CAPSIDIOL PRODUCTION FROM *NICOTIANA TABACUM* CV. XANTHI LIQUID CELL SUSPENSION CULTURES

by
Ümit Cem Derman

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APPROVED BY:

Assist. Prof. Dr. Bahar Soğutmaz Özdemir ……
(Thesis Supervisor)

Assist. Prof. Dr. Dilek Tekdal ……

Assist. Prof. Dr. Emrah Nikerel ……

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ABSTRACT

CAPSIDIOL PRODUCTION FROM *NICOTIANA TABACUM* CV. XANTHI LIQUID CELL SUSPENSION CULTURES

Plants are capable of producing a tremendous amount of secondary metabolites that can be used in chemical, food, pharmaceutical, and cosmetic industries. The purpose of these secondary metabolites which accumulate in plants is generally for defense and communication. Phytoalexins are antimicrobial compounds which are not produced in primary metabolism of a plant; however, as a result of stress or infection, phytoalexins accumulate. Capsidiol, a bicyclic sesquiterpene, is the main phytoalexin known to be accumulated in *Capsicum annuum* (pepper) and *Nicotiana tabacum* (tobacco) as a response to biotic fungal stress such as *Phytophthora infestans*. In general, these secondary metabolites are harvested from whole plants, yet the whole plant has a risk of contamination from various sources such as fertilizers, agrochemicals, pests, and diseases. The more relevant way to produce secondary metabolites is using plant cell suspension cultures. In this study, callus formation was achieved from different explant sources of the tobacco plant. Leaf explants demonstrated the highest callus generation rate with 95 per cent in 21 days. Growth parameters were optimized by measuring increasing fresh weight over time. Characterized calli were later regenerated into the whole plant via organogenesis and ad acclimatized in greenhouse conditions; The period of time needed to reach the flowering stage from callus culture was approximately four months, consistent with the previous studies. Callus-derived cell suspension culture was established and characterized by microscopic analysis of the cells and growth parameters via fresh weight measurements. In order to induce capsidiol production, five different elicitors were utilized. After organic solvent extraction, samples were analyzed for the availability of capsidiol with high performance liquid chromatography (HPLC) and mass spectrometry (MaS). Yeast extract (1 g/L) and salicylic acid (0.5 mM) are found to be the best elicitor candidates for capsidiol accumulation in tobacco cell suspension culture. This study will be a base for large scale production of capsidiol from tobacco cell suspension cultures.

ÖZET

NICOTIANA TABACUM CV. XANTHI SÜSPANSİYON KÜLTÜRLERİNDEN KAPSİDİOL ÜRETİMİ

Bitkiler çok fazla sayıda sekonder metabolit üretme kapasitesine sahiptirler ve bu metabolitler kozmetik, ilaç, gıda ve kimya endüstrilerinde kullanılmaktadırlar. Bitkiler tarafından üretilen bu sekonder metabolitler, genellikle bitki savunma ve iletişimi için kullanılır. Antimikrobiyal özelliğe sahip fitoaleksinler, bitkilerin birincil metabolizmasında yer almazlar, ancak bitki strese girdiği zaman ya da herhangi bir saldırıya maruz kaldığında üretilirler. Bisiklik sekuterpen olan kapsidiol, *Capsicum annuum* (biber) ve *Nicotiana tabacum* (tütün) bitkilerinde biyotik stres yaratan *Phytopthora infestans* gibi fungal patojenlere karşı üretilen ana sekonder metabolittir. Genellikle bu sekonder metabolitler gelişmiş tüm bitkiden elde edilirler. Ancak bütün bir bitkinin kullanımı çeşitli kaynaklardan (gübreler, tarım ilaçları, böcekler, hastalıklar vb.) gelen kontaminasyon riski taşır. Sekonder metabolitleri üretmenin daha uygun bir yolu da bitki hücre süspansiyon kültürleridir. Bu çalışmada, farklı eksplant kaynağı kullanılarak *Nicotiana tabacum* cv. Xanthi kallus kültürü oluşturulmuştur. Kallus oluşumu en fazla yaprak eksplantından yüzde 95 oranında 21 günde elde edilmiştir. Büyüme parametreleri artan ağırlığın zamanla ölçülmesiyle optimize edilmiştir. Karakterize edilen kallus daha sonra organogenez ile bütün bir bitki haline getirilip, sera koşullarında adaptasyonu sağlanmıştır. Çiçeklenme sürecine kadar geçen süre, yaklaşık dört ay, önceki çalışmalarla benzerlik göstermektedir. Kallus kültüründen hücre süspansiyon kültürü elde edilmiştir ve hücrelerin mikroskobik analizi ve yaş ağırlık ölçümüne dayalı büyüme parametreleri ile karakterize edilmiştir. Kapsidiol üretimini indüklemek için beş farklı elisitör kullanılmıştır. Örneklerin organik çözücü ile ekstraksiyonundan sonra yüksek performanslı sıvı kromatografisi (HPLC) ve kütle spektrometresi (MaS) ile analiz yapılmıştır. Tütün hücre süspansiyonunda kapsidiol üretimi için maya ekstraktı (1 g/L) ve salisilik asit (0,5 mM) en iyi elisitör adayları olarak bulunmuştur. Bu çalışma, tütün hücre süspansiyon kültüründen büyük ölçekli kapsidiol üretimi için bir temel oluşturacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABA	Abscisic acid
BAP	6-Benzylaminopurine
CH	Casein hydrolysate
ddH_2O	Double Distilled water
DMPF	Dimethylallyl pyrophosphate
EtOH	Ethanol
FPP	Farnesyl pyrophosphate
FDA	Florescein diacetate
GC	Gas chromatography
GB_5	Gamborg B5
HPLC	High Performance/Pressure Liquid Chromatography
IAA	3-Indoleacetic acid
IPP	Isopentenyl diphosphate
IBA	Indole-3-butyric acid
KN	Kinetin
LLE	Liquid-liquid extraction
LS	Linsmaier & Skoog
MS	Murashige & Skoog
MaS	Mass Spectrometry
MVA	Mevalonic acid

NAA	1-Naphthaleneacetic acid
NMR	Nuclear magnetic resonance
NtCIM	*Nicotiana tabacum* callus induction medium
NtE	*Nicotiana tabacum* elicitation
NtGM	*Nicotiana tabacum* germination medium
NtR	*Nicotiana tabacum* rooting
NtTR	*Nicotiana tabacum* tissue regeneration
OSE	Organic solvent extraction
PGRs	Plant growth regulators
SA	Salicylic acid
SPE	Solid phase extraction
UPLC	Ultra Performance/Pressure liquid chromatography
YE	Yeast extract

1. INTRODUCTION

In vitro plant tissue culture techniques had been a significant tool for both commercial and scientific studies [1]. In 1902, Harberlandt's experiments on single plant cells were considered as the theoretical basis for plant tissue culture technology. Even though most of his work was unsuccessful, the importance was the idea that he predicted: "totipotency of plant cells" [2]. Furthermore, Gautheret was the first who established plant tissue culture in 1934 [3]. After that, the discovery of several plant growth regulators with the application of numerous vitamins in the culture and establishment of carrot root tissues *in vitro* was done in 1939 [4]. Almost 40 years after, the first callus formation with the artificial nutrient medium in *in vitro* conditions was established by White in 1939 by using a hybrid *Nicotiana* species. Middle of the 20th century was an important era for plant tissue culture since the discovery of the coconut water provided many plants to be cultured in *in vitro* conditions [5]. Throughout those years, callus cultures from many plant species were developed [6]. In 1955, the discovery of Kinetin, a plant hormone, lead to advancements in plant tissue culture studies [7]. Murashige and Skoog proposed a new plant tissue culture salt composition by examining the tobacco callus ashes and this medium became the most popular readily available plant growth medium. It is still used for a wide range of plants in *in vitro* cultures [8].

Tissue culture technology was then started to be used for solving the problems in biology, horticulture, forestry, and agriculture. Main study areas were plant improvement, clonal propagation, pathogen-free plants, and cell behavior [1]. Even though plant tissue culture had been established in 1940's, the first improvement attempts performed in the 1970s [9]. With plant genetics, modifications in the genomic level were achieved in the early 1980s and then plant tissue culture has become an important tool in plant biotechnology [10].

Similarly, in the 1970s, plant cell suspension cultures emerged and thought to be the way of examining/producing secondary or primary metabolism/metabolites [11]. Chandler *et al*. (1972), grew photosynthetic *Nicotiana tabacum* callus cell suspension culture using 2,4-D and KN (Kinetin) as plant growth regulators [12]. Fossard *et al*. established callus culture by using a different cytokinin, BAP (6-Benzilaminopurine), rather than KN in 1974 [13]. Ali *et al*. (2007), found the best callus inducing plant growth regulators as NAA (1-

Naphthaleneacetic acid) and BAP for *N. tabacum* and exhibited that different hormones could be used to generate callus [14]. Callus has the potential to differentiate from every other plant tissue [15]. Owing to its unique property, callus culture has become an important plant science tool for different applications such as genetic manipulation, clonal propagation and secondary metabolite production [16].

Plants are capable of producing thousands of secondary metabolites which are used as pharmaceuticals, flavors, dyes, agrochemicals and more [17]. Producing those secondary metabolites from callus cell suspension is possible since plant cells in culture have complete genetic information to produce these chemicals [18]. Moreover, many studies showed that cell suspensions could produce more secondary metabolites in amounts than the plant itself [19]. Elicitation is a useful approach to produce desired molecules in *in vitro* cultures [20]. Elicitors are signal molecules activating the expression of appropriate genes for the biosynthesis of secondary metabolites [21]. With the appropriate elicitor, many plants are capable of producing the right phytoalexins. Phytoalexins can prevent an infection by stabilizing the spread of invading organism [22].

First Müller in 1958 [23] and later Van Den Ende in 1969 [24] showed evidence related to phytoalexin accumulation in *Capsicum frutescens*. Stoessl *et al*. gave the name of the phytoalexin as capsidiol since it was produced from capsicum and it was a sesquiterpenoidal diol. In 1972, they found out that *B. cinera*, *F. oxysporum*, *F. vasinfectum*, *C. herbarum*, *T. viride*, *P. frequentas*, and *M. fructicola* caused capsidiol accumulation, and also capsidiol had an antifungal effect on these fungi [25]. In 1985, Brooks *et al*. produced capsidiol by using cellulase and pectinase in pepper cell suspension culture [26]. Induction of pepper fruits with arachidonic acid also caused capsidiol production, and salicylic acid was given as possible defense inducer molecule [27]. Whitehead *et al*. demonstrated that 5-epi-Aristolochene was a precursor of capsidiol and showed capsidiol production via farnesyl diphosphate pathway [28]. In 1987, fungal elicitors were found to induce capsidiol accumulation in tobacco cell suspension cultures [29]. More specifically, cryptogein, a protein from *Phytophthora cryptogea*, was reported as a good elicitor to produce capsidiol from tobacco cells in 1991 [30]. In 1996, Egea *et al*. showed the antifungal effect of capsidiol on *Phytophthora capsici* [31]. Ralston *et al*. (2001) established a yeast expression system to characterize the 5-epi-Aristolochene-1,3-Dihydroxylase, which is an enzyme used in capsidiol biosynthesis and the source of the

gene was a tobacco plant [32]. Literakova *et al*. (2010) used Chandler's media [12] to produce cell suspension culture and demonstrated a capsidiol determination protocol for tobacco cells elicited via cryptogein using high pressure liquid chromatography (HPLC) - mass spectrometer (MS) [33]. Giannakopoulou *et al*., (2014) used the yeast expression system to observe the effect of capsidiol over *P. infestans* and *P. capsici*, the results showed that *P. infestans* was more sensitive to capsidiol than *P. capsici* [34].

1.1. PLANT TISSUE CULTURE

Plant tissue culture is a collection of methods that focus on manipulating the plant cells, organs, or whole plants under *in vitro* conditions with the combination of aseptic techniques [4]. Usually, plant tissue culture starts with surface sterilization, growing the tissues in artificial media under controlled environment and different plant tissue culture techniques are utilized depending on the application. Plant tissue culture conditions are controlled by manipulating different parameters such as nutrient supply, temperature, pH of the environment or humidity [35]. It is enough to have a small amount of tissue to achieve thousands of plants within a continuous process in a shorter period of time than its natural way [5].

Plant tissue culture is mainly used for mass propagation (large scale production) of pathogen free plants. However; plant tissue culture has many other application areas such as plant improvement, disease elimination, production of secondary metabolites or transgenic/cisgenic plants and conservation of endangered species [5,6] (Figure 1.1).

Medium selection is one of the most important factors in plant tissue culture since it is crucial for growth. Macronutrients, magnesium (Mg), calcium (Ca), sulfur (S), potassium (K), nitrogen (N), and phosphorus (P), are inorganic elements required in relatively large amounts. Micronutrients, nickel (Ni), zinc (Zn), iron (Fe), boron (B), molybdenum (Mo), copper (Cu), chlorine (Cl), are the elements required in relatively small amounts, sometimes in trace amounts. The carbon source is supplied as sugars such as glucose, sucrose, and maltose. A solidifying agent, agar or gel rite like substances is required to achieve solid media. There are also undefined supplement molecules (e.g. coconut milk) used in tissue culture medium [37]. The choice of the nutrient medium is the cause of successful plant tissue culture. Salt composition (macro- and micronutrients), vitamins,

carbon source and growth regulators are the main components of plant tissue culture medium. There are readily available salt compositions (MS [8], LS [38], GB_5 [39] and more) exist, however, the salt composition can be modified [39].

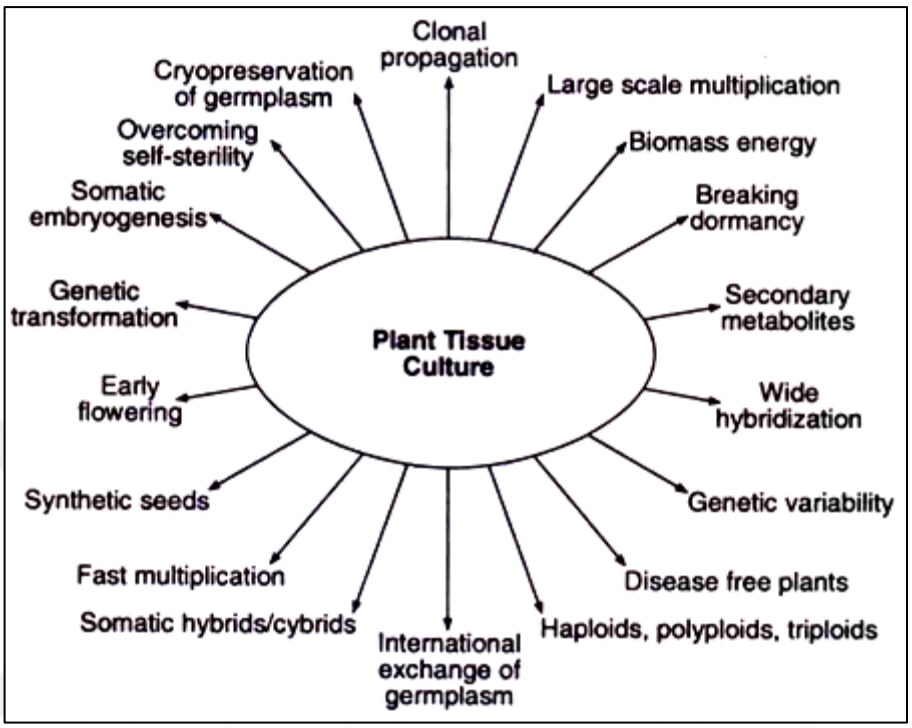

Figure 1.1. Applications of plant tissue culture [40].

Plant Growth Regulators (PGRs), plant hormones, are synthetic or naturally occurring molecules that have various effects on development processes of plants. They do not have any phytotoxic activity on plants below the threshold. The threshold depends on plant species and PGR type and combination, and also they do not have any nutritional value [41]. They function on fruit ripening, fruit drop, flowering, fruit formation or defoliation like processes so that they are essential for plants to respond to their external or internal stimuli and perform their regular development as well [42].

Five main groups of the plant growth regulators are cytokinins, gibberellins, auxins, ethylene and abscisic acid [42] (Figure 1.2). Lately, jasmonates and brassinosteroids have also been accepted as plant growth regulators after their effect on plant development was discovered. Polyamines and other several compounds are also thought to be involved in plant development, yet they are still controversial in the sense of their abundance [42].

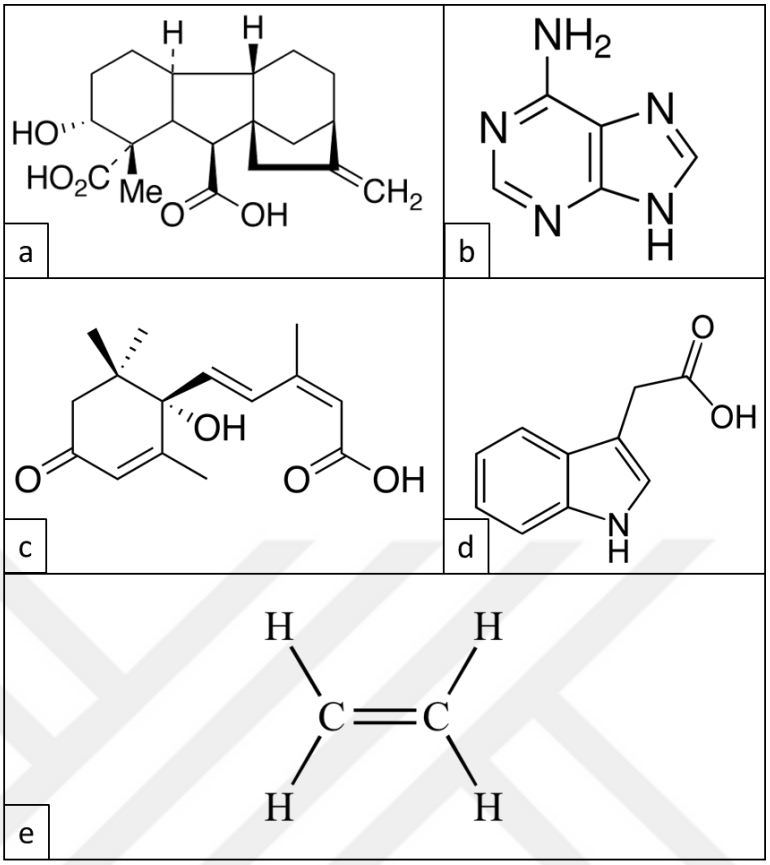

Figure 1.2. Plant hormones, (a) Gibberellin, (b) Auxin, (c) Abscisic Acid, (d) Cytokinin, and (e) Ethylene [43].

Auxins are naturally occurring substances that stimulate cell elongation, cell division in roots and cambium. 3-Indoleacetic acid (IAA), 1-Naphthaleneacetic acid (NAA), and 3-Indole butyric acid (IBA) are examples of auxins. Several auxins are also used as herbicides [44]. Cytokinins have both natural and synthetic forms. They participate in cell division, seed germination, bud formation, and decrease cell senescence rate. Zeatin is a natural cytokinin, however, its derivatives, Kinetin (KN) and 6-benzylaminopure (BAP) are synthetic cytokinins [44]. Gibberellins participate in seed germination, stem elongation, cone formation in several conifers, and flower promotion. In some plant species, gibberellins are essential for callus growth [45]. Abscisic acid (ABA) usually participates in inhibition processes. It plays role in seed and bud dormancy maintenance, slows down cell elongation, and inhibits auxin promoted cell wall loosening. One of the most important roles is closing of stomatal apertures to control water and ion levels in plants [46].

Ethylene plays role in leaf abscission, fruit senescence, and fruit ripening with a combination of various plant hormones [47].

1.2. PLANT METABOLITES

Metabolites are specialized products and/or substrates, which play an important role in crucial cellular actions. These molecules participate in all parts of metabolism. Even though metabolites are usually produced inside the cell, they can be taken up from the extracellular matrix where other organisms secrete their molecules [48]. Information in the living systems follows the flow in Central Dogma Theory. The metabolism, which is the last step of the flow, is controlled by the DNA itself and metabolites are the responsible elements in the metabolic actions, metabolic reaction networks (Figure 1.3) [49].

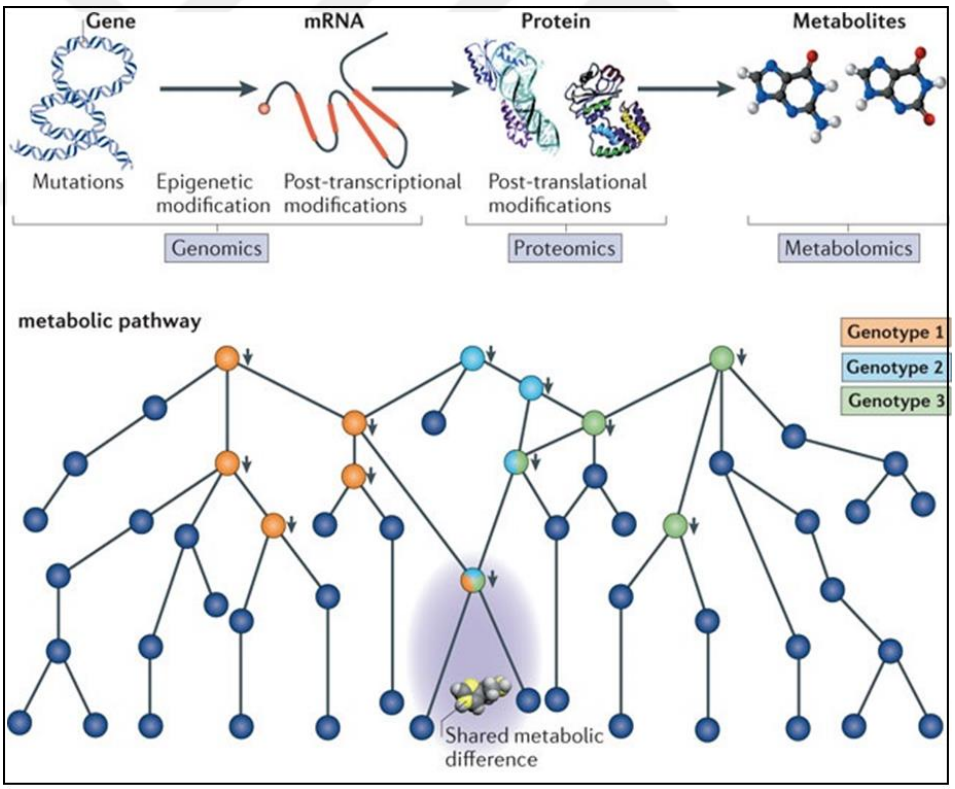

Figure 1.3. The flow of information and pathway differences among different genotypes with different results [50].

Due to their key actions in the living systems, metabolites are essential for the continuity of the living organisms. Acetyl-CoA, ATP, NAD^+ are the most abundant metabolites which

involve in most of the cellular events [51]. Metabolites also have the ability to modify the environmental parameters such as pH and hydrophobicity according to their chemical structure [52]. Plants are capable of producing a variety of biochemical compounds [53]. Furthermore, secondary metabolites in plants differ from species to species [54]. Yet, there is not much information about most of those molecules. Their functions and even the metabolites themselves are still unknown, owing to the fact that the biosynthesis pathways are also a mystery [55].

1.2.1. Primary Metabolites in Plants

Molecules that are found in the living organisms are classified as primary and secondary basically, and the primary metabolites are the ones that are used in the growth and performing basic cellular actions [56]. Primary metabolism is crucial for a plant to assimilate, degrade and synthesize organic compounds [57]. These metabolites are fundamentally proteins, carbohydrates, lipids, nucleic acids and pigments [58]. Even though secondary metabolites share many intermediate molecules with primary metabolites, they are relatively in low concentrations [59]. Primary metabolites in plants also act as precursors in the secondary metabolite production [57].

1.2.1. Secondary Metabolites in Plants

Apart from primary metabolites, secondary metabolites are usually produced in case of extreme conditions. Plants are sessile organisms; therefore they require some defense actions to protect themselves from the changing environmental conditions. In the evolutionary means, the stronger ones survived as they produce several secondary metabolites that come out by adaptation with mutagenesis [60]. Secondary metabolites are originated from primary metabolites with several modifications. Those modifications are usually glycosylation, methylation, and hydroxylation. Owing to their modifications, they are known to be more complex than primary metabolites [61].

Since secondary metabolites do not participate in normal plant growth and development [62], lacking secondary metabolites does not result in a cell death under normal growth conditions [59]. However, secondary metabolites participate in important plant defense

mechanisms by protecting the plants from biotic or abiotic stresses [63]. Due to changing environment and their sessile nature, plants have adapted to live together with their pathogens. There is no adaptive immune system in plants like in mammals so that they have their response as innate immunity [64,65]. There are two aspects in plant immunity; these are cellular defense pathways to stop the pathogen or slow the growth of the offensive parasite [66]. Plants have evolved in a way that they recognize pathogen's effector molecules by the help of their resistance proteins, and activate their effector-triggered immunity [67]. The resulting physiological changes might be the callose deposition, thickening of the cell wall, or secretion of biochemical molecules like reactive oxygen species [64]. Furthermore, as a defense metabolism, several defenses related to secondary metabolites (e.g. phytoalexins and/or proteins) can be produced systemically or locally [68]. Even though plants are capable of initiating specific defense mechanism based on the type of pathogen, the best defense is activating all possible mechanisms that can help to protect the plant against the infecting pathogen at once [69]. Due to the antiviral, antibiotic and antifungal activities of secondary metabolites, various secondary metabolites are used by plants to protect themselves from pathogens [70]. There are also UV absorbing compounds as secondary metabolites which protect the plant from excessive radiation damage [71].

Secondary metabolites derived from the plants are known as an extraordinary source for flavors, pharmaceuticals, food additives and several other products in industrial applications [21]. For the human consumption, they are recreational drugs and medicines [59]. Secondary metabolites are classified according to their chemical structure or the biosynthetic pathway that they are evolved [72]. There are three major families classified in secondary metabolites so far, and these families are alkaloids, terpenes and steroids, and phenolics [73].

Terpenes are the first group. Actual biosynthesis of terpenes is quite hard since their building block is isopentenyl diphosphate (IPP) [74] which is produced by two different pathways. These pathways are mevalonic acid (MVA) pathway and methylerythtiolphosphate pathway [75]. Some terpenes are known to inhibit weed growth around the mother plant and some of them work as attractive molecules to attract pollinators or to kill the insects. Abscisic acid (ABA) is also produced via terpene pathway [56].Phenolics are the most abundant plant molecules and flavonoids are produced from

phenolics [76]. Plants are known to have evolved from aqueous environment and that evolutionary adaptation was eased by the production of phenolic compounds. Phenolics helped in several ways and the first one was defending the plant. Barks or woods of plants differ from one another from species to species due to their altered phenolic compound-complex. The coumarins, stilbenes, and furanocoumarins are effective against fungal and bacterial pathogens. Flower color or flavors are chemically based on phenolic compounds [74].

Alkaloids are the nitrogen-containing metabolites and they are diverse among plant species. Alkaloids, derived from plants, are pharmacologically active [76]. There are different ways to consume alkaloids by humans. The plant extracts are used as stimulants, narcotics, medicines, and poisons for several thousand years. Several abusive drugs are also alkaloids such as nicotine and caffeine. Morphine is used both in medicine and as an abusive drug [76], where berberine is utilized as an antibacterial agent [77]. The biosynthesis of alkaloids is from aromatic amino acids [78]. Alkaloids are known to have a bitter taste and have potential physiological effects on mammals [74].

1.3. PLANT CELL SUSPENSION CULTURES FOR SECONDARY METABOLITE PRODUCTION

Since plants are rich sources for metabolite production, in particular, secondary metabolites, there is a technological tendency to increase plant products. Two different plant liquid culture techniques have emerged to produce plant-based useful compounds, callus cell suspension cultures, and organ cultures. Among the organ cultures, the most promising one is hairy root culture [77,79]. Hairy root cultures have higher growing ability than plant cell cultures and they do not require hormones in the liquid media. However, in order to establish a hairy root culture, the plant should be transformed with *Agrobacterium rhizogenes* [80].

Callus tissues are masses of uncategorized cells and usually produced when there is a pathogen infection or wounding [15]. The cell mass in callus is disorganized and every cell is capable of differentiating into desired plant tissue, which is termed as totipotency [81,82]. There are different types of callus classified according to their physiological and morphological characteristics. Callus without any conspicuous organ regeneration is called

friable callus. If there is an organ regeneration occurring on the callus, they are named after the forming organ such as embryogenic, rooty or shooty callus [83,84]. Explant source and media composition are important factors for the success of plant tissue culture. In order to establish a friable callus, root tips, shoot tips or non-dormant buds in dicots and in monocots roots and mesocotyl can be used as explant sources [39]. Friable callus cultures are preferred in establishing cell suspension cultures due to their embryogenic potential [85].

The amounts of potassium and nitrogen are the key elements to sustain and achieve healthy development of callus [86,87]. In order to establish a regular callus, ammonium and nitrate concentrations need to be elevated around 60 mM [88]. Transferring a callus from one medium to another, usually resulted in decreased growth rates for a couple of subcultures, later cells adapt and return back to their original growth ratio [89]. Casein hydrolysate is an essential compound for some species and resulted with increasing growth rate, especially while inducing callus formation [39].

In plant tissue culture, cytokinins and auxins that applied exogenously are the key points of inducing a callus. In general, the equivalency between these two plant hormones is referred to create a suitable environment to induce a friable callus. The ratio between auxin and cytokinin is known to promote shooting when cytokinin amounts are higher or promote rooting when auxin amounts are higher [90]. The other way to induce callus is wounding. Callus obtained from wounding mostly accumulate pathogen-related proteins and phytoalexins [91]. They are capable of preventing infections and more resistant to water loss. Vascular cells, pith cells, and cortical cells are most suitable cells for wound-inducing callus establishment. Wound-induced callus has increased the ability to generate new tissues [92]. Suspension cultures obtained from the callus cells have a perfect culture homogeneity compared to organ cultures and this gives rise to ease in scale-up processes [77,79].

To produce secondary metabolites, cell division and differentiation must be controlled in cell suspension cultures. Several secondary metabolites might not be produced in the dedifferentiated state so that media can be manipulated with several plant growth regulators. An elevated auxin level usually results with the increase in cell division, however, in several situations, it decreases the secondary metabolite production [93]. In that case, the cell division rate can be reduced by using low phosphate media or several

DNA synthesis inhibitory agent applications and the agents should be chosen according to their effect on the production rate of desired products. When callus cultures are compared with callus cell suspension cultures, suspension cultures demonstrate a faster growth rate [74].

1.3.1. Elicitation

Molecules have different effects on living cells. Several molecules, also called signal molecules, produced from pathogens can trigger the total defense mechanism on their targets. Triggering the responding mechanism with those molecules to host cell is called elicitation. Most of the elicitors are defense regulated [94,95]. There are two different types of elicitor recognition so far. First, one is universal, triggering several defense mechanisms and is not specific to species. The second one is more specific and defense mechanism only activated by encountering pathogenic strains [96]. The elicitor cryptogein which is a *Phytophthora cryptogea* protein is a specific elicitor triggering the capsidiol production in pepper and tobacco plants [33].

1.4. *NICOTIANA TABACUM* AND ITS SECONDARY METABOLITES

The Solanaceae family has more than three thousand species, including potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) (Figure 1.4), and peppers (*Capsicum annuum*) [97]. The Solanaceae family is considered as the third most economically important plant taxon since it contains agriculturally edible/medicinal plant species. The taxon contains important model plants for fruit development (tomato and pepper) [98], and for plant defense (tomato tobacco) [99].

Nicotiana tabacum have originated from the hybridization of *Nicotiana sylvestris* and *Nicotiana tomentosiformis* which are diploid autogamous species [100]. Tobacco is grown commercially in many countries. *Nicotiana tabacum* is a perennial herbaceous plant which can reach between one to two meters [101]. *N. tabacum* is quite sensitive to ground humidity, type of soil and temperature. Around 20 to 30^{o}C is the optimal temperature for normal growth [102]. Humidity requirements are between 80 to 85 per cent [103].

Figure 1.4. *Nicotiana tabacum* [104].

Tobacco has an easy transformation procedure and a quite short generation time and used as a model plant [105]. Pollen tube development [106], pathogen response [107], cell cycle [108] and several phytochemical biosyntheses [109] studies and far more were being conducted with a tobacco plant.

There are plenty of phytochemicals produced by tobacco. As alkaloids, nicotine is the most common secondary metabolite and covers a big spectrum [110]. Nornicotine, anatabine, and anabasine were discovered in *Nicotiana tabacum* by gas chromatography. Several of those alkaloids can also be found in different plant species, however, their amounts vary from species to species [96]. Several growth regulators are also found to elevate or decrease the amount of alkaloid production. For instance, a synthetic auxin, 2,4-dichlorophenoxyacetic acid, decreased the nicotine production in tobacco [111].

Flavonoids such as chalcone and several anthocyanins also exist in cultivated tobacco plants [112]. Isoprenoids from tobacco is an important compound group. They are used in regular growth and also these phytochemicals give its original aroma of tobacco plant [113]. Sesquiterpenes in tobacco were found to exhibit phytoalexins activity [114].

Phytotuberin, phytuberol, and capsidiol are the sesquiterpenes produced by tobacco cells [115].

1.4.1. Capsidiol and its Biosynthesis

Capsidiol (Figure 1.5), a bicyclic sesquiterpene, is mostly produced by *Nicotiana* and *Capsicum* species as an important phytoalexin. Phytoalexins are antimicrobial phytochemicals. Capsidiol is found in several plant species in the Solanaceae family, in particular, tobacco and pepper. Since it is a phytoalexin, when the plant is attacked by a pathogen, it accumulates and neutralizes the growth of species in Phytophthora family, especially *Phytophthora infestans* [116,117].

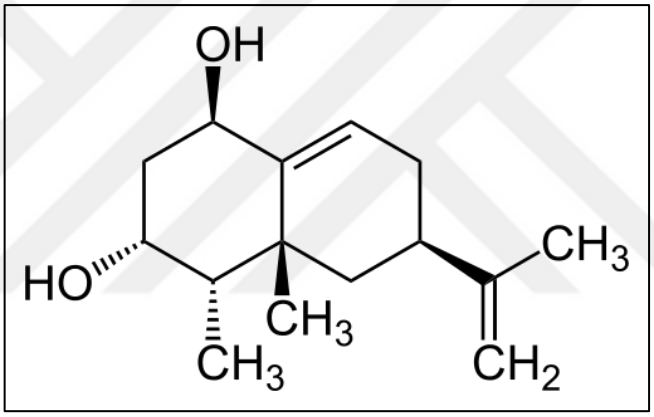

Figure 1.5. Chemical structure of capsidiol [33].

Capsidiol produced via isopentenyl pyrophosphate (IPP) pathway in plants [116,117] (Figure 1.6). IPP and its allylic isomer dimethylallyl pyrophosphate (DMPF) are combined to produce farnesyl pyrophosphate (FPP). Later farnesyl diphosphate is used to produce capsidiol (Figure 1.7).

FPP is an important precursor in terpenoid and sterol biosynthesis [118]. In plant cells, IPP is produced in the cytoplasm via MVA (mevalonate) pathway where the acetyl CoA is the precursor molecule [117]. MVA pathway is conserved in almost all eukaryotic cells. At the same time, it is the main metabolic pathway for secondary metabolite production that derived from sterol and many other isoprenoids [119].

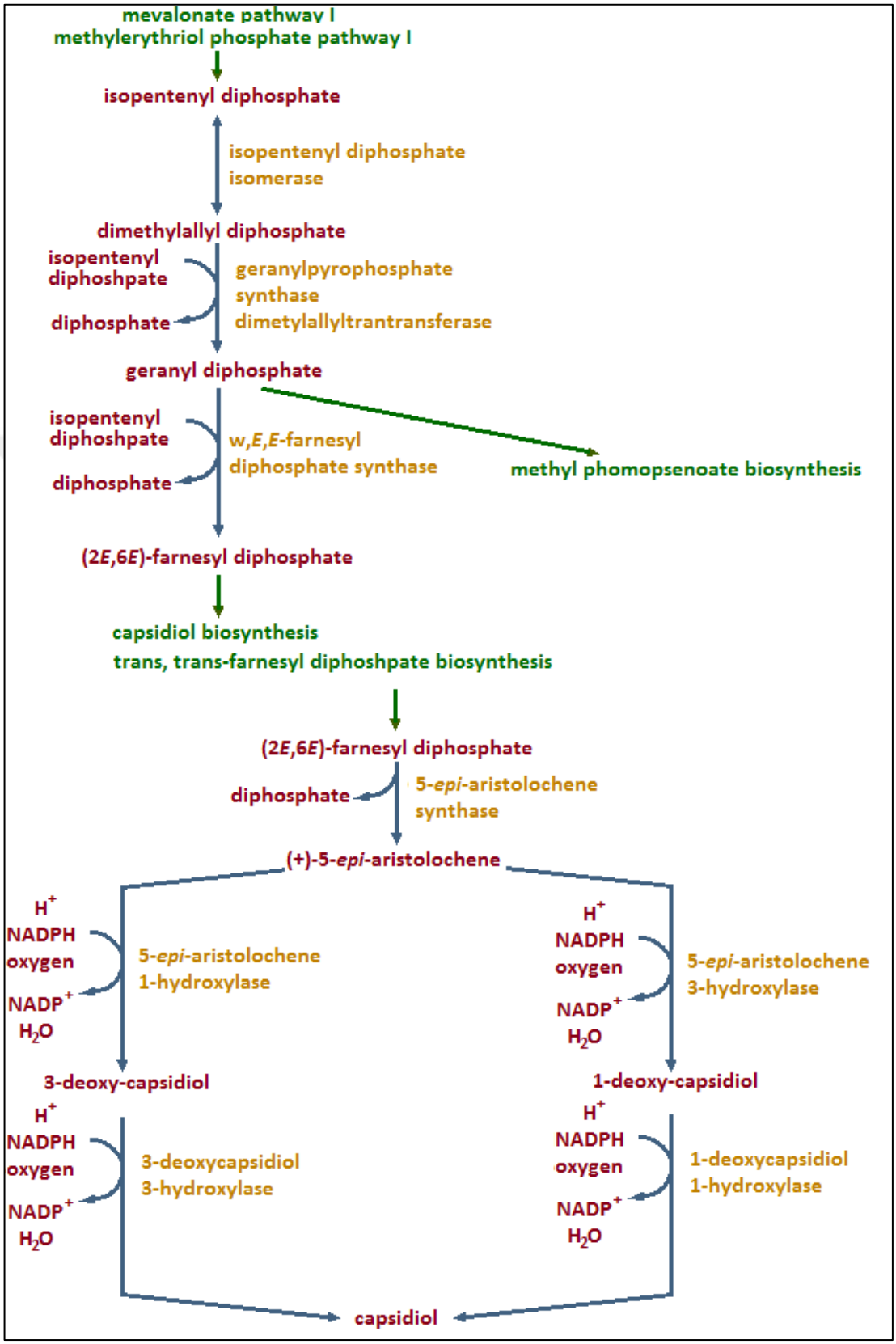

Figure 1.6. Capsidiol production pathway. Showing the production of capsidiol from mevalonate (MVA) pathway [120].

1.5. PHYTOPHTHORA INFESTANS

Phytophthora species are originated from oomycete genus. Oomycetes are the eukaryotic microbes that contain filaments. They have a destructive effect on many plant species, including the economically important ones. *Phytophthora* species are not capable of producing their own sterols due to lack of this metabolism. For this reason, they attack plants and they obtain sterols from their hosts [121,122]. Previous studies demonstrated that 200 μM capsidiol was enough for *Phytophthora infestans*, and 3000 μM capsidiol was enough for *Phytophthora capsici* in order to deactivate them [34]. In 1996, there is more than three million dollar damage caused by *Phytophthora infestans* worldwide [123]. They cause late blight disease on potato and tomato [124]. In spite of several *Phytophthora* species, *P. infestans* can cause damage on the stem, leaf, tubers of potato, and tomato fruit instead of only rotting the root [125].

In 2014, a late blight disease spread in potato fields in Turkey and a congress was organized to focus on *Phytophthora infestans* and its prevention [126]. In 2015, in Balikesir city, there was another late blight incidence happened; this time the target was tomato plants, and the fields were immediately treated with chemical fungicides [127]. In 2017, in Niğde, which is the highest potato producing city in Turkey, the potato production decreased because of late blight problems, yet the solution was again chemical fungicides. Without the fungicides, the loss of yield could increase to 80 per cent [128]. Some of those chemical fungicides contain azoxystrobin which causes bile duct inflammation and biliary toxicity in human [129]. Another dangerous molecule used in fungicide mixtures is Captan and it is toxic in different aspects including neurotoxicity. Apart from human health, Captan is also known to be toxic to aquatic organisms, in other words, a dangerous poison for the ecosystem [129].

Several studies were conducted in Turkey to overcome and enlighten the mechanism of late blight disease. Özgönen and Erkılıç (2007) found out that concentration greater than 1000 ppm of salicylic acid and 2000 ppm of beta amino butyric acid completely inhibited the spore germination by forcing pepper plant to produce capsidiol [130]. In 2014, Aydin *et al*. focused on choosing *Phytophthora* resistant tomato plants to cultivate around Balikesir city [131]. In 2016, there were decline symptoms reported by Kurbetli *et al*. caused by a *Phytophthora* fungus on young almond trees and they found out that

Phytophthora was sensitive to mefenoxam which is a dangerous fungicide [132]. Mefenoxam is carcinogenic and neurotoxic and has an acute toxicity on human health [129].

1.6. METABOLITES AND ANALYTICAL TECHNIQUES

Metabolites are small molecules (<1500 Da) existing in cells, tissues or organisms [133]. High resolution analytical techniques are used for the analysis of metabolites [134], and an analyte must be prepared before the analysis. LLE (Liquid-Liquid Extraction), SPE (Solid-Phase Extraction), and PPT (Precipitation) are common methods to obtain the analyte. Depending on the analyte, the right extraction is chosen. LLE is common when the analyte solubility among different liquids is considered. However, SPE shares similar separation idea with chromatographic techniques [135]. The high resolution analytical techniques commonly used are NMR (Nuclear Magnetic Resonance), GC (Gas Chromatography), HPLC (High Performance/Pressure Liquid Chromatography), UPLC (Ultra Performance/Pressure Liquid Chromatography), and MaS (Mass Spectrometry) [136]. Analytical techniques provide a measurement of metabolites and alteration in metabolite amounts under specific conditions [137]. Due to the complex metabolite composition of the cells or organs, most of the time advanced separation techniques are coupled with MaS. LC-MaS is highly applicable for most of the semi-polar molecules including the secondary metabolites, whereas GS-MaS only applicable to volatile compounds. Identification of the metabolite is the key point while studying metabolomics. Due to the high sensitivity of MaS, and its ability to analyze a wide range of metabolites, MaS has an important role in metabolomic research [136]. To determine capsidiol, early methods used were thin-layer chromatography and gas chromatography after chloroform or dichloromethane extraction [29,30]. SPE was also used to extract capsidiol by using absorption with C_{18} and polyamide columns [138]. For extraction of capsidiol, also supercritical CO_2 extraction was used [139]. However, in 2010, Literakova *et al*. demonstrated capsidiol determination with reversed-phase HPLC by using a C_8 column with diode array detector (DAD) at a 210 nm wavelength. They also compared OSE and SPE and difference was not statistically significant. With that method, they were able to detect a minimum concentration of 0.1 mg/L capsidiol [33].

1.7. AIM OF THE STUDY

The objectives of this study are to 1) establish a callus culture and a callus-derived cell suspension culture from tobacco (*Nicotiana tabacum* cv. Xanthi) plants and characterize these cultures using microscopic analysis and growth (fresh weight) measurements, 2) set-up a workflow for regeneration (and acclimatization) of tobacco plants in *in vitro* conditions using indirect organogenesis, 3) induce the secondary metabolite, capsidiol, production in tobacco cell suspension cultures via use of different elicitors, and 4) determine the capsidiol accumulation in elicitated suspension cultures using HPLC and MaS analysis.

2. MATERIALS

2.1. CHEMICALS

6-Benzylaminopurine (Duchefa Biochemie, Lot# 011210.01), 1-Naphthaleneacetic acid (Sigma Aldrich, Lot# SLBM5342V), Indole-3-butyric acid (Lot# 0112130.01), Indole-3-acetic acid (Duchefa Biochemie, Lot# 011973.04), Kinetin (Lot# 0112120.01), Murashige & Skoog medium including vitamins (Duchefa Biochemie, Lot#P11839.01), Murashige & Skoog basal salt medium (Duchefa Biochemie, Lot#P11781.01), Sucrose (Duchefa Biochemie, Lot# 012100.06), Agar plant tissue culture tested (Sigma Aldrich, Lot#BCBR3228V), Agar plant tissue culture tested (Duchefa Biochemie, Lot#B010856.10), Agar plant tissue culture tested (Phytotech, Lot#15H0111141G), Casein hydrolysate (Duchefa Biochemie, Lot#011718.03), Charcoal (Duchefa Biochemie, Lot#010823.02), Nutrient Agar (Conda pronadiga, Lot#907231), Potato dextrose broth (Sigma Aldrich, Lot#SLBH2045V), Agar microbiology (Sigma Aldrich, Lot#BCBC4369V), Methanol (Sigma Aldrich, Lot#STBG3718V), Dichloromethane (Sigma Aldrich, Lot#STBG0874V), Acetonitrile (Sigma Aldrich, Lot#STBF5101V), Hydrochloric acid (Sigma Aldrich, Lot#SZBE2640V), Sodium hydroxide (EMD Millipore, Cas#1310-73-2), L-Salicylic acid (Duchefa Biochemie, Lot#012263.01), Tween 20 (Sigma Aldrich, Lot#SZBE2460V), Yeast extract (Sigma Aldrich, Lot#BCBK7991V), Hydrogen peroxide (Sigma Aldrich, Lot#SZBE1010V), Ethanol (Duzey Lab, Cas#64-15-5), Ethanol (Umay Lab, Cas#64-15-5).

2.2. GLASSWARE & CONSUMABLES

Glass measuring cylinder 50mL, 250mL, 500mL and 1000mL, Pipette Fillers For 10mL, and 25mL (pi-pump), Micropipettes 0.1-10uL, 20-200uL, 100-1000uL, Micropipette tips 10uL, 200uL, 1000uL, glass bottles borosilicate 250mL, 500mL, and 1000mL, Serological Pipettes Polysterylene 10mL, Pasteur Pipettes Polyethylene, Erlenmeyer's flasks 50mL, 100mL, 250mL, 500mL, 1000mL, Sash Bottles, Centrifuge Tubes Conical Bottom 15mL and 50mL, Tube Racks Polypropylene for 15mL and 50mL, Forceps, Scalpel Handles,

Scalpel Blade no:10 and 11, , Tubing's Silicone, Cling Film, Sealing, Foil Roll Aluminum, Glass Beads, Glass Flask Pear Shape, , Flask Filter Glass Side Arm, Glass Funnels Gooch with Sintered Glass Disc 125mL porosity 3 and 4, Funnel Separating Squib Graduated 250mL, Weighing dishes, , Syringe filters sterile, Syringe polypropylene sterile, Glass Vials screw cap N8, caps septas N8, Inserts glass N8, Eppendorfs tubes 1.5mL and 2mL, Microtube racks for 1.5mL and 2mL, Plant culture box, Vitrovent magenta boxes, Petri dishes 9mm, Cover glasses 20x20mm, Slides cut edges plain, Inoculation loops, HPLC column (water, C18 Xselect 25 cm 5um HSS GH186004775).

2.3. EQUIPMENT

Centrifuge (Eppendorfs, S#5811AK563617), Incubator (Emmert), High Performance Liquid Chromatography with UV detector (Schimatzu, LC20AD XR), plant growth rooms (GROTECH, GR94 and GR74), Shaker (Daihanscientific, thermostable IS-10RL), Incubator (Wisd, Thermostable IR-150), Vertical Flow Hood (Esco, class II BSC), Dry Bead Sterilizer (Swiss made, steri 350), Climatic Chambers (Aralab, S#1799, 1877, 1778), pH meter (Hanna pH211), pH meter (Mettler Toledo, Sevencompact), Oven Natural Flow (Wisd, WON-50), Autoclave (Wisd, MeXterile 60), Analytical Balance with 0.0001 and 0.001 precision (Shimadzu), Vacuum/Pressure pump 15 L/min (IsoLab, Serial#1500081), Rotary Evaporator (Sartorius), Magnetic Stirrer With Hot Plate (SciLogex MS-H280-Pro), Vortex Mixer (WiseMix, Wisd, VM-10).

2.4. BIOLOGICAL MATERIAL

Nicotiana tabacum cv. Xanthi was used as plant material throughout the study and their seeds were kindly provided by Assoc. Prof. Halil Erdem, Gaziosmanpaşa University, Tokat, Turkey. *Aspergillus niger*, filamentous fungi, was used for functional analysis of capsidiol and they were kindly provided by Assist. Prof. Emrah Nikerel, Yeditepe University, İstanbul, Turkey.

3. METHODS

3.1. MEDIA COMPOSITIONS

Various medium compositions used throughout this study are given in Table 3.1. NtCIM1 composition was taken from Murnilawati *et al*. by modifying NAA amounts [140].

Table 3.1 Compositions of the media used in *Nicotiana tabacum* tissue culture experiments.

Media Name	MS	Sucrose	Casein Hydrolysate	Agar	Charcoal	NAA	BAP	IAA	IBA	KN
NtCIM1	4.4 g/L	30 g/L	-	8 g/L	-	3 g/L	0.2 mg/L	-	-	-
NtCIM2	4.4 g/L	30 g/L	-	8 g/L	-	-	-	2 g/L	-	2 g/L
NtCIM3	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	3 g/L	0.2 mg/L	-	-	-
NtCIM4	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	2 g/L	0.2 mg/L	-	-	-
NtCIM5	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	2.5 g/L	0.2 mg/L	-	-	-
NtCIM6	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	1.6 g/L	0.1 mg/L	-	-	-
NtCIM7	4.4 g/L	30g/L	250 mg/L	8 g/L	-	1.3 g/L	0.05 mg/L	-	-	-
NtTR1	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	-	-	-	-	-
NtTR2	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	2 g/L	0.2 mg/L	-	-	-
NtTR3	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	2.5 g/L	0.2 mg/L	-	-	-
NtTR4	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	3 g/L	0.2 mg/L	-	-	-
NtTR5	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	2 g/L	-	-	-	-
NtTR6	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	2.5 g/L	-	-	-	-
NtTR7	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	3 g/L	-	-	-	-
MS1BA	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	-	1 g/L	-	-	-
MS1BACH	4.4 g/L	30 g/L	250 mg/L	8 g/L	-	-	1 g/L	-	-	-
NtGM	4.4 g/L	30 g/L	-	8 g/L	-	-	-	-	-	-
NtGM2	4.4 g/L	30 g/L	-	8 g/L	1 g/L	-	-	-	-	-

NtGM3	4.4 g/L	30 g/L	250 mg/L	8 g/L	1 g/L	-	-	-	-	-
NtR1	4.4 g/L	30 g/L	250 mg/L	8 g/L	1 g/L	4 g/L	-	-	-	-
NtR2	4.4 g/L	30 g/L	250 mg/L	8 g/L	1 g/L	-	-	-	4 g/L	-
NtR3	4.4 g/L	30 g/L	250 mg/L	8 g/L	1 g/L	-	-	4 g/L	-	-

3.2. GERMINATION OF *NICOTIANA TABACUM* CV. XANTHI

Seeds of the *Nicotiana tabacum* cultivar Xanthi were produced in the greenhouse and stored at +4^{o}C. Upon need, they were surface sterilized for culturing. In order to germinate tobacco seeds, NtGM medium was used. NtGM represents *Nicotiana tabacum* germination medium. Later medium was improved with the addition of casein hydrolysate and charcoal. 3 different germination media, (NtGM), were used.

Tobacco seeds were surface sterilized inside the laminar flow with 70 per cent EtOH for 8 minutes, washed with autoclaved ddH_2O twice for 1 minute. Then they are further sterilized with 2 per cent NaOCl solution for 5 minutes and washed with autoclaved ddH_2O for 6 times. Seeds were transferred to fresh sterile ddH_2O and waited for 30 minutes. Then the seeds were transferred to the NtGM2 medium by placing 5 seeds in each magenta box.

3.3. CALLUS FORMATION FROM *NICOTIANA TABACUM* CV. XANTHI

Callus induction of *Nicotiana tabacum* cv. Xanthi was accomplished by using the explants of previously germinated tobacco seeds (Figure 3.1). For callus induction, the media named NtCIM1 and NtCIM2 were used.

Leaf explants were prepared by cutting into 2-5 mm in size. For root explants, root tips were excised. For hypocotyl explant, the parts between the roots and the first leaves were excised whereas for epicotyl explant, the parts between first and second leaves, were taken. Finally, for petiole explants, the part of the plant leaf connecting the stem was taken. Callus induction performed at dark and temperature was set to 25^{o}C. Approximately after 12-15 days, the plant material cut down and subcultured into new petri dishes with NtCIM1 or NtCIM3 media. Approximately after 7-11 days, the calli were divided into

smaller pieces and subcultured in the middle of the petri plates containing fresh media. Newly growing callus was subcultured every 2-3 weeks.

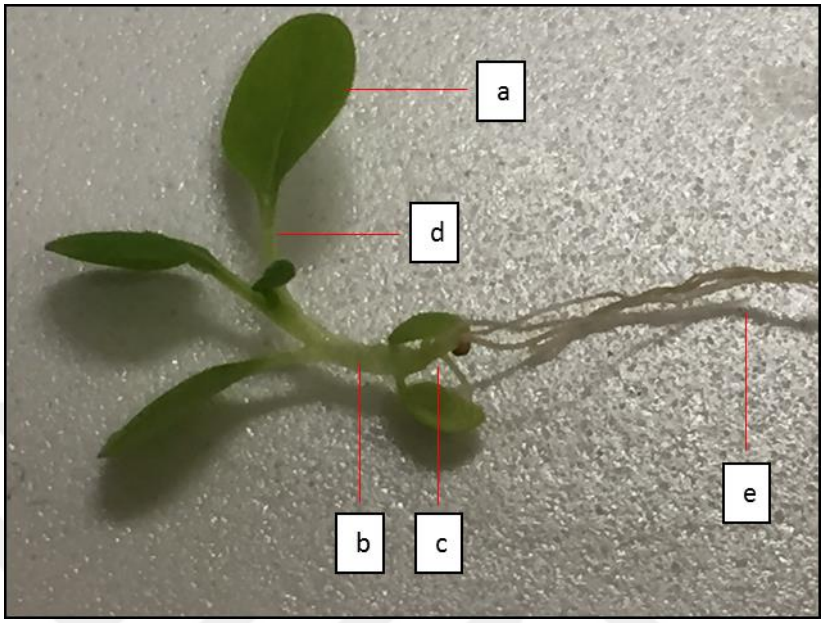

Figure 3.1. *Nicotiana tabacum* cv. Xanthi parts (explants) used to induce callus. (a) Leaf, (b) Epicotyl, (c) Hypocotyl, (d) Petiole, (e) Root.

After 2 months with regular subculturing of the callus within 2-3 week intervals, they were transferred under visible light radiated environment. Within 1 week, callus regained its chlorophyll content and became green. The culturing conditions were as follows: 25ºC, 16h/8h light/dark period, with 65 per cent humidity.

3.4. CALLUS FRESH AND DRY WEIGHT ANALYSIS

Frequently sub-cultured callus was used in this experiment. After the second week of the subculture, excess calli that would not be sub-cultured was used for dry weight measurements. Weighing dishes were weighed in an analytical scale. Approximately one gram of callus placed into each weighing dish and the weight of fresh callus was measured. Weighing dishes containing fresh tissue were put into 70ºC in drying oven for overnight (~16h). The dried callus was measured by weighing the dishes on the analytical scale. Average dry weight was divided into average fresh weight to find the amount of dry matter in fresh tissue.

3.5. INDIRECT ORGANOGENESIS OF *NICOTIANA TABACUM* CV. XANTHI

Nicotiana tabacum cv. Xanthi callus culture previously initiated with the specific media (NtCIM1 and NtCIM3) was used. Regularly subcultured callus in NtCIM3 was used to initiate organogenesis. Approximately 1.0 g callus was placed inside the magenta boxes containing NtTR2, 3, 4, 5, 6, 7 media (How many replicates?). After 45 days, regenerated plantlets were separated and placed in a new medium called MS1BACH for 9 weeks. The plantlets were subcultured in every 3 weeks.

3.6. MICROPROPAGATION OF *NICOTIANA TABACUM* CV. XANTHI

Nicotiana tabacum cv. Xanthi plantlets obtained from the organogenesis continuously subcultured in the MS1BACH medium. After 3 weeks, multiple plantlets obtained from single explant were counted to find out the multiplication rate. At the same time, 3-week-old plantlets were observed under a stereo microscope.

3.7. ROOTING AND ACCLIMATIZATION OF *NICOTIANA TABACUM* CV. XANTHI

Plants that are multiplied in MS1BACH medium were selected for rooting, and 3 different media were prepared in order to induce rooting (NtR1, NtR2, and NtR3). The differences among the media were their source of auxin. 4 mg/L IBA, 4 mg/L NAA and 4 mg/L IAA were used in rooting media separately. For the different media, 5 different magentas were prepared. Total 45 plantlets were placed in rooting media (9 plants per magenta). Longer plants (approximately 1-1.5 cm in length) were placed in rooting media. Relatively small plantlets were placed in MS1BACH media to continue micropropagation. On the 12^{th}, 16^{th}, 19^{th}, 22^{nd}, 26^{th}, and 29^{th} days of cultivation in the rooting media (NtR1, 2, and 3), rooting of the plantlets was observed. After 1.5 months, the plants were acclimatized in greenhouse conditions. While transferring plants into the greenhouse, pots were treated with distilled water, and antifungal chemical to prevent any possible fungal contamination. Plants were removed from the magenta boxes with their medium and placed into the pot containing water and antifungal agent. Roots were cleaned from the media and planted into viol. A

mixture of peat and perlite (1:1) was used as soil material. A total of 19 plants from NtR1, 19 plants from NtR2, and 16 plants from NtR3 were selected from the magenta boxes. If the roots were damaged, they were not used in acclimatization process. After 19 days of plantlet growth at high humidity, they were transferred into pots. The soils tested, were perlite and peat (1:1) and regular soil in a ratio of 2:1. Total 10 plants were transferred to soil from each rooting media.

3.8. PRELIMINARY STUDIES WITH *NICOTIANA TABACUM* CV. XANTHI CALLUS CELL SUSPENSION CULTURE

Nicotiana tabacum cv. Xanthi liquid culture started from callus culture obtained from leaves and grown under the light. They were put into 250 ml Erlenmeyer just after cutting in a sterile petri dish to 1 mm sizes and flasks (100 ml medium/flask) were prepared; for NtCIM1 and NtCIM3. They were put under light at 25^{o}C with shaking at 110 rpm and subcultured after 15 days. Subculturing was performed via the vacuum filtration system, and the liquid culture obtained from 15-day old plants were transferred to sterile falcon tubes for further analysis of capsidiol availability. Tissues collected in a vacuum filtration system was put in new sterile 250 ml Erlenmeyer flasks including 100 ml liquid media. After another 15 days, liquid cultures were subcultured. Subculture performed via transferring to the new flask by pouring, not filtration via the vacuum filtration system. Tissues were transferred into sterile 250 ml Erlenmeyer flasks and required liquid media were added until it has 100 ml media with callus mixture, NtCIM1, and NtCIM3. They were subcultured weekly and transferred into new sterile media by vacuum filtration system.

3.9. ESTABLISHMENT OF *NICOTIANA TABACUM* CV. XANTHI CALLUS CELL SUSPENSION CULTURE

Nicotiana tabacum cv. Xanthi liquid cell suspension cultures were prepared as performed on preliminary data. In order to prevent the aggregated big calli transfer to fresh medium, callus filters were used. Callus filters are stainless steel 0.5 mm^2 – 1.0 mm^2 hole containing meshes. While subculturing cell suspension cultures, calli were filtered with these callus

filters, remaining big particles were discarded. A new medium called NtCIM4, NtCIM5, NtCIM6, NtCIM7, NtCIM8, and NtCIM9 were prepared and during subculturing cells were transferred from NtCIM3 to these new media. Also, hypocotyl-derived callus cultures grown in the dark was used to induce cell suspension culture with similar methods.

3.10. CONSTRUCTION OF GROWTH CURVE FOR CALLUS CELL SUSPENSION CULTURE

After the sixth week of subculture, the cell suspension was used to initiate growth curve construction. Conditions were set at 25^{o}C, 16h/8h light/dark with orbital shaking at 110 rpm. 10 x 50 mlml glass Erlenmeyer flasks with NtCIM3 medium were used. 10 mlml of media and approximately 1g of callus were put in each flask. In every other day, calli were weighed after filtration. Results were recorded to construct a growth curve.

3.11. MICROSCOPICAL OBSERVATIONS OF *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURES

3.11.1. Single Cell Observations under A Light Microscope

With sterile tips, inside the vertical flow sterile hood, liquid samples were taken from the continuously growing cells into a 1.5ml Eppendorf tubes. The sample prepared by placing one drop into a slide, covered with cover slide and cells were observed under a light microscope with different magnifications.

3.11.2. Sample Preparation for Scanning Electron Microscopy (SEM)

Two different methods with methanol were conducted in order to achieve fixed cells. Firstly, from cell suspension culture, samples were taken and placed onto the slide. One drop of methanol was dropped every 15 seconds (until every drop slightly dries) totally for one hour. Secondly, callus sample was placed inside methanol and waited until one hour. After an hour, cells were placed on the slide with the help of micropipette and waited until

remaining methanol dried out. Samples later coated with 17 nm layer gold in a vacuum environment and observed under the scanning electron microscope.

3.11.3. Viability Test via A Fluorescent Microscopy

Viability test was performed with fluorescein diacetate (FDA). The stock was prepared by dissolving 5 mg of FDA in 1 mL Acetone. With sterile tips, 1ml of liquid sample was taken from the continuously growing suspension cells into 2 mL Eppendorf tubes. 950 µL of fresh medium, NtCIM5, was mixed with the suspension cells in the same 2ml Eppendorf tubes. 50 µL of stock FDA, freshly prepared, was added to the mixture and Eppendorf tubes were covered with aluminum foil and waited for 5 minutes in order to obtain fluorescent molecule release as esterase enzyme works inside the cells. Later samples were placed onto the slide and covered with a cover slide. Illumination from fluorescent molecule was observed under a fluorescent microscope with UV detector and alive cells were selected.

3.12. PRELIMINARY WORK #1: ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI, ORGANIC SOLVENT EXTRACTION AND HPLC ANALYSIS

Frequently sub-cultured callus was used in this experiment. After the seventh week of the subculture, suspension callus was used in elicitation. Media were NtE1, NtE2, and NtE3. 20 mL of medium was used. 2g of *Nicotiana tabacum* cv. Xanthi cells were collected via filtration system and measured in the laminar flow, previously sterilized with 70 per cent EtOH and UV, and transferred to 20 mL liquid media containing 100ml Erlenmeyer flasks. The experiment performed with three flasks; there were no repeats since it was considered as a preliminary work before method optimization. From the initial day, cells were grown at 25^{o}C, with constant light stimulation and shaking at 110 rpm. After four days, cells were removed with filtration and filtrated media was put in 50 mL sterile tubes and placed at -20^{o}C freezer until used for organic solvent extraction.

Since dichloromethane was used in organic solvent extraction, following steps were performed in a fume hood. Elicitated medium was taken from the freezer and stored on ice until it slightly defrosted. Media was transferred into a liquid separating funnel and the

same amount of CH_2Cl_2 was poured on top of it. Separating funnel was mixed and waited a couple minutes until liquids separated into two phases. Later, organic phase (at the bottom) was taken and placed inside pear-shaped glass balloons. Balloons were kept on ice until the extraction completed. Then balloons were placed in a rotary evaporator at 37ºC temperature and waited until all the organic phase evaporated and lastly collected in a waste balloon. Balloon with the extract was taken and 1000 µL methanol was put onto it and dissolved by pipetting. Dissolved extracts were transferred into 2 mL Eppendorf tubes. 100 µL was taken and transferred to HPLC sampling vials.

HPLC column was Agilent Poroshell 120 EC-C18 (3.0 x 150 mm 2.7-Micron), Mobile phase was gradient among the run (Table 3.2), conditioning performed with 70 per cent methanol and 30 per cent water mixture. While the sample was running, methanol was increased to 80 per cent gradient from 70 per cent. UV/Vis detector was used wavelength was 210 nm. The column was 0.9ºC.

Table 3.2. HPLC mobile phase and flow rate table.

Time (min)	% Methanol	% Water	Flow (mL/min)
0.00	70.0	30.0	0.3
50.00	80.0	20.0	0.3
51.00	70.0	30.0	0.3
55.00	70.0	30.0	0.3

3.13. PRELIMINARY WORK #2: ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI WITH HCL AS ELICITOR

Frequently sub-cultured cell suspension was used in this experiment. After the 9th week of the subculture, three 250mL Erlenmeyer flasks used for regular subculture but noted as HCl elicitation. 10 grams of suspension culture was put in 50 mL media, and shank regularly 110 rpm for 7 days, and then 0.25 mL of 2 M HCl was added to the first Erlenmeyer flask.<<Final concentration was 10 mM HCl. 0.50 mL of 2 M HCl was added to the second Erlenmeyer flask and final concentration was 20 mM. Finally, 1.00 mL of 2 M HCl was added to the third Erlenmeyer flask and final concentration was 40 mM. All of the experiments performed inside the laminar flow and sterile environment. Cells were

exposed that much HCl for 3 days. After 3 days, cells were collected via filtration system and were taken away with filtration. Filtrated media was put in 50 mL Eppendorf and placed in a Freezer at -20^{o}C.

After a day, organic solvent extraction performed as it was written in the previous method. However, the final methanol dissolving phase was different. Extract containing balloon was taken and put 500 µL methanol. Dissolved by pipetting, to get everything while pipetting walls were cleaned with methanol to dissolve every molecule stick there.

HPLC column was Agilent Poroshell 120 EC-C18 (3.0 x 150 mm 2.7-Micron) Mobile phase was gradient among the run, conditioning performed with 70 per cent Methanol and 30 per cent Water mixture. Conditioning was 1 hour this time. Gradient increased among 24 minutes.

3.14. PRELIMINARY WORK #3: ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI WITH DIFFERENT ELICITORS

10 x 50 mL Erlenmeyer flasks were prepared. 10 mL of medium with 1g of callus was put inside Erlenmeyer flasks and callus source was leaf. At the same time, 5x 100 mL Erlenmeyer flasks were prepared. 20 mL of medium with 2 g of callus was put inside Erlenmeyer flasks and callus source was hypocotyls. Broad range of elicitors were prepared. Firstly, 470 µL from 35 per cent H_2O_2 solution taken, and completed to 50 mL to achieve 0.033 per cent. Later used as stock of hydrogen peroxide. Secondly, 8.26 mL from 37 per cent HCl solution taken, and completed to 50 mL to achieve 2M. Later used as a stock hydrochloric acid. Thirdly, 85 mg of Salicylic acid was measured and dissolved into 50 mL to achieve 0.013M. Later used as a stock salicylic acid. Fourthly, 5g of Yeast extract was measured and dissolved in 50 mL to achieve 0.1 g/mL stock solution. Finally, tween20 was used as a stock solution. Yeast extract was autoclaved, and other elicitors were cold sterilized using 20 um filters. HCl and tween20 were not sterilized. Elicitors were added to the media at 7th day. The final concentration of H_2O_2 was 1 mM in the media. 1mM for HCl in media, 1mM for SA in media, 0.02 mg/mL for YE in media, and 0.025 per cent (v/v) tween20 in the media. Media were elicited until the 11th day. Later media were collected by filtering and cells were thrown away. Organic solvent extraction was performed.

HPLC column was XSelect HSS C18 Column, 100Å, 5 µm, 4.6 mm x 250 mm, 1/pkg. The mobile phase was gradient among the run, and (Table 3.3.) conditioning performed with methanol and water same as the mobile phase. While samples (Table 3.4.) were running methanol was increased to 80 per cent gradient from 70 per cent. UV/Vis detector was used at wavelength 210 nm. Run time was 16 minutes. Injection volume was 10 µL.

Table 3.3. HPLC mobile phase and flow rate table.

Time (min)	**% Methanol**	**% Water**	**Flow(mL/min)**
0.00	70.0	30.0	1.0
16.00	80.0	20.0	1.0
20.00	70.0	30.0	1.0
20.50	70.0	30.0	1.0

Table 3.4. Extracts and sample names & injection information.

Sample #	**Sample Name**	**Callus tissue/Standard**	**Elicitor**
1	A1	STD	YE(Yeast extract)
2	A2	*N. tabacum* leaf callus 1	YE(Yeast extract)
3	A3	*N. tabacum* leaf callus 2	YE(Yeast extract)
4	A4	*N. tabacum* hypocotyl callus	YE(Yeast extract)
5	B1	STD	SA (Salicylic acid)
6	B2	*N. tabacum* leaf callus 1	SA (Salicylic acid)
7	B3	*N. tabacum* leaf callus 2	SA (Salicylic acid)
8	B4	*N. tabacum* hypocotyl callus	SA (Salicylic acid)
9	C1	STD	HCl
10	C2	*N. tabacum* leaf callus 1	HCl
11	C3	*N. tabacum* leaf callus 2	HCl
12	C4	*N. tabacum* hypocotyl callus	HCl
13	D1	STD	H_2O_2

14	D2	*N. tabacum* leaf callus 1	H_2O_2
15	D3	*N. tabacum* leaf callus 2	H_2O_2
16	D4	*N. tabacum* hypocotyl callus	H_2O_2
17	E1	STD	Tween20
18	E2	*N. tabacum* leaf callus 1	Tween20
19	E3	*N. tabacum* leaf callus 2	Tween20
20	E4	*N. tabacum* hypocotyl callus	Tween20
21	F	STD	Solo-Medium
22	G	STD	BAP
23	H	STD	NAA
24	A1/NE	STD-non extracted	YE (Yeast extract)
25	B1/NE	STD-non extracted	SA (Salicylic acid)
26	C1/NE	STD-non extracted	HCl
27	D1/NE	STD-non extracted	H2O2
28	E1/NE	STD-non extracted	Tween20
29	G2	STD-non extracted low [C]	BAP
30	G3	STD-non extracted high [C]	BAP
31	H2	STD-non extracted low [C]	NAA
32	H3	STD-non extracted high [C]	NAA

3.15. ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI WITH SALICYLIC ACID

10 x 50 mL Erlenmeyer flasks were prepared. 10 mL of medium with 1 g of callus was put inside those Erlenmeyer flasks and callus sources were leaf and hypocotyl evenly distributed. At the same time, 10 x 100 mL Erlenmeyer flasks were prepared. 20 mL of medium with 2 g of callus was put inside Erlenmeyer flasks and callus sources were leaves and hypocotyls evenly distributed. Salicylic acid was used as elicitor source. 85 mg of Salicylic acid was measured and dissolved into 50 mL to achieve 0.013 M. Elicitor

sterilized with 20 μM filters. Samples and elicitors are demonstrated in Table 3.5 with their final concentrations.

Table 3.5. Elicitor applications in *Nicotiana tabacum* cv. Xanthi leaf and hypocotyl cell suspension cultures with varying salicylic acid concentrations.

Sample #	**Sample Name**	**Callus Organism/Tissue**	**Elicitor (SA) Concentration**
1	B5	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	1.0 mM
2	B6	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	2.0 mM
3	B7	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	3.0 mM
4	B8	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	4.0 mM
5	B9	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	5.0 mM
6	B10	*Nicotiana tabacum* cv. Xanthi Leaf 20 mL	0.5 mM
7	B11	*Nicotiana tabacum* cv. Xanthi Leaf 20 mL	1.0 mM
8	B12	*Nicotiana tabacum* cv. Xanthi Leaf 20 mL	1.5 mM
9	B13	*Nicotiana tabacum* cv. Xanthi Leaf 20 mL	2.0 mM
10	B14	*Nicotiana tabacum* cv. Xanthi Leaf 20 mL	2.5 mM
11	B15	*Nicotiana tabacum* cv. Xanthi Hypocotyl 10 mL	1.0 mM
12	B16	*Nicotiana tabacum* cv. Xanthi Hypocotyl 10 mL	2.0 mM
13	B17	*Nicotiana tabacum* cv. Xanthi Hypocotyl 10 mL	3.0 mM
14	B18	*Nicotiana tabacum* cv. Xanthi Hypocotyl 10 mL	4.0 mM
15	B19	*Nicotiana tabacum* cv. Xanthi Hypocotyl 10 mL	5.0 mM
16	B20	*Nicotiana tabacum* cv. Xanthi Hypocotyl 20 mL	0.5 mM
17	B21	*Nicotiana tabacum* cv. Xanthi Hypocotyl 20 mL	1.0 mM
18	B22	*Nicotiana tabacum* cv. Xanthi Hypocotyl 20 mL	1.5 mM
19	B23	*Nicotiana tabacum* cv. Xanthi Hypocotyl 20 mL	2.0 mM
20	B24	*Nicotiana tabacum* cv. Xanthi Hypocotyl 20 mL	2.5 mM

Media were elicited until the 11th day. Later media were collected by filtering and cells were thrown away. Organic solvent extraction performed.

HPLC column was XSelect HSS C18 Column, 100Å, 5 μm, 4.6 mm x 250 mm, 1/pkg. The mobile phase was gradient among the run, and conditioning performed with methanol and

water same as the mobile phase. Injection volume was 10 µL. The similar protocol used as in the 3rd preliminary HPLC analysis. Three replicates were run.

Smaller range of salicylic acid also experimented as follows: 10 x 50 mL Erlenmeyer flasks were prepared. 10mL of medium with 1g of callus was put inside those Erlenmeyer flasks and callus source was a leaf. Same stock SA was used as elicitor source. Samples and elicitors are demonstrated in Table 3.6 and 3.7 as their final concentrations.

Sample named B5, which was 0.5mM salicylic acid treated *Nicotiana tabacum* cv. Xanthi leaf was used with different gradients of methanol and acetonitrile mobile phases to optimize mobile phase.

Table 3.6. Different methanol and acetonitrile applications. Mobile phase range demonstrates the gradient increase of methanol or acetonitrile amounts in a 20-minute run.

Sample #	**Mobile Phase (Mobile Phase Nomenclature)**	**Mobile Phase Range**
1	Methanol (MeOH_100)	90-100%
2	Methanol (MeOH_90)	80-90%
3	Methanol (MeOH_80)	70-80%
4	Methanol (MeOH_70)	60-70%
5	Methanol (MeOH_30)	20-30%
6	Methanol (MeOH_20)	10-20%
7	Acetonitrile (ACN_100)	90-100%
8	Acetonitrile (ACN_90)	80-90%
9	Acetonitrile (ACN_80)	70-80%
10	Acetonitrile (ACN_70)	60-70%
11	Acetonitrile (ACN_60)	50-60%
12	Acetonitrile (ACN_50)	40-50%

Table 3.7. Elicitor applications in *Nicotiana tabacum* cv. Xanthi leaf cell suspension cultures with varying salicylic acid concentrations.

Sample #	**Sample Name**	**Callus Organism/Tissue**	**Elicitor (SA) Concentration**
1	B25	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	Control 0 mM
2	B26	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	Control 0 mM
3	B27	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.125 mM
4	B28	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.125 mM
5	B29	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.250 mM
6	B30	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.250 mM
7	B31	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.375 mM
8	B32	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.375 mM
9	B33	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.500 mM
10	B34	*Nicotiana tabacum* cv. Xanthi Leaf 10 mL	0.500 mM

Media were elicited until the 11th day. Later mediums were collected by filtering and cells were thrown away. Organic solvent extraction performed. HPLC column was XSelect HSS C18 Column, 100Å, 5 µm, 4.6 mm x 250 mm, 1/pkg. The mobile phase was gradient

among the run, and conditioning performed with methanol and water same as the mobile phase. Injection volume was 10 µL. A similar protocol as in the 3rd preliminary HPLC analysis was used. Three replicates were run.

Different amounts of hormones also prepared. From the stock 1 mg/1mL dilutions were done to obtain 0.1 mg/L, 0.2 mg/L, 0.5 mg/L, 1 mg/L, 2 mg/L, 4 mg/L, and 8 mg/L BAP and 1 mg/L, 2 mg/L, 4 mg/L, and 8 mg/L NAA samples. Those samples were run with HPLC to obtain a standard curve shown in Table 3.8.

Table 3.8. Standard curve applications for NAA and BAP hormones.

Sample Name	**Hormone**	**Concentration**
G4	BAP	0.1 mg/L
G5	BAP	0.2 mg/L
G6	BAP	0.5 mg/L
G7	BAP	1.0 mg/L
G8	BAP	2.0 mg/L
G9	BAP	4.0 mg/L
G10	BAP	8.0 mg/L
H4	NAA	1.0 mg/L
H5	NAA	2.0 mg/L
H6	NAA	4.0 mg/L
H7	NAA	8.0 mg/L

Finally, from the sample named B5, peak at the retention time 3.29 was collected for MsS and function analysis.

3.16. ACTIVITY ANALYSIS OF THE EXTRACTS

HPLC samples and direct extracts were dropped in sterile filter papers inside the laminar flow and waited to get dry. Than *Aspergillus niger* was cultivated in petri dishes (NA). Extract absorbed papers were placed in the middle of the petri dish. Extracts were from Preliminary #1 experiments. Whole extracts and HPLC purified peaks were used. NtE1, NtE2, and NtE3 extracts directly dropped, also pure methanol as a control, and 13th-minute peak, 16th-minute peak and 18th-minute peak were used in order to test the effect of antimicrobial activity over *Aspergillus niger*.

3.17. MAS ANALYSIS OF THE EXTRACTS

Extracts that were used in activity analysis were also run in MaS analysis in order to detect putative capsidiol by using Bruker Compact Electrospray-quadruple time of flight. Ion polarity was positive and the capillary was 4000V, charging voltage was 2000V and end plate offset was -500V. Nebulizer had 0.6 bar pressure. The dry heater was 250^{o}C, the gas flow rate was 4.0 L/min. Samples ran in the mass spectrometry analysis were NtE1, NtE2, and NtE3 as the whole extract, and peaks that were collected at 6th minute, 9th minute, 13th minute, 16th minute, and 18th minute, based on Literakova *et al*. [33].

4. RESULTS AND DISCUSSION

4.1. GERMINATION OF *NICOTIANA TABACUM* CV. XANTHI

There are several factors helping the germination of *Nicotiana tabacum* in *in vitro* tissue culture techniques. In the basal salt mixture, it can germinate more than 50 per cent of the seeds. However, with several additions to the media, germination rates can be elevated. Preliminary works demonstrated the highest germination rate with the NtGM3 medium as 84 per cent, compared to NtGM which is a basal MS salt mixture with sucrose, and NtGM2, basal MS salt, 3 per cent sucrose, and containing 250 mg/L casein hydrolysate. Even though NtGM and NtGM2 were demonstrated similar germination rates. Couple-month-old leaves of NtGM was whitened due to lack of nitrogen, yet NtGM2 leaves were green. Nitrogen requirements of the tobacco plant are higher than the basal MS salt [38]. In addition, adding charcoal to the media was found the reason for increased germination. 10 NtGM3 with every magenta had five seeds planted.

In this study with the addition of 1 g/L charcoal and 250 mg/L casein hydrolysate, 84 percent germination rate was achieved and shown in Figure 4.1. Charcoal was used to mimic the soil as it gives a dark environment and casein hydrolysate was used to compensate for the nitrogen requirement.

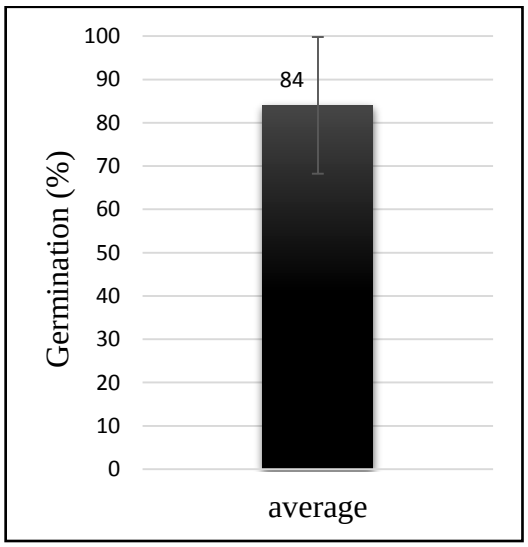

Figure 4.1. Total germination of *Nicotiana tabacum* cv. Xanthi after two months period.

4.2. CALLUS FORMATION OF *NICOTIANA TABACUM* CV. XANTHI

After germination, callus was obtained from the different parts of the plants. Plant growth regulators play a significant role in callus induction [42], as well as the environment. In order to achieve a good callus generation, the usually dark environment is preferred in order to increase the plant's media dependency. Without photosynthesis plant cells are forced to get nutrients from the media. By forcing plants to get more nutrient from the media, plant growth regulators become more effective.

Two media were used to induce callus initially. Preliminary work was done with NtCIM1 and NtCIM2 media and callus was generated with NtCIM1 medium only, shown in Figures 4.2., 4.3., and 4.4. Abnormalities were observed on NtCIM2 media shown in Figure 4.6. NtCIM2 demonstrated direct organogenesis results, and the cause of direct organogenesis might because of the cytokinin levels used in NtCIM2 medium because of the ratio of auxin to cytokinin rates (1:1). On the other hand, NtCIM1 has the rate of auxin: cytokinin as 15:1. Usually, the rate of auxin and cytokinin needs to be 1:1 for callus formation [141], however plants produce several amounts of hormones by themselves. Despite the externally added hormones, final hormone concentration is much more important. 21 days of exposure to the callus induction medium are enough to achieve callus formation from tobacco explants. Best results were obtained from *Nicotiana tabacum* leaves with 95 per cent callus formation in 21 days (Figure 4.2), however, callus formation rate increased 5 per cent in 7 days.

It was observed that 14 days are enough to generate callus. If callus is formed from explants, it continues to grow after 14 days, yet if there is not any callus observed at 14^{th} day, the tobacco explant can be considered dead, or not forming callus due to other reasons, such as wrong plant growth regulators or intracellular contamination. But 21 days are essential to achieving sufficient amounts of callus mass to start subculturing.

Fully grown calli after 21 days were collected into the middle of the petri dishes. They subcultured in the middle of the petri on the upcoming subcultures, demonstrated elevated growing rates shown in Figure 4.3.

Metabolism of the plants alters due to the light exposure. Several metabolites are produced in green tissues (light exposed environment) (Figure 4.4), and some of them in white (dark

environment) (Figure 4.5). In this study, half of the callus was transferred to light, and the other half left in the dark and used accordingly.

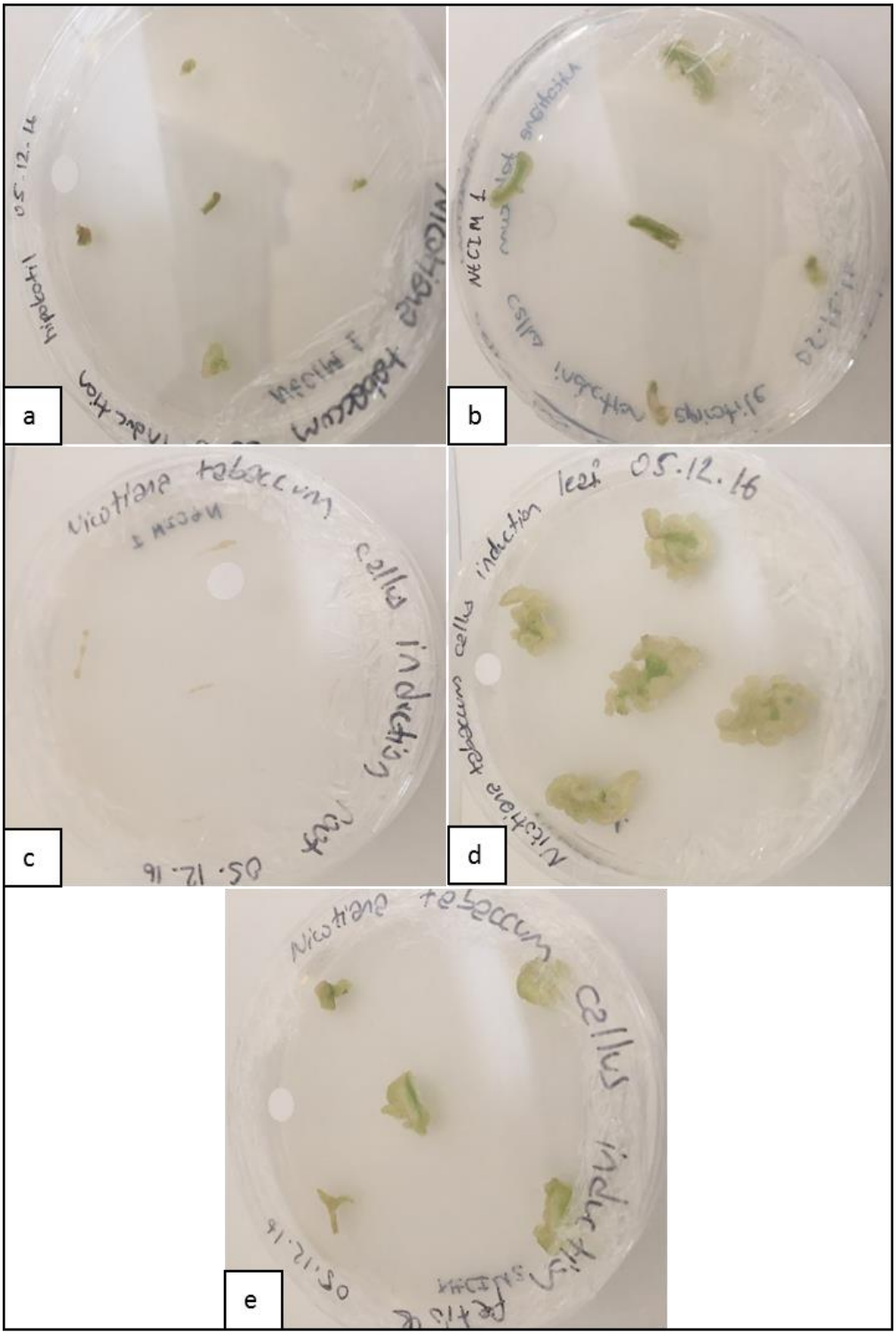

Figure 4.2. Two weeks old *Nicotiana tabacum* cv. Xanthi growing callus, on the NtCIM1 medium. (a) Hypocotyl, (b) Epicotyl, (c) Root, (d) Leaf, (e) Petiole.

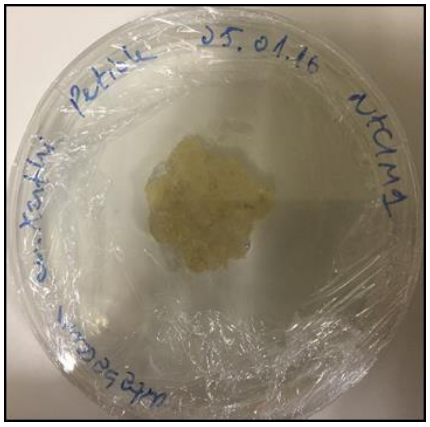

Figure 4.3. Regularly subcultured petiole callus culture, all collected in the middle and growing as one.

Figure 4.4. Green growing callus under visible light, green color observed after transferring callus into light containing conditions.

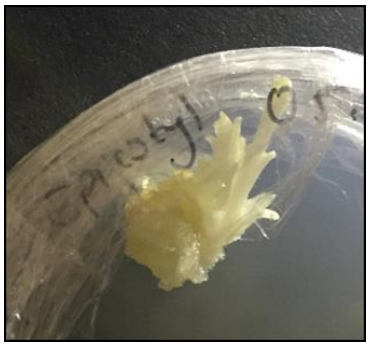

Figure 4.5. Grown abnormality while callus induction with NtCIM2 media.

However, long exposure to medium NtCIM1 was not good for the tobacco had found out, so that NtCIM3, which contained casein hydrolysate as extra nitrogen source was found a better resulting medium. And new callus induction was performed with NtCIM3 medium and callus generation data was obtained through 21 days shown in Figure 4.6.

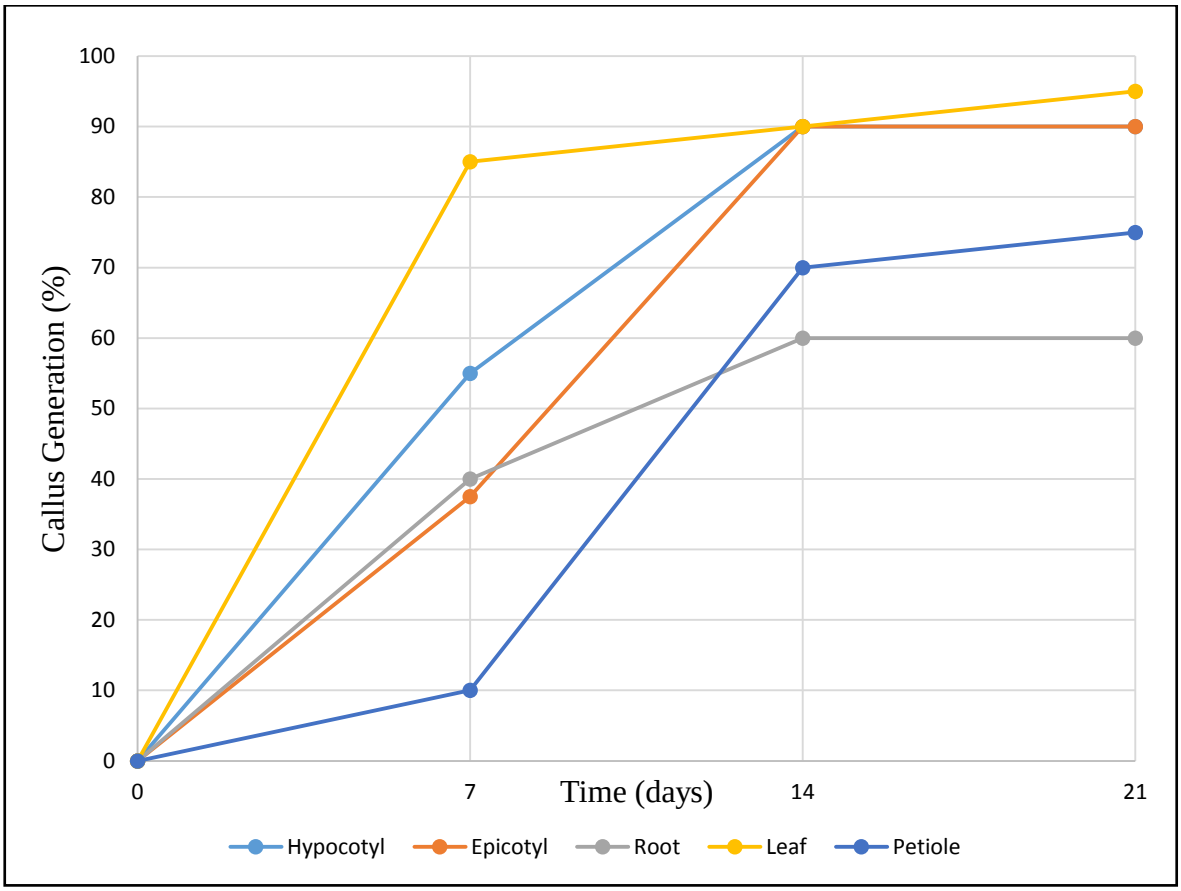

Figure 4.6. Dynamics of callus generation of different tissues of *Nicotiana tabacum* cv. Xanthi.

Leaf explants have the highest callus generation rate at 21 days with 95 per cent, and it is followed by hypocotyl and epicotyl with 90 per cent. Petiole has 75 per cent callus generation rate and roots generated 60 per cent callus in NtCIM3 media. 15 days of exposure to callus induction media is approximately sufficient to achieve callus formation from tobacco (Figure 4.6). Since the callus induction performed in the dark, formed callus tissue showed whitish yellow color owing to the lack of chlorophylls. When the white callus transferred to the 8/16 dark/light photoperiod conditions, its chlorophyll content increased in approximately in 14 days.

4.3. CALLUS GROWTH

After the establishment of callus, its growth curve constructed to have a better understanding of callus' growth potential. Increasing fresh matter amount of *Nicotiana tabacum* cv. Xanthi leaf callus was observed for a 15 day period shown in Table 4.2. The growth curve was prepared is increasing logarithmically. A growth equation was constructed according to the graph produced shown in Figure 4.7. 1 gram of callus grew into 5.3 grams in 15 days, whereas it became only 3 grams in 10 days when it was cultured in semi-solid media supplemented with 3.0 mg/L NAA and 0.2 mg/L BAP. According to the growth curve, it was observed that the lag phase ended in the seventh day. For the first 7 days, it had barely doubled the culturing amount, however, following 3 days it tripled the initial amount.

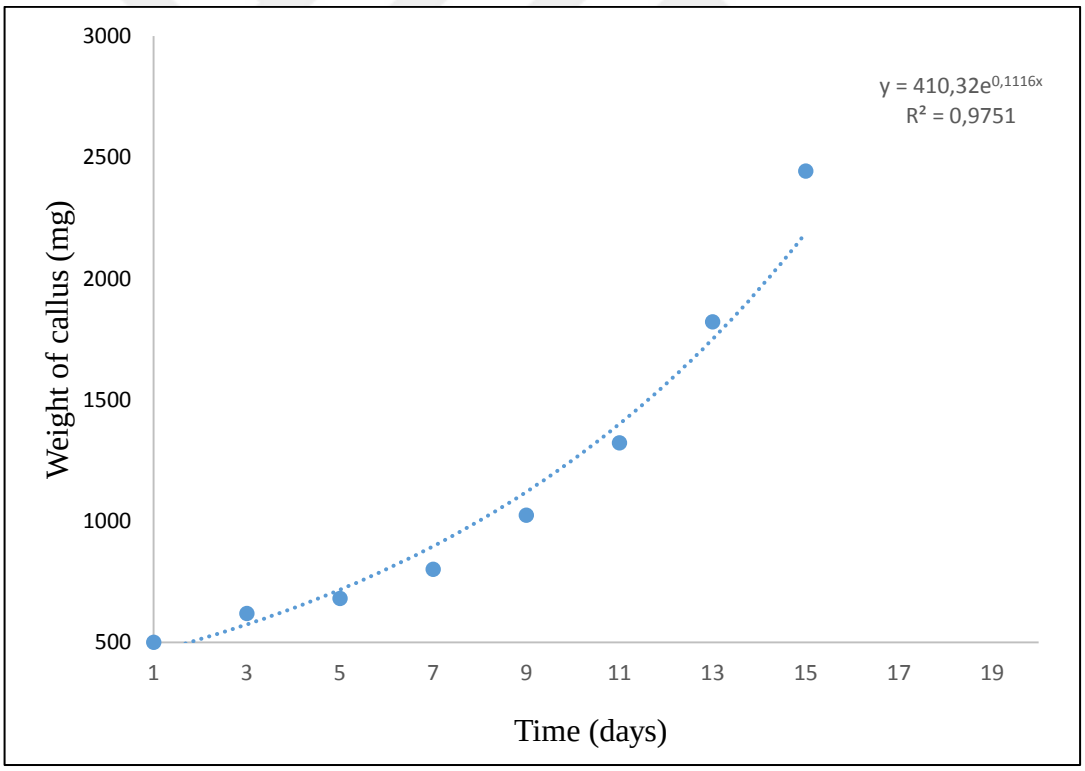

Figure 4.7. Growth curve of *Nicotiana tabacum* cv. Xanthi leaf callus.

Y-axis represents the weight of callus (in mg) and the x-axis represents the time (in days). A trendline equation was fitted to predict the growth of leaf callus over time;

$$Growth(as\ miligrams) = initial\ mg\ of\ callus\ \times\ 0.821e^{0.1116\times(Days\ after\ culturing)}$$

4.4. CALLUS FRESH AND DRY WEIGHT ANALYSIS

To have more understanding about its physiology, dry weight analysis was performed; it was found that only 4.6 per cent of the callus was dry weight and the rest was water. Dry weight analysis was performed with approximately 1g of fresh callus and data obtained from 20 samples. The standard deviation was 0.3 per cent and average dry weight over fresh weight ratio was 4.6 per cent (Figure 4.8).

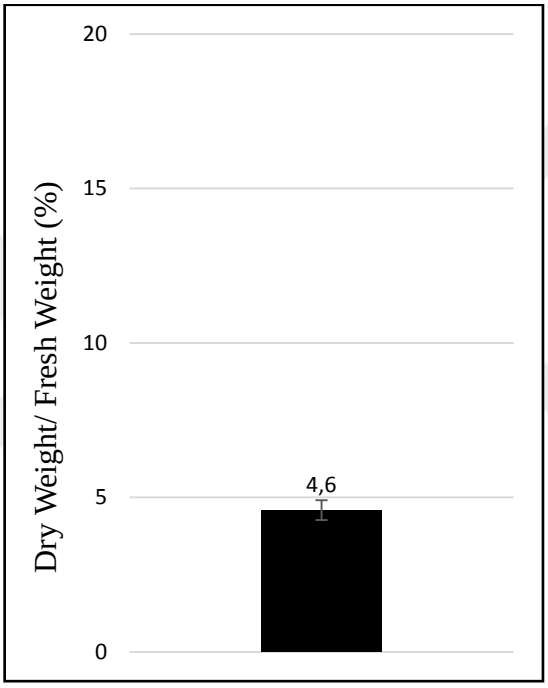

Figure 4.8. Callus dry weight percentage analysis.

4.5. INDIRECT ORGANOGENESIS OF *NICOTIANA TABACUM* CV. XANTHI

As the preliminary work, organogenesis was performed under both light and dark conditions. Preliminary studies demonstrated that in 45 days, plantlets regenerated from organogenesis under the light condition, however, there was not any plantlet observed where there was no light shown in Figure 4.9; 4.10.

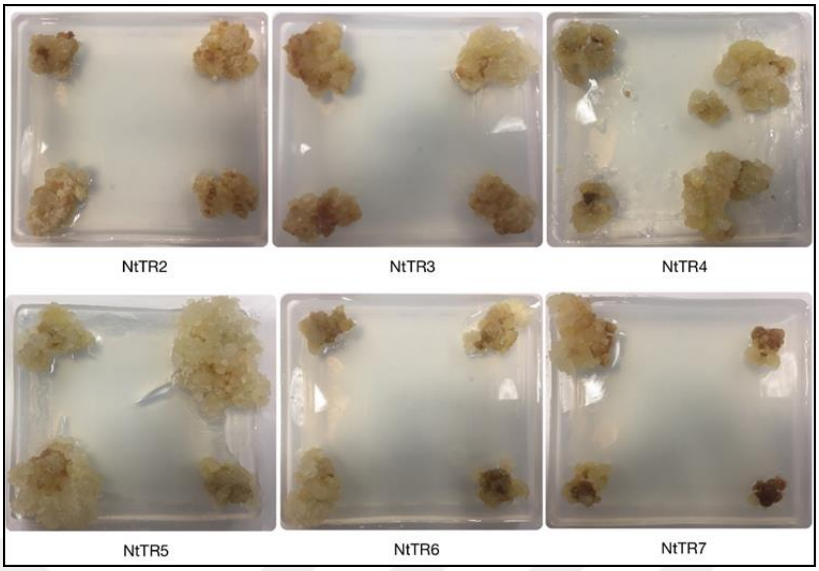

Figure 4.9. Preliminary organogenesis in dark environment. There was no plantlet observed.

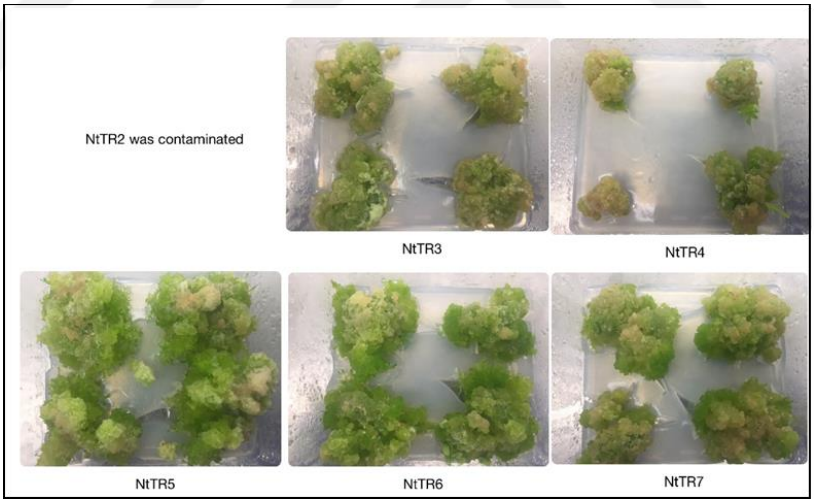

Figure 4.10. Preliminary organogenesis in light environment. Several calli demonstrated plantlet grown in 45 days.

With a wide range plant growth, regulators were utilized to optimize the organogenesis media. Plants had become 0.1-1 cm length in 30-40 days. There were 1 mm plantlets obtained from callus which were also enough to start subculturing into micropropagation media. After preliminary data, the experiment was conducted in light and plantlets were observed as shown in Figure 4.11.

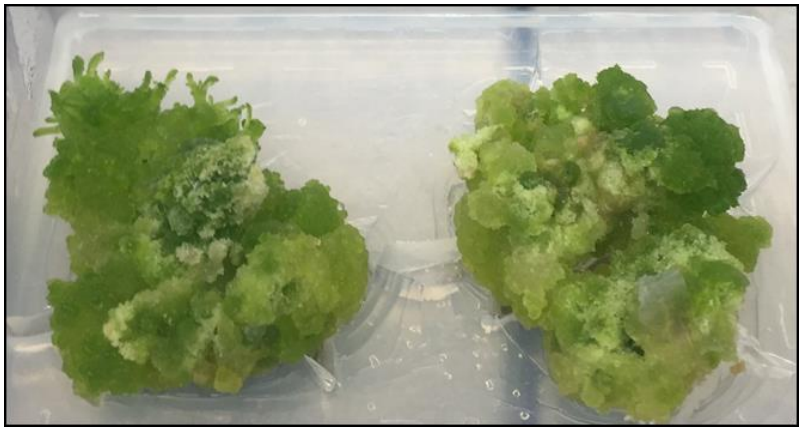

Figure 4.11. Organogenesis of *Nicotiana tabacum* cv. Xanthi, 45 days old callus-plantlet intermediate.

The most plantlet formation was obtained from NtTR6 media containing 2.5 mg/L BAP without auxin, and 110 plantlets were counted in total. NtTR5 and NtTR4 followed the highest plantlet formation in 81 and 80 plantlets respectively shown in Figure 4.12. However, NtTR2, 3, and 7 had demonstrated statistically lower plantlet formation rate where $p<0.1$.

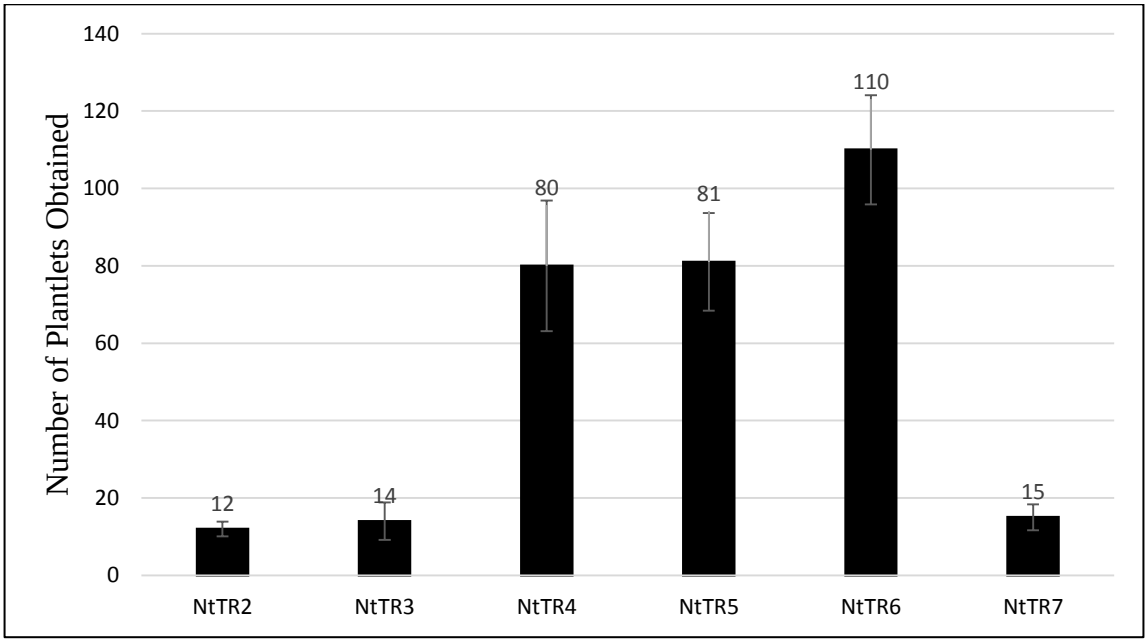

Figure 4.12. Plantlet formation among different media. According to ANOVA PostHoc test NtTR4, NtTR5, and NtTR6 were significantly different then NtTR2, NtTR3, and NtTR7 as $p<0.1$).

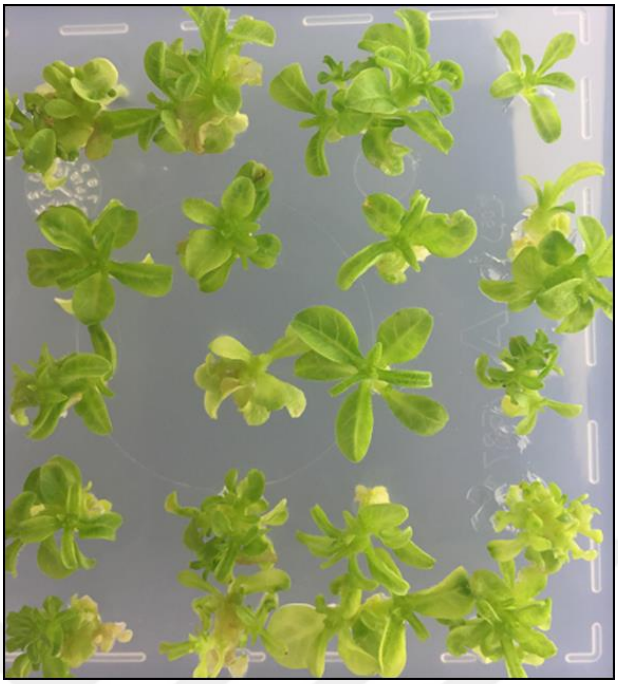

Figure 4.13. Regularly subcultured tobacco plantlets, ready for rooting process.

NtTR7's low organogenesis rate might be caused by cytokinin levels. For NtTR2, and 3 low amounts of cytokinin: auxin rate is the potential reason for small numbers of plantlets. The rates were respectively 10:1, 12.5:1 compared to NtTR4, 5, and 6 which were 15:1. 15:1 ratio of cytokinin: auxin can be the threshold to achieve multiple plantlets. Multiple plantlets later separated from each other and placed in MS1BACH media.

Counted micropropagation rates for three weeks old MS1BACH exposed plantlets were shown in Figure 4.12. Presently, high multiplication rates were obtained, and the reason might be the cytokinin: auxin ratio on shooting media. Micropropagation rate was more than 20 for the plantlets obtained from NtTR4, 5, and 6. However, it was less than 15 for the tissues obtained from NtTR2 and 3 as shown in Figure 4.14. Finally, after roughly 2 months with 3-week interval subculturing, multiplication rates decreased. For each plant multiplication rate was approximately 10 to 12, data not shown. All of the plantlets subcultured observed healthy without browning or crystallizing, and all of them were ready to be used in rooting.

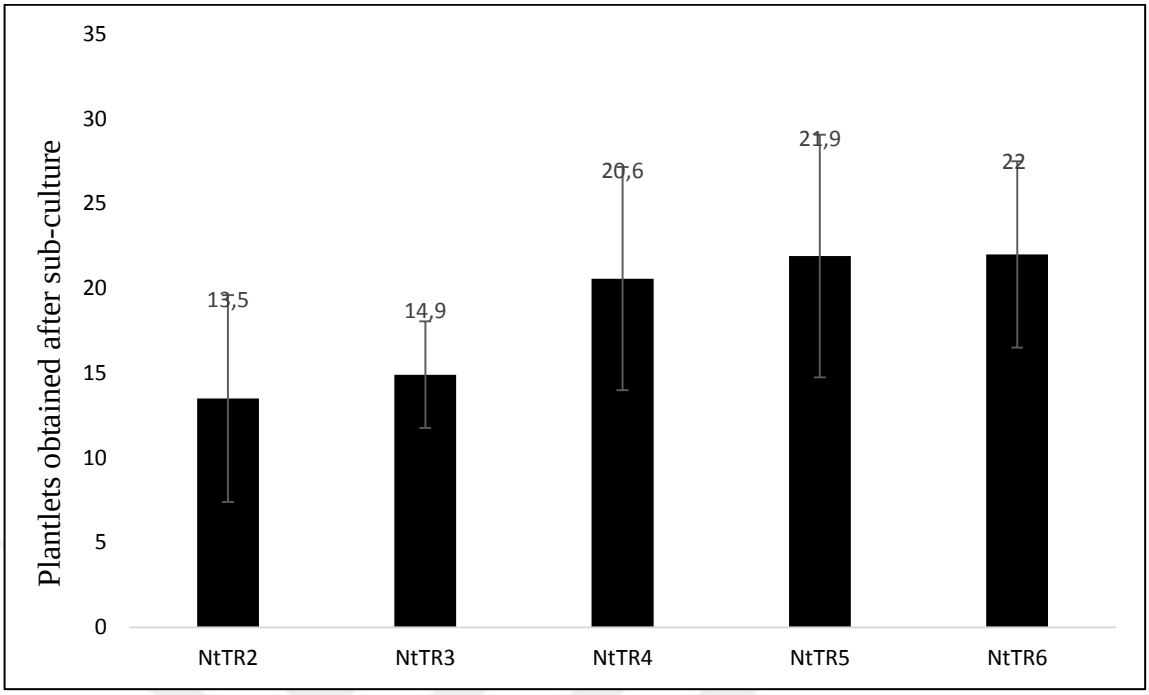

Figure 4.14. Plantlets obtained after micropropagation, numbers representing the amount of plantlets.

Figure 4.15. Stereo microscope image of the plantlets after three weeks of 1.0 mg/L BAP exposure.

A sharp decrease of cytokinin (BAP) concentration did not affect the tissue immediately shown in Figure 4.15. In the figure 4.15, from the single plant, there are more than 15 plantlets observed. Several subcultures are required to achieve a regular multiplication rate such as 10 to 12 plantlets from a single explant in three weeks. The absorbed cytokinin from the organogenesis media might stimulate plants for two more subcultures.

4.6. ROOTING AND ACCLIMATIZATION OF *NICOTIANA TABACUM* CV. XANTHI

By using totipotency of calli, to demonstrate somaclonal variation did not affect tobacco fertility, after shooting, tobacco shoots were rooted to observe the growth cycle. 3 mg/L NAA with 0.2 mg/L BAP was used in forming and maintaining the callus, by increasing the hormone concentrations to 4 mg/L's of NAA, IBA, and IAA, plantlets were induced rooting. Plants chosen for the rooting process were approximately 1 cm lengthened, and they adapted easily into the rooting media. All three hormones, NAA, IBA, and IAA demonstrated similar results of rooting with approximately 80 per cent in 30 days of the period shown in Figure 4.16.

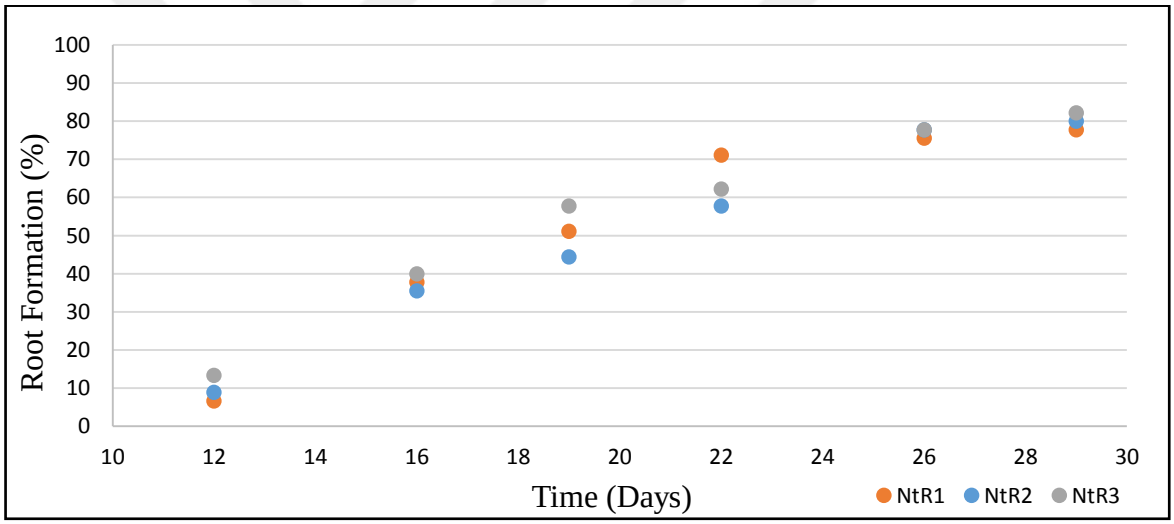

Figure 4.16. Rooting of the *Nicotiana tabacum* cv. Xanthi with different mediums.

Even though 30 days found enough to observe roots, 15 more days have waited to achieve bigger plants to ease acclimatization. After the 45 days in total, plants were grown, and roots were established. They were ready for acclimatization process.

Figure 4.17. Plants just before they proceeded to acclimatization process.

Due to the high auxin levels, some plants demonstrated root generation from above-rooting media. Again to mimic the soil, charcoal was used in rooting procedure.

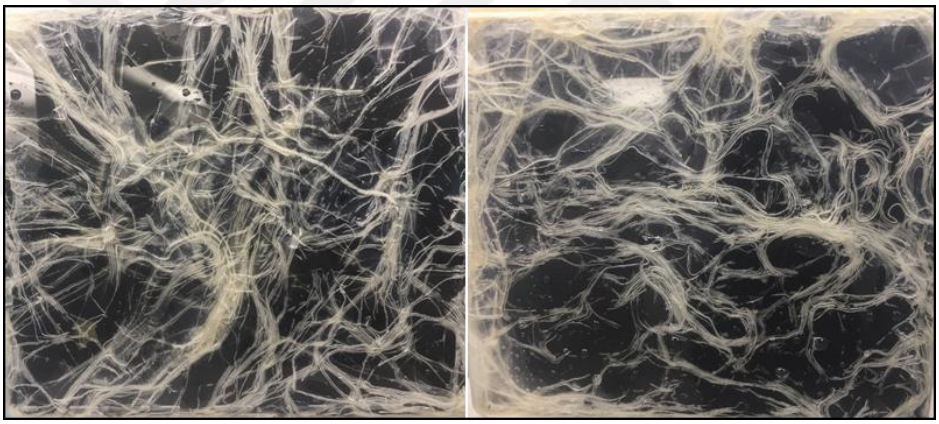

Figure 4.18. Rooted tobacco plants, bottom view of the rooting magenta.

Their stem size was approximately 10 cm as well as the roots (Figure 4.17 and Figure 4.18). Roots were gently removed from the media under distilled water, being gently is important, since roots are one of the most sensitive parts of the plant. The physiological properties of the whole plants looks similar to 2-month-old germinated seeds. While transferring plants, and clearing media, root strength was tested and roots generated with NtR3 media, containing 4 mg/L IAA, had the strongest roots among three media and almost all of the roots were healthy, however plants rooted in NtR2 and NtR1 medium lost most of their roots, they broke into small pieces. Not only rooting was successful, but also plants produced a lot of roots. Having too many roots attached to the media caused

difficulties in the separation process. Even though plants regenerated, look so healthy in Figure 4.19. In the Figure 4.20. some of the plants are seen with broken roots. As plants were growing, they absorbed the water from the media, waiting too much (45 days) in rooting, lead media to dry out which made the cleaning process difficult too.

Figure 4.19. Plants in the distilled water ready to clear remaining media from their roots.

Figure 4.20. Rooted tobacco plants ready to transfer into a mixture of peat and perlite.

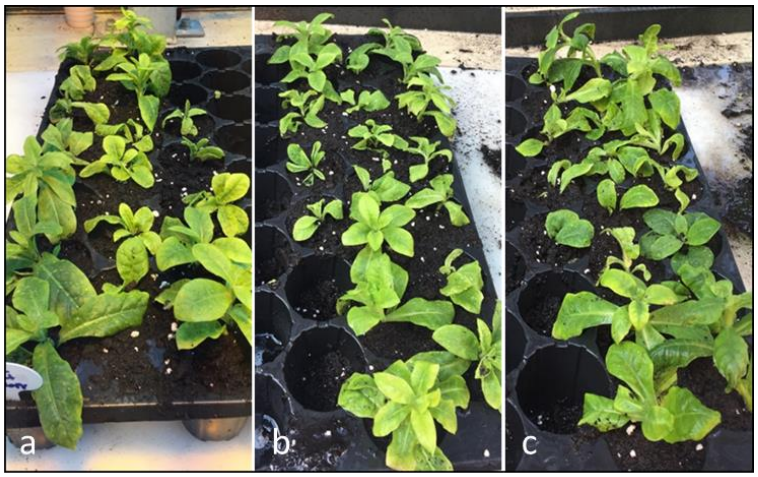

Figure 4.21. Tobacco plants transferred into soil mixture. (a from NtR1, b from NtR2, and c from NtR3).

A total of 19 plants from NtR1, 19 plants from NtR2, and 16 plants from NtR3 were selected, preferably healthy roots (Figure 4.21.).

In order to adapt harsh soil conditions after *in vitro* culture high humidity environment was preferred with the perlite, peat mixture with soil in a ratio of 2:1. Humidity gradually decreased from 90 per cent to 70 per cent in 15 days. After 15 days, approximately 95 per cent of the tobacco plants were adapted to soil and plants transferred to bigger pots shown in Figure 4.22.

Figure 4.22. Tobacco plants transferred into pots. (a, NtR1, b, NtR2 and c, NtR3).

Acclimatized tobacco plants started budding in 58th day after they were transferred to greenhouse conditions shown in Figure 4.23 and flowered at 66th day shown in Figure 4.24.

Figure 4.23. Budding of the *Nicotiana tabacum* cv. Xanthi coming from callus.

Figure 4.24. The flowering of the *Nicotiana tabacum* cv. Xanthi coming from callus.

To flower *Nicotiana tabacum* 4 months are enough, and by the time they were acclimated they were 2 months old, and 2 more months to flowering, all things considered, the life cycle was completed in regular time.

4.7. PRELIMINARY *NICOTIANA TABACUM* CV. XANTHI CALLUS CELL SUSPENSION CULTURE

Plant callus cell suspension cultures were produced with the aim of the secondary metabolite production. Initially, calli were grown on dark. 50 mL media with approximately 2.5-3 grams of callus were put in a 250 mL Erlenmeyer flask without cutting. Low amounts of liquid medium and not dissociation of calli resulted with big clumps of callus growing inside the suspension media. Calli absorbed the water in one week, almost all of the extracellular water was taken by the calli shown in Figure 4.25. Calli particles were continuously growing, which is an unwanted condition since cell suspension culture must consist of cells, but not cell clumps due to increasing surface area and secondary metabolite secretion. Changing the shake conditions from 80 to 100 did not result in cell suspension either.

Figure 4.25. Two weeks old *Nicotiana tabacum* cv. Xanthi leaf callus suspension culture.

Filling only twenty per cent of the flask was found not enough to achieve a cellular growth so that the liquid media amount increased to forty per cent. Furthermore, calli were cut down to achieve small pieces shown in Figure 4.26. This resulted with the more homogeneous mixture and small particles of callus, however, calli continued to grow in size.

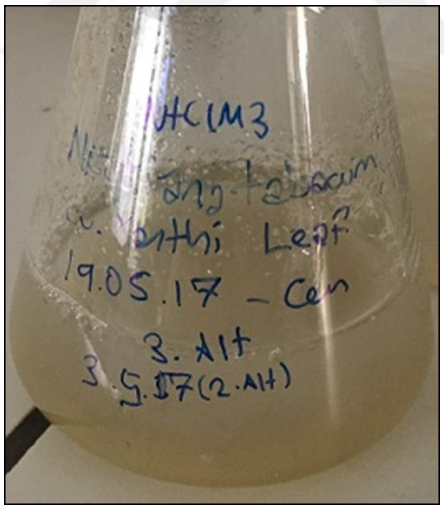

Figure 4.26. Callus had been cut into small pieces, more homogeneously distributed.

When plants were introduced to light, two different media used NtCIM1 and NtCIM3. In NtCIM1 which lacks casein hydrolysate, callus grown observed as lighter green, however, in NtCIM3 media callus grown had dark green color, demonstrating more chlorophyll production. Yet the growing as clumps was similar problem shown in Figure 4.27 and

4.28. After deciding to reduce calli size, green callus placed in media shown in Figure 4.29.

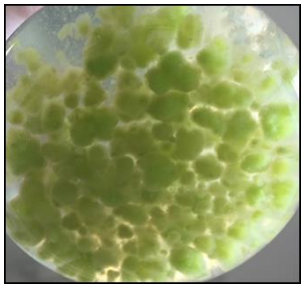

Figure 4.27. *Nicotiana tabacum* cv. Xanthi leaf cell suspension callus in NtCIM1 media.

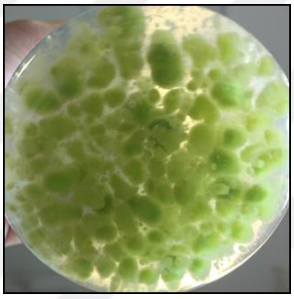

Figure 4.28. *Nicotiana tabacum* cv. Xanthi leaf cell suspension callus in NtCIM3 media.

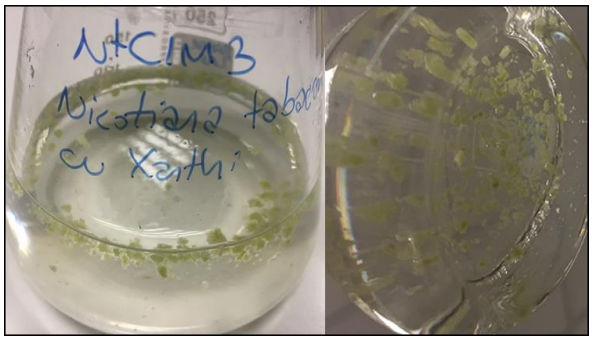

Figure 4.29. Callus cut into smaller pieces before placing it into the cell suspension culture. Top of flask photograph on the left, the bottom of the flask photograph on the right.

Even though liquid medium amount increased to forty per cent of the flask, calli were still absorbing the medium. However, the amount was enough to continue homogenously dispersion. Clump growing of the calli was still an issue, size increments were continued,

which disrupts homogeneity in another level, even though there is enough liquid. Calli cut down into small pieces to solve the issue while callus cell suspension exposed to light. It did not result in cell suspension either shown in Figure 4.30. Owing to the fact that big clusters needed to be eliminated.

4.8. ESTABLISHMENT OF *NICOTIANA TABACUM* CV. XANTHI CALLUS CELL SUSPENSION CULTURE

Because of the calli grow in size (Figure 4.30.), it was decided to find a way to get rid of big callus particles.

Figure 4.30. Slightly big calli grown in cell suspension media.

Callus filters were used to get rid of those big particles. After the big calli removed from the suspension culture, small calli were subcultured shown in Figure 4.31.

Small callus was obtained via filtration through a 0.5 to 1mm^2 circular mesh, however, after every 3 subcultures (approximately 21 days), some of the calli begin to grow as clumps again, so that, every 3-week callus filters were used to keep big cell clumps away from suspension.

Figure 4.31. After filtration via callus filters to get rid of big callus, small callus is ready to be subcultured.

After filtering cells via callus filters the big clumps were removed and, small callus particles are shown in Figure 4.32. The effect of auxin levels, inducing cell elongation, was a consideration as the reason of cell clumps. While growing the callus in solid media 3.0 mg/L NAA, and 0.2 mg/L BAP was used. The callus surface area that in contact with the medium of the cell suspension culture is higher than the callus surface area in contact with the medium in solid cultures. Because of that decreased auxin and cytokinins were tried to overcome a clumping problem.

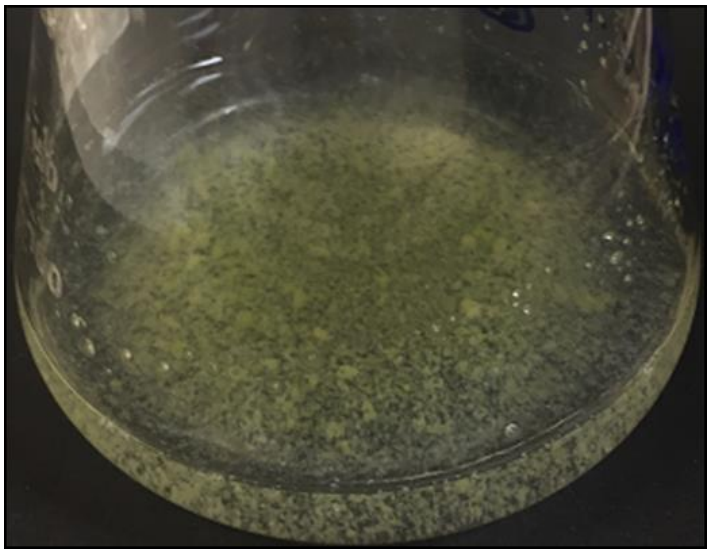

Figure 4.32. Callus after filtrating through callus filter.

However, decreasing the plant growth regulator concentrations more than 0.5 mg/L resulted in browning or decreased growing. So that, plant growth regulator concentrations are decreased gradually. Different media were used instead of NtCIM3 to observe plant growth regulators effect with NtCIM4, NtCIM5, NtCIM6, NtCIM7, NtCIM8, and NtCIM9. However, the only successful one was NtCIM5, after 20th subculture instead of NtCIM3 a medium, NtCIM5 containing 2.5 mg/L NAA and 0.2 mg/L BAP was used. Found out 2.5 mg/L NAA was a good decrease and callus started to grow as cells instead of clusters shown in Figure 4.33.

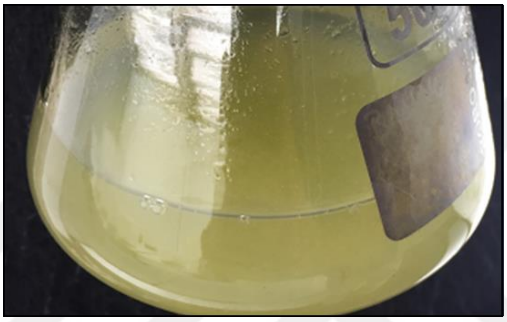

Figure 4.33. *Nicotiana tabacum* cv. Xanthi cell suspension culture in NtCIM5 medium, all cells were homogeneously and approximately single-celled.

The cellular suspension was achieved. With all the knowledge obtained from the leaf callus, hypocotyl callus cell suspension cultures were also established.

4.9. GROWTH OF CALLUS CELL SUSPENSION OF *NICOTIANA TABACUM* CV. XANTHI

The growth curve of callus was obtained through 11 days of growing cells and measuring fresh weights after filtration. Lag phase took 4 days, the 5th day is the initial of exponential phase and exponential phase ends around the 8th day. Tobacco cells were getting into stationary phase around the 11th day of subculturing shown in Figure 4.34. Whitehead *et al.*, found the application of elicitor for 4 days is optimum to produce secondary metabolites [22]. The best elicitation and yield of the desired molecule can be achieved after adding the molecule at late exponential phase, since the number of cells are a lot, and there is not any problem occurred due to lack of nutrition. In a crisis, plants usually

activate all defensive mechanism, it was thought to elicitate cells at 7th day until 11th day is the perfect combination.

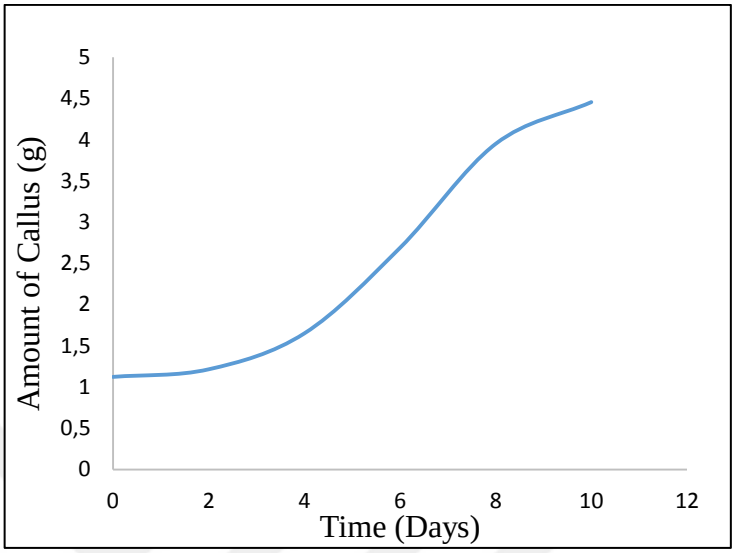

Figure 4.34. Growth curve of *Nicotiana tabacum* cv. Xanthi cell suspension culture.

4.10. MORPHOLOGICAL AND MICROSCOPICAL OBSERVATIONS OF *NICOTIANA TABACUM* CV. XANTHI CELL SUSPENSION CULTURES

4.10.1. Single Cell Observations under Light Microscope

Furthermore, microscopic examinations of tobacco cell suspension performed under several different microscopes. Firstly, under the light microscope suspension sample was observed, cell clumps observed as well as single cells. There were tangled-like structures. Elongated cells, thought the reason of high auxin concentrations. However, the results were matching with the previous studies Langbecker *et al*. [142] shown in figure 4.35. Cell clusters were seen and some cells were observed to be elongated.

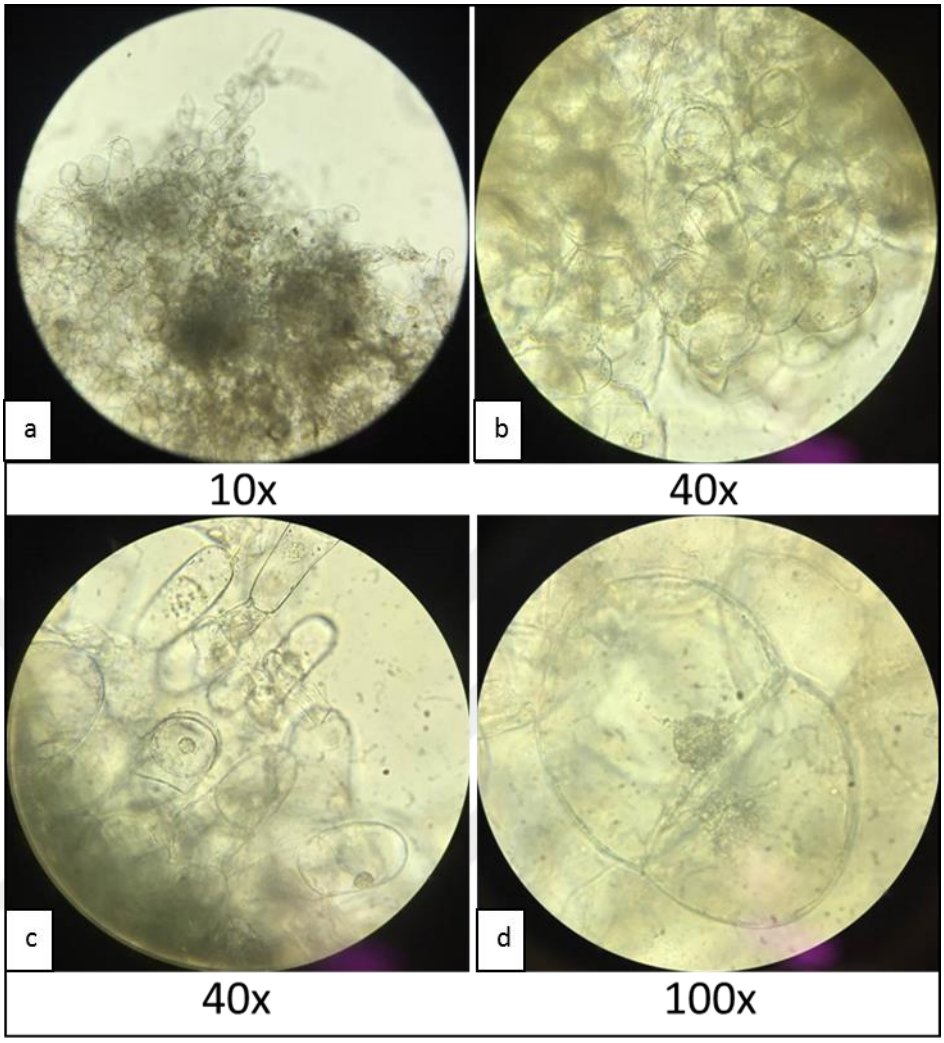

Figure 4.35. Light microscope images of *Nicotiana tabacum* cv. Xanthi cell suspension culture, (a) clustered cells all together in 10x magnification, (b) round shapes of 40x magnified clustered cells, (c) elongated and cylindrical shaped 40x magnified tobacco cells, (d) singe cell dividing in 100x magnification.

4.10.2. Scanning Electron Microscopy (SEM) Observations

Two different methods were tried to achieve fixation, drop by drop and constant methanol and scanning electron microscope photographs are shown in Figure 4.36., Figure 4.37 and Figure 4.38.

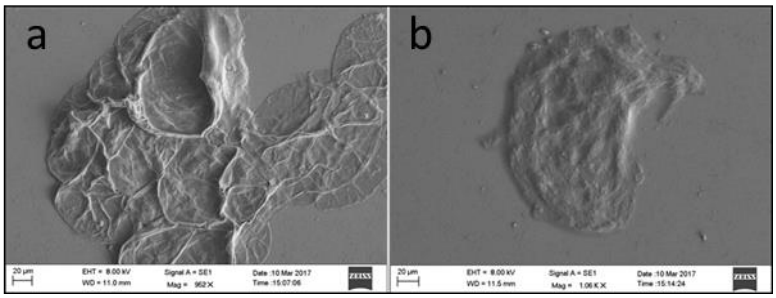

Figure 4.36. One drop of methanol in every 15-20 seconds fixation for 1 hour. a) Cell clusters, and b) single cell shown.

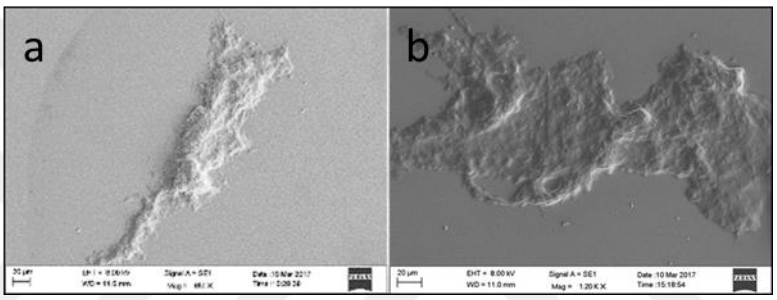

Figure 4.37. Constant methanol fixation for 1 hour. a) Singe-cell, b) three cells together.

The shape of the cells is more protected in constant fixation with methanol, yet shrinking due to vacuum environment while coating with gold is a common problem with SEM, rather Environmental-SEM can be used or new fixation techniques can be tried.

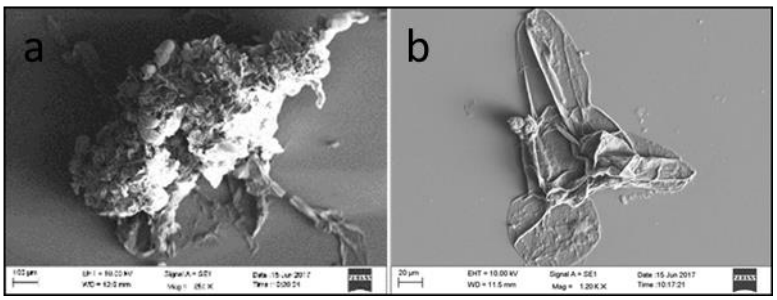

Figure 4.38. Constant methanol fixation for 1 hour. a) big cell cluster, b) 4-5 cells as a cluster.

Fixation performed with two different methods with methanol. Both methods demonstrated similar results. The constant exposure of methanol for one hour, slightly decreased the

shrinking rather than dropping methanol in every fifteen seconds, however, was not effective.

4.10.3. Viability Test via. Fluorescent Microscopy

The cells are tested whether they were dead or not with fluorescein diacetate test. Green cells were observed under a fluorescent microscope that demonstrated their viability as shown in Figure 4.39.

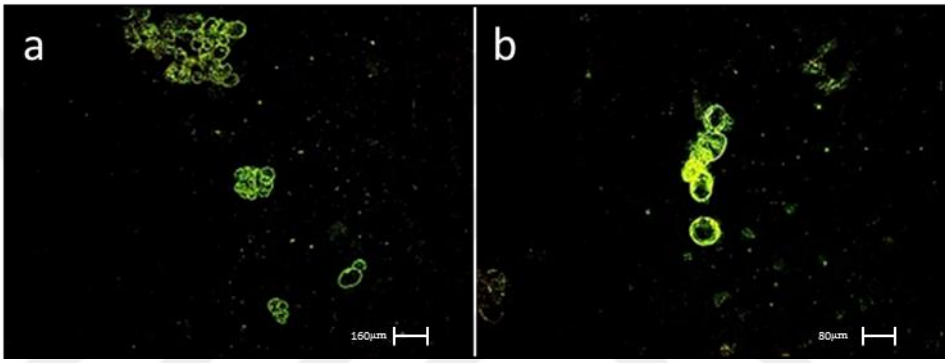

Figure 4.39. FDA test applied *Nicotiana tabacum* cv. Xanthi cell suspension cultures, showing alive cells, both clustered and single-celled.

Esterase's inside living cells digest FDA and release the fluorescein molecule which illuminates under UV light. The photographs of living cells were obtained from the fluorescent microscope. Also, dead cells were observed as they do not fluorescence, yet they were in a very small amount. By that way, cells were considered alive and functioning.

4.11. PRELIMINARY WORK #1: ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI, ORGANIC SOLVENT EXTRACTION AND HPLC ANALYSIS

After elicitation cells were separated from the media and media was collected. 1:1 media and CH_2CL_2 mixed and extracted, later dissolved as the final volume of 1 mL methanol. After running on HPLC results shown in following Figure 4.40. and Table 4.1.

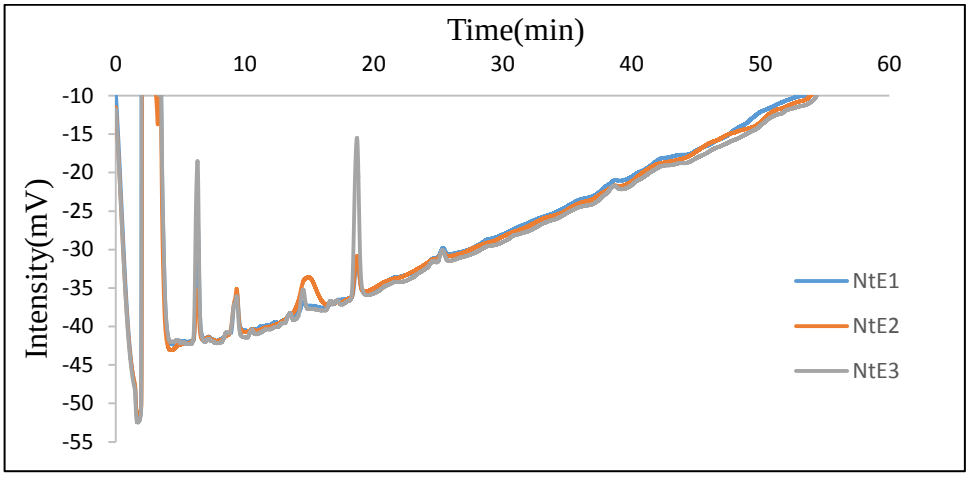

Figure 4.40. NtE1, NtE2, and NtE3 extracted chromatograms run in HPLC column was Agilent Poroshell 120 EC-C18.

Table 4.1. NtE3 injection and peaks with area under the curve results.

#	Time (min)	Area
1	6.341	445.4
2	9.383	219.6
3	13.542	30.9
4	16.674	46.6
5	18.729	550.9

The peaks obtained from NtE3 elicitated cell suspension media after extraction were collected for further MaS and activity analysis. After mass spectroscopy analysis 18.7th minute peak found out capsidiol. All NtE1, NtE2, and NtE3 treated cells demonstrated the peak at the 18.6th minute, first two media chromatogram results are not shown. And the peak at 18.7th minute followed an increasing pattern with the increase of yeast extract concentration.

4.12. PRELIMINARY WORK #2: ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI WITH HCL AS ELICITOR

The second elicitation was done with HCl as elicitor. Peaks observed with HPLC was showing differences. A similar column was used with shorter protocol, peaks shown in

figure 4.41. Due to the modifications on flow rate, the expected capsidiol peak must be around 9th minute. There were peaks observed at 9th minute, and 10 mM HCl did not produce a potential capsidiol peak. This might be because of high concentration. Similar, result obtained from 20 mM as well as 40 mM final concentration HCl induction. Not only, not inducing cells to produce capsidiol, HCl is killing to the cells with high concentrations.

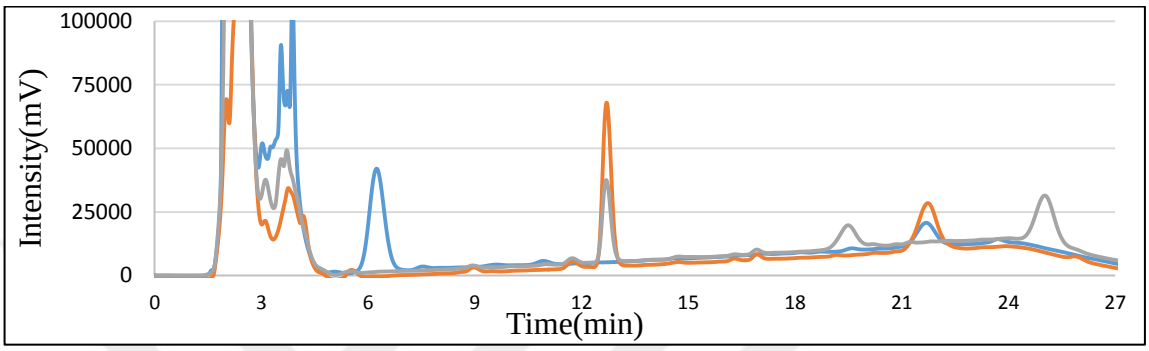

Figure 4.41. Combined image of different chromatograms, Blue 10 mM HCl, Orange 20 mM HCl, and Grey 40 mM HCl final concentrations.

4.13. PRELIMINARY WORK #3: ELICITATION OF *NICOTIANA TABACUM* CV. XANTHI DIFFERENT ELICITORS

The reason of using broad range to find effective elicitors, first elicitor used was yeast extract, there were different peaks observed similar to preliminary results.

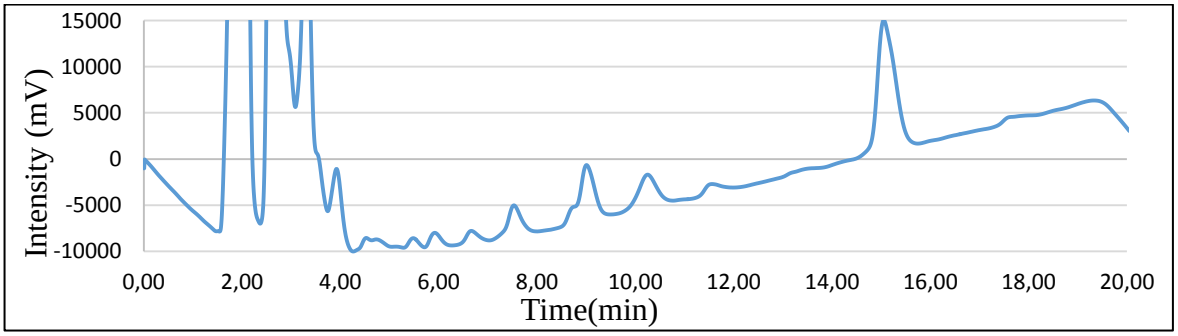

Figure 4.42. 20mL Hypocotyl cell suspension culture elicited with yeast extract 0.02 mg/mL final concentration chromatogram, after organic solvent extraction and run on HPLC.

The second elicitor used was salicylic acid; following Figures demonstrate different chromatograms of leaf and hypocotyl cell suspension cultures affected by 1mM SA.

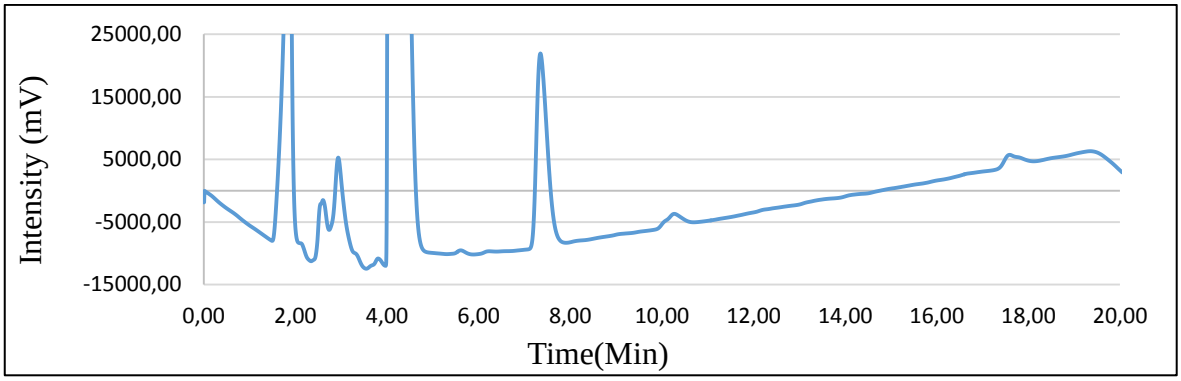

Figure 4.43. 10mL leaf cell suspension culture elicited with salicylic acid 1 mM final concentration, chromatogram, after organic solvent extraction and run on HPLC.

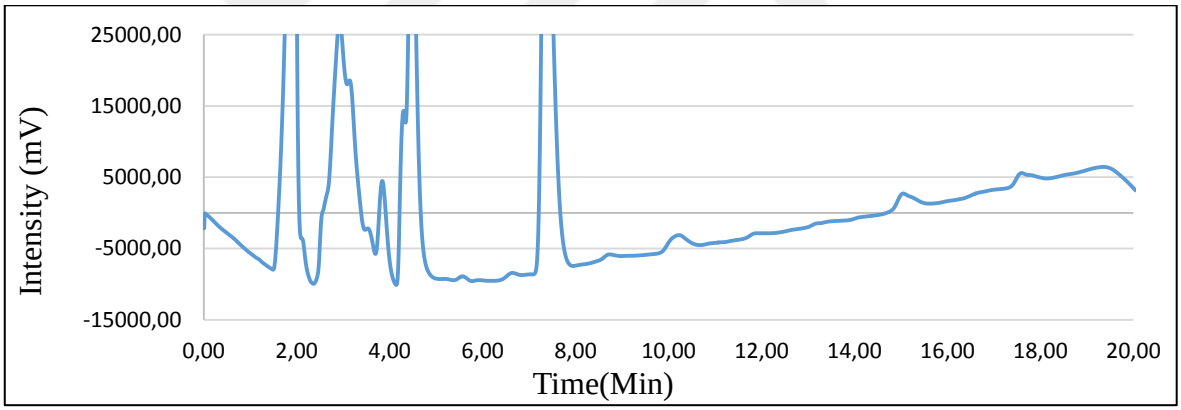

Figure 4.44. 20mL Hypocotyl cell suspension culture elicited with salicylic acid 1 mM final concentration, chromatogram, after organic solvent extraction and run on HPLC.

According to Literakova *et al*. capsidiol observed with HPLC has retention time at the 6th minute. However, their column was C8 which is less hydrophobic than C18, and particle sizes were bigger. Peak coming at 7th minute was considered as potential capsidiol compared to Literakova *et al*. Hydrochloric acid, tween 20, and hydrogen peroxide did not found as effective as yeast extract and salicylic acid while inducing capsidiol. After elicitors, the media itself was extracted and observed. As a preliminary work, low and highly concentrated (were not calculated) plant growth regulators analyzed. Peaks observed from hormones, for media, there was not a significant change observed.

4.14. ELICITATION WITH SALICYLIC ACID, STANDARDS, AND MOBILE PHASE OPTIMIZATIONS

Different methanol trials were done to optimize mobile phase. As similar with the literature [33], best methanol gradient found gradually increasing from 70 per cent to 80 per cent. Acetonitrile trials demonstrated best acetonitrile gradient is from 80 to 90 percent.

When acetonitrile was used as mobile phase, sharper peaks observed, and best peak found acetonitrile gradually increasing from 80 per cent to 90 per cent. Owing to that results all following SA elicited HPLC analysis performed with 80-90 ACN. Hormones are also soluble in organic solvents so that from the media they are thought to come to extract mixture. BAP and NAA were used with different gradients with 80-90 per cent acetonitrile mobile phase to obtain standard curve and an equation to observe the exact amounts of hormones left in the media (Figure 4.45 and Figure 4.46).

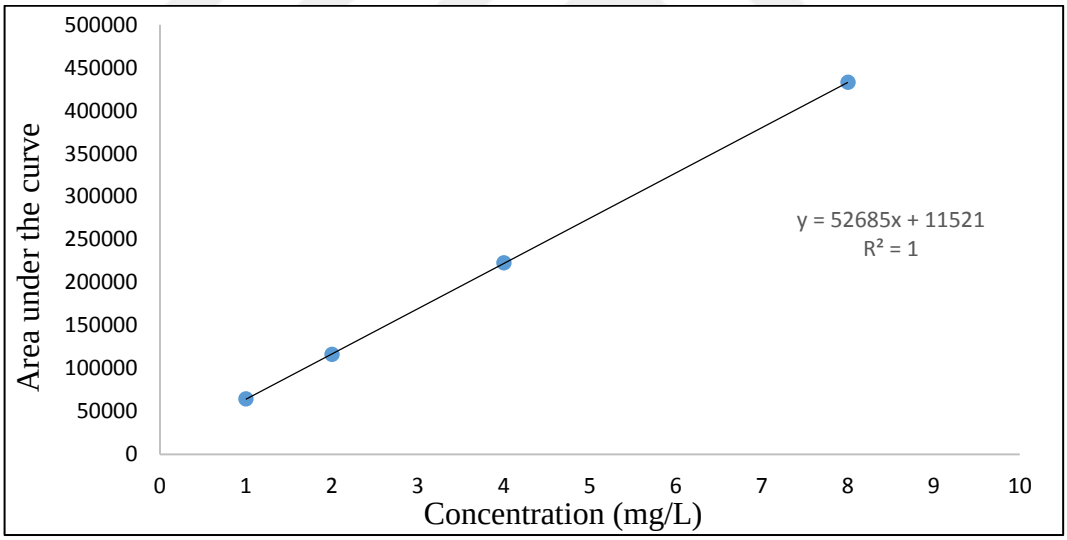

Figure 4.45. NAA concentration vs. area under the curve, calibration curve and equation.

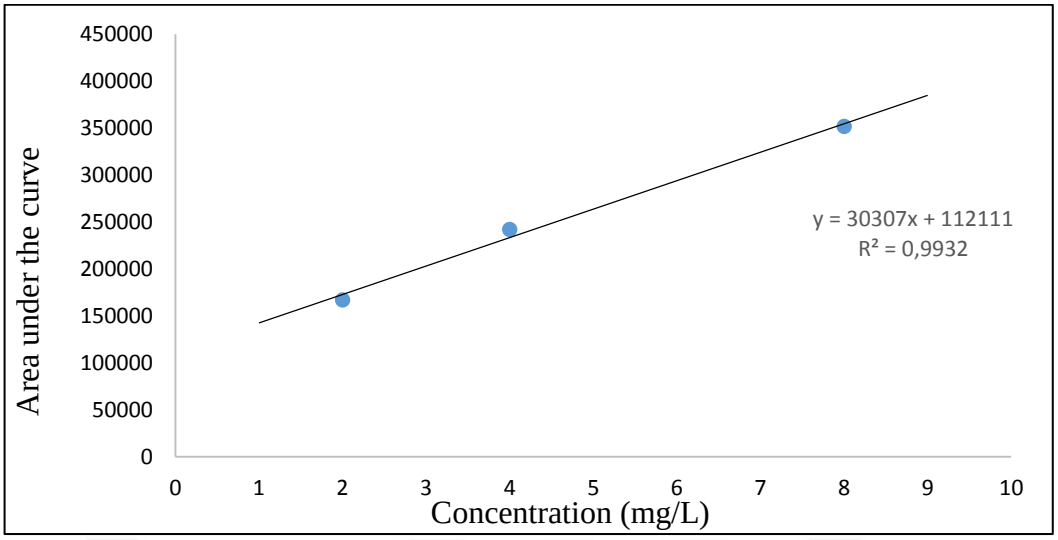

Figure 4.46. BAP concentration vs. area under the curve calibration curve and equation.

Different NAA and BAP concentrations prepared to obtain a graph to measure the amounts of hormones if needed. After the runs with 80-90 ACN, all hormones observed to come before minute 3. The salicylic acid produced the potential capsidiol. Owing to that, different salicylic acid concentrations were experimented on cell suspension to find the best capsidiol producing concentration.

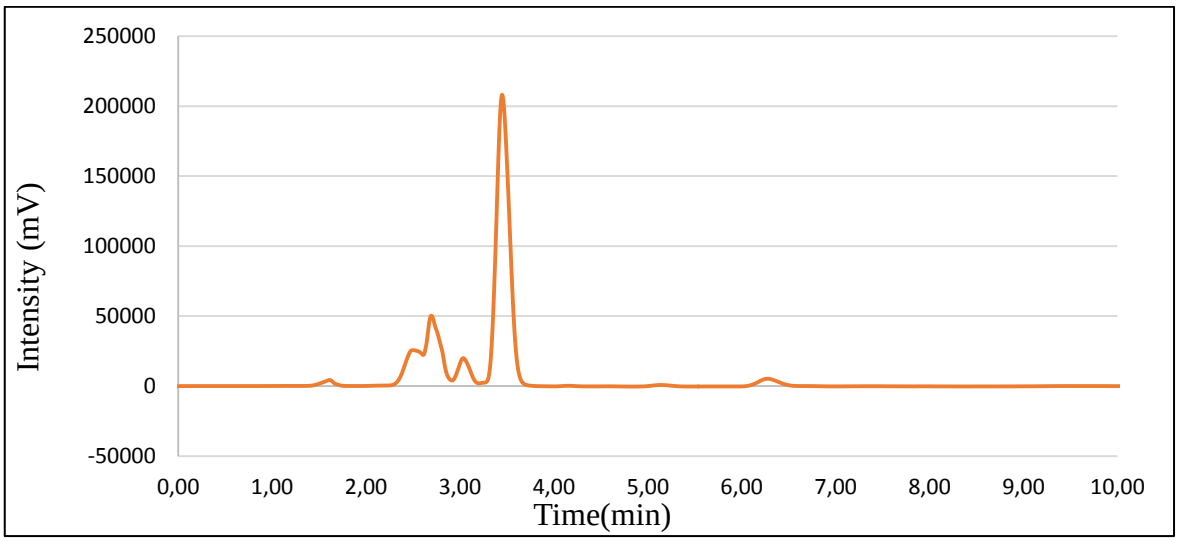

Figure 4.47. *Nicotiana tabacum* cv. Xanthi leaf cell suspension culture treated with 0.5 mM SA.

The best production of the potential capsidiol was obtained from 0.5 mM salicylic acid (Figure 4.47) after covering a wide range of salicylic acid concentrations, peak coming at 3.3rd minute was considered as capsidiol. Previous peaks were hormones demonstrated in BAP and NAA characterization Figures. Concentrations lover than 0.5 mM SA demonstrated the low generation of capsidiol, and concentrations higher than 0.5 mM SA exhibit damaging effect on both plant and metabolites. With the last change in mobile phase potential capsidiols' retention time found the 3.3rd minute. With various trials, 0.5 mM SA concentration found the best capsidiol producing concentration. Different than the control, the rate of potential capsidiol production increased almost 10 times with 0.5 mM SA concentration than 0.125 mM SA concentration. However, after increasing the SA concentration more than 0.5 mM resulted in decreasing potential capsidiol concentration. High concentration of SA might kill the cells instead of inducing more potential capsidiol. In between 1 to 5 mM SA concentration cells also produced capsidiol, however, the amounts were 50 to 100 times less than 0.5 mM. 5 mM final concentration of SA considered as threshold concentration since it demonstrated similar results with a control.

4.15. ACTIVITY ANALYSIS OF THE EXTRACTS

Capsidiol has a fungicide effect over *B. cinera*, *F. oxysporum, F. vasinfectum*, *C. herbarum*, *T. viride*, *P. frequentas*, and *M. fructicola* [25] *P. Capsici*, *P. Infestans* [34]. Since there are many fungi capsidiol affects, A*spergillus niger* was tested. However, there was not any antifungal effect observed on *Aspergillus niger* since it does not belong Phytophthora family shown in Figure 4.48. And 4.49. Methanol control also did not effected (Figure 4.50).

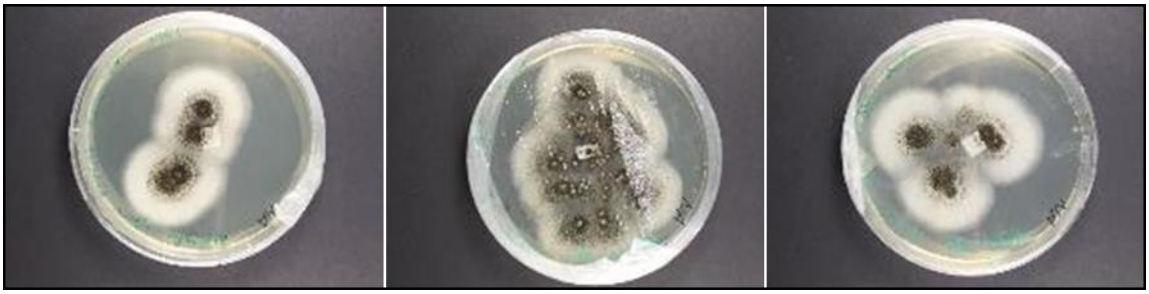

Figure 4.48. *Aspergillus niger* growth on LBA with soaked extract papers. From left to right, NtE1, NtE2, and NtE3.

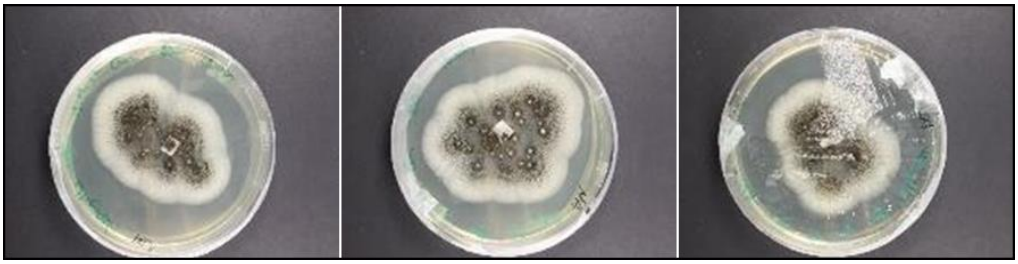

Figure 4.49. *Aspergillus niger* growth on LBA with soaked extract papers. From left to right, 13th minute of the chromatogram, 16th minute of the chromatogram, and 18th minute of the chromatogram.

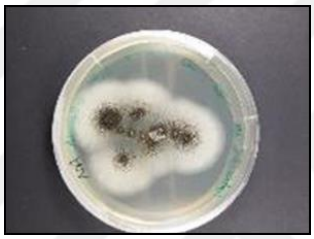

Figure 4.50. *Aspergillus niger* growth on LBA with soaked extract papers. Methanol control.

All fungi covered the capsidiol absorbed sterile filter papers. The amount of capsidiol might be the reason for the unsuccessful effect, or the fungi *Aspergillus niger* was not damaged by capsidiol.

4.16. MAS ANALYSIS OF THE EXTRACTS

MaS analysis was done for the samples obtained from preliminary #1 experiments demonstrated on the Figure 4.57., Figure 4.58., Figure 4.59., Figure 4.60., and Figure 4.61. With the increasing concentration of yeast extract, increased production of the peak at 18.7th minute observed. It was thought to be capsidiol. After analyzing all the peaks with MaS, m/z ratio 202 observed in all the collected peaks, was not considered as a part of capsidiol (Figures 4.51, 4.52, 4.53, 4.54). However, as a difference, peak at the 18th minute demonstrated m/z ratios of 173, 185, 201, and 225 shown in Figure 4.55. Those molecules prove that peak at 18th minute is possible capsidiol.

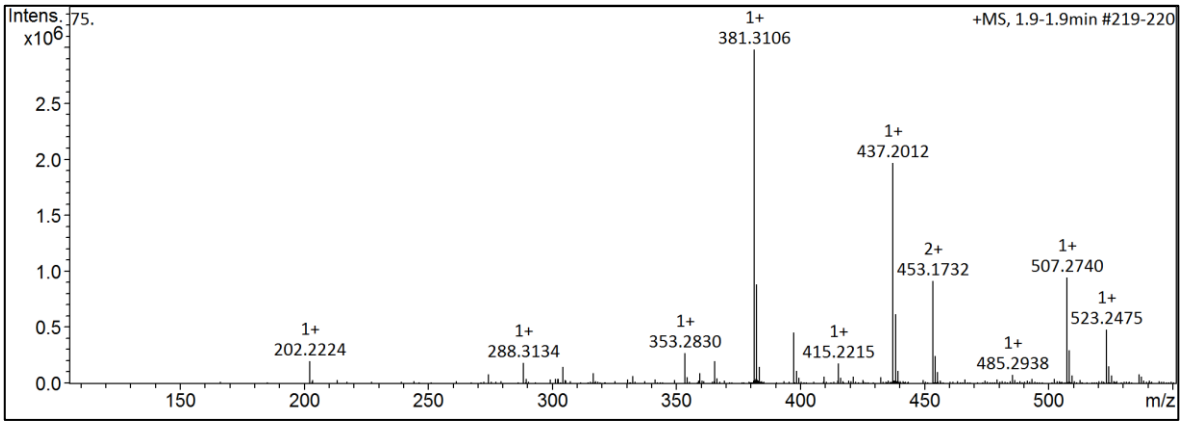

Figure 4.51. Yeast extract (1 g/L) elicited cell suspension extracts 6th minute HPLC peaks.

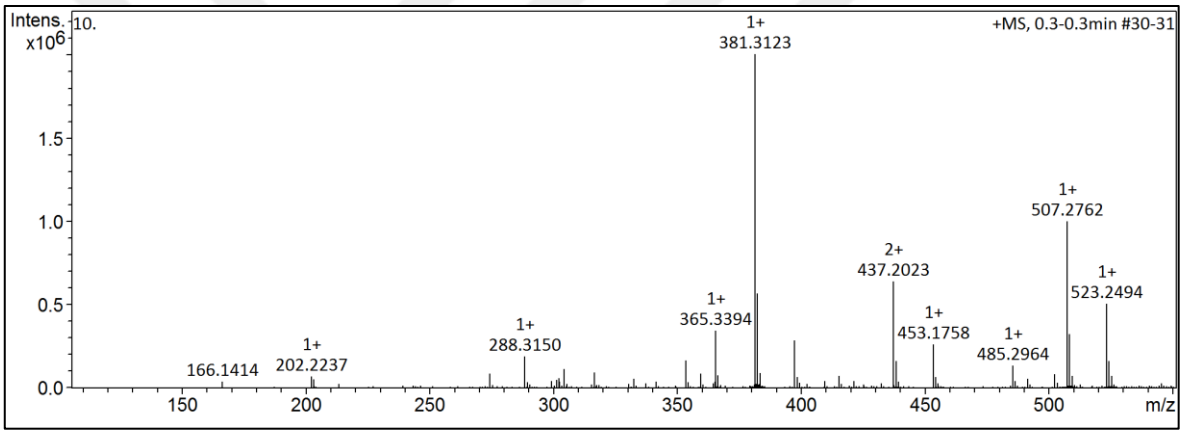

Figure 4.52. Yeast extract (1 g/L) elicited cell suspension extracts 9th minute HPLC peaks.

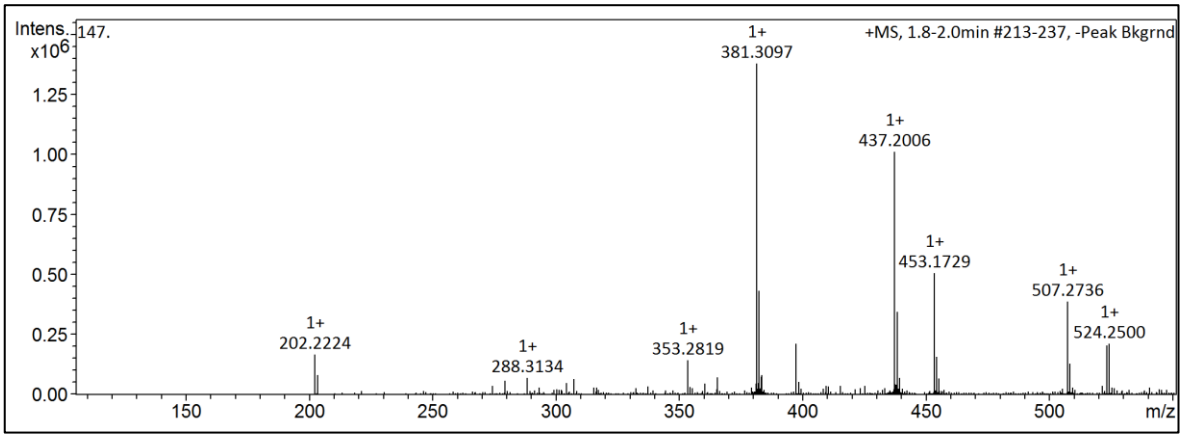

Figure 4.53. Yeast extract (1 g/L) elicited cell suspension extracts 13th minute HPLC peaks

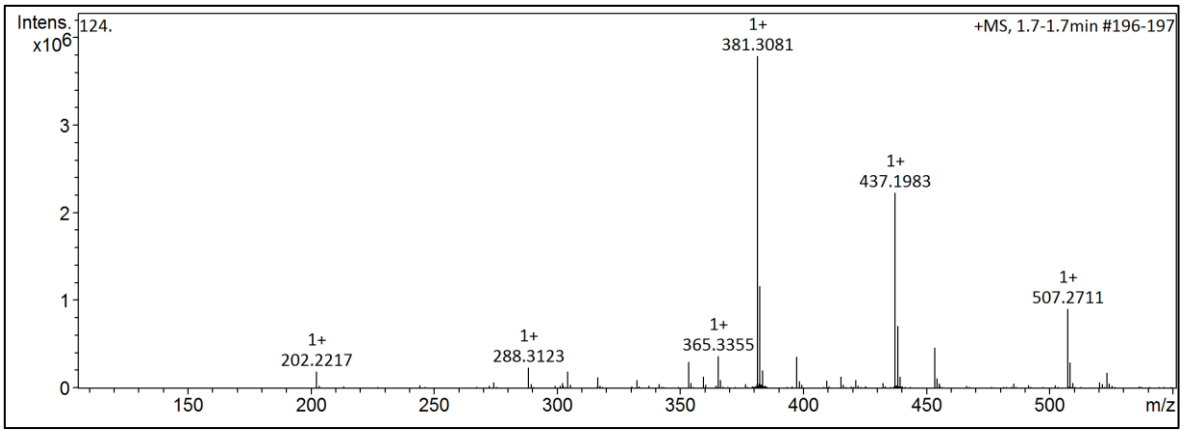

Figure 4.54. Yeast extract (1 g/L) elicited cell suspension extracts16th minute HPLC peaks.

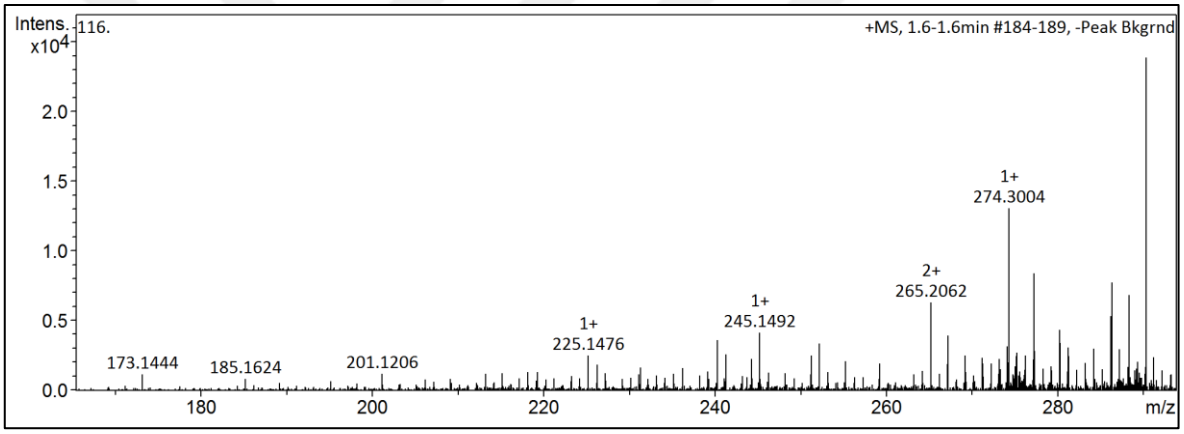

Figure 4.55. Yeast extract (1 g/L) elicited cell suspension extracts 18th minute HPLC peaks.

Literakova *et al*. (2010) found LC-MaS results as 173, 179, 201, and 219 m/z ratios, similar peaks observed in our results. According to Table 4.2. and Figure 4.56. m/z ratios obtained from the 18th-minute peak for 1 g/L yeast extract elicited tobacco cells are giving possible capsidiol.

Table 4.2. Possible MaS results' molecular weights and chemical formulas.

Molecular Weight (g/mol)		Chemical Formula
a	236.3513	$C_{15}H_{24}O_2$
b	219.3439	$C_{15}H_{23}O$
c	202.3365	$C_{15}H_{22}$
d	187.3018	$C_{14}H_{19}$
e	172.2671	$C_{13}H_{16}$
f	188.3098	$C_{14}H_{20}$
g	187.3018	$C_{14}H_{19}$
h	173.2751	$C_{13}H_{17}$
i	161.2644	$C_{12}H_{17}$
j	146.2297	$C_{11}H_{14}$

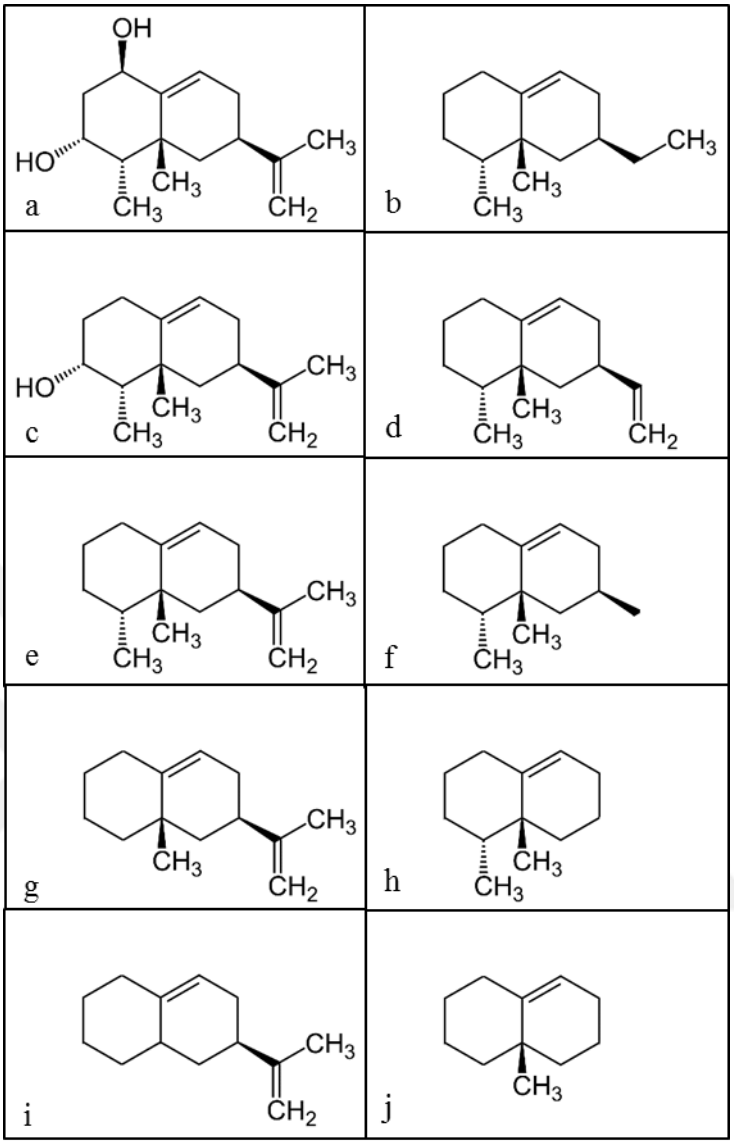

Figure 4.56. Possible MaS results.

After the examination, it was found out 18th-minute peak is potential capsidiol.

5. CONCLUSION AND FUTURE PROSPECTS

Nicotiana tabacum cv. Xanthi seeds demonstrated higher germination rates with the presence of charcoal and casein hydrolysate apart from only basal salt mixtures.

Callus induction with the application of auxin and cytokinin concentrations at 3 mg/L NAA and 0.2 mg/L BAP was achieved successfully. Moreover, casein hydrolysate addition into this callus induction medium produced softer and greener callus. Leaf explants demonstrated the highest callus generation rate with 95 per cent in 21 days. By weighing fresh tissue among time (fresh weight), the callus growth curve was constructed, providing better subculture timescale.

Indirect organogenesis was succeeded by reversing the concentrations of plant growth regulators and also slightly decreasing the cytokinin amount used in the callus formation medium. Rooting was achieved by increasing the auxin amounts to 4 mg/L. The three different plant growth regulators (IAA, IBA, and NAA) used in these experiments showed similar results with 80 per cent rooting rate.

With the adaptation (acclimatization) of regenerated plants in greenhouse conditions, ultimately tobacco plants completed their life cycle. The period of time needed to reach the flowering stage of callus culture was approximately four months.

Cell suspension culture was established by cutting the callus into small pieces and filtering through 'callus filters'. Environmental parameters, which were set to the constant shaking at 100 rpm and 25^{o}C under light (24h), maintained the growth of cell suspension culture. Cell viability test using FDA was applied on cell suspension culture and cells were observed with fluorescein molecule demonstrating the viable cells.

The growth curve of cell suspension culture was also constructed. Elicitor addition time was found as 7th day after subculture since the cells reached late logarithmic phase at that day. Yeast extract, hydrochloric acid, tween 20, salicylic acid and hydrogen peroxide were used as elicitors to induce the capsidiol production in cell suspension culture.

Organic solvent extraction was performed with dichloromethane from elicited cell suspension culture medium. Extract contents were separated on reversed phase HPLC with

C18 column and UV detector and analyzed via MaS with ESI. Yeast extract (1 g/L), and salicylic acid (0.5 mM) were found to be the best elicitor candidates for capsidiol accumulation in tobacco cell suspension culture. At 173, 179, 201, and 219 m/z ratios were obtained to demonstrate potential capsidiol production.

Since all of the tissue culture techniques are settled, this study can be a strong reference to future transgenic studies.

In further studies, new elicitors such as methyl jasmonate (MeJA) can be used to increase the production amount of capsidiol in tobacco cell suspension culture. With the more understanding about mevalonate pathway as a capsidiol production precursor, specific elicitors can be chosen to improve capsidiol production. Besides, *Phytophthora infestans* infected plants can be used together with the purified capsidiol to observe its antifungal effect.

There are many laboratories working on prevention of potato blight, and other *Phytophthora* family causing diseases. Capsidiol is found out as a biological response for *Phytophthora*-related diseases. Capsidiol might be the final organic solution for several diseases related to agricultural problems.

To sum up, this study will be a base for large-scale production of capsidiol from tobacco cell suspension cultures. Consequently, characterization of production and large-scale manufacturing of the capsidiol is the ultimate future goal.

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