“ENHANCED BIOACTIVE COMPOUNDS IN MEDICINAL PLANT USING PLANT TISSUE CULTURE”

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE
OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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CANDIDATE's DECLARATION

I hereby certify that the work which is presented in the research work entitled "Nutrigenomics in Cardiovascular disease" in fulfilment of the requirement for the award of Degree of Masters in Science in Biotechnology and submitted to the Department of biotechnology, Delhi technological university, Delhi is an authentic record of my own work, carried during a period from 7-jan-2021 to 28-may-2021, under the supervision of Dr. Navneeta Bharadvaja.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other university. This work has been communicated in SCI indexed journal with the following details:

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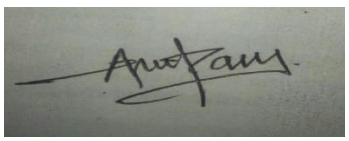

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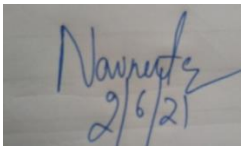
CERTIFICATE

I hereby clarify that the project dissertation titled "**Enhanced bioactive compounds in medicinal plants using plant tissue culture**" which is submitted by **Anupam Singla, 2K19/MSCBIO/16**, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

Place: Delhi

Date: 28 May, 2021



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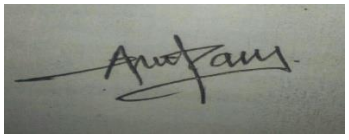

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Abstract

Bioactive chemicals are described as dietary components that affect people who consume those substances in their physiological as well as cellular function. Flavonoids, anthocyanins, tannins, berets, carotenoids, plant sterols and glucosinolates are all included. These may well be present most often in fruits and vegetables; offer anti - oxidant, anti-inflammatory but also antiviral activities; and therefore, can shield humans toward chronic disorders and metabolism. These favorable effects empower researchers to create novel functional foods containing prospective protective and healthful. Cardiovascular disease is the cardiac or blood vessel dysfunctional action. An inadequate heart and blood vessel function boosts the heart attack risk, heart failure, sudden death, stroke and heart rhythm disorders, resulting to diminished standard of health and a shorter life expectancy. Plant tissue culture is an effective venue for the generation of secondary metabolites along with its diverse implications. Diverse plant-based strategies including such callus or suspension cultivation are utilized generally in the synthesis of secondary plant metabolites. Several novel approaches which aim to have a rather significant and neglected influence on secondary metabolite synthesis.

CONTENTS

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Chapters 1

ORGANIZATION OF THESIS

The following thesis title as “Enhanced bioactive compounds in medicinal plants for cardiovascular disease” is basically reviewed information collected from various research and review article. The thesis focuses on the enhancement of bioactive compounds through biotechnological techniques.

The chapter 1 of thesis is an introduction of the bioactive compounds, their dormant role in therapeutics, brief of cardiovascular diseases and brief of plant tissue culture.

The chapter 2 is a review of literature which contains a deeper knowledge about the plant bioactive compounds and its types. Their beneficial roles in cardiovascular disease. It also consists of invitro production of bioactive compounds using micropropagation technique to increase the productiveness of bioactive compounds.

The chapter 3 is proposed methodology for the project and brief estimation about the techniques that can be used for detection of bioactive compounds followed by conclusion.

1.1 INTRODUCTION

The role of the bioactive compound in the diet is being defined in diet. These components are being identified as dietary components that have a significant health impact on physiological activities or molecular functions. "Bioactive compounds" seem to be additional nutrient components normally available in modest amounts in meals; in Greek ‘bios’ expresses as life while in Latin ‘activus’ expresses as the dynamic or full of energy; further differentiated as betalains, flavonoids, plant sterols , carotenoids, glucosinolates, , anthocyanins [1], [2]. These are indeed being widely studied in order to better understand their health impacts. The spur of all this study analysis has come from a large number of epidemiologic studies indicating the protecting influence on cardiovascular disease (CVD) and cancer of plant-based nutrition. The considerable growth range of bioactive components with significant health applications are being found. These components can really serve like antioxidants, inhibitors and inductors of the enzyme, receptor activity inhibitors and gene expression inducers and inhibitors, among

others. Flavonoids are often noticeably antioxidant and anti-inflammatory, cardiovascular health and anti-cancer-related ingredients. Anthocyanins offer favorable health impacts, yet deeper research is needed. In favour of significant impacts of eyesight and skin, carotenoids offer protection properties towards various types of cancer. The therapeutic impact of glucosinolates toward cancer and dementia is relevant. The role of bioactive compounds in one's nutrition is nonetheless immensely complex, requires considerable exploration as well as increased awareness, just to achieve maximal potential value to healthcare. In this field, the main concern seems to be the recognition of bioactive components and so forth their linked health impacts as well as accompanying mechanisms. The emergence of the phrase "functional foods" highlights the concept of consuming the right proportions and patterns of the diverse nutrients and bioactive substances. Functional foods being considered entire foods, supplemented or enhanced, whose ingestion delivers health benefits beyond their nutritive benefits in right amounts [3]. Because of its inclusion in many biological functions, fatty acids are being studied extensively and are also extremely beneficial in nourishment. In fact, many kinds of fatty acids are used, that are mostly monounsaturated and polyunsaturated. For example, polyunsaturated fatty acids are omega 3, show significant influence as anti-inflammatory, neuroprotective, neurodevelopment, and protective in cardiovascular and metabolic disorders.

Cardiovascular diseases (CVD) turn out to be the major cause of globally mortality [4], [5]. CVD is a vast spectrum of diseases related to heart dysfunction which includes high/low blood pressure, heart attack, angina, atherosclerosis. These diseases can either be transience or prolonging to life time of a person. The major reasons behind these diseases are anxiety, lack of physical activity, genetic inheritance, bad immune system, wrong diet, stress. When it comes to a person diet plan it really matters what the person is consume and how it's affecting the energy balance, cellular activities of body. Nutrition always shows the positive impact in the body. The treatment of CVD can be done naturally with the help of certain medicinal plants that contain particular bio-compound needed to treat such CVD. Some of the plants are artichoke, ashwagandha, garlic, ginkgo, guggul, hawthorn, lemongrass. Generally, these compounds show both toxicological and pharmacological influence in humans [1]. Bioactive compounds are effective because they have anti-glycemic, anti-hypertensive, anti-lipidemic,

anti-thrombotic, anti-atherogenic dormant action [6]. CVD factors varies from structural abnormalities, inflammation and heredity to infection.

The plant tissue culture relies on the fundamental of totipotency term; given by Haberlandt (1902) [7]. The main focus of this technique is to ameliorate the crop production with desire improvements. The specific plants or cells of plants offer morphological and physiological impact towards chemical and microbial factors invitro acknowledged as “ELICITORS” [8]. Since plant bioactive compounds have pharmacological as well nutrition additives impact, the elicitors application mainly aims to modify the production of such components in an effective way. Recognizing then isolating a highly valued bioactive component instantly creates a requirement for a continuous manufacturing technique. The secondary metabolite often is defined by its varied as well as complicated chemical composition, normally comprising various chiral centers and labile bonds [9]. Bioactive compounds components are indeed isolated quite regularly from their origin [9]. Recognizing then isolating a highly valued bioactive component instantly creates a requirement for a continuous manufacturing technique. The secondary metabolite often is defined by its varied as well as complicated chemical composition, normally comprising various chiral centers and labile bonds. Bioactive compounds components are indeed isolated quite regularly from their original origin. Tissue culture does seem as a potential biotechnological technique for such generation, notably to ensure the sustainable preservation and appropriate beneficial of Bioactive Compounds that will be implemented in a rather diverse location. The Phenolic molecules are more often generated as a security measure against a cellular invasion or stressful situations for instance temperature difference, different pH, light [10]. These substances are associated with secondary metabolites even though not having connection with the establishment as well as maintenance functionality of the tissue of plants and are usually located in tissues and organs in particular.

CHAPTER 2

2.1 REVIEW OF LITERATURE

Bioactive compounds are found in vegetables and fruits which have an effective impact on physiological as well as cellular activity. Bioactive compound is consisting of anthocyanins, tannins, betalains, carotenoids, plant sterol, glucosinolates. Flavonoids further distinguished as isoflavones, flavanonols, anthocyanin, flavanones, flavanols, flavones, and flavans. Sources of flavonoids are cabbage, ginger, legumes, carrot, apple, kale, lemon, parsley, buckwheat, and tomato. Flavonoids also known as phytonutrients or phytochemicals as it offers antioxidant as well as anti-inflammatory properties. They scavenge free radicals and tends to minimize the damage in cells, tissues. In many studies, it was that flavonoids can be used in order to ameliorate the characteristic of artery walls, can manage the performance of enzymes and cell receptors. Betalains found in amaranth, beetroot, chard, pear, red pitahaya, and cacti. This compound offer antioxidant, anti-cancer, anti-lipidemic, antimicrobial activities. They are essential to one's health as it take mitigate the oxidative stress, inflammation from cardiovascular disease. Anthocyanins present in red grape, cherry, berry, purple corn, blackcurrant, blueberry, acai [11]. It has anti-inflammatory as well as anti-oxidant properties. Tannins main sources are pomegranates, cranberries, thyme, teas, coffee, persimmons, grapes, chocolate, cinnamon, red wine, berries. Shows assertive impact on lowering blood pressure, speeding the process of blood clotting, prevent LDL oxidation also scavenge the free radicals from various physiological activities. Carotenoids sources are spinach, carrots, cantaloupes, plums, turnip greens, apricots, mangoes, kale, coriander, thyme, winter squash sweet potatoes [11]–[13]. Glucosinolates mainly found in cruciferous vegetables. Their dormant action is the elimination of cancer cells without affecting normal cells. Plant sterols can be found in vegetable oils, some functional food product like yogurt, margarine. It shows diminish the LDL cholesterol level in blood. Isothiocyanates are organically synthesized organosulphuric agents that seem to be extremely reactive and are accountable for the intense flavour, associated in cruciferous vegetables like as broccoli, radish, cauliflowers, sprouts. These chemicals, which do have antioxidant, anti-inflammatory, antibacterial, neuroprotective and cardioprotective characteristics, were found in recent studies to be therapeutic and preventive [4]. Different pathways of intracellular activity became revealed amongst their cardioprotective activities and

are commonly used to improve antioxidant responses. Quercetin is just a flavonol that may be found lemongrass, grapes, apples, citrus fruits, red wine, broccoli, tea [14]. Blood pressure in hypertensives was found to significantly reduce by supplementation of quercetin aglycone in diet. Quercetin may reduce blood pressure thus diminish animal and human hypertension incidence [15]. These processes are often a reduction in oxidative stress, a distortion of the renin-angiotensin-aldosterone system and/or an endothelium-dependent/ independent betterment in vascular efficiency. Mushrooms are often extremely protein-intensive, with a major amino acid concentration, but low in fat. Moreover, they offer a high number of carbs and vitamins as well as mineral components with a significant nutritional value mainly of Calcium, iron, Magnesium, Sodium, potassium, copper, manganese, selenium. The arrangement of fatty acids in food products appears to help lower blood cholesterol as hypocholesterolemia effect [16]. The analysis of the fatty acid profiles of several edible mushrooms revealed significant quantities of polyunsaturated fatty acid. The antioxidants significant action of pomegranate emerges from subcategory of tannin, ellagitannins (punicalagin and punicalin). In pomegranate, flavonoids were mostly anthocyanins that contribute the fruit juice color. Juice and peel contains (strong antioxidant potential) catechins [17]. Pomegranate flavonoids have antioxidant action combined via inflammatory marker suppression, such TNF-α. Pomegranate juice could inhibit oxidative stress macrophage, free radicals, fat peroxidation, as well as cell proliferation and trigger apoptosis. Garlic can indeed act as an antioxidant, enhance NO and sulfide (H2S) synthesis [18], and diminish the hypertension of angiotensin conversion enzymes. The consumption of garlic was shown to efficiently on lipoprotein cholesterol, hypertensive, total cholesterol.

Plants utilized as herbal remedies also aid to identify a new therapeutic use. Medicinal plant items are not as poisonous as synthetic medicines with minimal adverse effect. The numbers of medications that are discovered in natural assets are abundant in a wide diversity of components.

Table 1 several supplements according to their dormant action

Potential benefit	Supplement/ functional food	References

Anti-inflammatory action	EVOO (polyphenolics, oleic acid)	[19]
	Vitamin D	[20]
	Vitamin C	[21]
	Cruciferous vegetables	[4]
	Quinones	[4]
Anti-oxidant property	Vitamin C	[22]
	Fruits and vegetables	[23]
	Coenzyme Q10	[24], [25]
		[19]
	EVOO	[11], [26]
	Anthocyanins, flavanols, myricetin and quercetin	
Lowering blood pressure	Dark chocolate	[27], [28]
		[29]
	Green/ black tea	[30], [31]
	Omega 3 fatty acid	[23], [30]
	Fiber (legumes)	[4]
		[32]
	Ascorbic acid (citrus fruits)	[33]
	Ginseng	[26]
	Quercetin (garlic)	
	Grapes, wine	
Lowering blood cholesterol	Omega 3 fatty acid	[34]
	Pectin (fiber- fruits, vegetables)	[35]
	Vitamin C	[36], [37]
		[29], [38]
	Green tea	

		[30]
	Flavonoid (dark chocolate)	[11]
	Carotenoids (green leafy vegetables)	
Platelet aggregation	Folic acid, vitamin B6, and vitamin B12	[39], [40]
	Tomato, golden kiwi	[41]

The plants use in proposed project are *Withania somnifera* so called Ashwagandha, winter cherry and *Cymbopogon citratus* so called lemongrass.

Withania somnifera Dunal, ashwagandha medicinal plant offer vitality, longevity, rejuvenation impacts on health [42]. It is intended to soothe the mind, decrease the tiredness, boost the endurance and enhance your sleep. It enhances immunological activity, promotes critical fluid production as well as protects the lymph, blood, the semen and cells [43]. It also offers anti-oxidant, anti-inflammatory, anti-stress, antineoplastic properties [44]. The extracts from different parts of this plant have their own dormant action.

- Leaves: anthelmintic
- Leaf juice: anti-oxidant, enhances immunity, anti-inflammatory, anti-tumor, anti-stress
- Fruits: Blood purifier, growth promoter (infant)
- Root: used in tonic for cough, hiccup, memory loss.

Table 2*: W. somnifera* bioactive compounds

Bioactive compound	Present in part of plant
Withanolide	Roots, leaves
Withanferin A	Root

Withanolide A	Roots, leaves
Withanolide D	Root
Anaferine	Roots, seeds

Table 3: Properties of bioactive compounds in *W. somnifera*

Properties	Roles
Anti-inflammatory	Withania, withanolides as influential hinders the action of pro-inflammatory transcription factors NF-kB; AP-1 as therapeutic agent of inflammatory cascade diseases
Hypoglycemic	Adjust blood and urine glucose levels
Cardiotonic	Have similar effect like digoxin, hypercholesterolemia, Inflammation
Adaptogenic	Anti-stress as it leads to activation of sympathoadrenal and hypothalamic activity
Positive inotropic activity	lowering blood pressure via blocking functioning of autonomic ganglion, myocardial depressant effects
Anti-atherogenic and hypolipidemic	Scavenge free radicals, inhibit lipid peroxidation, ameliorate lipoprotein lipase enzyme release

The beneficial roles of *withania somnifera* are expanding as more study is done on it. The bioactive compound of withania have physiological impact and also show toxicity which is also under research [45]. For the best pharmacological use of its bioactive compound, we must study its negative impact as well.

Cymbopogon citratus also known as lemongrass belongs to a Poaceae family [46]. It have anti-inflammatory, anti-bacterial as well as anti-microbial [46], anti-obesity, antinociceptive, anxiolytic, anti-oxidant and antihypertensive properties[44]. Lemongrass has many major bioactive chemicals beneficial in a multitude of diseases. Myrcene, limonene, citrus, geraniol, citronellol, geranyl acetate, neral and nerol all compounds seem to be present in lemongrass [44]. While aromatic chemicals include myrcene as well as limonene, the citric and geraniol are also antibacterial and an insecticidal. Inflammation occurs scientifically if macrophages are incubated by lipopolysaccharides (LPS), which leads to the release of pro-inflammatory mediators, like nitric oxide and prostaglandin E2 (PGE2) [4]. Other induction agents involve reactive oxygen species (ROS), tumor necrosis factor - TNF-α, cytokines, interleukins as well as NF- B-cell protein up-regulation. The polyphenolic portions of the compounds are also used to diminish NO release and iNOS expression in LPS. Ethanolic lemongrass compound seems effective over LPS-induction inflammation of alveolar macrophages. The therapeutic effect is through an inhibition of NO production and indeed the TNF-α proinflammatory cytokine necrosis factor. The abundance of terpenes has demonstrated that lemongrass tea exhibits analgesic action mainly myrcene [14]. Biochemical factors such as cell membrane, cell lipids, protein and DNA are harmed by ROS, which are principal causes among many health problems such as atherosclerosis, rheumatoid arthritis and muscle loss. Antioxidants protect the body from the toxic consequences of the radicals' oxidation process. The far more potent antioxidant components in relation to phenolic acid component include tannin as well as flavonoid portions of oil free infusion extract. Anti-hypertensive substances, including the flavonoids and alkaloids, are said to complement the consequences of hypoglycemia in lemon grass .
Amid numerous essential oils, lemon grass oil seems to have a good possibility by preventing the proliferation of the fungal cells responsible in secretions of mycotoxins under agricultural purposes.

Elicitation a method ameliorates generation of secondary metabolites in plants to fortify their survival. The elicitors are used in plant tissue culture to enhance and modify the secondary metabolites in desired plants. There are two types Biotic, those are obtain from pathogen or

from plant and Abiotic, are mainly the chemical and physical factors [8]. The beneficial roles of elicitors are the consideration of -regular secondary metabolism, -plant defense action potential and enhance the target component. They are environmentally safe as the process aim is to initiate the naïve immune action of genes, A comprehensive as well as extended protection impact, numerous defense system cooperation, induced resilience, rendering it almost hard to adjust infections to protected plants; induction of non-specific fungal resistance, bacteria, viruses, nematodes, etc. Many plants have already been cultivated from old period as something of a bio-cultural asset and been continuously cited as the major manufacturers of natural assets from diverse plants, such as barks, stems, leaves and roots. Plants may sometimes create primary or secondary metabolites. It is relatively usual to project to determine for cultivating plant cells to yield secondary metabolites. Secondary metabolites as alkaloids, phenolics and essential oils serve a crucial in the metabolization of plants, namely pathogen, insect defensive performance, pigmentation, germination regulatory processes, advancement, as well as in the yield of efficient pharmaceutical drugs, dietary supplements, medicinal products. Various production methods – for the generation of secondary metabolites in plants, as with the manufacture of callus or suspension cultivation are utilized routinely. In plant tissue culture laboratory, far more important products or apparatus required are general glassware, chemical substances used mostly for medium preparation as magnetic stirrer, mineral nutrients, distilled water, weighing balance, pH meter, autoclave, freezer, microwave oven, temperature controller, [7]. A sterile air workstation known as laminar flow; to handle aseptic explants, media. Cultures can be incubated on light-fitted shelves. For culturing, MS media (Murashige and Skoog 1962) was used. Nutrition in media include carbon, inorganic nutrients, energy sources, vitamins, growth regulators {auxin: IAA; cytokinin: Kinetin}, organic supplements which are organic nitrogen, acid and complex substance. The elements those concentration is >0.5mmol/L are called macro/major-nutrient [N, K, P, Ca, S, Mg], while those is <0.5mmol/L are minor/micro-nutrient [Mo, Fe, Co, Mn, B, Cu, Zn, I]. The inorganic nutrients are:

i. Calcium: Component of cell wall
ii. Nitrogen: Essential for amino acids, vitamins, proteins, nucleic acid
iii. Magnesium: Factor of chlorophyll
iv. Iron, Molybdenum, Zinc: For enzymes

The kind of culture that should be developed depends on crop purposes, including the micropropagation, the secondary synthesis of metabolites, in vitro flora etc. Frequent subculture in all instances should be performed to restore nutrients but also remove harmful

parts of explant by trimming it. Subculture intervals can usually be 10–14 days for cell suspension cultures. Semi-solid nutrient culture should be moved once every four to six weeks to new medium. Shoot tip and meristem culture described as the shoot tips i.e., apical meristem shoot may be cultivated in vitro, generating clusters of axillary or adventitious buds. This may be utilized to proliferate clonally. In order to grow them on an artificial nutritional medium in glass vials under regulated aseptic circumstances, the Callus culture might acknowledged as generation or maintenance of an unstructured mass of proliferative cells of explant cells, tissue or organ [47]. Explant culture described as plant species variation in their shape, such as trees, herbs and grasses that display the fundamental morphological units, namely the root, stem and leaves. The other most flexible among all cells is Parenchyma. They can multiply and proliferate. Plants invitro culture techniques benefit the major fields of science but it mainly aims on micropropagation [47], somatic cell genetics and generation of desired transgenic plants that can be therapeutic. The production of plants in sterile condition benefits the plants with minimal possibilities of transmitting diseases, pathogens, pests. The new generated plantlet from the explant tends to have better production of flowers, fruits, desirable traits.

CHAPTER 3

Methodology

Withania somnifera was obtained from Delhi Technological University Nursery, while *Cymbopogon citratus* seeds were purchased from Allthatgrows.

3.1 For *Withania somnifera*:

PLANT MATERIAL COLLECTION

The explants were shoot, nodal, bud, root of the plant which were kept in the 1% saline solution after the excision.

3.1.1 Media preparation

INORGANIC CONSTITUENT

A.	MACRONUTRIENT	g/l
	Ammonium nitrate	1.65
	Potassium nitrate	1.9
	Calcium chloride dihydrate	0.44
	Magnesium sulphate heptahydrate	0.37
	Potassium dihydrogen orthophosphate	0.17

B.	MICRONUTRIENT	mg/l
	Boric acid	6.20
	Manganese sulphate	22.30
	Zinc sulphate	8.6
	Potassium iodide	0.83
	Molybdic acid Na salt	0.25
	Cupric salt	0.025

	Cobalt chloride	0.025
	Sodium EDTA	37.3
	Ferrous sulphate	27.8
C.	VITAMIN'S	mg/l
	Inositol	100
	Glycine	2
	Thiamine HCl	0.1
	Pyridoxine HCl	0.5
	Nicotinic acid	0.5
D.	GROWTH REGULATORS	µl
	IAA	175
	Kinetin	220

After addition of above-mentioned nutrients, 30g of sucrose was added and stirred. The pH was calibrated to 5.5-5.7 by using dilute HCl or NaOH. Followed by the addition of 8g of agar. We use autoclave to sterilize the media. After cooling it down to mild warm temperature, we add growth hormones/regulators indole 3-acetic acid (auxin) and kinetin (cytokinin) After stirring the media carefully, we pour the media in the test tubes. Then, we kept it aside so that media will solidify.

3.1.2 Preparation of stock solution

To prevent the time consuming, tedious process of weighing individual salts each time, concentrated solution of desired chemicals is prepare known as stock solution. Stored in 4℃ after being prepared in distilled water.

We prepared the stock solution 1mg/l of micronutrients and vitamins except for inositol. After, weighing 40 mg of each micronutrients in falcon tube, 40ml of distilled water is added and stirred so chemical dissolve properly. For inositol stock, we dissolved 1g inositol in 10ml of dissolved water. To add growth regulators, we make stock solution of 0.2mg/20ml. IAA was weighed 0.2mg and dissolved in 20ml of absolute ethanol in falcon tube. After constant stirring, we use syringe filter to prevent any precipitate in the solution. Kinetin was weighed 0.2mg and

dissolved in 20ml of absolute ethanol in falcon tube. After constant stirring, we use syringe filter to prevent any precipitate in the solution.

3.1.3 Sterilization of explants

Explants were submerged in 70% alcohol for 1-2 mins. Followed by the treatment with 20% sodium hypochlorite for 5-10mins. Then in sterile laminar flow hood chemicals were discarded and explants were washed with autoclaved distilled water.

3.1.4 Procedure for culture

i. Proper trimming of explant.
ii. Surface sterilization of explant.
iii. Explants were washed in sterilized distilled water.
iv. Preparation of aseptic culture media for explants and sterilized in autoclave.
v. Inoculation of explant in the culture media under aseptic condition.
vi. Incubation under suitable condition for proper culture.
vii. Subculturing till contamination free culture was obtain.
viii. From contamination free culture, further subculturing proceeded with different carbon and nitrogen sources which are sucrose, glucose, fructose and potassium nitrate, sodium nitrate, ammonium nitrate, respectively.
ix. Plantlet transfer.
x. Detection of bioactive compounds in the plantlet.

3.1.5 For detection of withanolides:

Chemicals and reagents: n-Hexane, chloroform, Methanol, Acetic acid, Distilled water

Preparation of plant extract and detection of withanolides-

i. The leaves and roots from the cultured plantlet were finely powder separated in liquid nitrogen in mortar and pestle and kept overnight in 20 ml methanol water (25:75, v/v) at room temperature and filtered.
ii. Later, the filtrate was assembled and the residue extracted.

iii. Followed by pooling of filtrate and withdraw by n-Hexane (3x60ml).
iv. Discard of n-hexane fraction. Methanol water proportion was further withdrawn by chloroform (3x60ml).
v. Chloroform proportion were pooled and concentrated to dry powder.
vi. 10mg obtained dry powder and mixed in HPLC grade methanol (1ml).
vii. Proceeded with HPLC analysis.

3.2 For *Cymbopogon citratus*, seeds.

3.2.1 Sterilization of seeds

First, seeds were washed in running tap water in tea strainer or nylon net pouch. In petri dish, seeds were submerged in 70% alcohol for 2 mins. Afterwards, seeds were decanted in a beaker containing 30% bleach (sodium hypochlorite) along with 2-3 drops of Tween-20 for 10-20 mins with constant stirring. In sterile laminar flow hood, chemicals were discarded in an empty beaker and seeds were washed 5 times with autoclaved distilled water.

3.2.2 Media preparation

INORGANIC CONSTITUENT

A. MACRONUTRIENT	g/l
Ammonium nitrate	1.65
Potassium nitrate	1.9
Calcium chloride dihydrate	0.44
Magnesium sulphate heptahydrate	0.37
Potassium dihydrogen orthophosphate	0.17

B. MICRONUTRIENT	mg/l
Boric acid	6.20
Manganese sulphate	22.30
Zinc sulphate	8.6
Potassium iodide	0.83

	Molybdic acid Na salt	0.25
	Cupric salt	0.025
	Cobalt chloride	0.025
	Sodium EDTA	37.3
	Ferrous sulphate	27.8
C.	VITAMINS	mg/l
	Inositol	100
	Glycine	2
	Thiamine HCl	0.1
	Pyridoxine HCl	0.5
	Nicotinic acid	0.5
E.	GROWTH REGULATORS	µl
	IAA	175
	Kinetin	220

After addition of above-mentioned nutrients, 30g of sucrose was added and stirred. The pH was calibrated to 5.5-5.7 by using dilute HCl or NaOH. Followed by the addition of 8g of agar. We use autoclave to sterilize the media. After cooling it down to mild warm temperature, we add growth hormones/regulators indole 3-acetic acid (auxin) and kinetin (cytokinin) After stirring the media carefully, we pour the media in the test tubes. Then, we kept it aside so that media will solidify.

3.2.3 Preparation of stock solution

To prevent the time consuming, tedious process of weighing individual salts each time, concentrated solution of desired chemicals is prepare known as stock solution. Stored in 4℃ after being prepared in distilled water.

We prepared the stock solution 1mg/l of micronutrients and vitamins except for inositol. After, weighing 40 mg of each micronutrients in falcon tube, 40ml of distilled water is added and stirred so chemical dissolve properly. For inositol stock, we dissolved 1g inositol in 10ml of dissolved water. To add growth regulators, we make stock solution of 0.2mg/20ml. IAA was

weighed 0.2mg and dissolved in 20ml of absolute ethanol in falcon tube. After constant stirring, we use syringe filter to prevent any precipitate in the solution. Kinetin was weighed 0.2mg and dissolved in 20ml of absolute ethanol in falcon tube. After constant stirring, we use syringe filter to prevent any precipitate in the solution.

3.2.4 Procedure for culture

i. Surface sterilization of seeds.
ii. Seeds were washed in sterilized distilled water.
iii. Preparation of aseptic culture media and sterilized in autoclave.
iv. Inoculation of seeds in the culture media under aseptic condition.
v. Incubation under suitable condition for proper culture.
vi. Subculturing till contamination free culture was obtain.
vii. From contamination free culture, further subculturing proceeded with different carbon and nitrogen sources which are sucrose, glucose, fructose and potassium nitrate, sodium nitrate, ammonium nitrate, respectively.
viii. Cultured plantlet transfer.
ix. Detection of bioactive compounds in the plantlet.

3.2.5 For detection of total phenolic content:

i. First, plant extract was prepared.
ii. The leaves from cultured plantlet were taken and dried at 45℃ for 48 hours.
iii. 0.5ml of extract with addition of 0.5ml Folin-Ciocleu reagent.
iv. Incubated for 5 mins at 22℃
v. Followed by addition of 2ml of 20% sodium carbonate.
vi. Absorbance taken at 650nm.
vii. Phenolic content was measured while GALLIC ACID used as standard.

3.3 RESULT & DISCUSSION

Carbon poses the framework of many plants, notably starches and cellulose, bio-molecules. The carbon synthesis is fixed by photosynthesis and produces a fraction of the carbohydrates which thus store energy in the plant. It also shows potential growth rate in plantlet. Generally, 2-4% sucrose is best for any micropropagation [33]. All proteins are made from nitrogen. The scarcity of nitrogen usually quite often leads to slow development. The impact of different carbon and nitrogen source on bioactive compounds of *Withania somnifera* and *Cymbopogon citratus* can be observed in our proposed project work.

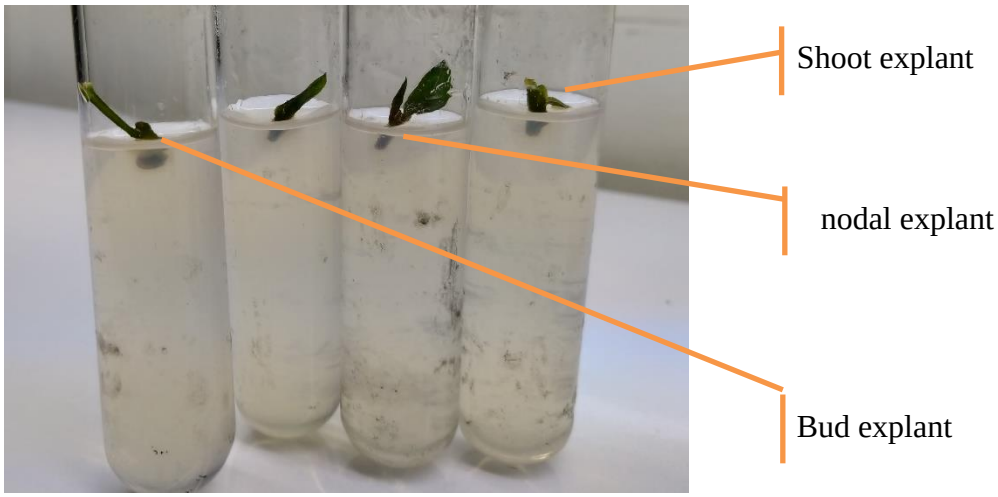

Figure 1 Withania somnifera culture

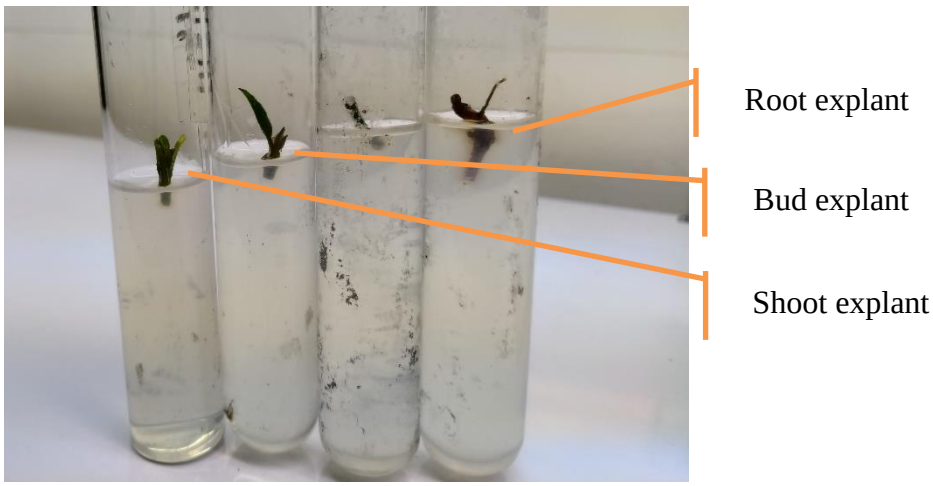

Figure 2 Withania somnifera culture

Withania somnifera belongs to Crassulaceae family. It is highly rich in withanolides offers beneficial properties like anti-stress, anti-inflammatory, anti-oxidant [48]. Even though, the plant is enriched in bioactive compounds still shoot culture are highly enrich in withanolides. In HPLC analysis, we can observe different withanolides present leaves and roots [49]. Withanolides are mostly synthesized by aerial portions observed within those hairy root culture. We study production of withanolide A in *Withania somnifera* culture shoot, the aerial sections of the natural plant's tissue culture counterparts [50]. *Cymbopogon citratus* is also medicinal plant belongs to Poaceae family. In addition to the impact of other parameters like type of cultivation, time of harvest, controlled oxidation and fermentation, withering circumstances, many studies in lemongrass extracts are made by infusion, distillation, or organic solvent extraction. The diverse medicinal properties of this plant can be attributable to the bioactive compounds found [46]. Our study also focuses at optimizing the bioactive compounds in *Withania somnifera* and *Cymbopogon citratus*. After approximately 30 days the cultured plantlets were seen proliferating Out of the different.

3.4 CONCLUSION

Culture of plant tissue assures to remain as a significant tool in this study regarding the development of plant, physiology, cellular activities as well as technological enhancement of crops. From ancient period plants were embraced as medicinal reasons [47]. The growing implementation of sustainable and quality naturally occurring phytochemicals paves the way for massive cloning through plant tissue crop techniques. In vitro methods have developed a wide range of medicinal plants and associated compounds in a quite limited timeframe compared with standard procedures. Bioactive natural compounds regarding their beneficial effects on health are being described and studied [1]. These offer additional antioxidant, anti-inflammatory and anti-carcinogenic characteristics that could safeguard against numerous chronic conditions, such as diabetes, CVDs, cancer, etc. Their use in diets with relevant benefits for their health makes compounds a viable candidate for the establishment of novel, functional foods with possible protection and conservative characteristics. These are typically abundant in fruit and vegetables. Bioactive compounds utilization within medicinal goods, scents, pigments, and so on by the technology of plant cultivation, is considered as commercially important.

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