination of cytokinin used for shoot proliferation in suspension culture

S.NO	BAP (mg/L)	KIN (mg/L)
C	0	0
T_1	1.0	0
T_2	1.5	0
T_3	2.0	0
T_4	0	1.0
T_5	0	1.5
T_6	0	2.0
T_7	0.5	1.0
T_8	1.0	0.5

3.2.5. Mass production of *Withania somnifera* using bioreactor

Bioreactor is particularly used for the mass production of *Withania somnifera* under *in vitro* condition. Bioreactor was autoclaved at temperature at 121°C for 20 minutes at 15 lbs pressure. The plant material from suspension culture were inoculated into bioreactor under sterile condition in laminar airflow chamber. After 15 days the plant growth and biomass will be observed.

RESULTS AND DISCUSSION

4.0. RESULTS AND DISCUSSION

The result of the present study entitled the "**Effect of different plant growth regulators on biomass accumulation of *Withania somnifera***" are presented and discussed under the following headings

4.1. Influence of different concentrations of BAP on biomass growth in shoot suspension cultures of *Withania somnifera*

4.2. Influence of different concentration of KIN on biomass growth in shoot suspension cultures of *Withania somnifera*

4.3. Influence of varying concentration of BAP and KIN on biomass growth in shoot suspension cultures of *Withania somnifera*

4.4. Biomass accumulation of *Withania somnifera* in bioreactor cultures

In the present study, *in vitro* shoot cultures of *Withania somnifera* was used for the identification of influence different cytokinin namely, BAP and Kin at different concentration (1, 1.5 & 2 mg/L) and different time periods (0, 7, 14, 21 & 30 days). Here, BAP and KIN hormones used for shoot proliferation and biomass induction purposes.

4.1. Influence of different concentrations of BAP on biomass growth in shoot suspension cultures of *Withania somnifera*

BAP (6 benzyl aminopurine) is a shoot multiplication hormone used for *in vitro* shoot proliferation for a number of plant species. Generally, BAP hormone was supplemented to the nutrient medium i.e., Murashige and Skoog (MS) basal medium for plant growth. In this study, BAP hormone at the concentration of 1, 1.5 & 2.0 mg/L was supplemented with MS medium for the treatment of in vitro shoots of *Withania somnifera* in suspension culture.

From the **Plate 4.1**, the biomass content was increased gradually in control (T_0) from 0.786g to 3.726g on o to 30 days time period. The leaves of T_1 shoots at 14^{th} day were broad and healthy compared to control shoots. However, T_1 shoots maintained for 30 days had the increased shoot branching and higher elongated shoots compared to T_2 and T_3 shoots. Therefore, among BAP treated shoots (T_1-T_3), T_1 shoots maintained for 30 days time period had the highest biomass (9.983g). The biomass accumulation was decreased at 30^{th} day of T_3 and the color of the leaves became yellow. On the other hand, in T_2 shoots, thickened and healthy leaves and highest biomass (5.41) observed at 21 days treatment which is higher than control shoots (2.65g) but lower than T_1 shoots (8.6g).

Among the various concentration of BAP treatment, the maximum biomass was observed in the media supplemented with 1.0mg/L BAP on comparing the treatments T_1, T_2 and T_3. Among which T_1 showed maximum growth index **(12.79±9.51)**. The maximum biomass accumulation was observed in T_1 (9.98g) among different concentration of BAP treatments

Treatments	0 day	7 days	14 days	21 days	30 days
T_0 Control					
T_1 (1.0 mg/L BAP)					
T_2 (1.5 mg/L BAP)					
T_3 (2.0 mg/L BAP)					

Plate 4.1. Different Concentration of BAP on biomass content in *in-vitro* suspension cultures of *Withania somnifera*

From the **Table 4.1**and **Plate 4.1,** it is observed that the increasing concentration of BAP alone has showed a significant increase in the shoot multiplication. A mounting effect was observed till 30 days from the period of inoculation. The growth index was calculated from the fresh weight of shoot in suspension culture.

$$\textbf{Growth index} = \frac{\textbf{Initial weight (g) - Final weight (g)}}{\textbf{Initial weight (g)}}$$

Table 4.1. Effect of plant growth hormones on biomass accumulation in *Withania somnifera in vitro* shoots

S. no.	BAP (mg/L)	KIN (mg/L)	Growth index after 30 days in fresh weight
T_0	MS	MS	4.02±0.97
T_1	1.0	0	12.79± 9.51
T_2	1.5	0	3.32± 2.59
T_3	2.0	0	3.43± 1.15
T_4	0	1.0	1.98± 0.11
T_5	0	1.5	8.62± 0.80
T_6	0	2.0	6.06± 1.61

T_0- MS basal medium with absence of plant growth hormones is taken as control
*Data represents mean±SE of 3 replication

From the **figure 4.1** it is evident that T_1 show maximum growth index among all the treatments compared to control T_0.

Study conducted by Parameswari *et al.,* 2017 revealed that maximum biomass growth was observed in medium with 4.44µM BAP by inoculating 2g fresh weight of shoot. The biomass growth observed in the study is (38g/l) in bioreactor. In the present study, the maximum biomass (9.98g) and growth index (12.79± 9.51) were observed in the T_1 supplemented with 1.0mg/L BAP. 0.86g 0f fresh shoot inoculum were transferred from BAP solid culture to find out the maximum biomass growth and growth index

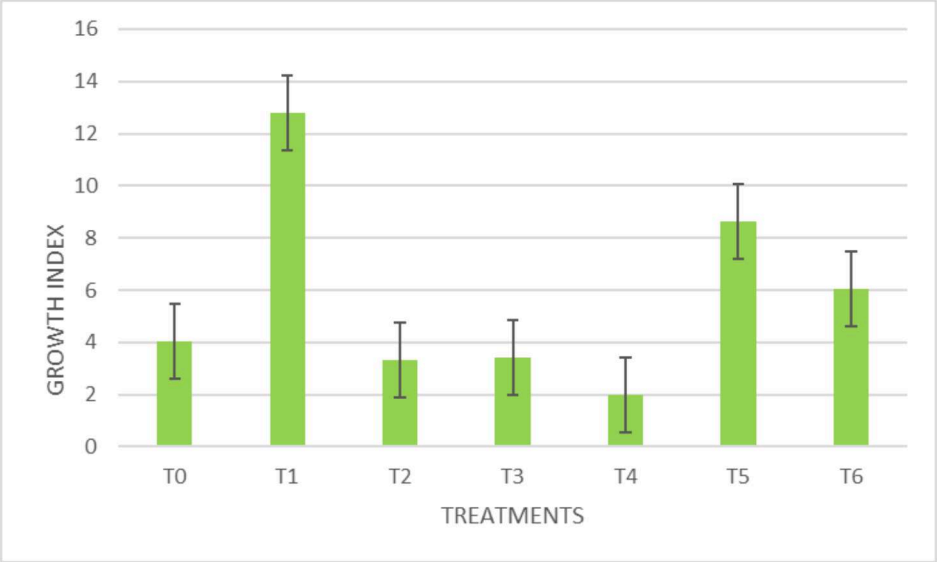


Figure 4.1. Growth index of *Withania somnifera* at different concentration of BAP and Kinetin

4.2. Influence of different concentration of KIN on biomass growth in shoot suspension culture

KIN (kinetin) is type of cytokinin hormone that is used for *in-vitro* shoot proliferation and elongation in a number of plant species. Generally, KIN hormone was supplemented to the nutrient medium i.e., Murashige and Skoog (MS) basal medium for plant growth. In this study, KIN hormone at the concentration of 1, 1.5 & 2.0 mg/L was supplemented with MS medium for the treatment of in vitro shoots of *Withania somnifera* in suspension culture.

From the **Plate 4.2**, the biomass content was increased gradually in control (T_0) from 0.786g to 3.726g on o to 30 days time period. The shoots were elongated and more branches was observed in T_4 at 21st days compared to control and highest biomass accumulation was observed at 30th days (3.84g) when compared to control. The shoots were healthy and leaves were elongated at 7 days in T_5 and maximum biomass accumulation was observed at 30 days (10.54g) which is lower compared to control. The shoots were elongated and leaves became thin at 21st days in T_6 and maximum biomass accumulation was observed at 30 days when compared to control. Among the kinetin treatments (T_4, T_5, T_6) at different concentration, the maximum biomass accumulation was observed at 30th day in treatment T_5 (10.54g) which is lower than control

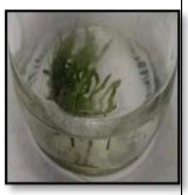
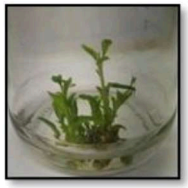
Treatments	0 day	7 days	14 days	21 days	30 days
T_0 Control					
T_4 (1.0 mg/L KIN)					
T_5 (1.5 mg/L KIN)					
T_6 (2.0 mg/L KIN)					

Plate 4.2. Varying concentration of KIN on biomass content in suspension cultures of *Withania somnifera*

From the **Table 4.1** and **Plate 4.2,** it is observed that the increasing concentration of KIN has promoted the cell division thereby increasing the elongation of the shoot as the days progressed. A mounting effect was observed till 30 days from the period of inoculation. Among the various concentration of KIN treatment, the maximum growth index was observed in the media supplemented with 1.5mg/L KIN concentration. On comparing T_3, T_4 and T_5 where different concentration of KIN was supplemented to the medium, T_5 shoots treated with KIN 1.5mg/L were found to given higher biomass/growth index. The maximum biomass (10.543) and

growth index (8.62±0.80) was observed in T_5 among different concentration of KIN treatments in suspension culture.

From the table 4.1.a it is evident that T_1 shows maximum biomass at 7th day with a maximum fold increase of 5.05 and the shoot cultures were healthy at this day. T_5 shows maximum growth at 14th day with a maximum fold increase of 3.02 and the leaves were broad and healthy at this day

Table 4.1a Fold increase observed in the biomass content of different concentration of BAP and KIN

	0th day (wt/g)	**7th day(wt/g)**	**14th day(wt/g)**	**21stday(wt/g)**	**30thday(wt/g)**
Control (T_0)	**0.786±0.10**	**1.1±0.567**	**2.20±1.304**	**2.65±1.41**	**3.726±1.73**
T_1	0.863±0.275	6.623±3.55	7.766±4.974	8.603±4.808	9.983±5.62
Fold increase	**0.09**	**5.02**	**2.53**	**2.24**	**1.68**
T_2	0.926±0.313	3.58±2.403	5.25±2.885	5.416±2.982	5.8±2.89
Fold increase	**0.17**	**2.25**	**1.38**	**1.04**	**0.56**
T_3	0.826±0.185	2.04±0.235	2.773±0.279	2.94±0.135	3.983±0.144
Fold increase	**0.05**	**0.85**	**0.25**	**0.10**	**0.06**
T_4	0.943±0.313	1.17±0.378	2.286±0.527	2.776±0.560	3.84±1.039
Fold increase	**0.19**	**0.06**	**0.03**	**0.04**	**0.03**
T_5	1.196±0.339	3.523±0.930	8.906±2.036	9.243±2.126	10.543±2.527
Fold increase	**0.52**	**2.20**	**3.04**	**2.48**	**1.83**
T_6	0.986±0.342	1.74±0.340	4.07±1.424	4.816±1.334	6.603±1.083
Fold increase	**0.20**	**0.58**	**0.85**	**0.81**	**0.77**

Fold increase was maximum at 7th day 0f concentration of 1.0mg/L BAP and at 14th day of concentration of KIN at 1.5mg/L

Culture medium containing only cytokinins have been found to be ineffective for growth and development of suspension culture of *Withania somnifera.* The lower level of BAP

(0.5mg/L-2.0 mg/L) exhibited slight better effect on growth of embryogenic tissues (Jhankare *et al.,* 2011).

From the figure 4.1a it is evident that T_1 and T_5 shows maximum fold increase. $T_2$and T_6 shows moderate fold increase. T_3 and T_4 shows minimum fold increase among all the other treatments.

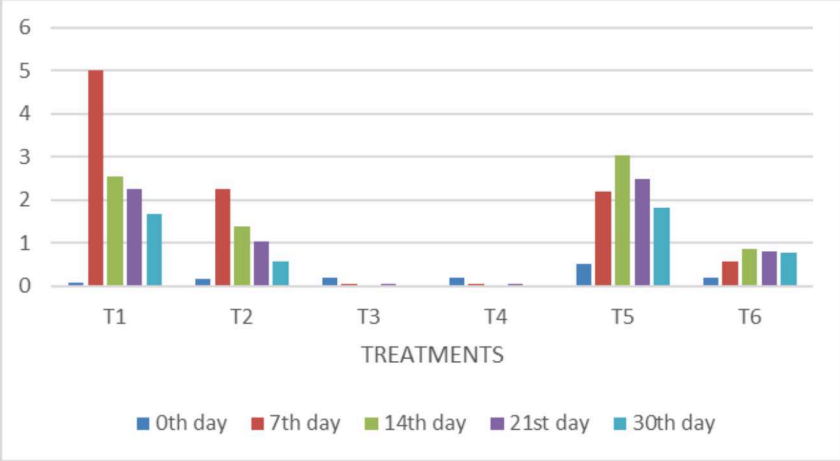


Figure 4.1a Fold increase observed for 30 days in *Withania somnifera*

4.3 Influence of varying concentration of BAP and KIN on biomass growth in shoot suspension culture

KIN (kinetin) is type of cytokinin hormone that is used for *in-vitro* shoot proliferation and elongation in a number of plant species. Generally, KIN hormone was supplemented to the nutrient medium i.e., Murashige and Skoog (MS) basal medium for plant growth. BAP (6 benzyl aminopurine) is a shoot multiplication hormone used for *in vitro* shoot proliferation for a number of plant species. Generally, BAP hormone was supplemented to the nutrient medium i.e., Murashige and Skoog (MS) basal medium for plant growth. In the present study, BAP at 1mg/L and KIN at 1.5mg/L showed maximum growth index further the studies were proceed for the combination of BAP and KIN.

From the **Table 4.1** and **Plate 4.3** it is observed that the increasing combination of BAP and KIN has showed a significant increase in the shoot multiplication rate and promoted the cell division thereby increasing the elongation of shoots as the days progressed. A mounting effect

was observed till 14 days from the period of inoculation. On comparing T_7 and T_8 where different concentration of BAP and KIN were supplemented to the medium, it was observed that T_7 shows maximum growth index than T_8 (1.73±1.07).

Table 4.3. Effect of plant growth regulators in combination of BAP and KIN

S.no	BAP	KIN	Growth index after 15 days in fresh weight
T_0	MS	MS	0.89±0.69
T_7	1.0	0.5	1.17±0.43
T_8	0.5	1.0	0.71±0.15

T_0- MS basal medium with absence of plant growth hormones is taken as control
*Data represents mean±SE of 3 replication

From the plate 4.3, the biomass content was increased gradually in T_0 from 0.59g to 3.03g on 0 to 14 days. The leaves were thickened and broad at 14days and maximum biomass accumulation was observed at 14th day in T_7 (2.46g) which is higher when compared to control. Fast growth was observed in T_8 from 0 to 14 days when compared to control and maximum biomass accumulation in T_8 was observed at 14 days (1.29g).

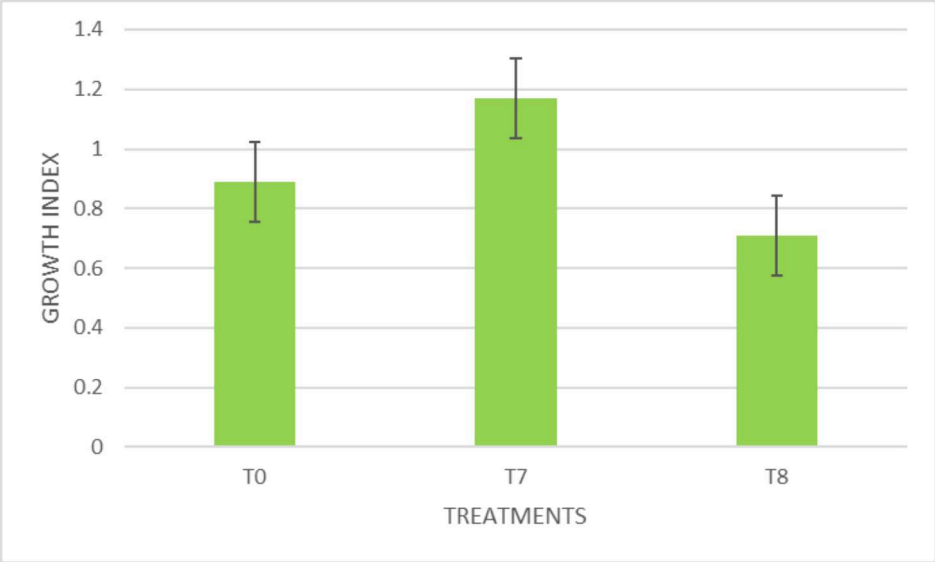


Figure 4.3 Growth index of *Withania somifera* at different combination of cytokinins

From the plate 4.3, the biomass content was increased gradually in T_0 from 0.59g to 3.03g on 0 to 14 days. The leaves were thickened and broad at 14days and maximum biomass

accumulation was observed at 14th day in T_7 (2.46g) which is higher when compared to control. Fast growth was observed in T_8 from 0 to 14 days when compared to control and maximum biomass accumulation in T_8 was observed at 14 days (1.29g).

Table 4.3a Fold increase observed in the biomass content of different combination of BAP and KIN

	0th day	7th day	14th day
Control (biomass)	0.59±0.25	0.80±0.11	1.01±0.03
T_7	1.13±0.04	1.38±0.14	2.46±0.46
Fold increase	**0.91**	**0.72**	**1.43**
T_8	0.76±0.13	0.95±0.13	1.29±0.15
Fold increase	**0.28**	**0.18**	**0.27**

From the table 4.3a it is evident that T_7 shows maximum biomass at 14th day with a maximum fold increase of 1.43 and the shoot cultures were healthy during this day. T_8 showed minimum fold increase when compared to T_7. Minimum fold increase was observed in T_8 (0.28) and maximum fold increase was observed in T_7(1.43).

Treatment	0 day	7 days	14 days
T_0 (Control) P			
T_7 (1.0 mg/L BAP and 0.5mg/L KIN)			
T_8 (1.0 mg/L KIN and 0.5mg/L BAP)			

Plate 4.3. Varying combination of BAP and KIN on biomass content in *Withania somnifera*

Sabir *et al*., (2014) reported that the good growth was observed in the suspension culture obtained from callus culture at a concentration of 2,4-D (3mg/L) and kinetin (0.5mg/L). In the present study, the maximum growth index was observed in T_7 of concentration (1.0mg/L BAP + 0.5 mg/L KIN).

4.4 Biomass accumulation of *Withania somnifera* in bioreactor

Figure 4.4 Production of *Withania somnifera* in bioreactor culture

T_5 at concentration 1.5mg/L with maximum biomass accumulation from suspension culture was transformed into bioreactor for large scale production and growth was observed. Sivanandhan *et al*., (2014) studied the biomass production using elicitors. Maximum withanolides were produced in bioreactor and shows 1 to 3 folds higher than the control. In the present study, maximum biomass production was observed using plant growth regulators at different concentration and combination of KIN

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

From the results of the present study entitled **"Effect of different plant growth regulators on biomass accumulation of *Withania somnifera*"** is summarized as follows

- *In-vitro* shoots of *Withania somnifera* JA-20 grown in MS basal medium supplemented with different concentrations and combination of cytokinin (BAP and KIN) were used to standardize the optimal concentration of cytokinin for *Withania somnifera* shoot growth in suspension culture under *in- vitro* condition.
- The maximum biomass accumulation was observed under the influence of growth regulators at the concentration of 1.0 (mg/L) BAP, 1.5 (mg/L) KIN followed by the combination of 1.0 (mg/L) BAP and 0.5 (mg/L) KIN
- To establish the suspension culture, one month old *in-vitro* explants were sub-cultured into the suspension culture containing MS basal media supplemented with different concentration (1.0mg/L, 1.5mg/L and 2.0 mg/L BAP) and (1.0mg/L,1.5mg/L and 2.0mg/L KIN) and combination of BAP and KIN (1.0mg/L BAP + 0.5mg/L KIN and 0.5mg/L BAP + 1.0 mg/L KIN) and allowed them to grow in orbital shaker.
- The increase in growth index of 12.79± 9.515 was observed in concentration at 1.0 mg/L BAP (T_1). The increase in growth index of 8.62± 0.80 was observed in concentration at 1.5mg/L KIN (T_5)
- The increase in growth index of 1.17±was observed in different combination of BAP and KIN (1mg/L+0.5mg/L) (T_7)
- Adventitious shoots grown in T_5 medium was further transferred to bioreactor for large scale production

To conclude, our results confirmed that *in-vitro* shoot culture under suspension culture supplemented with different concentrations of cytokinin showed maximum growth index at T1, T_5 and T_7 of concentration 12.79±9.51, 8.62±0.80 and 1.17±0.43. The suspension culture with high biomass content were used as an explant for bioreactor culture. This proves that the effect of plant growth regulators, their concentration and time period had positive effective on *in-vitro* shoots of *Withania somnifera*

BIBLIOGRAPHY

6.0. BIBLIOGRAPHY

- A Dar, P., R Singh, L., A Kamal, M., & A Dar, T. (2016). Unique medicinal properties of *Withania somnifera*: Phytochemical constituents and protein component. *Current pharmaceutical design*, *22*(5), 535-540.

- Abdelwahed, M. T., Hegazy, M. A., & Mohamed, E. H. (2023). Major biochemical constituents of *Withania somnifera* (ashwagandha) extract: A review of chemical analysis. *Reviews in Analytical Chemistry*, *42*(1), 20220055.

- Ara, T., & Choudhary, A. (2014). An Attempt to Propagate and Conserve *Withania somnifera* (L). Dunal: A Polypharmaceutical Plant Through In Vitro Culture. *Impact: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)*, *2*, 159-164.

- Autade, R. H., Fargade, S. A., Savant, A. R., Gangurde, S. S., Choudhary, R. S., & Dighe, S. S. (2016). Micropropagation of Ashwagandha (*Withania somnifera*). *Biosci Biotech Res Comm*, *9*, 88-93.

- Badyal, S., Singh, H., Yadav, A. K., Sharma, S., & Bhushan, I. (2020). Plant secondary metabolites and their uses. *Plant Arch*, *20*(2), 3336-3340.

- Betül, A. K. I. N. (2020). Tissue culture techniques of medicinal and aromatic plants: History, cultivation and micropropagation. *Journal of Scientific Reports-A*, (045), 253-266.

- Bhasin,S., Singh,M.,& Singh,D.(2019).Review on bioactive metabolites of *Withania somnifera*.(L.) Duna and its pharmacological significance.*J.Pharmacogn.phytochem*, 8(3),3906-3909.

- Bhoyar, p., suryawanshi, m., & krishnasamy, v. (2015). Callus induction and est us induction and est us induction and establishment of suspension ablishment of suspension culture from ture from ture from *Withania somnifera Withania somnifera Withania somnifera*: the indian ginseng: the indian ginseng.

- Bisht,P., & Rawat,V. (2014). Antibacterial activity of *Withania somnifera* against gram positive isolates from pus samples.*Ayu,*35(3),330

- Chandana, B. C., Nagaveni, H. C., Lakshmana, D., Kolakar, S. S., & Heena, M. S. (2018). Role of plant tissue culture in micropropagation, secondary metabolites production and conservation of some endangered medicinal crops. *Journal of Pharmacognosy and Phytochemistry*, *7*(3S), 246-251.

- Dhar, N., Razdan, S., Rana, S., Bhat, W. W., Vishwakarma, R., & Lattoo, S. K. (2015). A decade of molecular understanding of withanolide biosynthesis and in vitro studies in

- *Withania somnifera* (L.) Dunal: prospects and perspectives for pathway engineering. *Frontiers in plant science*, *6*, 1031.
- Durg, S., Dhadde, S. B., Vandal, R., Shivakumar, B. S., & Charan, C. S. (2015). *Withania somnifera* (Ashwagandha) in neurobehavioural disorders induced by brain oxidative stress in rodents: A systematic review and meta-analysis. *Journal of Pharmacy and Pharmacology*, *67*(7), 879-899.
- Dutta, R., Khalil, R., Green, R., Mohapatra, S. S., & Mohapatra, S. (2019). *Withania somnifera* (Ashwagandha) and withaferin A: Potential in integrative oncology. *International journal of molecular sciences*, *20*(21), 5310
- Ergun, K. A. Y. A., GALATALI, S., GÜLDAĞ, S., ÖZTÜRK, B., CEYLAN, M., ÇELİK, O., & AKTAY, İ. (2018). Mass production of medicinal plants for obtaining secondary metabolite using liquid mediums via bioreactor systems: SETISTM and RITA®. *Türk Bilimsel Derlemeler Dergisi*, *11*(2), 5-10.
- Ezez, D., Mekonnen, N., & Tefera, M. (2023). Phytochemical analysis of *Withania somnifera* leaf extracts by GC-MS and evaluating antioxidants and antibacterial activities. *International Journal of Food Properties*, *26*(1), 581-590.
- Fatima, N., & Anis, M. (2012). Role of growth regulators on in vitro regeneration and histological analysis in Indian ginseng (*Withania somnifera* L.) Dunal. *Physiology and molecular biology of plants*, *18*, 59-6
- Gaurav, H., Yadav, D., Maurya, A., Yadav, H., Yadav, R., Shukla, A. C., ... & Palazon, J. (2023). Biodiversity, Biochemical Profiling, and Pharmaco-Commercial Applications of *Withania somnifera*: A Review. *Molecules*, *28*(3), 1208.
- Gaurav, N., Kumar, A., Tyagi, M., Kumar, D., Chauhan, U. K., & Singh, A. P. (2015). Morphology of *Withania somnifera* (distribution, morphology, phytosociology of *Withania somnifera* L. Dunal). *Int J Current Sci Res*, *1*(7), 164-173.
- Girme, A., Saste, G., Pawar, S., Balasubramaniam, A. K., Musande, K., Darji, B., ... & Hingorani, L. (2020). Investigating 11 withanosides and withanolides by UHPLC–PDA and mass fragmentation studies from Ashwagandha (*Withania somnifera*). *ACS omega*, *5*(43), 27933-27943.
- Halder, T., & Ghosh, B. (2015). Phytochemical and pharmacological activity of *Withania somnifera* (L.) Dunal. *International Journal of Economic Plants*, *2*(4), 192-196.
- Hussain, M. S., Alqahtani, S. S., Ahmad, S., & Iqbal, M. R. (2023). A Comprehensive Scientific Claims on Ethnobotany, Phytochemical Pharmacological and Toxicology of *Withania somnifera* (L.) Dunal. *Journal of Drug Delivery and Therapeutics*, *13*(3), 194-207.
- Jain, R., Kachhwaha, S., & Kothari, S. L. (2012). Phytochemistry, pharmacology, and biotechnology of *Withania somnifera* and Withania coagulans: A review. *Journal of Medicinal Plants Research*, *6*(41), 5388-5399.

- Jana, S. N., & Charan, S. M. (2018). Health benefits and medicinal potency of *Withania somnifera*: a review. *Int J Pharm Sci Rev Res*, *48*(1), 22-29.
- Javadian, F., Sepehri, Z., Saeidi, S., & Hassanshahian, M. (2017). Antifungal effects of the extract of the *Withania somnifera* on Candida albicans. *Advanced Herbal Medicine*, *3*(1), 31-37.
- Jhankare, a., tripathi, m., tiwari, g., pandey, g., baghel, b., & tiwari, s. Short communication establishment of embryogenic cell suspension cultures and plantlet regeneration in ashwagandha (*Withania somnifera* l.).
- John, J. (2014). Therapeutic potential of *Withania somnifera*: a report on phyto-pharmacological properties. *International Journal of Pharmaceutical sciences and research*, *5*(6), 2131-2148.
- Kaur, K., Dolker, D., Behera, S., & Pati, P. K. (2022). Critical factors influencing in vitro propagation and modulation of important secondary metabolites in *Withania somnifera* (L.) dunal. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *149*(1-2), 41-60.
- Kaur, K., Singh, P., Kaur, K., Bhandawat, A., Nogia, P., & Pati, P. K. (2021). Development of robust in vitro culture protocol for the propagation of genetically and phytochemically stable plants of *Withania somnifera* (L.) Dunal (Ashwagandha). *Industrial Crops and Products*, *166*, 113428.
- Kaur, P., & Tiwari, A. (2018). Use of Abiotic Elicitors to enhance Secondary Metabolites Production in *Withania somnifera*. *Research Journal of Pharmacy and Technology*, *11*(11), 5139-5142.
- Khanal, P., Chikhale, R., Dey, Y. N., Pasha, I., Chand, S., Gurav, N., & Gurav, S. (2022). Withanolides from *Withania somnifera* as an immunity booster and their therapeutic options against COVID-19. *Journal of Biomolecular Structure and Dynamics*, *40*(12), 5295-5308.
- Khanchandani, N., Shah, P., Kalwani, T., Ardeshna, A., & Dharajiya, D. (2019). Antibacterial and antifungal activity of Ashwagandha (*Withania somnifera* l.): A review. *Journal of Drug Delivery and Therapeutics*, *9*(5-s), 154-161.
- Krutika, J., Tavhare, S., Panara, K., Kumar, P., & Karra, N. (2016). Studies of ashwagandha (*Withania somnifera* dunal). *Int J Pharm Biol Arch*, *7*(1), 1-11.
- Kukreti, C., Rajwar, G. S., & Uniyal, P. L. (2013). Effect of growth regulators on growth and yield of *Withania somnifera* Dunal. *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, *5*(2), 66-70.
- Kumar, V., Dey, A., Hadimani, M. B., Marcovic, T., & Emerald, M. (2015). Chemistry and pharmacology of *Withania somnifera*: An update. *Cellmed*, *5*(1), 1-1.
- Mandal, A., & Reddy, J. M. (2017). A review on potential therapeutic uses of *Withania somnifera*. *World J Pharm Res*, *6*, 846-860.

- Martin, Y., & Vermette, P. (2005). Bioreactors for tissue mass culture: design, characterization, and recent advances. *Biomaterials*, *26*(35), 7481-7503.
- Meena, L. K., Gupta, A. K., Patel, J., Khan, M. Y., & Kumar, S. Ashwagandha (*Withania somnifera* L.).
- Meher, S. K., Das, B., Panda, P., Bhuyan, G. C., & Rao, M. M. (2016). Uses of *Withania somnifera* (Linn) Dunal (Ashwagandha) in Ayurveda and its pharmacological evidences. *Research Journal of Pharmacology and Pharmacodynamics*, *8*(1), 23-29.
- Mikulska, P., Malinowska, M., Ignacyk, M., Szustowski, P., Nowak, J., Pesta, K., ... & Cielecka-Piontek, J. (2023). Ashwagandha (*Withania somnifera*)—Current Research on the Health-Promoting Activities: A Narrative Review. *Pharmaceutics*, *15*(4), 1057.
- Mir, B. A., Mir, S. A., & Koul, S. (2014). In vitro propagation and withaferin A production in Withania ashwagandha, a rare medicinal plant of India. *Physiology and Molecular Biology of Plants*, *20*, 357-364.
- Motolinía-Alcántara, E. A., Castillo-Araiza, C. O., Rodríguez-Monroy, M., Román-Guerrero, A., & Cruz-Sosa, F. (2021). Engineering considerations to produce bioactive compounds from plant cell suspension culture in bioreactors. *Plants*, *10*(12), 2762.
- Munir, N., Mahmood, Z., Shahid, M., Afzal, M. N., Jahangir, M., Ali Shah, S. M., ... & Yousaf, F. (2022). *Withania somnifera* Chemical Constituents' In Vitro Antioxidant Potential and Their Response on Spermatozoa Parameters. *Dose-Response*, *20*(1), 15593258221074936.
- Namdeo, A. G., & Ingawale, D. K. (2021). Ashwagandha: Advances in plant biotechnological approaches for propagation and production of bioactive compounds. *Journal of Ethnopharmacology*, *271*, 113709.
- Narinderpal, K., Junaid, N., & Raman, B. (2013). A Review on Pharmacological Profile of *Withania somnifera* (Ashwagandha). *Research & Reviews: Journal of BotanicalSciences*.
- Nefzi, A. (2016). Aydi Ben Abdallah R. *Jabnoun-Khiareddine H, Medimagh-Saïdana S, Haouala R, et al*, 144-150.
- Ojha, S. K., & Arya, D. S. (2009). *Withania somnifera* Dunal (Ashwagandha): a promising remedy for cardiovascular diseases. *World J Med Sci*, *4*(2), 156-158.
- Oseni, O. M., Pande, V., & Nailwal, T. K. (2018). A review on plant tissue culture, a technique for propagation and conservation of endangered plant species. *International journal of current microbiology and applied sciences*, *7*(7), 3778-3786.
- Pandey, V., Ansari, W. A., Misra, P., & Atri, N. (2017). *Withania somnifera*: Advances and implementation of molecular and tissue culture techniques to enhance its application. *Frontiers in Plant Science*, *8*, 1390.

- Pant, B. (2014). Application of plant cell and tissue culture for the production of phytochemicals in medicinal plants. In *Infectious Diseases and Nanomedicine II: First International Conference (ICIDN–2012), Dec. 15-18, 2012, Kathmandu, Nepal* (pp. 25-39). Springer India.

- Parameswari, M., Estri Laras, A., Joni, K., Valizadeh, M., & Kalaiselvi, S. (2017). A study on the influence of plant growth regulator on shoot multiplication and evaluation of major withanolides in *Withania somnifera*. *World J. Pharm. Pharm. Sci.*, *6*, 842-853.

- Patnaik, N. (2015). Role of Brahmi and Ashwagandha in Anti-Ageing. *Journal of Biosciences and Medicines*, *3*(10), 102.

- Patel, P., & Krishnamurthy, R. (2013). Feasibility studies on in vitro mass-scale propagation of Indian Ashwagandha (*Withania somnifera*) cultivars for commercial purpose. *Journal of Pharmacognosy and Phytochemistry*, *2*(2), 168-174.

- Paul, S., Chakraborty, S., Anand, U., Dey, S., Nandy, S., Ghorai, M., ... & Dey, A. (2021). *Withania somnifera* (L.) Dunal (Ashwagandha): A comprehensive review on ethnopharmacology, pharmacotherapeutics, biomedicinal and toxicological aspects. *Biomedicine & Pharmacotherapy*, *143*, 112175.

- Polumackanycz, M., Petropoulos, S. A., Śledziński, T., Goyke, E., Konopacka, A., Plenis, A., & Viapiana, A. (2023). *Withania somnifera* L.: Phenolic Compounds Composition and Biological Activity of Commercial Samples and Its Aqueous and Hydromethanolic Extracts. *Antioxidants*, *12*(3), 550.

- Ragini,P., & Sailaja,K.(2021).An overall review on *Withania somnifera* and its applications.*Aditum Journal of Clinical and Biomedical and Research,*2(2),1-4

- Saidulu, C., Venkateshwar, C., & Rao, S. G. (2014). Morphological studies of medicinal plant of *Withania somnifera* (L.) Dunal grown in heavy metal treated (contaminated) soil. *Journal of Pharmacognosy and Phytochemistry*, *3*(1), 37-42.

- Saleem, S., Muhammad, G., Hussain, M. A., Altaf, M., & Bukhari, S. N. A. (2020). *Withania somnifera* L.: Insights into the phytochemical profile, therapeutic potential, clinical trials, and future prospective. *Iranian Journal of Basic Medical Sciences*, *23*(12), 1501.

- Santhanu, K., & Senthil, K. (2021). Therapeutic potential of *Withania somnifera* (Linn) Dunal (Ashwagandha) in historical perspective and pharmacological evidence. *Annals of Ayurvedic Medicine*, *10*(2), 135-135.
- Sarangi, A., Jena, S., Sarangi, A. K., & Swain, B. (2013). Anti-diabetic effects of *Withania somnifera* root and leaf extracts on streptozotocin induced diabetic rats. *Journal of Cell and Tissue Research*, *13*(1), 3597.

- Sangwan, N.S, Sabir, F., Mishra, S., Bansal, S., & S Sangwan, R. (2014). Withanolides from *Withania somnifera* Dunal: development of cellular technology and their production. *Recent Patents on Biotechnology*, *8*(1), 25-35.

- Sabir, F., Sangwan, R. S., Singh, J., Misra, L. N., Pathak, N., & Sangwan, N. S. (2011). Biotransformation of withanolides by cell suspension cultures of *Withania somnifera* (Dunal). *Plant Biotechnology Reports*, *5*, 127-134.

- Scarpa, G. M., Prota, V., Schianchi, N., & Manunta, F. (2022). In Vitro Cultures for the Production of Secondary Metabolites. In *Secondary Metabolites*. IntechOpen.

- Sengupta, P., Agarwal, A., Pogrebetskaya, M., Roychoudhury, S., Durairajanayagam, D., & Henkel, R. (2018). Role of *Withania somnifera* (Ashwagandha) in the management of male infertility. *Reproductive biomedicine online*, *36*(3), 311-326.

- Sharifi-Rad, J., Quispe, C., Ayatollahi, S. A., Kobarfard, F., Staniak, M., Stępień, A., ... & Cho, W. C. (2021). Chemical Composition, Biological Activity, and Health-Promoting Effects of *Withania somnifera* for Pharma-Food Industry Applications. *Journal of Food Quality*, *2021*, 1-14.

- Sharma, V., Sharma, S., & Pracheta, R. P. (2011). *Withania somnifera*: a rejuvenating ayurvedic medicinal herb for the treatment. *Int J PharmTech Res*, *3*(1), 187-192.

- Shelke,P., Sapkal,S., Prashad, P., & Pandhure,P.(2020. Tissue culture studies in *Withania somnifera* (L.) Dunal, *Bioscience Discovery,*11(3), 178-182.

- Shukla, D. D., Bhattarai, N., & Pant, B. (2010). In-vitro mass propagation of *Withania somnifera* (L.) Dunal. *Nepal Journal of Science and Technology*, *11*, 101-106.

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

- Singh, N., Bhalla, M., de Jager, P., & Gilca, M. (2011). An overview on ashwagandha: a Rasayana (rejuvenator) of Ayurveda. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(5S).

- Singh, V., Shah, H. H., & Guillemin, G. J. (2017). Neuroprotective Effect of Ashwagandha (roots of *Withania somnifera*): The rejuvenator. *Canad J Clin Nutr*, *5*(2), 34-51.

- Sivanandhan, G., Selvaraj, N., Ganapathi, A., & Manickavasagam, M. (2014). Enhanced biosynthesis of withanolides by elicitation and precursor feeding in cell suspension culture of *Withania somnifera* (L.) Dunal in shake-flask culture and bioreactor. *PloS one*, *9*(8), e104005.

- Supe, U., Dhote, F., & Roymon, M. G. (2011). A review on micro propagation of *Withania somnifera*–A medicinal plant. *Journal of agricultural technology*, *7*(6), 1475-1483.

- Tandon, N., & Yadav, S. S. (2020). Safety and clinical effectiveness of *Withania somnifera* (Linn.) Dunal root in human ailments. *Journal of ethnopharmacology*, *255*, 112768.

- Thakur,S., Kaurav,H., & Chaudhary,G.(2021). A review on Ashwagandha (*Withania somnifera*): mind and body rejuvenator.*Int.J.Ayurveda Pharm,* 12(2),2229-3566.
- Tripathi, N., Shrivastava, D., Mir, B. A., Kumar, S., Govil, S., Vahedi, M., & Bisen, P. S. (2018). Metabolomic and biotechnological approaches to determine therapeutic potential of *Withania somnifera* (L.) Dunal: A review. *Phytomedicine*, *50*, 127-136.
- Ullah, N., Zahoor, M., & Farhat, A. (2014). A review on general introduction to medicinal plants, its phytochemicals and role of heavy metal and inorganic constituents. *Life Science Journal*, *11*(7s), 520-527.
- Umadevi, M., Rajeswari, R., Rahale, C. S., Selvavenkadesh, S., Pushpa, R., Kumar, K. S., & Bhowmik, D. (2012). Traditional and medicinal uses of *Withania somnifera*. *The pharma innovation*, *1*(9, Part A), 102.
- Visweswari, G., Christopher, R., & Rajendra, W. (2013). Phytochemical screening of active secondary metabolites present in *Withania somnifera* root: role in traditional medicine. *International journal of pharmaceutical sciences and research*, *4*(7), 2770.

APPENDIX

APPENDIX-1

COMPOSITION OF MS MEDIUM

INGREDIENTS	COMPOSITION (mg/L)	STOCK SOLUTION(W/V)(g)
MS MACRO I (10X)		
NH4NO3	1650	16.5
KNO_3	1900	19
MgSO4.7H_2O	370.6	3.7
KH_2PO_4	170	1.7
100 ml		
MS MACRO II (10X)		**1000 ml**
Cacl2.2H2O	439.8	4.398
100ml		
Fe-Na EDTA		100 ml
Fe-Na EDTA	36.7	36.7
1ml		
MICRO NUTRIENTS (1000X)		**100 ml**
$NaMoO_4.7H_2O$	0.25	0.025
$CuSO_4.5H_2O$	0.025	0.0025
$CoCl_2.2H_2O$	0.025	0.0025
$MnSo_4.4H_2O$	13.2	1.32
$ZnSO_4.4H_2O$	8.6	0.86
H_3BO_3	6.2	0.62
1ml		
KI (1000X)	0.83	100 ml
MYO-INOSITOL(100X)		100ml
Myoinositol	100mg	
10 ml		
MS VITAMINS (1000x)		**100 ml**
Nicotinic acid	0.5	0.05
Pyridoxine HCL	0.5	0.05
Thiamine HCL	0.1	0.01
Glycine		
1ml		