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Production of Phenolic Compounds in Cell Suspensions of Arctic Bramble and Cloudberry

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Monet teollisuuden haarat ovat kiinnostuneita fenoliyhdisteistä, joita löytyy eriävissä määrin ja laaduin monista kasvilajeista. Suuria pitoisuuksia näitä yhdisteitä on havaittu etenkin pohjoismaalaisista marjoista. Näiden luonnolliset sadot ovat kuitenkin erityisen haavoittuvaisia kasvuympäristön muutoksille, minkä seurauksena mahdollisia menetelmiä arvokkaiden fenoliyhdisteiden tuottamiseksi bioreaktorikasvatuksissa tutkitaan. Fenoliyhdisteiden biosynteesireittejä ei kuitenkaan tunneta hyvin, ja niiden pitoisuudet ovat hyvin riippuvaisia kasvuolosuhteista sekä käytetystä solulinjasta. Vaikkakin monien marjalajien fenoliprofiilit ovat varsin hyvin määritelty aiemmissa tutkimuksissa, ovat monien marjalajien solususpensiolinjojen profiilit pitkälti kartoittamatta. Solususpensioiden edut fenoliyhdisteiden valmistuksessa ovat huomattavat verrattuna perinteiseen kasvatukseen, mutta eri solulinjojen tuotantokyvyt tarvitsevat tutkimusta, sekä tarvitaan parempi ymmärrys fenolien biosynteesireiteistä, ennen kuin niitä kyetään tehokkaasti hyödyntämään teollisuudessa.

Opinnäytetyössä toteutettiin perustutkimusta koskien useamman mesimarjan (*Rubus arcticus*) sekä lakan (*Rubus chamaemorus*) solususpensiolinjan kasvuominaisuuksia, fenoliyhdisteprofiileja, sekä fenolien biosynteesireittien muokkaamista. Biosynteesireittejä muokattiin kahdella eri elisiittorilla, metyylijasmonaatilla sekä etefonilla. Fenoliyhdisteet analysoitiin kasvusyklien ja elisitaatioiden eri vaiheissa UPLC-MS –menetelmällä, jotta saatiin kuvaavaa tietoa fenoliprofiilien muutoksista ajan suhteen.

Saadut tulokset osoittavat huomattavaa vaihtelua kasvuominaisuuksissa ja fenoliprofiileissa myös samaa lajia edustavien solususpensiolinjojen välillä. Molempien elisiittoreiden havaittiin lisäävän fenolituotantoa mesimarjan solususpensiossa. Etefonilla elisitoidessa havaittiin myös muodostuneen yksi uusi ennalta tuntematon yhdiste. Tarkastelluista solususpensiolinjoista mesimarjan todettiin poikkeuksetta sisältävän enemmän fenoliyhdisteitä kuin lakan.

Avainsanat	lakka, mesimarja, UPLC-MS, fenoliyhdisteet

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Phenolic compounds are of interest for various industries, and can be found in differing qualities and quantities between plant species, with particularly high concentrations observed in Nordic berries. The natural yields of these berries are highly vulnerable to environmental conditions, and consequently methods for producing these valuable compounds in bioreactors are being investigated. However, the phenolic content in all cultures is highly dependent on growth conditions and the used cell line, and the synthesis pathways leading to these compounds are poorly understood. Although the profiles of phenolic contents for certain berry species have been well characterized in literature, the profiles for cell suspension lines are largely unknown. Cell suspension cultures have significant advantages for production of phenolic compounds over traditional agricultural methods, and more research on the cell suspensions' production capabilities and a better understanding of the biosynthesis pathways is needed before utilization in the industries can be achieved.

In this thesis, basic research was made on several arctic bramble (*Rubus arcticus*) and cloudberry (*Rubus chamaemorus*) cell suspension lines regarding their growth characteristics, profiles of phenolic compounds, and modification of the synthesis pathways. The pathways were stimulated with two elicitors, methyl jasmonate and ethephon. The phenolic compounds were analysed by UPLC-MS during different stages of the growth cycle and the elicitations, to display representative changes in the profiles of the different cell lines over time.

The obtained results show substantial variations in the growth rates and the profiles of phenolic compounds even within cell suspension lines of the same species. Both elicitors were found to increase production of phenolic compounds, and one unidentified novel phenolic compound was found to be synthesized by stimulation with ethephon in an arctic bramble cell suspension line. Of the two species, arctic bramble was invariably found to contain more phenolic compounds than cloudberry.

Keywords	cloudberry, arctic bramble, UPLC-MS, phenolic compounds

Content

Abbreviations

AU	absorbance unit
BEH	ethylene bridged hybrid
CA	cellulose acetate
DAD	diode array detection
DMSO	dimethyl sulfoxide
DW	dry weight
ESI	electrospray ionization
ET	ellagitannin
FDA	fluorescein diacetate
FW	fresh weight
GFP	green fluorescent protein
GH	greenhouse
KAS ###	VTT plant cell line code
KN	kinetin and 1-naphtaleneacetic acid
LC	liquid chromatography
MeJa	methyl jasmonate
MS	mass spectrometry
NAA	1-naphtaleneacetic acid
RT	retention time
UPLC	ultra-performance liquid chromatography
UV	ultraviolet

1 Introduction

This bachelor's thesis was carried out as a part of a larger, Academy of Finland-funded BERRYTANNIN-project performed at VTT Technical Research Centre of Finland Ltd. The main objective of the BERRYTANNIN-project is to characterize and increase biodiversity of ellagitannins (ETs), a class of polyphenols found in cloudberry and arctic bramble, and to understand the molecular mechanisms that regulate their synthesis by using cell and organ cultures as research tools.

Arctic bramble and cloudberry are part of a ubiquitous *Rubus* genus of the *Rosaceae* family, which is commonly known as the rose family of plants. They are flowering plants that produce bramble fruits, which are aggregations of stone fruits. Both arctic bramble and cloudberry are distributed throughout the northern hemisphere, and in Europe they can be found in the Baltic countries and Fennoscandinavia. The demanding growth conditions of the north, where the plants are exposed to alternating weather conditions and daylight cycles, result in abundant production of secondary metabolites, i.e. molecules that are not directly involved in plants' basic life functions. One group of these phytochemicals is the phenolic compounds: a group that has been associated with a variety of bioactivities not only helpful for the plant, but also for human health. There is evidence that phenolic compounds can help in prevention of several cancers and cardiovascular diseases, and have been found to act as antimicrobial and antioxidative agents [1-8]. Phenolic compounds have also been found to modulate intestinal bacterial population of humans, and to inhibit the growth of a variety of intestinal pathogens [9-11]. Evidence suggests that the health benefits of phenolic compounds could be attributed to their subsequent bioactive metabolites produced by the individual's gut microbiota [11, 12].

With all the health benefits associated with phenolic compounds, it becomes evident that berries rich in phenolic content are an important raw material for medical, food, beverage and cosmetic industries. The natural yield and crop quality of Nordic berries of the Rubus genus are, however, highly vulnerable to fluctuating temperatures, weather, and right timing of pollination. Arctic bramble and cloudberry are sensitive to pests and pathogens [13, 14], and modern forestry and agriculture have additionally contributed to a reduced natural habitat of the plants. Furthermore, plants from particular biotopes can be hard to grow outside of their local ecosystems, and cannot withstand large field cultivations due to sensitivity to pathogens [15].

The importance of phenolic compounds to various industries, and the susceptibility of the berry yields have resulted in an interest in biotechnological production of the valuable compounds, and the development of cultivars more tolerant towards environmental changes.

For the biotechnological production of phenolic compounds, it is important to understand how the plants synthesize these molecules. Many of the pathways leading to certain phenolic end products, including ETs, however, are largely uninvestigated. As the secondary metabolites are synthesized for protection, the metabolic pathways can be promoted by imposing stressful growth conditions onto the plant by biotic or abiotic inducers. Useful platforms for the research of the pathways are plant cell suspensions, in which the phenolic compounds are moderately easy to elicitate, extract and identify. The chromophores of the phenolic compounds –their aromatic structures and hydroxyl groups, result in distinctive ultraviolet (UV) spectra. As phenolic compounds also form complex conjugates with sugars and organic acids, they are commonly analyzed by their mass spectrometric (MS) data and UV spectra, in combination with reversed-phase chromatographic separation due to their high polarity. [16-18]

The objective of this thesis was to characterize profiles of phenolic compounds and their changes in multiple cell suspension lines of arctic bramble and cloudberry throughout their growth cycle, as well as to study parameters of the growth cycle itself. In addition, the production of phenolic compounds in one cell suspension line of arctic bramble was elicited by methyl jasmonate (MeJa) and ethephon, and its phenolic profile likewise characterized under these modified growth conditions. The phenolic compounds were extracted with methanol, and the sample contents were analyzed by UPLC-DAD/ESI^+-MS. The theoretical part of the thesis focuses on general information about cloudberry and arctic bramble, their phenolic compounds, and biomodification of the secondary metabolism.

THEORETICAL PART

2 Nordic Berries of the Rubus Genus

Rosaceae family, commonly known as the rose family, are flowering plants that are distributed globally, but are most diverse in the Northern hemisphere. Widely known species of *Rosaceae* include apples, cherries, and plums. One of the genera classified into the family is the *Rubus* genus that includes many of the edible berries found in Finland. *Rubus chamaemorus* (cloudberry), *Rubus arcticus* (arctic bramble), and *Rubus idaeus* (raspberry) to name a few, have traditionally been staple seasonal foods in the North.

Figure 1. Cloudberry [19] and arctic bramble [20].

The cloudberry is a perennial plant found all over Finland. It grows in wet habitats, including bogs and marshes. They can withstand temperatures as low as -40 º Celsius, and consequently are one of the northernmost flowering plants. Cloudberry is sensitive to dry and salty conditions.

Arctic bramble thrives in more acidic conditions than cloudberry. The plant is distributed throughout Finland, with largest yields found in Northern Ostrobothnia and Eastern Finland. Arctic bramble is widely regarded as one of the tastiest berries found in Fennoscandinavia.

3 Calli and Cell Suspensions

Plant calli (singular *callus*) are biochemically totipotent, unstructured masses of parenchymal cells. Usually, callus is induced from somatic plant cells by culturing injured,

surface sterilized plants in the presence of growth regulating phytohormones, e.g. cytokinins and auxins. Calli retain all of the parent plants genetic information, and are thus able to synthesize the whole range of the chemicals found in the parent plant, and can even regenerate again into a whole plant.

Induced callus cultures are known to undergo so-called somaclonal variation, where differences inherent in the cultured cells can cause erratic production of metabolites [21]. The callus will usually acquire genetic stability after several subculture cycles, and will become a homogeneous aggregate [15]. After the stability has been reached, little to no variation in metabolism should occur between the subcultures.

The homogeneity and other attributes of calli are exceedingly useful for the production and research of plant metabolites compared to conventional agricultural methods. Foremost, the cell suspensions are independent of various geographical, seasonal, and environmental factors, and their homogeneity ensures uniform quality and yield. These attributes allow for reproducibility of conditions, which is significant for the research of metabolism, as it further allows procurement of comparable results. Secondly, the cell mass can be grown rapidly, and the products are easier to recover. There is also evidence that novel compounds not normally found in the parent plant can be produced in cell suspensions. [16]

4 Secondary Metabolites in Berries

The concept of secondary metabolite in biology is usually attributed to a German biochemist Albrecht Kossel. In the turn of the 20th century, Kossel defined these metabolites as opposed to primary metabolites, which are compounds directly associated with photosynthesis, respiration, and growth and development, to act in a vital role of the plants adaptation to its environment, and interaction with the ecosystem. Some secondary metabolites appear to have a key role in protecting the plants from herbivores and microbial infection, as attractants for pollinators and seed-dispersing animals, and as allelopathic agents, UV protectants, and signal molecules in the formation of nitrogen-fixing root nodules in legumes. Secondary metabolites are divided into three major groups based upon their biosynthetic pathways: i) phenolics, ii) terpenes and steroids, iii) nitrogen-containing alkaloids and sulphur-containing compounds (Figure 2). [15, 22]

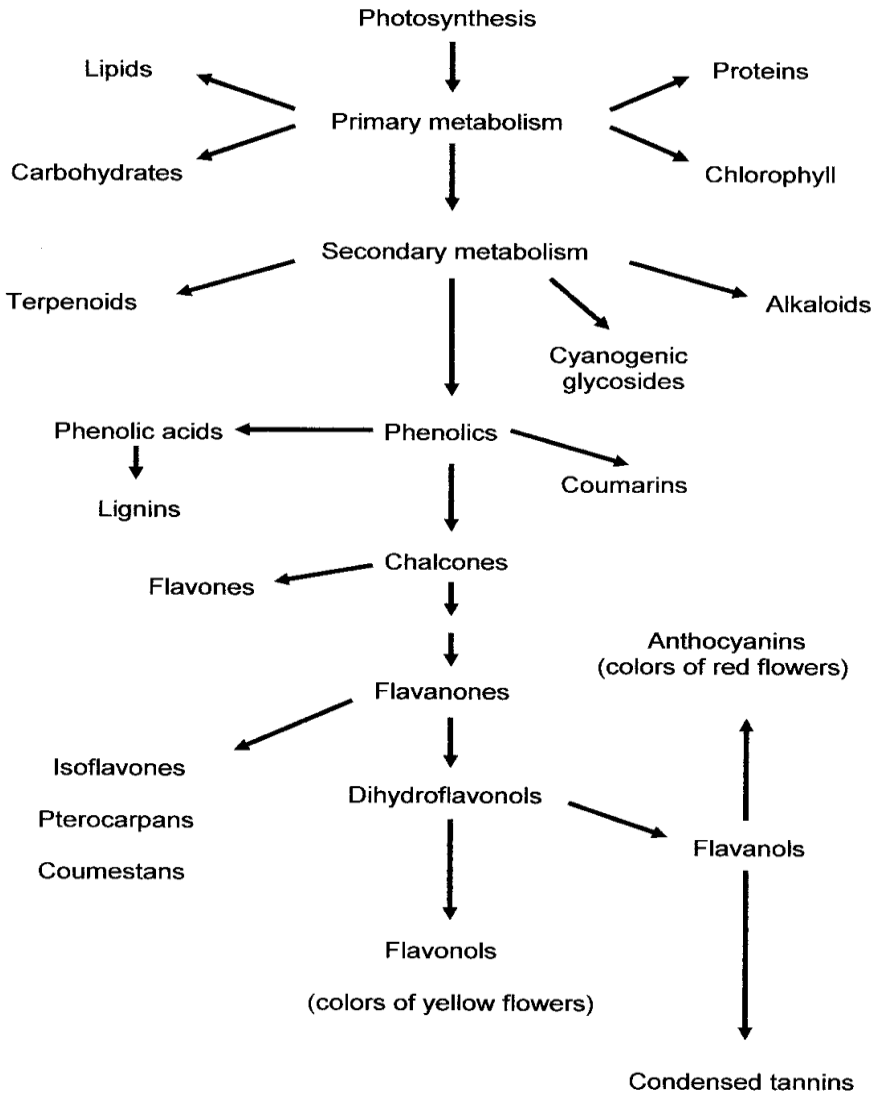


Figure 2. The primary and secondary metabolism in plants. [23]

Historically, traditional medicine has used these metabolites for many purposes, and in recent times in western countries, natural plants are the origin of as much as 25% of the molecules used by the pharmaceutical industry [24]. Considering that only a minority of plant metabolite contents is characterized, the higher plants will probably continue to provide novel drugs, antibiotics, insecticides and herbicides for use. The secondary metabolites are also of interest because of their many industrial uses, such as dyes, fibers, glues, oils, waxes, flavoring agents and perfumes. [15, 22, 25]

4.1 Phenolic compounds

The phenolic compounds are a family of secondary metabolites common to all higher plants. In Nordic plants, berries have been found to be especially rich in phenol content compared to vegetables and other fruit [22, 23, 26]. The compounds are usually produced in the plant as a response to stresses from the outside, such as microbial infections, injury, and chemical or physical stress [27]. In their simplest, phenolic compounds are substances, which possess an aromatic ring bearing a hydroxyl substituent, including functional derivatives [18]. Phenols are often found in dimerized or polymerized forms called polyphenols. Solubility can principally be considered characteristic for phenols, even though the smaller phenols can be volatile, and the largest polyphenols hardly dissolve into any common solvents. Phenols are weakly acidic, and can form hydrogen bonds with proteins and other macromolecules.

In nature, phenols are predominantly found in alkylated or esterified forms, and are usually conjugated with sugars and organic acids [22]. The sugar conjugants of the phenolic compounds are most commonly glucose, galactose, rhamnose, arabinose, and the most common acid conjugants are formic, malonic, succinic, p-hydroxybenzoic, and hydroxycinnamic acids. Some phenolic compounds can also exist in free forms, albeit rarely. The free-form phenols can be toxic for the plant, and conjugation can at least partly detoxify them [23]. The biomodification of the phenols causes considerable complications in the analytics and identification of the phenolic compounds. Methods exist to remove the conjugants and to reveal the aglycon, the phenolic compound with glycosides replaced by hydrogen. A well-known process to remove the conjugants is acid hydrolysis, as presented by Määttä-Riihinen et al. (2004) [10].

The plant phenols can be grouped into multiple families depending on their chemical composition, and many compounds from each family may be present in a single plant. For example, studies have described over 6000 different flavonoids found in plants [4]. In Finnish plants, ellagitannins (ETs), which are one subgroup of phenols, can be found in the green parts and roots of over 50% of plants [28]. In *Rubus* plants, commonly only two ETs (sanguiin H-6 and lambertianin C) are reported to be found, but as the more detailed analyses of Kähkönen et al. (2012) suggest by revealing altogether 26 ET compounds, many ellagitannic compounds are probably still to be found [29]. There is evidence that ETs with molecular weight of up to 5606 Da exist in the *Rubus* species [30].

4.1.1 Bioactivity and health benefits of phenolic compounds

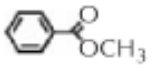
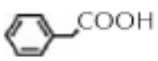
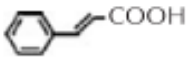
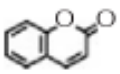
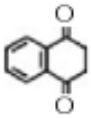
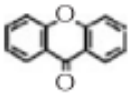
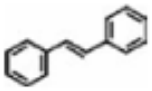
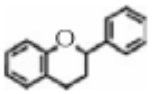
Phenolic compounds are one of the prime candidates for explaining the health benefits and protective effects from eating berries, fruit, and vegetables. Reliable data on the polyphenol contents of plants' are still scarce, but advances are being made to alleviate this, as the attained health benefits are highly dependent on the amount of phenols consumed. Ellagitannins' role as protective dietary constituents against the onset of many human chronic diseases, such as cardiovascular diseases, diabetes mellitus, and even some tumors, has been highlighted by Serrano & Puupponen-Pimiä (2009), Giménez-Bastida et al. (2012), and Neto et al. (2006) [6, 31, 32]. In studies made at VTT, remarkable antimicrobial properties against severe human pathogens and spoilage bacteria have been implicated to ETs [9, 33].

Two methods for the phenols to exert their biological effects have been proposed: the phenols can act either locally in the gastrointestinal tract, or as absorbable metabolites. It has also been suggested that tannins may chemically apply the effects in three ways: i) formation of complex with metal ions, ii) by antioxidant and radical scavenging activities, or iii) through their ability to complex with other molecules, such as proteins [34]. As the catabolism of the compounds starts in the gastrointestinal tract, the bioavailability of the phenols appears to be dependent on the composition of the gut microbiota [35, 36]. Furthermore, phenols, and especially ETs, have been show to affect the gut microbiota positively and to alleviate gastric inflammation [11, 12, 32, 36, 37]. In some cases, as with curcumin – a natural phenol, the anti-inflammatory action has been found to prevent cognitive deficits and Alzheimer's disease [38, 39].

4.1.2 Phenolic composition

As the phenolic compounds are common to all higher plants, and with more than 8000 phenolic structures of varying complexity reported, they are typically classified based on the number and arrangement of carbon atoms [22, 40] (Table 1). Furthermore, the compounds can be roughly divided into two groups: flavonoids and non-flavonoids, with flavonoids being the most numerous group [41].

Table 1. Structures of phenolic and polyphenolic compounds without phenolic hydroxyl groups [22].

Number of carbons	Skeleton	Classication	Example	Basic structure
7	C_6–C_1	Phenolic acids	Gallic acid	
8	C_6–C_2	Acetophenones	Gallacetophenone	
8	C_6–C_2	Phenylacetic acid	*p*-Hydroxyphenyl-acetic acid	
9	C_6–C_3	Hydroxycinnamic acids	*p*-Coumaric acid	
9	C_6–C_3	Coumarins	Esculetin	
10	C_6–C_4	Naphthoquinones	Juglone	
13	C_6–C_1–C_6	Xanthones	Mangiferin	
14	C_6–C_2–C_6	Stilbenes	Resveratol	
15	C_6–C_3–C_6	Flavonoids	Naringenin	

Flavonoids

Most flavonoids are pigments for flower coloration, but some also have an effect on taste and toxicity, act as messengers in nitrogen-fixing nodules, and contribute to disease resistance [42]. The flavonoids comprise of a general structure of fifteen carbon atoms, with two phenyl rings (A and B) and a heterocyclic ring (C), that are connected by a carbon bridge [22].

The substituents in the heterocyclic ring C, and the varying forms of the ring, are the basis for flavonoid classification [43]. Flavonoids are divided into six major subclasses: flavones, flavonols, isoflavones, flavanones, anthocyanidins, and flavan-3-ols, which

are commonly known as catechins (Figure 3). Multiple substituents can be present in the structural skeleton, with hydroxyl groups normally in 4', 5 and 7 positions.

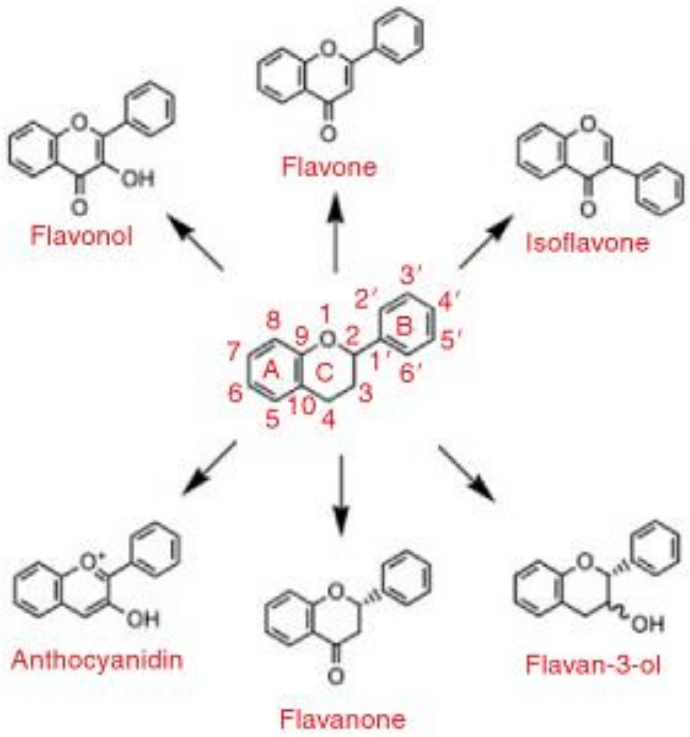


Figure 3. Generic structures of the major flavonoids [22].

Flavonols are the most widespread group of the flavonoids and have presence in most of the plant kingdom, with the exception of algae and fungi. Most flavonols can be found as O-glycosides, as is common with the prevalent quercetin, myricetin, and kaempferol [43]. Flavones, unlike the flavonols, are not widespread, and significant amounts occur only in few plants. Most of the flavones are found as 7-O-glycosides. Flavan-3-ols, or catechins, are the most complex of flavonoids. The absence of double bond between their C2 and C3 carbons forms an asymmetric center, breaking the planarity of the molecules. The isomers (**+**)-catechin and (**-**)-epicatechin are widespread, whereas (**-**)-catechin and (**+**)-epicatechin are comparatively rare. Catechins have the tendency to couple and create proanthocyanidins [43], which for example induce defense mechanisms in strawberries [44]. Anthocyanidins are widely dispersed particularly in flower tissue and fruits. They attract pollinators and participate in UV protection of the plant. Anthocyanidins are the cause for colors of red, purple and blue of the organs. In tissue, they are regularly found as sugar conjugates known as anthocyanins. The anthocyanins often form conjugates with hydroxycinnamates or organic acids. [22]

Non-flavonoids

Phenolic acids, also known as hydroxybenzoates, stilbenes, and hydroxycinnamates are the most notable groups in non-flavonoids. Gallic acid, a phenolic acid, is a common precursor for another notable non-flavonoid group, the hydrolysable tannins.

Tannins are phenolic polymers, that can be divided into four major groups by their chemical structure: proanthocyanidins or condensed tannins, hydrolysable tannins, phlorotannins found in marine brown algae, and complex tannins [31]. Tannins are responsible for the astringency in plant food, and are prevalent especially in unripe fruits, seeds, and leaves. They can also bind to the collagen of animal hides, and have been the basis of the tanning industry, as their name suggests [22]. Hydrolysable tannins, i.e. gallotannins and ellagitannins, can easily be degraded down to their constituents, gallic acid and ellagic acid (Figure 4). The gallo- and ellagitannin monomers can also be oxidized further to form polymers.

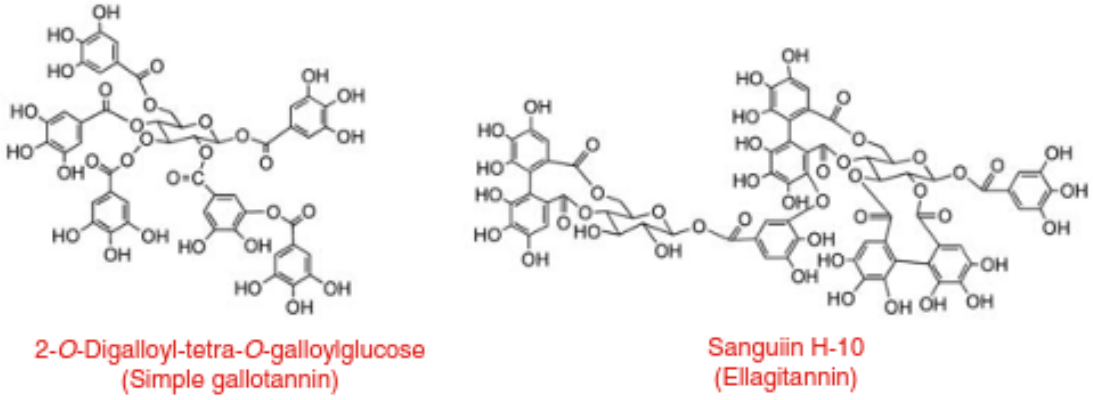


Figure 4. Structures of one gallotannin, and a dimeric ellagitannin [22].

The hydroxybenzoates and hydroxycinnamates share a similar aromatic ring backbone with variations in methoxylation and hydroxylation. Cinnamic acid is the main component for the hydroxycinnamates, also known as phenylpropanoids. The hydroxycinnamates form a diverse group, which is present in vegetables, fruits, berries, and coffee alike [22, 26]. Most common hydroxycinnamic acids are p-coumaric, caffeic, ferulic, and sinapic acids, and respectively p-hydroxybenzoic, protocatechuic, vanillic, and syringic acids for the hydroxybenzoics [45].

The stilbenes are phytoalexins, compounds that are synthesized as a response to microbial infections. They are phenylpropanoids, and share the common precursor phenylalanine. Resveratrol is the most common stilbene, and can especially be found in grapes.

4.1.3 Biosynthesis

Although phenolic compound biosynthesis pathways are complex, all phenolic compounds share the same intermediate, phenylalanine, or its precursor shikimic acid [18]. The synthesis of phenolic compounds is based on three pathways: i) shikimate, ii) phenylpropanoid, and iii) flavonoid pathways (Figure 5).

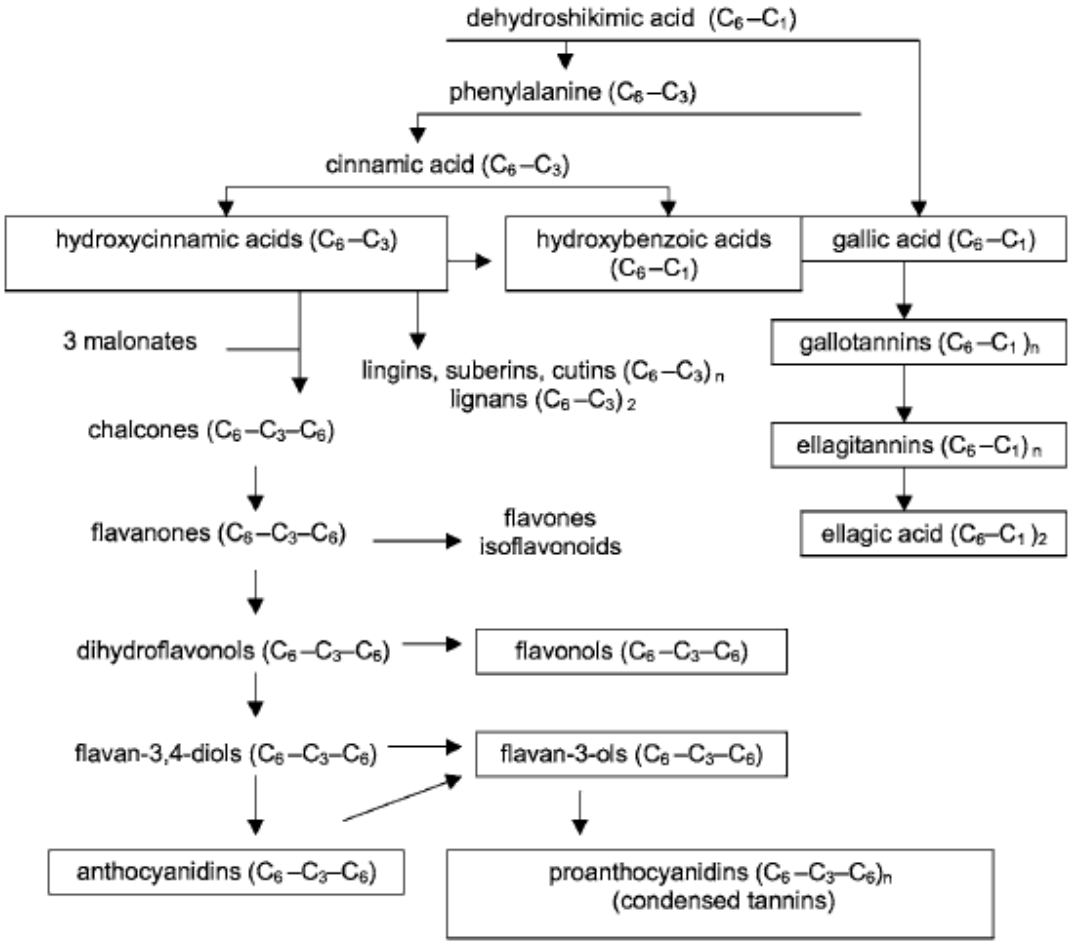


Figure 5. Biosynthetic relationships of phenolic compounds [46].

The shikimate pathway, which leads to shikimic acid, is the pivotal pathway in the formation of phenols. It is started by substrates from carbohydrate metabolism, namely phosphoenolpyruvate and erythrose-4-phosphate. The pathway not only produces the

phenylalanine that is required by the other pathways, but is a crucial part in the production of aromatic amino acids [47], and also produces hydroxybenzoates. Gallic acid, the precursor for hydrolysable tannins, is synthesized from an intermediate compound of the shikimate pathway, called dehydroshikimic acid [48, 49]. The downstream phenylpropanoid pathway produces the hydroxycinnamate derivatives. The phenylpropanoid pathway produces p-coumaroyl-CoA, which is the precursor for flavonoid pathway. The flavonoid pathway has been characterized comparatively well for many plants, but due to its complexity and the gaps in knowledge, applications have been difficult to achieve. The flavonoid pathway, besides flavonoids, also leads to the synthesis of condensed tannins [23]. [22, 40, 47, 50]

5 Modification of Secondary Metabolism

Since plant cell cultures were first introduced in the 1960s, a wide range of methods and strategies to produce and research secondary metabolites have become available. For various reasons, despite the advances, plant biotechnology has led to few commercial successes [15]. This can mainly be explained by the lack of understanding of the metabolite synthesis, and by certain characteristics of the plants themselves. For example, most metabolites synthesized by plant cells are stored within the cells organs, hindering continuous and efficient production in bioreactors [17]. Biotransformation has consequently become an important tool for metabolite production in plant cell systems [51].

Multiple platforms and approaches to the modification of secondary metabolism exist. In recent times, the recombinant DNA technology has made direct manipulation of the genes associated with the biosynthesis possible [15]. Feeding the cultures with precursors or intermediates of the secondary metabolite production has been found to significantly improve the end product yields, as demonstrated for example by Zabetakis et al (1997) with strawberry callus cultures [51, 52]. The synthesis pathways have also been manipulated by more indirect methods, as is the case with Ossipov et al (2003), where the addition of glyphosate to block the shikimate pathway in birch leaves resulted into nearly 100% increase of ET synthesis [49].

One of the platforms in use for metabolite production is hairy root cultures. These transformed plants have been valuable for the study of the biosynthesis pathways. Hairy roots are generated from the parent plant by *Agrobacterium Rhizogenes* infec-

tion, and are biochemically stabile, possess high growth rate, and are known to produce secondary metabolites on levels comparable to intact plants [53]. Some metabolites are even known to accumulate in much higher amounts in plant cell and hairy root cultures than in their parent plants. In a study by Petersen and Simmonds (2003), *Plectranthus scutellarioides* (formerly *Coleus blumei*) hairy root and cell cultures' production of rosmarinic acid was nine fold to that of an intact plant [54].

The pathways can also be affected by altering the cultivation conditions or by transformation, in such a way, that a continuous release of product into the growth medium is achieved. The excretion of the metabolites can be further enhanced by altering cells' membrane permeability, or by introduction of absorbents [17].

5.1 Elicitation

As secondary metabolites are defense mechanisms for the plant, certain stimuli that stress the organism can result in an increased or modified biosynthesis. Elicitation is the process, where these stresses are imposed onto the organism to activate molecular synthesis. One of the key uses of elicitation is to increase enzymatic activity within the secondary metabolite pathways [52]. Common elicitors include polysaccharides, mechanical wounding, physicochemical stressors, heat-killed fungi and bacteria or their fractions, and phytohormones directly involved in the relevant stress response of the plant [55].

The potential of elicitation is well established, but research concerning Rubus berries is rare. Furthermore, the effects of the elicitation are highly dependent on the concentration of the elicitor, and the used cell line within the species. Consequently, it is imperative to select the most efficient line for optimization of target molecule synthesis [53].

5.1.1 Ethephon

Ethephon, 2-Chloroethylphosphonic acid (Figure 6a), is the most common plant growth regulator in industrial use. The plants metabolize ethephon into ethylene, which is a gaseous ripening agent and a senescence controlling phytohormone. In terrestrial plants, ethylene induces thickening of stems and inhibits elongation. The biosynthesis of ethylene in plants is induced by the plants stage of development, phytohormonal activity, and environmental changes, e.g. injury or red light [56]. Ethephon is a widely

used elicitor for metabolite production in plants. In *Vitis vinifera*, common grape vine, ethephon has been shown to increase anthocyanin content over 2-fold [57]. In ethephon elicitations performed on a cell suspension line of arctic bramble by Nissilä at VTT (2008), increases in phenolic contents, but no new compounds were revealed [58].

5.1.2 Methyl jasmonate

Methyl jasmonate (MeJa, Figure 6b) is a phytohormone, and a common organic compound found in plants. Varieties of biotic and abiotic stresses can induce the synthesis of methyl jasmonate. In plants, methyl jasmonate is known to regulate flowering and senescence of seeds and fruit. The compound also induces the production of several antimicrobial agents like phytoalexins. Observations have been made, that MeJa, as a volatile compound can disperse via air currents to nearby non-stressed plants, inciting their defense mechanisms [59]. Methyl jasmonate has also been recorded to inhibit cold injuries in plant cells, and there is evidence of proapoptotic processes against cancer cells. [60]

In *Rubus idaeus*, red raspberry, elicitation with MeJa resulted into 2 to 3 fold increase in benzalacetone and ketone yields [61]. In red arctic bramble cell cultures, MeJa was found to increase the amount of some hydroxycinnamates [58].

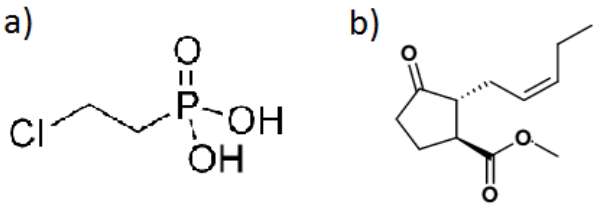


Figure 6. Selected elicitors a) ethephon b) methyl jasmonate

6 Liquid Chromatography and Mass Spectrometry Analytics

Liquid chromatography and mass spectrometry (LC-MS) combination is a widely used method for qualitative and quantitative plant metabolite studies. Preparation of the samples is generally straightforward; the samples need only be dissolved into solvents and filtered before injection. The method of extraction of the analytes from the sample can vary. Some plant derived samples may include abundant concentrations of unsolicited lipids, proteins, and polysaccharides, and the selection of solvent, and possible purification methods should be carefully considered. Phenolic compounds are commonly extracted by methanol and purified from conjugants by acid hydrolysis. Different phenolic compounds have characteristic UV spectra that allow cursory identification of the compounds with LC alone, but combined with MS fragment data, the compounds can be determined decisively.

6.1 Ultra-performance liquid chromatography

Advances in the LC technology in the 21st century have led to fast and high-resolution analyses. In ultra-performance liquid chromatography (UPLC), the pressure of mobile phase can reach over 1000 bar. In combination with a small particle size of the packing material in the column, 1.7-1.8 µm, the pressure enables run times measurable in minutes.

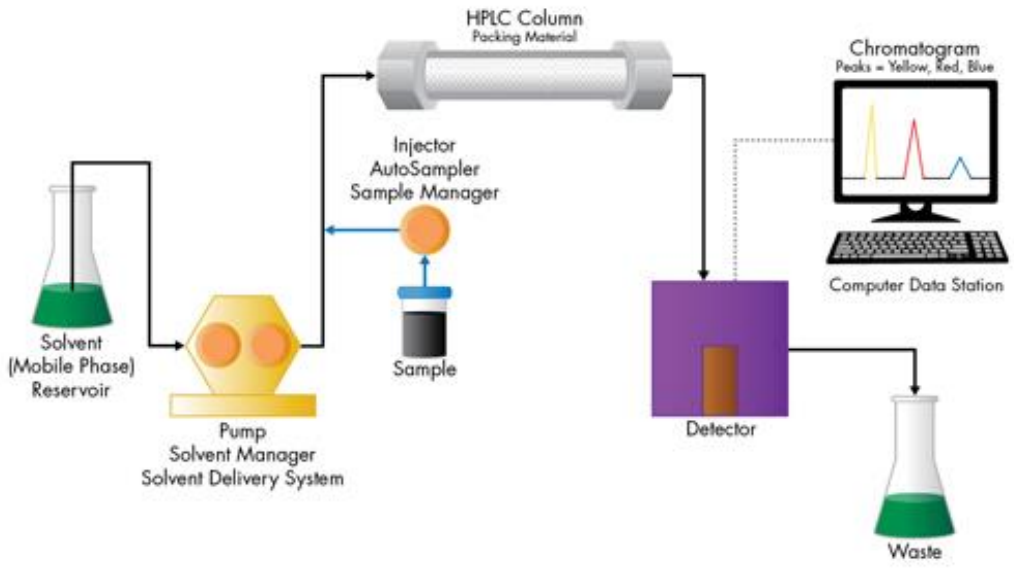


Figure 7. Representation of liquid chromatography. [62]

The sample is first injected into a mobile phase that usually consists of two solutions of differing polarity. The ratio of the solutions in the mobile phase is usually set to change as a function of time to form a gradient. The mixture of the mobile phase and the sample is then pumped into the column, where differences in compound polarity and size cause them to have varying speeds. Consequently, the compounds are separated into detectable bands. After the column, the analyte molecules are passed into a detector. In the study of the phenolic compounds, diode array detection (DAD) is a commonly used detection method. In DAD, the transmitted light is observed in a range of wavelengths. The gradient bands of the analyte are converted into absorbance peaks by the detector, relative to the base line that is formed by the absorbance of the mobile phase. [62]

The phenolic compounds are easily degraded in the column oven, and hence the temperatures are usually kept mildly over the room temperature [63].

6.2 Mass Spectrometry

In its basic, a mass spectrometer consists of an ion source, a mass analyzer, a detector, and a system for data handling. The sample enters the mass spectrometer as a stream from the inlet, into the ion source where the molecules become ionized. The ionized molecules become separated in the mass analyzer according to their mass-to-charge ratio (m/z), and pass on to the detector.

Multiple types of ion sources are used in mass spectrometry to generate ions for the analyzable gas-phase. One of these is an atmospheric pressure ionization method called electrospray ionization (ESI), which can be readily coupled with LC. The ESI method is especially suitable for polar and ionic biomolecules, such as the phenolic compounds, which are highly polarized because of their hydroxyl and carboxyl substitutes. A prerequisite for the usage of ESI is that the sample molecules need to be dissolvable. Weak organic acids and bases are commonly used to enhance the formation of ions. The ionization is achieved by spraying the dissolved analyte from an electrically charged capillary by using backpressure from the UPLC pump. The applied potential is approximately 4kV, either positive or negative depending on the wanted ion mode. The charged droplets of the spray then travel towards a lower-potential counter electrode, at the same time losing solvent and breaking into smaller droplets with the assistance of heated desolvation gas. From the counter electrode orifice, the ions are transmitted

to the ion tunnels, separated in a quadrupole, and analyzed. ESI is a concentration dependent process, and the optimal sensitivity can theoretically be achieved with low flow rates, high concentration, and low sample volume. The method can be used for a wide array of analytes, with the masses ranging from less than 200 Da to larger than 100 kDa. Depending on the molecular size, the compounds in the analyte either form singly charged $[M+H]^+$ or $[M-H]^-$ fragments depending on the ion mode, or series of multiply charged ions, allowing the formation of molecular weight profile for each of the compounds. [64, 65]

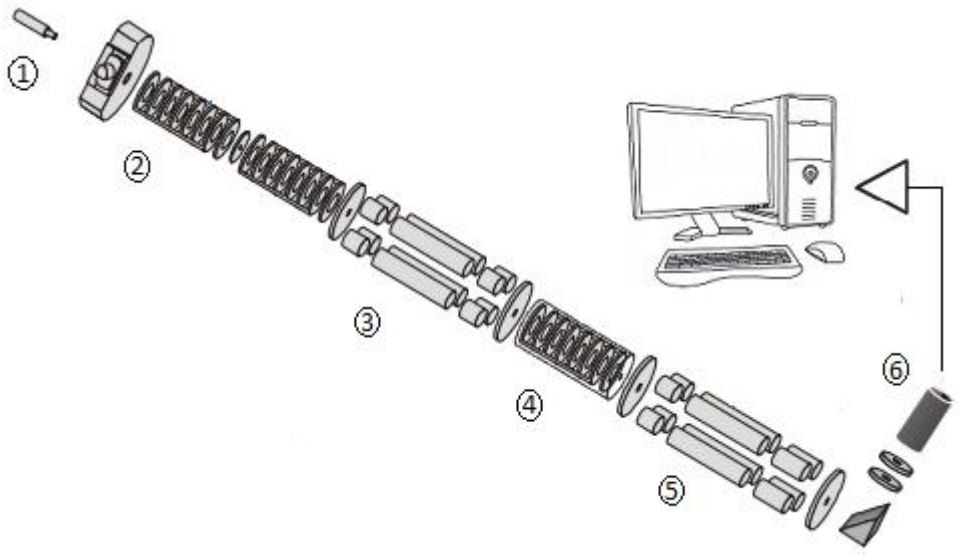


Figure 8. Overview of tandem quadrupole MS: 1) inlet and ionization, 2) sampling of ions through series of orifices, 3) ions are filtered by their m/z in the quadrupole, 4) collision-induced decomposition of the mass in the collision cell, 5) fragment filtration in the second quadrupole, and 6) detection of the ions. (Modified from [65]).

7 Aim of the Thesis

Several cell cultures of arctic bramble and cloudberry were studied in this thesis. The aim was to evaluate their capacity for phenolic compound production. Elicitation was used as a stimulant to increase biosynthesis of phenolic compounds. The effects of two elicitors, methyl jasmonate and ethephon, were compared on one arctic bramble cell line.

RESEARCH PART

8 Materials and Methods

8.1 Plant material

Two cell suspension lines of cloudberry (*Rubus chamaemorus*) and three cell suspension lines of arctic bramble (*Rubus arcticus*) were used in this thesis. These included one yellow suspension culture of cloudberry (KAS 371/2), and two suspension cultures of arctic bramble, red arctic bramble (KAS 341/15) and green arctic bramble (KAS 341/10) (Figure 9). Additionally, cell lines of cloudberry (KAS 371/2 GFP) and arctic bramble (KAS 341/8 GFP) that had been transformed to carry green fluorescent protein –gene (GFP gene) by *Agrobacterium tumefaciens* infections (VTT, unpublished data) were used, both yellowish in color.

Callus formation was induced on both cloudberry and arctic bramble. The induction was performed two times with arctic bramble leaves, and once with cloudberry leaves obtained from multiple greenhouse plants (KAS 341/1 & KAS 341/2, KAS 450/1 & KAS 450/2, respectively).

All work with plant material was done aseptically in a laminar flow cabinet.

Figure 9. Green arctic bramble (KAS341/10), yellow cloudberry (KAS 371/2) and red arctic bramble (KAS 341/15).

8.2 Growth media

The plant material was grown on modified Murashige and Skoog (MS) medium variants of either solid or liquid form, depending on cultivation. The used media were based on two MS variants: MS 300 KN for cloudberry and MS 301 KN for arctic bramble, where KN stands for added phytohormone concentrations of 0.1 mg L^{-1} kinetin (Kin) and 1.0 mg L^{-1} 1-naphtaleneacetic acid (NAA). NAA was added to all of the media before autoclaving, and kinetin, which was sterilized by syringe filtering (Whatman 0.2-µm cellulose acetate, GE Healthcare Life Sciences, UK), was added after autoclaving. The difference between the two base media variants was only their acidity, with pH 5.8 in MS 300 KN and pH 4.0 in MS 301 KN. Solidification of the media for plate cultures was done with 3.0 g L^{-1} of gelrite.

The GFP-strains of cloudberry and arctic bramble were grown on their species-specific media, containing 25 ppm kanamycin. The compositions and instructions to prepare all of the media are included in Appendix 5.

8.3 Measurement of growth curves

The growth curves were measured for all of the plant cell lines: yellow cloudberry KAS 371/2, red arctic bramble KAS 341/15, green arctic bramble KAS 341/10, yellow cloudberry KAS 371/2 GFP and yellow arctic bramble KAS 341/8 GFP.

8.3.1 Maintenance of cell suspensions

The cell suspensions were maintained in 250 ml Erlenmeyer flasks sealed by aluminum foil, with 50 ml of MS 300 KN for cloudberry, or 50 ml of MS 301 KN growth medium for arctic bramble. The cell suspensions were grown in an incubation room at 24 ºC under a white fluorescent light (irradiation ca. 50 µmol/m^2sec) with light/dark regime of 16 and 8 hours, on an orbital shaker (NOVOTRON TR-225, Infors HT, CH) set to 110 rpm. Subculturing was done approximately every two weeks by dividing the suspension into 2-3 Erlenmeyer's depending on thickness of the suspension. As the plant cells aggregate easily, open-ended pipettes were used for dividing the cell suspensions to help ensure homogeneity between the divided flasks. After division, the Erlenmeyer's were sucked dry of the old growth medium with pipettes, and 50 ml of new growth medium was added into each flask. For all of the used cell lines, smaller 125 ml Erlen-

meyer flasks and only 30 ml of growth media were used in the growth curve measurement experiments.

8.3.2 Growth curve procedure

The growth curves were measured similarly for all of the cell lines. The measured variables were pH, fresh weight, dry weight, and viability. Sampling was done on days 0, 4, 7, 11, and 14 of the culturing. Three parallel samples were taken at each sampling time point.

The homogenous inoculum for the experiment was prepared by pouring four 250 ml Erlenmeyer flasks with 10-day-old cultures into a 500 ml sterile beaker. The beaker was then emptied of liquid by pipetting. Approximately 2-2.3 grams of fresh weight cell material was weighed into each of the Erlenmeyer flasks and 30 ml of MS 300 KN or MS 301 KN was added. Three of the flasks were sampled immediately, and the rest at the other sampling points during the two-week period. From the samples, 1 ml of cell suspension was taken by pipette for fluorescein diacetate (FDA) staining to estimate the cells' viability, and the rest of the samples proceeded to filtration.

8.3.3 Measurement of fresh and dry weight

The samples were filtered using a Büchner funnel with Miracloth. The cell material was collected into pre-weighed falcon tubes, and then weighed (92SM-202A or EP520A, Precisa Gravimetrics, CH) to obtain fresh weight (FW). The samples were frozen at -80 ºC for 30 minutes and kept stored in -20 ºC until freeze-dried over weekend with freeze dryer (ALPHA 1-4 LD plus, Christ, DE). After the lyophilization, the tubes with sample were weighed again to obtain dry weight (DW). The filtered growth media of the samples were measured for pH in room temperature.

The measured FW and DW also allow growth rate, specific growth rate, and doubling time to be calculated using the following equations:

Growth rate:

$$r_x = \frac{(FW(t_2) - FW(t_1))}{(t_2 - t_1)}$$

Specific growth rate:

$$\mu = \frac{(\ln FW(t_2) - \ln FW(t_1))}{(t_2 - t_1)}$$

Doubling time:

$$t_d = \frac{\ln 2}{\mu}$$

where FW (t_1) = fresh weight at the beginning of sampling; FW (t_2) = fresh weight at the end of sampling; t_1 = beginning of the time range; t_2 = end of the time range

8.3.4 Viability staining

Fluorescein diacetate (FDA) solution was prepared by diluting 0.5% FDA/acetone stock solution into growth medium to achieve final concentration of 0.01% FDA. Then, 0.5 ml of solution was combined with an equal amount of cell suspension sample, and allowed to stand for 5 minutes. Viability was estimated by sight with a fluorescence microscope (Olympus IMT-2, Olympus Corporation, JP). The cell aggregation hampers quantitative measurement, making estimations grossly directional. Photographs were taken from some representative samples with a camera (MicroPublisher 5.0 RTV, QImaging, CA) and Q-capture Pro 7 (v7.0.5) imaging software linked to the microscope.

8.3.5 Extraction and analyses of phenolic compounds

The extraction was performed to all of the samples after lyophilization. The samples were ground, and 100 mg was weighed into 10 ml KIMAX test tubes. Phenolic compounds were then extracted in room temperature by 2 ml of analytical grade methanol (MeOH) for one hour with 250 rpm magnetic stirring followed by centrifugation (Universal 30F, Hettich Lab Technology, DE) at 3000 rpm for 5 minutes. The supernatants were collected into Eppendorf tubes, and filtered through hydrophilic polypropylene (GHP) syringe filter (0.2-µm GHP Agrodisc 13 mm, Pall Corporation, US) into a Waters LC/MS glass vial. The extracted compounds were either analyzed immediately, or stored in -20 ºC.

An additional test extraction was carried out on GFP cloudberry samples of days 11 and 14. The extraction was performed otherwise as before, but with a second 2 ml MeOH extraction, with 30 minutes of magnetic stirring. The supernatants were combined, and phenolic compounds were concentrated by evaporating the methanol by nitrogen gas-vortexing (TurboVap LV, Caliper Life Sciences, US) for 1 hour in 40 ºC bath. Compounds were then dissolved again into 2 ml of methanol using ultrasonic cleaner (Laborette 17, Fritsch, DE) for 20 minutes. Filtering was performed with 0.2-µm GHP filters as before, into LC/MS glass vials.

The samples were analysed for phenolic compounds by ultra-performance liquid chromatography with diode array detection (UPLC-DAD) and electrospray ionization mass spectrometry in positive mode (ESI^+-MS). The used UPLC-DAD hardware configuration was Acquity ultra-performance LC (Waters Inc., US) equipped with eLambda 800 nm detector, and BEH C18 1.7 µm (2.1 x 100mm) column. Run time was 12 minutes including reconditioning, as presented on Table 2. Injection volume was 5.0 µl, and the solvents used were 0.1% formic acid (HCO_2H) in water and acetonitrile (CH_3CN) with a flow rate of 0.430 ml/min. The compounds were detected at a range of 210-600 nm, and the data was processed on four 2D channels at 280, 320, 360, and 520 nm for possible classification of different phenolic compounds. Sampling rate was 20 points/sec with a resolution of 1.2 nm.

Table 2. UPLC/MS run conditions. A= 0.1% HCO_2H in H_2O; B= CH_3CN. Reconditioning starts after step 4.

Run conditions				
Step	Time(min)	Flow Rate(ml/min)	%A	%B
1	Initial	0.43	95	5
2	1.13	0.43	90	10
3	5.67	0.43	60	40
4	9	0.43	10	90
5	10	0.43	95	5
6	12	0.43	95	5

Micromass Quattro Micro (Waters Inc., US), a tandem quadrupole mass spectrometer with Zspray electrospray injection was used in combination with the UPLC set-up to analyse the samples. Capillary voltage of 2.50 kV and cone voltage of 40 V were used. Desolvation gas flow was 800 L/h and cone gas flow 30 L/h. Source and desolvation temperatures were 125 ºC and 350 ºC, respectively.

The UPLC-DAD/ESI+-MS hardware set-up and analysis data were handled with MassLynx Software (v4.1, Waters Inc., US).

8.4 Analysis of LC-MS data

Found phenolic compounds were compared to, and identified through literature references [10, 63, 66]. Certain phenolic compounds can be identified by their characteristic absorption maximum: some phenolic acids and catechins at 280 nm, flavonoids and other phenolic acids at 324 nm, and anthocyanins at 520 nm. The retention times (RT) of the analytes are highly dependent on the run conditions, e.g. column packing material and mobile phase solutions. Thus, even though the retention order is usually retained, the RT data is exactly comparable only to LC analyses done with similar conditions. When scrutinizing the accrued data, it is noteworthy that esterification and glycosylation cause bathochromic (shift to longer wavelength) and hypsochromic shifts (shift to shorter wavelength), respectively, in the absorption spectra as compared to their aglycons [10]. The fragments of the ESI-MS are distinctive to the used ion mode, which should be acknowledged when comparing to literature data.

8.5 Elicitation experiments of arctic bramble suspension culture

The elicitations were performed with the green arctic bramble cell suspension line (KAS 341/10). Three separate elicitation series were performed for this thesis. In addition to elicitations, a negative control (solvent of MeJa, DMSO) and a control with no added substances were performed. All ethephon elicitations were done on the same date, but methyl jasmonate elicitations were separated into two different occasions, as shown on Table 3.

Table 3. Elicitation experiments. Concentrations of elicitors in liter of cell suspension, used elicitor stock solution, and amount of stock solution added. The solvent for MeJa is DMSO, and for ethephon H_2O.

Series, starting date	Elicitor	Stock solution	Solution / suspension
#1, Feb 9th 2015	MeJa 11.2 mg L^{-1}	5,6 mg/ml	60 µl/30 ml
	DMSO	-	120 µl/30 ml
#2, Mar 2nd 2015	MeJa 2.24 mg L^{-1}	5,6mg/ml	12 µl/30 ml
	MeJa 22.4 mg L^{-1}	5,6mg/ml	120 µl/30 ml
#3, Mar 16th 2015	Ethephon 100 mg L^{-1}	20 mg/ml	150 µl/30 ml
	Ethephon 20 mg L^{-1}	20 mg/ml	30 µl/30 ml
	Ethephon 200 mg L^{-1}	20 mg/ml	300 µl/30 ml

8.5.1 Elicitation procedure

The elicitation experiments were carried out using 125 ml Erlenmeyer flasks as in the growth curve measurement, with an inoculum of similar size. Two parallel samples were produced for each sampling time point for the controls, 4 parallels for MeJa elicitations, and 3 for ethephon elicitations. The started 125 ml flask cultures were cultivated as before. The measured growth curves were used in determining the elicitation time point. The elicitors were added on the 7th day of cultivation, at the beginning of exponential growth phase. The negative control was done by adding the greatest amount of DMSO used in elicitations – 120 µl, into the control samples at the same time as elicitors were added. The samples were taken every 24 hours for five consecutive days, starting from right after the addition of elicitor. Color of the growth medium was monitored during sampling as an indication of secreted compounds.

8.5.2 Extraction and analysis

The phenolic compounds were extracted and analyzed with the same method as when determining the growth curves, with the exception that an internal standard of 100 µg morin (M-4008, Sigma, US) / 1 ml MeOH was added to the extraction solvent (Figure 10). The internal standard was included to allow cursory quantification of the detected compounds, and to provide a standard between samples for statistical analysis. A more sensitive Xevo TQ-S tandem quadrupole MS was used instead of the earlier Micromass Quattro Micro MS.

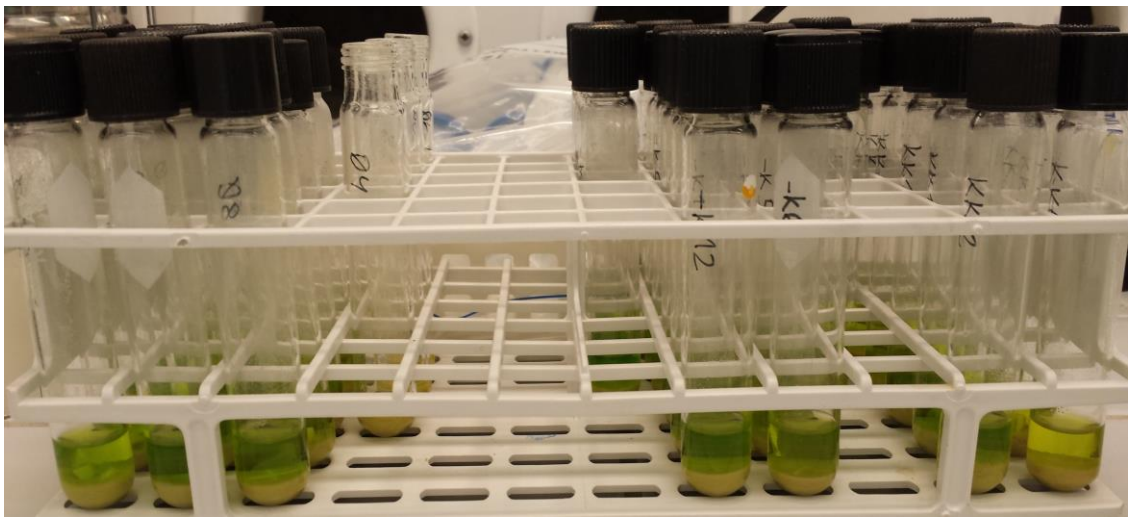


Figure 10. Samples from the 1st elicitation, after extraction and centrifuging.

8.6 Establishment of callus cultures

The callus cultures were established from several greenhouse plants, including KAS 341, KAS 450/1, KAS 450/2.

8.6.1 Callus induction procedure

Leaves were selected and cut from the plants, leaving some leaf stalk. Surface sterilization was then performed by aseptically submerging the leaves as follows:

1) 200 ml of 70% ethanol for 1 minute, 2) 300 ml of 0.6% hypochlorite with two drops of Tween 20 for 10 minutes with light magnetic stirring, 3) washing in three beakers with 300 ml of sterile water in succession, and then 4) gently drying the leaves between sterile papers for approximately one hour.

Hypochlorite treatment of only 2.5 minutes was used for the cloudberry leaves because of their fragility.

After drying, the leaf fragments were first wounded by cutting off the outer edges of the leaves with a scalpel. The wounded fragments were then transferred to culture plates containing their species-specific growth medium, with NAA and Kin hormones that promote the formation and proliferation of calli. The leaves were cultivated at 27 ºC in the dark.

9 Results and Analysis

9.1 Growth curves

The growth curves were measured in part to determine the right time for elicitation, which is usually in late logarithmic growth phase. The growth rates were slower than expected, and for the determination of exact time of plateauing of growth, the stationary phase, one more sampling time point would have been necessary. The results were sufficient for the determination of exponential phase, and consequently the selection of the elicitation time point. In the future, the inoculum mass could be moderately increased to speed growth. Only minor deviation was found between parallel samples.

Of the used cell suspension lines, red arctic bramble KAS 341/15 was distinctly the fastest growing in terms of both fresh and dry weight . The GFP transformed cultures were slower to grow and produced the lowest yields of dry weight, probably due to their modified metabolism. Green arctic bramble KAS 341/10 reached the highest dry weight of the cell suspensions, 14.7 g L^{-1}, even though its growth had not plateaued by the end of sampling. The second highest dry weights were measured in 7-day-old red arctic bramble KAS 341/15 cultures, 14.2 g L^{-1}. Overall, the fresh and dry weights increased through the sampling, with the exception of KAS 341/15 of which dry weight dropped after day 7.

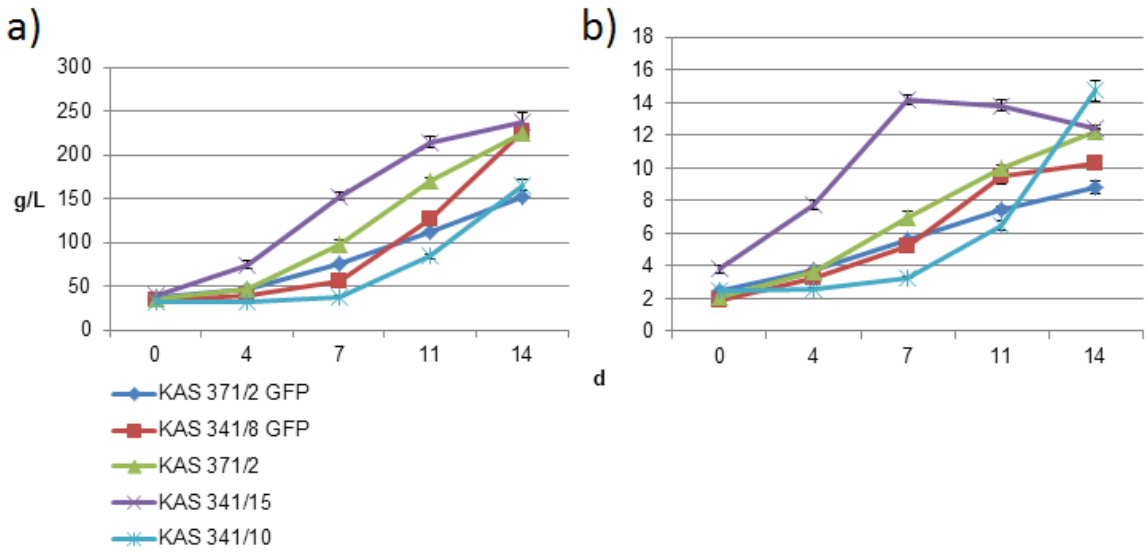


Figure 11. Growth curves of the cell lines expressed as a) fresh weight, and b) dry weight. Error bars depict standard deviation.

After the first elicitation series, the growth rate of green arctic bramble cultures became substantially faster. When comparing the ethephon elicitation cultures to the growth curve cultures of green arctic bramble, fresh weight was nearly double and dry weight triple at day 7 of culturing. By the end of the elicitation experiments on 12th day of cultivation, the dry weight attained was ~16.3 g L^{-1} with similar results from all of the later elicitations. This is a clear indication of a change in the metabolism of the green arctic bramble after the growth curve and 1st elicitation cultures. Due to the changed growth, the growth curve and MeJa 11.2 mg L^{-1} LC-MS data were disqualified from comparison. Seasonal variation of growth is often described in various plant cell culturing systems, and the probable explanation for the sudden shift in the growth rate is that the culturing was dated at the start of spring.

PH increased slightly during the culturing with all cell suspensions, with the exception of arctic bramble KAS 341/8 GFP, which varied between pH 4.27 and 5.19 (Figure 12). The low viability of GFP arctic bramble samples also correlates with the pH changes. The growth medias lack of kanamycin, which is used as a selection method for the GFP transformed cells, might have contributed to the loss of viability through allowing unwanted cell growth. The viability of GFP arctic bramble decreased to 63%, but stayed well above 80% in the other cell suspension lines throughout measurement (Figure 13). The suspension cell lines grown in MS 300 KN medium experienced a slight drop in pH through days 0 to 4, from circa 5.15 to pH 4.90. For optimal growth rates, it can be deduced, that the pH of the growth medium MS 300 KN could perhaps be slightly higher, around 6.00 before autoclaving.

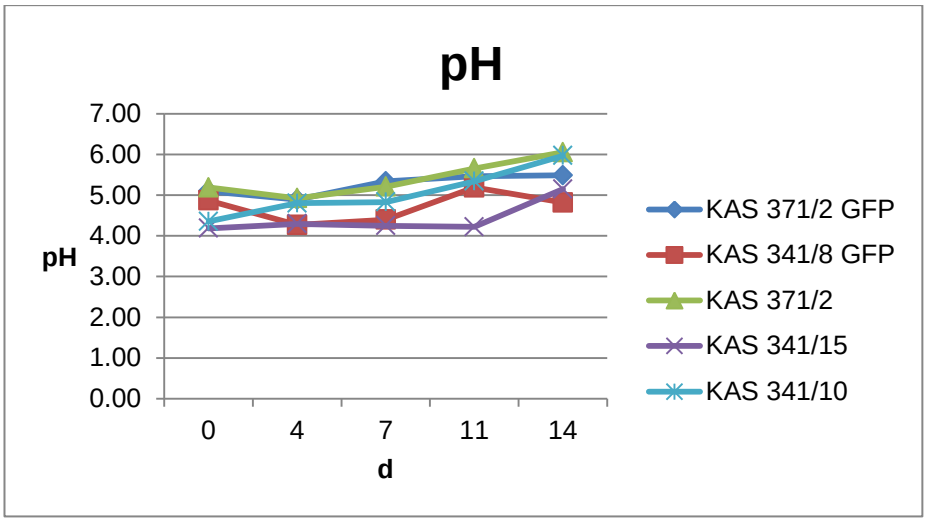


Figure 12. pH of the growth curve experiments.

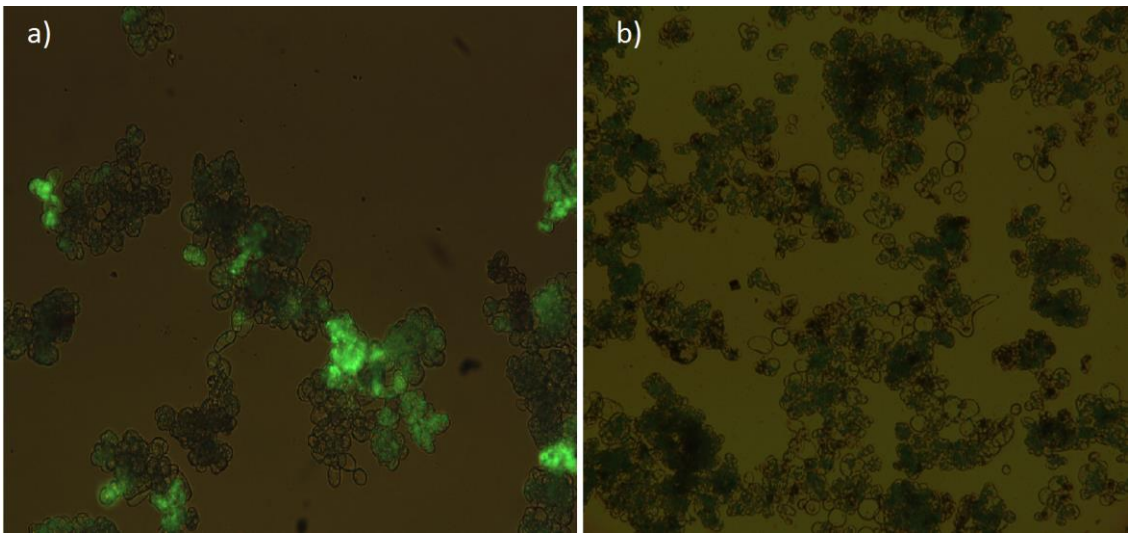


Figure 13. Viability stained suspension cultures. a) 11-day-old stained GFP cloudberry (KAS 371/2 GFP) cell suspension culture, b) 14-day-old stained red arctic bramble (KAS 341/15) cell suspension culture. The difference of color and intensity of fluorescence between the images is only due to background lighting and magnification.

The results of the growth curves can be found in Appendix 1.

9.2 Phenolic compounds

Overall, 86 distinct peaks were taken under examination; 16 in KAS 371/2, 18 in KAS 341/15, 13 in KAS 371/2 GFP, 23 in KAS 341/8 GFP, and 16 in KAS 341/10 (Figure 14). Small concentrations and the lack of purification caused multiple minor peaks to be left out of closer examination, as the peaks were hardly distinguishable from background. The lack of purification especially thwarted attempts to identify the mass fragments of most compounds due to massive background noise. Therefore, all identifications are tentative.

VTTs red arctic bramble KAS 341/15 has been previously shown to be rich in phenolic compounds [58], and highly polymerized procyanidins have also been detected (Salminen, Puupponen-Pimiä, Nohynek, VTT unpublished data). In the growth curve measurements of this study, the red arctic bramble had significantly higher peak intensities than any of the other cell suspension cultures, with absorbance units (AU) reaching double to that of the others. Red arctic bramble was the only cell suspension line where anthocyanins were detected, as was presumed by the colors of the suspensions. The two anthocyanins detected in the KAS 341/15 samples were found to be

cyanidins, and peak 4 (RT 3.04) was further tentatively identified as cyanidin-3-rutinoside.

Estimating by the UV spectra, most numerous compounds overall were galloyl esters. In each cell suspension culture, many of the compounds with low retention times (RT) were found to be phenolic acids and their esters. Mass fragments 139 and 163, which can be associated with quinic acid, were mainly found on cloudberry KAS 371/2. A compound with RT of around 7.00, and identified as galloyl ester by UV spectra, was found in all of the cell suspension lines except for KAS 341/10, where a similar compound was found at a lower RT of 6.39. Common MS fragments between the samples found at that peak are 133 and 395 m/z. The compound is found in substantial amounts, and only on the first day of culturing, disappearing completely by the second sampling time point. This leads to the conclusion that the compound might be produced before the subculturing, and is associated with high cell density. The peak of the morin internal standard used in green arctic bramble samples was found to be stacked with at least three other compounds. These compounds were identified to contain glycosides by MS fragments.

Some differences in profiles of phenolic compounds can be seen within the two cloudberry cell lines. The GFP cloudberry seems to have none of the peaks found after RT 7.00 in KAS 371/2, but seems to have increased peaks in the lower retention times. In the case of arctic bramble, the profiles of phenolic compounds differ exceedingly between cell lines, and few similar peaks were observed. This result supports the presumption that the profiles of phenolic compounds are largely dependent on the used cell line within a species.

The additional test extraction carried out on GFP cloudberry produced considerably lower peak intensities compared to the original extraction from the same samples. This can probably be attributed to long gas-vortexing due to faulty machine, long storage in methanol, or insufficient ultrasonic cleaning. In the case of arctic bramble cell lines,

The full list of examined peaks, their UV_{max} , mass fragments and tentative identification can be found in Appendix 3.

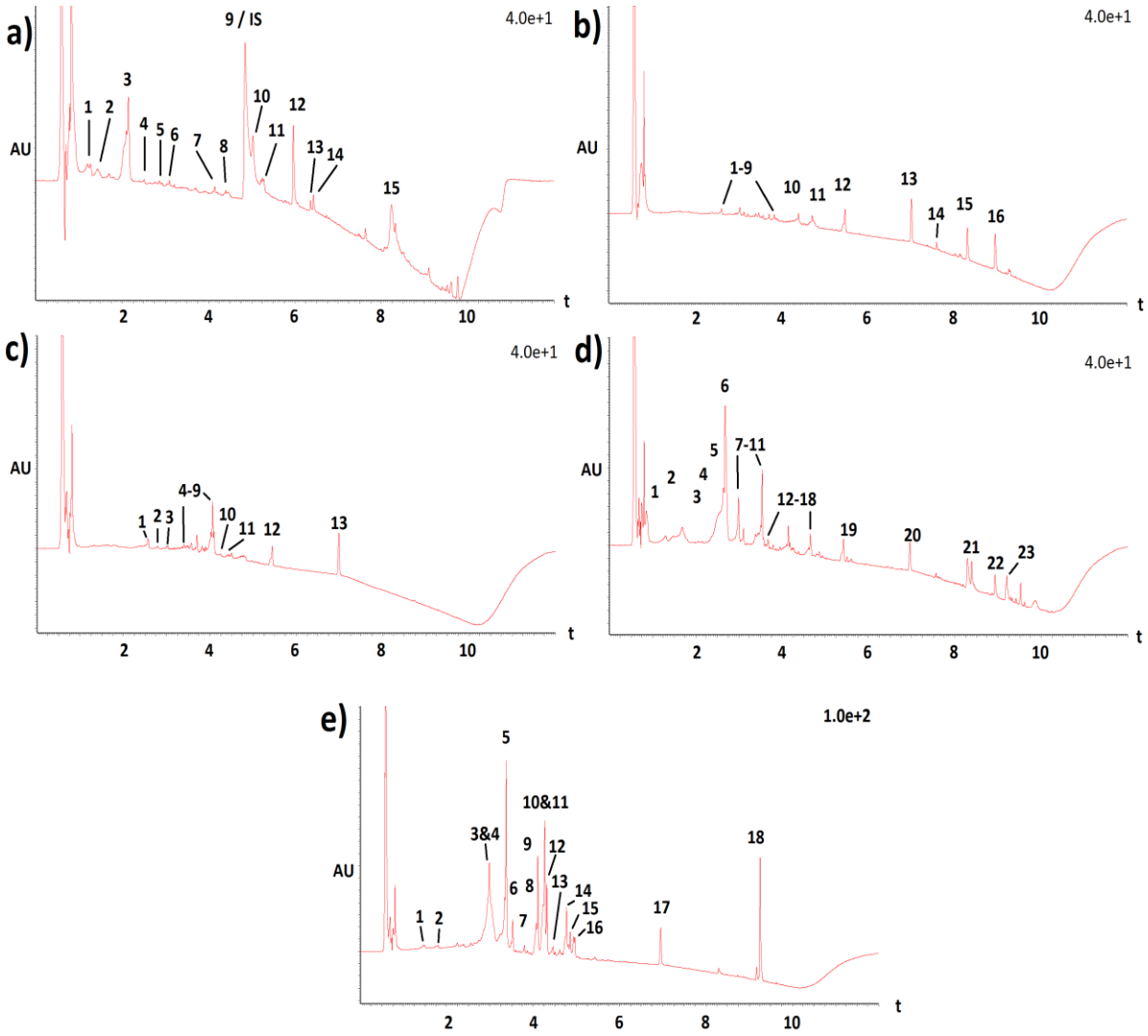


Figure 14. UPLC-DAD chromatograms from samples run for growth curves. a) KAS 341/10, b) KAS 371/2, c) KAS 371/2 GFP, d) KAS 341/8 GFP, and e) KAS 341/15. The intensities on KAS 341/10 are higher than on other samples. The first visible peaks are from methanol. IS (in a) = internal standard, morin. Peak numbers correspond to those used in Appendix 3.

9.3 Elicitations

Both elicitors were found to increase peak intensities. Ethephon was found to be most effective in higher concentrations and methyl jasmonate in lower. One unforeseen peak was formed during the ethephon elicitations. The sudden growth change in green arctic bramble also affected the DMSO control, and it is not directly comparable to the two consequent elicitation series. However, compared to the growth curve, and to MeJa 11.2 mg L^{-1} elicitation that was performed at the same time, no changes in the LC-MS -data could be attributed to DMSO.

Growth curve data can be found on Appendix 2, peak UV_{max}, mass fragments, and tentative identification on Appendix 3, and LC-DAD –graphs on Appendix 5.

9.3.1 Ethephon

The samples of ethephon elicitations were kept stored at -20ºC for one week after the methanol extraction and before LC-MS run. The LC graphs show clear degradation in morin peak intensities in all of the samples, probably caused by the longer storage. No visible degradation was noticed on any other peaks. This result questions the validity of morin as an internal standard in KAS 341/10, especially when considering that the peak of morin also stacks with multiple major peaks.

Only the highest concentration of ethephon, 200 mg L^{-1} caused considerable changes in the growth of fresh and dry weight. On the 24h sampling, the dry weight had dropped from 8.4 g L^{-1} to 5.8 g L^{-1}, but recovered back to levels similar to the control and other elicitation samples after 72 hours. The fresh weight growth of ethephon 200 mg L^{-1} elicitation lagged behind, and at final sampling point was 178.6 g L^{-1}, more than 20 g less than its comparisons. Ethephon had an effect on pH relative to its concentration, but the acidity was greatly mitigated after 24 hours. The cell suspensions pH was able to return to near normal levels still at the ethephon concentration of 100 mg L^{-1}. Viability of the cultures stayed around 90% throughout the experiment. (Figure 15b)

One unidentified peak (peak 16, RT 3.93) was detected at the highest elicitor concentration after 72 hours. This peak was not found on any other samples. Peak 3 (RT 2.20) and some of its stacked compounds went through increase during the experiments. The increase was detected on all samples, and was positively correlated to ethephon concentration. All of the compounds in this composite peak seem to be galloyl esters, as the UV spectra is characteristic and stays constant throughout the peak.

9.3.2 Methyl Jasmonate

The elicitation with MeJa concentration of 11.2 mg L^{-1} was disqualified from comparison due to the changed growth rates.

High concentration of methyl jasmonate had a negative effect on the growth of fresh and dry weight. The fresh and dry weight of MeJa 2.24 mg L^{-1} at the end of sampling

were 119.9 g L^{-1} and 10.9 g L^{-1}, compared to 113.2 g L^{-1} and 8.0 g L^{-1} of MeJa 22.4 mg L^{-1}, respectively. The growth rates experienced a moderate decline after 24 hours of elicitation due to orbital shaker being off for approximately 14 hours. The lack of agitation caused pH to rise in both elicitations to around 6.10 at 48h sampling, but resumed to normalcy at around pH 5.5 in the following sampling points. Viability of the cultures also declined by approximately 10% at the 48h sampling, and slightly recovered towards the end.

Increases in peak intensities were the highest at the lowest MeJa concentration of 2.24 mg L^{-1}. Most of the peaks that experienced increase could not be identified. Peak 1 (RT 1.27) was increased at the start of the elicitation on all of the MeJa samples, and lessened with time. The disqualified MeJa 11.2 mg L^{-1} elicitation and growth curve also had similar increases, and in consequence, the changes are unlikely to be caused by MeJa. As in ethephon elicitation, Peak 3 (RT 2.20) and some of its stacked compounds increased during the experiments. Conversely, the highest intensities were attained with lower concentration of MeJa. Slightly later retention time of the MeJa elicited galloyl esters in the composite peak suggests that they differ from the compounds elicited by ethephon. Peaks 10 (RT 5.03) and 11 (RT 5.27), which are stacked with the peak of morin, saw an increase after 72 hours. The compounds are likely to be glycosides, based on the glycosidic mass fragments found on the composite peak. Major decrease in intensity was found at peak 15. The intensity is similar to the samples with the lower growth rate, and is unlikely to be a result of MeJa elicitation. (Figure 15c)

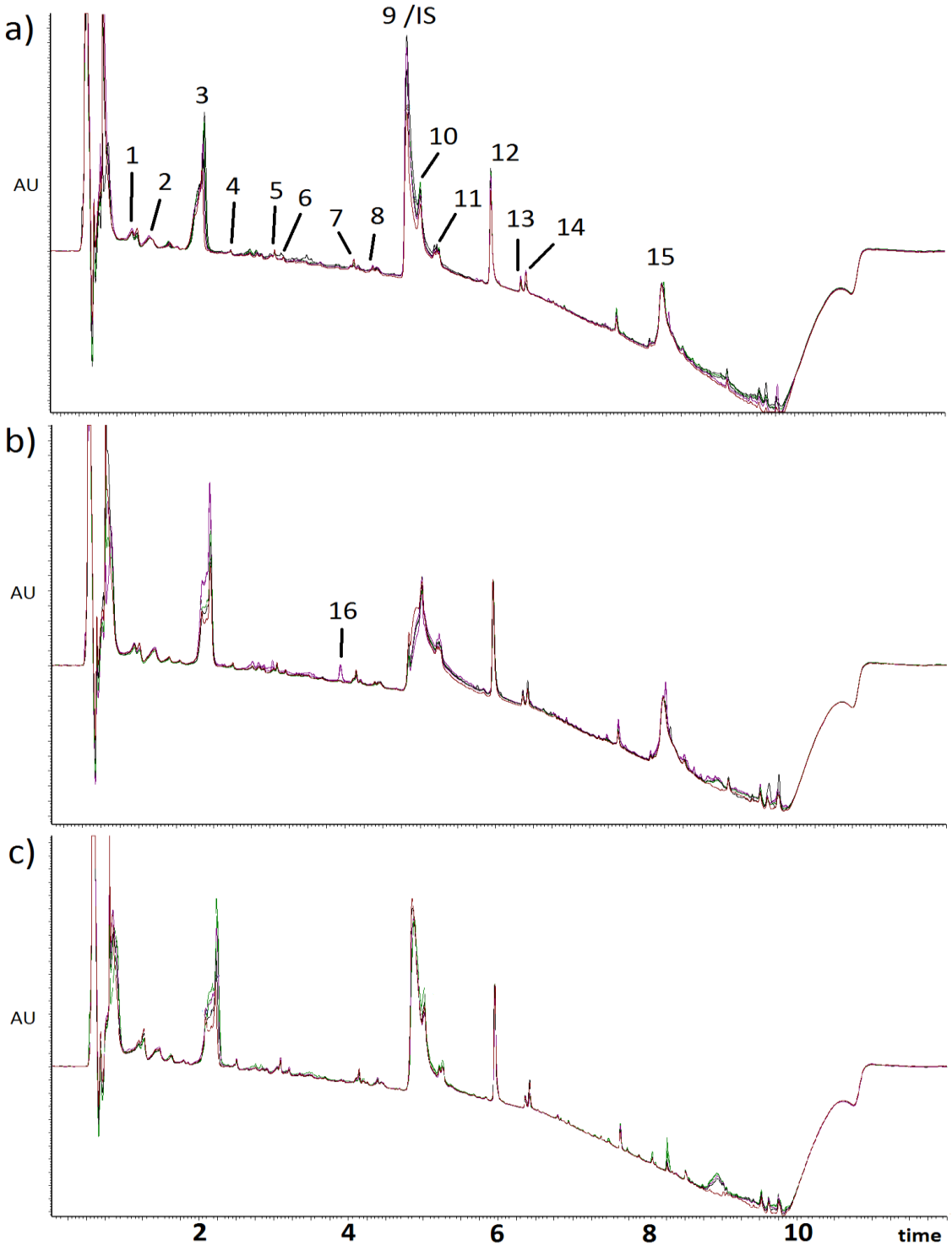


Figure 15. Overlay graphs (UPLC-DAD) of some of the elicitations and the control. a) KAS 341/10 control, b) ethephon 200 mg L^{-1}, and c) MeJa 2.24 mg L^{-1}. Each graph has one sample from each sampling time point overlaid, showing variations in peak intensity between the samples relative to time. IS = internal standard, morin.

9.4 Establishment of callus cultures

A sufficient amount of callus for transfer had usually formed on the leaf fragments after a few weeks. The callus was transferred from the leaves onto fresh culture plates and cultivated in 24 ºC, at a light/dark regime of 16 and 8 hours respectively, and subcultured to new media approximately once a month.

Callus was obtained from all of the arctic bramble plants (Figure 16a). Yellow coloration started to form immediately after the transfer to fresh culture plates. On some of the KAS 341/1 and KAS 341/2 derived calli, red coloration started to occur after two subculture cycles. The red coloration is a sign of anthocyanin synthesis. This colored calli experienced deterioration after subculturing, and their viability is uncertain.

No calli were transferred from the cloudberry leaves, as the surface sterilization was too stressful even in short hypochlorite solution immersion of 2.5 minutes. Minuscule callus formation did occur, but the leaves perished before sufficient amount had formed for transfer (Figure 16b). Alternate forms of surface sterilization are suggested to be explored for further studies.

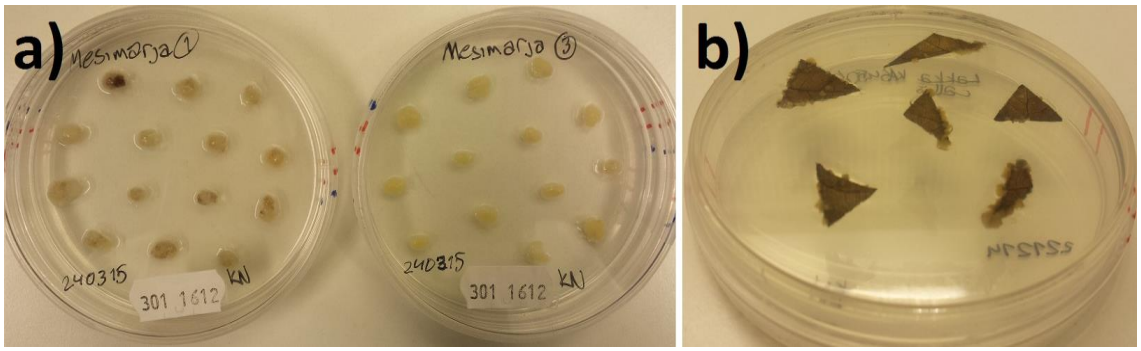


Figure 16. Generated callus cultures. a) arctic bramble calli, b) unviable cloudberry KAS 450/2 leaf fragments and calli. In the upper-left corner of arctic bramble plates, some red coloration caused by anthocyanin production can be seen.

10 Discussion and Conclusion

The growth curves were successfully measured for all of the used cell suspension lines. One more sampling time point would have been necessary for complete measurement of growth cycles. The only cell suspension that plateaued was red arctic bramble KAS 341/15. Despite green arctic bramble KAS 341/10 had not ended its growth, it had the highest dry weights of all the suspension lines. Even so, red arctic bramble had the highest peak intensities and thus probably contained the highest phenolic compound contents. Cloudberry cultures had considerably lower peak intensities than arctic bramble cultures, as was expected on the basis of previous research. The GFP cultures grew relatively slowly compared to their non-transformed counterparts, probably due to varied metabolism caused by the GFP gene. Problems arose with the green arctic bramble cultures from a sudden shift in growth rates, leading to decreased comparability between samples.

Lack of purification and low concentrations of compounds hindered the identification of compounds due to high background noise. Consequently, the identifications on this thesis should be considered tentative. Overall, 86 peaks were taken under examination. Most numerous group of phenolic compounds found in the cultures were galloyl esters with 19 peaks identified by their UV spectra. In the case of several compounds, UV data was supported by detected MS fragments, which were compared to literature. Red arctic bramble was the only culture to produce anthocyanins, as was expected by the suspensions' colors and earlier research. Phenolic acids were found in all of the cultures, mostly in low retention times. Red arctic bramble was found to contain at least nine flavonoid glycosides. Two of these were further identified as quercetin-3-hexosides, and one as kaempferol-3-glycoside.

The elicitors had only minor effects on cell growth rates. Both ethephon and MeJa increased peak intensities, and caused no definitive decreases. No conclusive identifications were attained for the increased compounds, although evaluating by context, some are most likely galloyl esters and some are glycosidic compounds. One unidentified new compound was produced by ethephon after 72 hours of elicitation in green arctic bramble suspension culture

Selected cell culture samples will be analyzed in more detail later during the BerryTannin project by UPLC-MS/MS.

The validity of morin as an internal standard for green arctic bramble KAS 341/10 is questioned as obvious degradation was found to happen during storage of ethephon elicitation samples, and it was discovered that its LC-DAD peak stacks with several other peaks. Although degradation was observed only in morin, a revision of storage conditions is suggested, perhaps storing the extracted samples at -80 ºC instead of -20 ºC.

Callus was successfully formed from arctic bramble leaves, but the surface sterilization seems to be too harsh for cloudberry leaves even in short amounts. Consequently, alternative methods for sterilization should be tried out in further studies.

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Growth curves

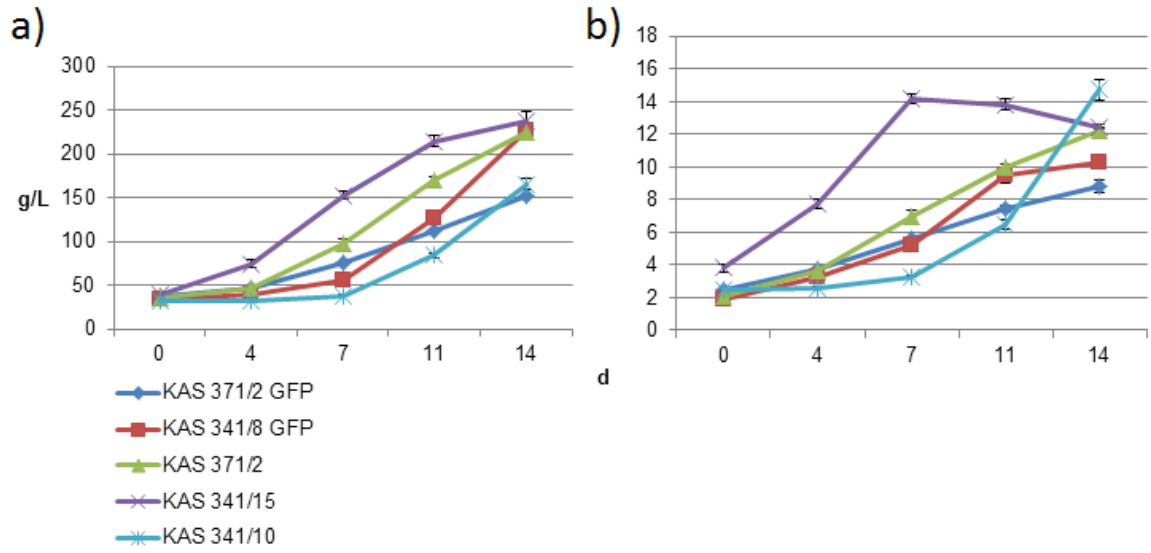

Figure A- 1. Growth curves of the cell lines expressed as a) fresh weight, and b) dry weight. Error bars depict standard deviation.

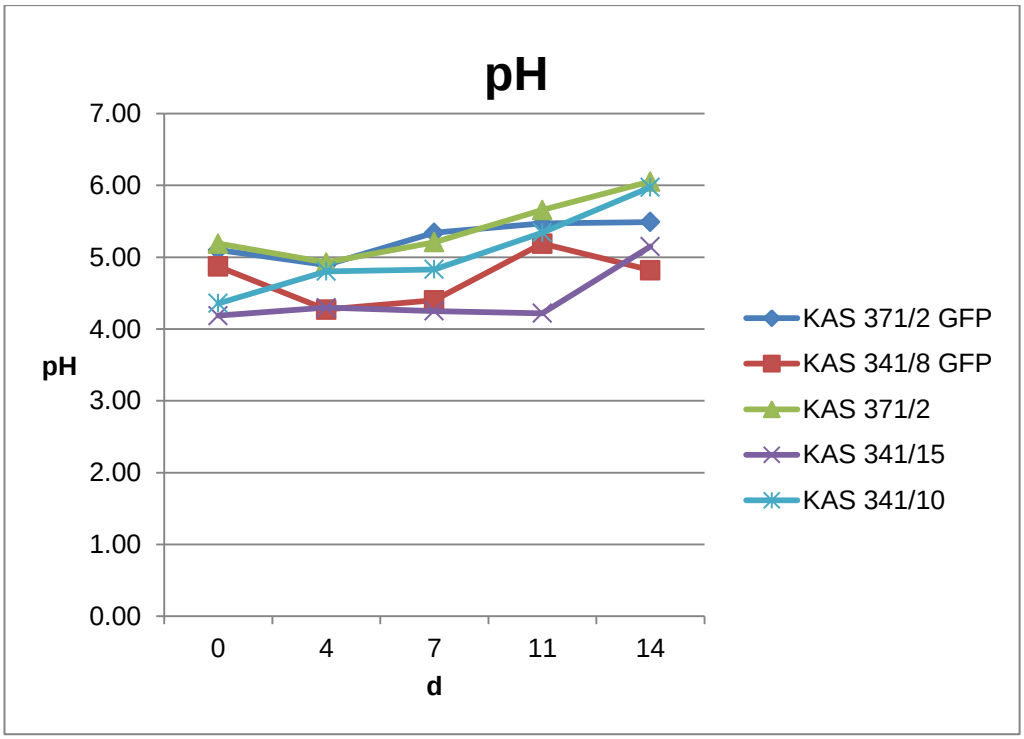

Figure A- 2. pH of the growth curve experiments.

Table A- 1. Viability at different time points by cell line.

KAS	Viability				
KAS 371/2 GFP	>0,95	0,9	0,9	0,9	0,9
KAS 341/8 GFP	>0,95	0,75	0,63	0,80	0,75
KAS 371/2	>0,95	0,95	0,95	0,95	0,95
KAS 341/15	>0,95	0,95	0,95	0,83	0,83
KAS 341/10	>0,95	0,92	0,92	0,80	0,95
d	0	4	7	11	14

Table A- 2. Growth characteristics of fresh and dry weights, from indicated sampling time point to the following. r_2 = growth rate, μ = specific growth rate, and t_d = doubling time.

Fresh weight	growth characteristic r_2			
KAS #	0d	4d	7d	11d
KAS 371/2 GFP	0,07	0,28	0,28	0,39
KAS 341/8 GFP	0,05	0,16	0,54	1,00
KAS 371/2	0,08	0,51	0,55	0,55
KAS 341/15	0,26	0,78	0,47	0,24
KAS 341/10	0,00	0,05	0,35	0,81

Dry weight	growth characteristic r_2			
KAS #	0d	4d	7d	11d
KAS 371/2 GFP	0,01	0,02	0,01	0,01
KAS 341/8 GFP	0,01	0,02	0,03	0,01
KAS 371/2	0,01	0,03	0,02	0,02
KAS 341/15	0,03	0,06	0,00	-0,01
KAS 341/10	0,00	0,01	0,02	0,08

Fresh weight	growth characteristic μ			
KAS #	0d	4d	7d	11d
KAS 371/2 GFP	0,05	0,15	0,10	0,10
KAS 341/8 GFP	0,04	0,11	0,21	0,19
KAS 371/2	0,06	0,25	0,14	0,09
KAS 341/15	0,16	0,24	0,09	0,04
KAS 341/10	0,00	0,05	0,20	0,23

Dry weight	growth characteristic μ			
KAS #	0d	4d	7d	11d
KAS 371/2 GFP	0,11	0,13	0,07	0,06
KAS 341/8 GFP	0,14	0,15	0,15	0,03
KAS 371/2	0,14	0,22	0,09	0,07
KAS 341/15	0,18	0,20	-0,01	-0,04
KAS 341/10	0,01	0,08	0,17	0,27

Fresh weight	growth characteristic t_d			
KAS #	0d	4d	7d	11d
KAS 371/2 GFP	12,85	4,50	6,76	6,94
KAS 341/8 GFP	16,91	6,06	3,37	3,60
KAS 371/2	11,28	2,80	4,95	7,40
KAS 341/15	4,41	2,90	8,12	19,77
KAS 341/10	415,58	14,61	3,47	3,07

Dry weight	growth characteristic t_d			
KAS #	0d	4d	7d	11d
KAS 371/2 GFP	6,51	5,22	9,76	11,98
KAS 341/8 GFP	5,01	4,48	4,64	24,98
KAS 371/2	5,08	3,21	7,74	10,15
KAS 341/15	3,91	3,42	-97,19	-19,69
KAS 341/10	47,69	9,15	4,09	2,53

Table A- 3. Growth characteristics of the entire 14-day growth time. r_2 = growth rate, μ = specific growth rate, and t_d = doubling time.

Fresh weight growth characteristics 0-14d			
KAS #	r_2	μ	t_d
KAS 371/2 GFP	0.245	0.099	6.994
KAS 341/8 GFP	0.414	0.136	5.088
KAS 371/2	0.406	0.131	5.303
KAS 341/15	0.426	0.128	5.416
KAS 341/10	0.284	0.116	5.972

Dry weight growth characteristics 0-14d			
KAS #	r_2	μ	t_d
KAS 371/2 GFP	0.017	0.092	7.569
KAS 341/8 GFP	0.018	0.121	5.716
KAS 371/2	0.022	0.125	5.525
KAS 341/15	0.018	0.085	8.193
KAS 341/10	0.026	0.128	5.436

Table A- 4. Numerical data of pH and fresh and dry weight (FW and DW). All sampling time points are separated by the color of table; 3 parallel samples in each time point. The shown data are measurements of the obtained mass from each 30 ml sample.

KAS 371/2 GFP			
#	FW (g)	DW (g)	pH
1	1,165	0,071	
2	1,198	0,075	
3	1,058	0,075	5,10
4	1,436	0,112	
5	1,327	0,106	
6	1,481	0,121	4,89
7	2,229	0,160	
8	2,330	0,170	
9	2,178	0,174	5,34
10	3,535	0,235	
11	3,269	0,205	
12	3,348	0,230	5,47
13	4,512	0,283	
14	4,527	0,280	
15	4,658	0,233	5,49

KAS 341/8 GFP			
#	FW (g)	DW (g)	pH
1	1,000	0,053	
2	0,922	0,058	
3	1,111	0,059	4,87
4	1,192	0,099	
5	1,141	0,094	
6	1,241	0,102	4,27
7	1,571	0,147	
8	1,666	0,157	
9	1,799	0,166	4,40
10	3,480	0,244	
11	4,098	0,303	
12	3,892	0,308	5,19
13	6,467	0,319	
14	6,503	0,300	
15	7,457	0,310	4,82

KAS 341/10			
#	FW (g)	DW (g)	pH
1	0,938	0,072	4,36
2	0,929	0,071	4,35
3	1,062	0,079	4,36
4	0,984	0,083	4,81
5	0,974	0,073	4,80
6	0,990	0,079	4,80
7	1,117	0,097	4,83
8	1,099	0,095	4,79
9	1,182	0,104	4,87
10	2,667	0,216	5,34
11	2,285	0,180	5,32
12	2,597	0,186	5,37
13	4,781	0,415	5,94
14	4,602	0,413	5,98
15	5,485	0,496	6,00

KAS 371/2			
#	FW (g)	DW (g)	pH
1	1,066	0,055	
2	1,045	0,069	
3	1,149	0,066	5,19
4	1,375	0,110	4,83
5	1,414	0,113	4,97
6	1,379	0,107	4,98
7	3,265	0,235	5,39
8	2,531	0,182	4,81
9	2,968	0,212	5,43
10	5,445	0,312	5,91
11	4,887	0,289	5,50
12	5,012	0,298	5,56
13	7,471	0,372	6,05
14	6,212	0,352	6,02
15	6,641	0,380	6,09

KAS 341/15			
#	FW (g)	DW (g)	pH
1	1,327	0,115	4,22
2	0,982	0,099	4,15
3	1,266	0,129	4,19
4	1,905	0,209	4,28
5	2,285	0,249	4,34
6	2,514	0,239	4,27
7	4,614	0,427	
8	4,222	0,405	
9	4,888	0,448	4,25
10	6,414	0,410	
11	6,925	0,440	
12	5,968	0,394	4,22
13	6,391	0,362	
14	7,051	0,377	
15	8,007	0,381	5,15

Elicitation growth curves

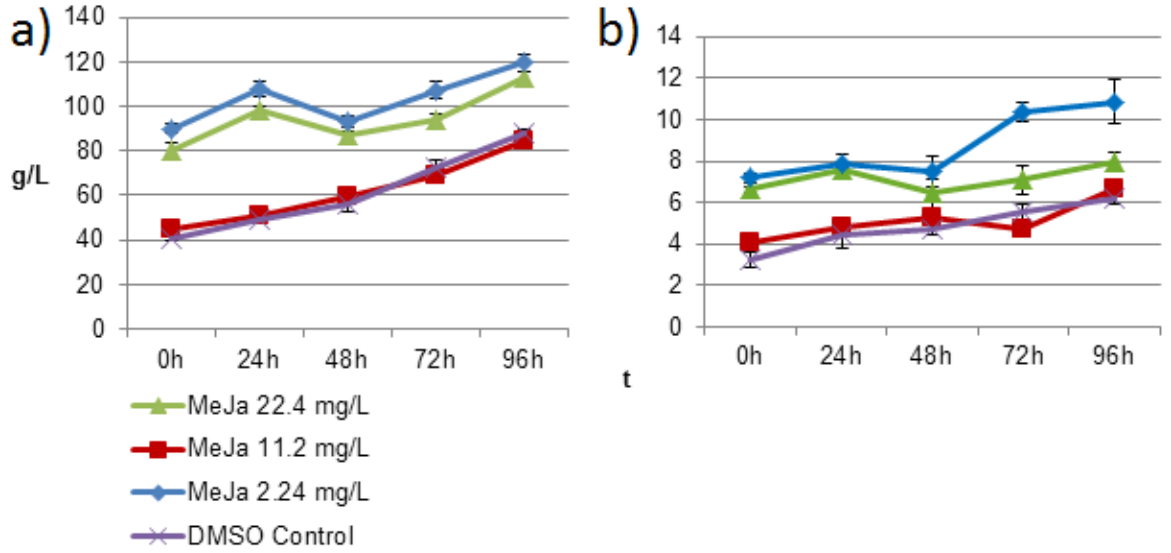

Figure A- 3. Growth curves during MeJa elicitations expressed as a) fresh weight, b) dry weight. Error bars depict standard deviation.

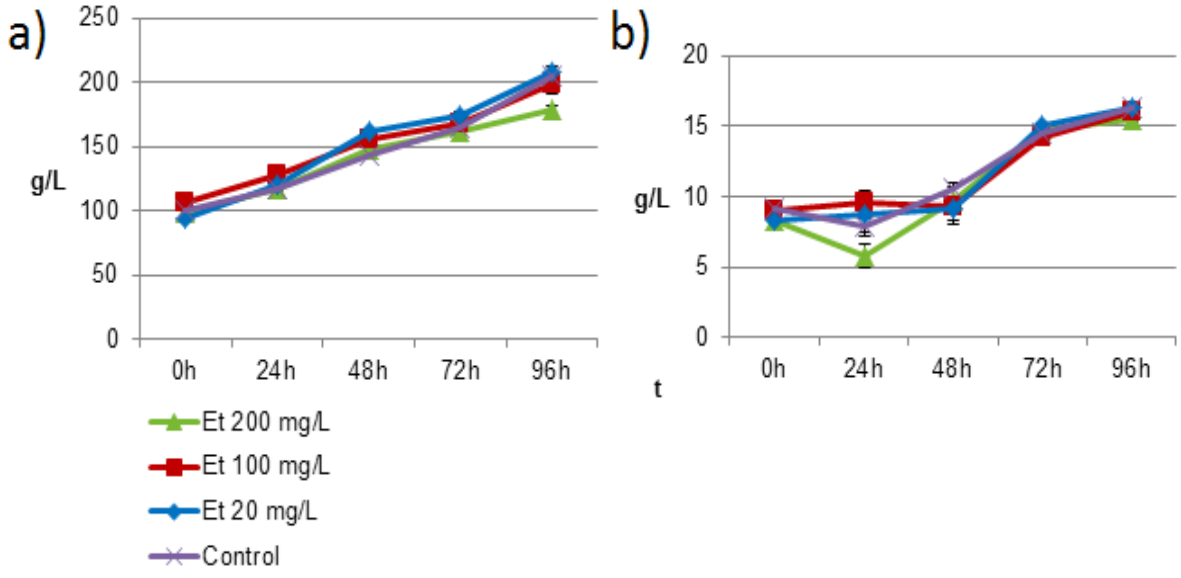

Figure A- 4. Growth curves during ethephon elicitations expressed as a) fresh weight, b) dry weight. Error bars depict standard deviation.

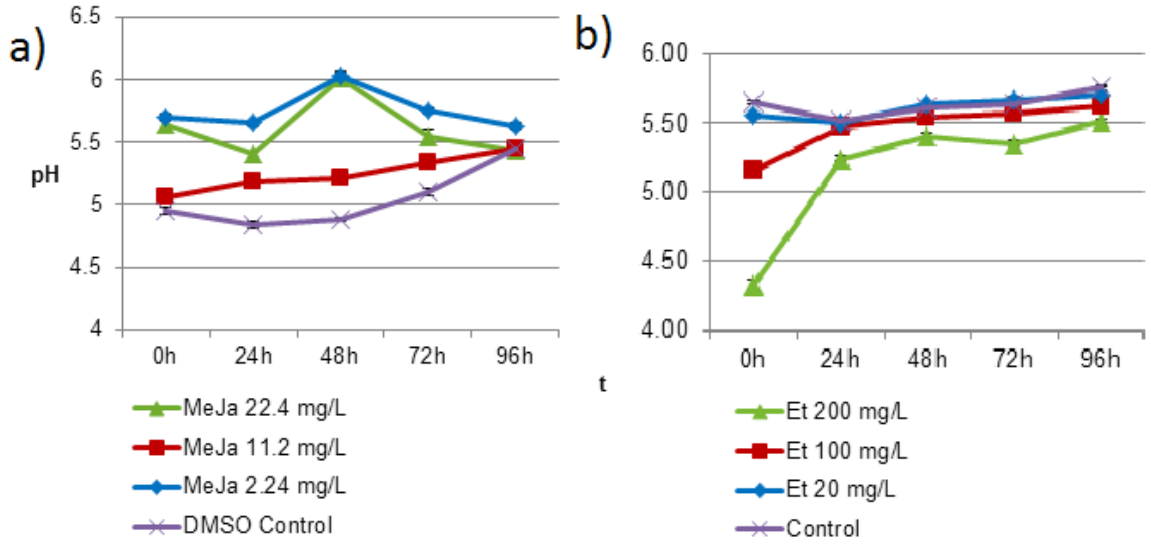

Figure A- 5. pH of a) MeJa, and b) ethephon elicitation experiments.

Table A- 5. Viability at different time points by elicitation.

Viability	Ethephon elicitations			MeJa elicitations			Control
t	200 mg	100 mg	20 mg	22.4 mg	11.2 mg	2.24 mg	
0h	0.95	0.95	0.95	0.95	0.95	0.95	0.95
24h	0.95	0.93	0.95	0.95	0.91	0.95	0.93
48h	0.88	0.92	0.93	0.85	0.94	0.86	0.95
72h	0.95	0.95	0.92	0.85	0.89	0.86	0.85
96h	0.95	0.93	0.90	0.88	0.85	0.90	0.88

Sample peak intensities and mass fragments

Table A- 6. Compound peak intensities and mass fragments of interest in cloudberry KAS 371/2 and red arctic bramble KAS 341/15. Sh = shoulder.

Cloudberry KAS 371/2				
#	RT	Peak UV_{max} (nm)	MS Fragments (m/z)	Tentative identification
1	2.59	220, 270sh, 278, 287	146, 188, 144, 118	Galloyl ester
2	2.76	220, 270sh, 278, 287	146, 188, 144, 118	Galloyl ester
3	3.03	<210, 228, 277	147, 139, 119	galloyl/gallic
4	3.22	232, 306	147	
5	3.47	233, 275	133, 163, 130, 105	
6	3.53	231, 277	139, 123, 104, 133	
7	3.7	229, 271	167, 575, 104, 327	
8	3.82	232, 272	105, 459, 497, 133, 167	
9	4.09	240, 282, 325sh	120, 206	
10	4.38	235, 272	177, 103, 133, 147, 130	
11	4.69	236, 274	175, 167, 193, 163, 341, 353	
12	5.44	224, 281	141, 134	Galloyl ester
13	7.02	223, 281	133, 395, 130, 105	Galloyl ester
14	7.58	256	503, 485	
15	8.3	<210, 250	205, 201, 407	
16	8.95	<210, 283	423, 405	

Red arctic bramble KAS 341/15				
#	RT	Peak UV_{max} (nm)	MS Fragments (m/z)	Tentative identification
1	1.65	<210, 293	120, 103	
2	2.64	219, 279, 288sh	146, 188, 144	Galloyl ester
3	2.97	<210, 230sh, 282, 517	287, 449	Anthocyanin (cyanidin)
4	3.04	<210, 230sh, 280, 517	287, 449, 595	Anthocyanin (cyanidin-3-rut)
5	3.36	239, 332	207, 175, 119, 147	Hydroxycinnamic acid derivative
6	3.51	<210, 230sh, 297	207, 175, 119, 225	
7	3.58	231, 258, 346	133, 130, 177, 159	
8	3.79	<210, 228sh, 256, 370	303, 465	Quercetin-3-hexoside
9	4.1	<210, 230sh, 256, 270sh, 354	303, 465	Quercetin-3-hexoside
10	4.19	<210, 230sh, 256, 270sh, 354	303, 465, 287	Flavonoid glycoside
11	4.26	<210, 225sh, 255, 270sh, 350	303, 287	Flavonoid glycoside
12	4.31	<210, 225sh, 254, 270sh, 348	287, 449	Flavonoid glycoside
13	4.62	230sh, 261, 285sh, 363	303, 465	Flavonoid glycoside
14	4.77	211, 224, 245sh, 268, 337	287, 449	Flavonoid glycoside
15	4.85	225, 252, 270sh, 347	301, 463	Flavonoid glycoside (hexose)
16	4.95	242, 268, 338sh	449, 287	Kaempferol-3-glycoside
17	6.97	223, 281	141, 353	Galloyl ester
18	9.26	242, 290sh, 324	435, 177, 453, 201, 189, 133	Hydroxycinnamic acid

Table A- 7. Compound peak intensities and mass fragments of interest in cloudberry KAS 371/2 GFP and arctic bramble KAS 341/8 GFP. Sh = shoulder.

Cloudberry KAS 371/2 GFP				
#	**RT**	**Peak UV_{max} (nm)**	**MS Fragments (m/z)**	**Tentative identification**
1	2.57	220, 270sh, 278, 290sh	188, 146, 136	Galloyl ester
2	2.75	219, 278, 287sh	188, 146, 205	Galloyl ester
3	3.02	<210, 229, 278	117, 104, 127	Ellagic acid derivative
4	3.38	233, 277, 320sh	371, 133, 130, 177	
5	3.54	230sh, 258	149, 133, 415, 307	
6	3.67	224, 272	137, 297, 345, 524	
7	3.79	230, 273	459, 476	
8	3.99	234, 277, (330sh)	520, 385, 503, 133, 103, 402	
9	4.07	234, 286, 324	206, 120	
10	4.21	240, 381	375, 419	Hydroxycinnamic acid derivative
11	4.26	241, 381	419, 375	
12	5.42	224, 281	134, 141, 175, 302, 324, 284, 365	Galloyl ester
13	6.99	223, 270sh, 281, 290sh	395, 133, 514, 130, 239	Galloyl ester

Arctic bramble KAS 341/8 GFP				
#	**RT**	**Peak UV_{max} (nm)**	**MS Fragments (m/z)**	**Tentative identification**
1	1.48	223	111, 120	
2	1.7	<210, 258	120	
3	2.36	222, 288, 307sh	188	
4	2.56	218, 288, 329	188, 146, 205	
5	2.64	220, 279	188, 205, 111	Galloyl ester
6	2.69	219, 278	188, 146	Galloyl ester
7	2.99	226, ~291	147, 207	
8	3.1	224, 291	147, 165	gallic acid derivative
9	3.29	234, (290sh), 319	147, 163, 207, 371	p-coumaric acid sugar ester
10	3.38	236, 323	371, 409, 133, 147, 388	p-coumaric acid sugar ester
11	3.53	<210, 224, 291	207, 225, 175	Galloyl ester
12	3.6	220sh, 233, 295sh, 326	415, 432, 453	Ferulic acid derivative
13	3.78	233, 272	459, 476	
14	3.97	229, 276	520, 385, 103, 503	
15	4.15	231, 312	446, 429, 207	Hydroxycinnamic acid derivative
16	4.27	240, 280sh, 324, 379	490, 473	Flavonol
17	4.52	233, 280sh, 305	304, 187, 147	
18	4.66	<210(sh), 224, 273	355, 175, 193, 341, 167, 533	Phenolic acid
19	5.42	223, 281	134, 141, 302, 175	Galloyl ester
20	6.97	223, 270sh, 281, 293sh	395, 396	Galloyl ester
21	8.31	<210, 247	425, 407, 205, 201	
22	8.93	<210, 245sh, 290	469, 451, 423, 487, 405, 407, 470	
23	9.2	243sh, 295sh, 311	520, 521	

Table A- 8. Compound peak intensities and mass fragments of interest in green arctic bramble KAS 341/10, and one new compound formed in ethephon elicitation. Sh = shoulder.

Green arctic bramble KAS 341/10				
#	**RT**	**Peak UV_{max} (nm)**	**MS Fragments (m/z)**	**Tentative identification**
1	1.27	225, 257	255, 456	
2	1.42	218, 257	343, 327	Gallic acid derivative
3	2.2	224, 279, 290sh	581, 582, 341, 359, 394	Galloyl ester
4	2.51	225, 257sh, 283	429, 413	Gallic acid derivative
5	3.1	229, 291	363, 342, 379, 476, 459, 481	Galloyl ester
6	3.21	233, 291, 322sh	523, 403	Galloyl ester
7	4.15	242, 293, 340sh	666, 671, 649	
8	4.26	224, 243sh, 272sh 281, 295sh	549, 710, 349	Galloyl ester
9	4.85	225, 253, 265sh, 353	456, 439, 284	Morin
10	5.03	249, 264sh, 293, 330sh	500, 483	
11	5.27	252, 263sh, 294, 355	632, 519, 615, 501, 473	
12	5.96	245sh, 265, 295sh, 325sh, 366	287, 288	
13	6.39	225, 270sh, 281, 293sh	455,437, 473, 519, 541, 409	Galloyl ester
14	6.43	(245sh), 266, 298	639, 456	
15	7.63	252, 290sh	471, 453, 407, 425, 489	

3.93	229, 247sh, 254sh, 261, 266sh, 293	362, 561, 321, 371, 201	Appears on 4th day of ethephon elicitation

Stock solutions & growth media

Both basal media used in this thesis, MS 300 KN and MS 301 KN, contain the following stock solutions (a.-d.) and Murashige & Skoog basal salt mixture M 0221 (e.). The MS 300 KN and MS 301 KN do not differ compositionally from each other, but only by their pH.

a. MSf Vitamin stock solution

Table A- 9. Composition of vitamin stock solution for the growth media.

m-Inositol	10 000 mg/L
Nicotinic acid	50 mg/L
Thiamine hydrochloride	10 mg/L
Pyridoxine hydrochloride	50 mg/L
UHP-water	1000 ml

Instructions:

Dissolve everything separately in water. Combine, and rinse containers with UHP-water. Add the rinsing water to the mixture, and fill to a litre. Divide into 10 ml batches. Stored at -20 ºC. 10 ml of stock solution used per 1000 ml of medium.

b. MSg Amino acid stock solution

Table A- 10. Composition of amino acid stock solution for the growth media.

Glycine	200 mg/L
UHP-water	1000 ml

Instructions:

Dissolve and fill to a litre. Divide into 10 ml batches. Stored at -20 ºC. 10 ml of stock solution used per 1000 ml of medium.

Hormone stock solutions:

c. A-Naphtalenacetic acid (NAA)

- NAA 25 mg/100 ml

Instructions:

Dissolve NAA into small amount of ethanol, add boiling UHP-water to evaporate ethanol, and fill to final volume when cooled. Store at +4 ºC.

d. Kinetin (Kin)

- Kinetin 50 mg/100 ml

Instructions:

Dissolve Kin into 0.5 M HCl, fill to final volume with UHP-water. Store at +4 ºC.

e. M 0221 Murashige & Skoog medium contents:

- Micro Elements

Table A- 11. Micro element composition of the basal MS medium.

	mg/l	µM
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
FeNaEDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91

- Macro Elements

Table A- 12. Macro element composition of the basal MS medium.

	mg/l	mM
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61

Total concentration of Micro and Macro elements: 4302.09 mg/l

1. MS 300 KN growth medium

MS 300 KN, is a modified Murashige & Skoog medium used for cloudberry (*Rubus Chamaemorus*) cultivation.

Table A- 13. Composition of the modified MS medium, MS 300 KN.

M0221 Murashige & Skoog medium	4.3 g/L
MSf Vitamins	10 ml/L
MSg Amino acids	10 ml/L
Sucrose	30 g/L
NAA	1.0 mg/L
Kin	0.1 mg/L

For solid medium:

- Gelrite 3 g/L

For GFP-appropriate medium:

- Kanamycin 25 mg/L

Instructions:

Dissolve solid reagents into UHP-water. Add NAA and MSf & MSg solutions. Fill nearly to final volume and adjust pH to 5.8 using 0.1 M potassium hydroxide (KOH). Fill to final volume. If making plate culture medium, weigh gelrite into autoclavable bottles. Divide medium into the bottles and sterilize by autoclaving 20 min at 120 ºC. After autoclaving, filter kinetin with 0.2 µm cellulose acetate filter, and add aseptically to the medium before use.

2. MS 301 KN growth medium

MS 301 KN, is a modified Murashige & Skoog medium used for arctic bramble (*Rubus Arcticus*) cultivation.

Table A- 14. Composition of the modified MS medium, MS 301 KN.

M0221 Murashige & Skoog medium	4.3 g/L
MSf Vitamins	10 ml/L
MSg Amino acids	10 ml/L
Sucrose	30 g/L
NAA	1.0 mg/L
Kin	0.1 mg/L

For solid medium:

- Gelrite 3 g/L

For GFP-appropriate medium:

- Kanamycin 25 mg/L

Instructions:

Dissolve solid reagents into UHP-water. Add NAA and MSf & MSg solutions. Fill nearly to final volume and adjust pH to 4.0 using 0.1 M hydrochloride (HCL). Fill to final volume. If making plate culture medium, weigh gelrite into autoclavable bottles. Divide medium into the bottles and sterilize by autoclaving 20 min at 120 ºC.After autoclaving, filter kinetin with 0.2µm cellulose acetate filter, and add aseptically to the medium before use.

Final composition of both MS 300 KN and MS 301 KN growth media:

Table A- 15. Composition of the basal growth media.

Glycine	2.00 mg/L
m-Inositol	100.00 mg/L
Nicotinic acid	0.50 mg/L
Thiamine hydrochloride	0.10 mg/L
Pyridoxine hydrochloride	0.50 mg/L
$CoCl_2.6H_2O$	0.025 mg/L
$CuSO_4.5H_2O$	0.025 mg/L
FeNaEDTA	36.70 mg/L
H_3BO_3	6.20 mg/L
KI	0.83 mg/L
$MnSO_4.H_2O$	16.90 mg/L
$Na_2MoO_4.2H_2O$	0.25 mg/L
$ZnSO_4.7H_2O$	8.60 mg/L
$CaCl_2$	332.02 mg/L
KH_2PO_4	170.00 mg/L
KNO_3	1900.00 mg/L
$MgSO_4$	180.54 mg/L
NH_4NO_3	1650.00 mg/L
Sucrose	30 g/L
NAA	1.0 mg/L
Kin	0.1 mg/L

Gelrite	3 g/L
Kanamycin	25 mg/L

LC-DAD –data

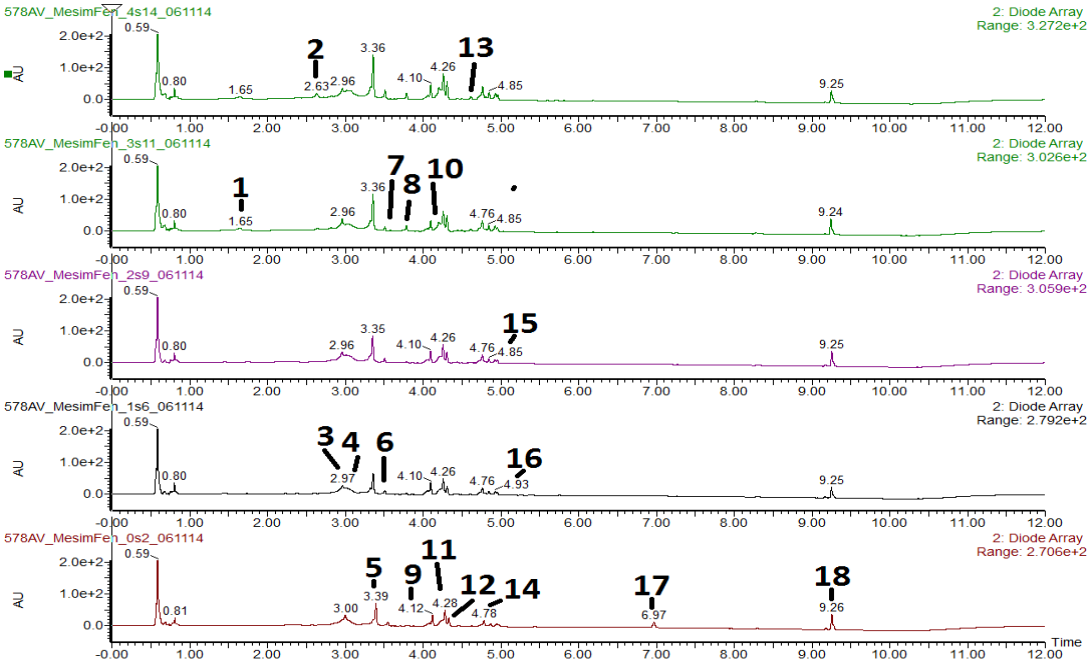

Figure A- 6. LC-DAD –data of KAS 341/15. From bottom to top: days 0, 4, 7, 11, and 14. Peak numbers correspond to those found in Appendix 3.

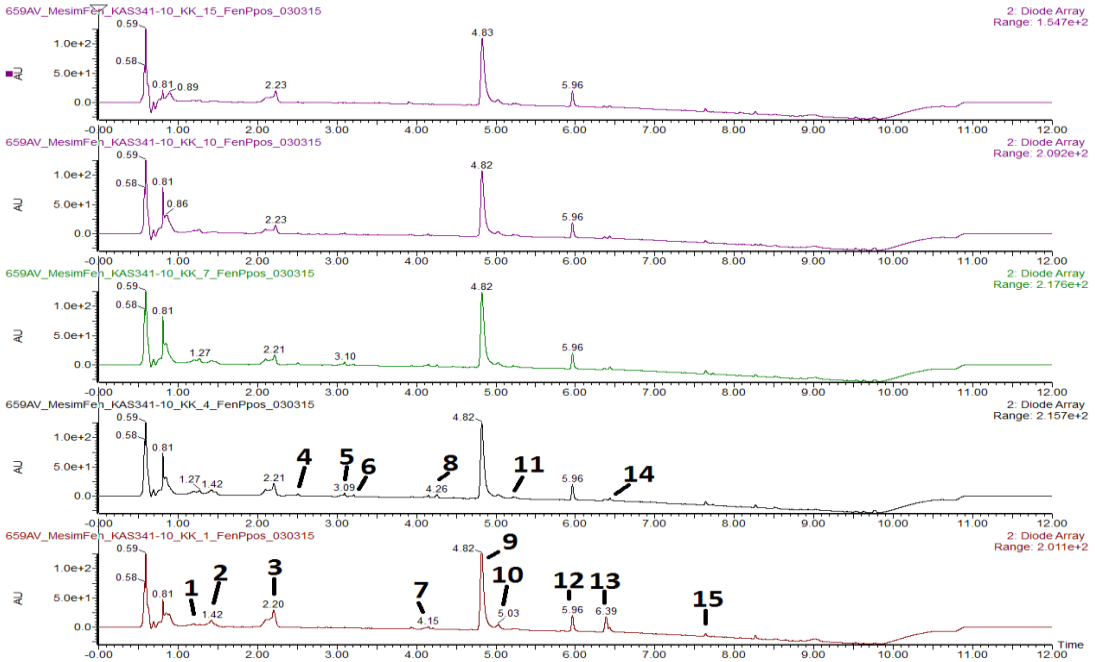

Figure A- 7. LC-DAD –data of KAS 341/10. From bottom to top: days 0, 4, 7, 11, and 14. Peak 9 is the internal standard, morin. Peak numbers correspond to those found in Appendix 3

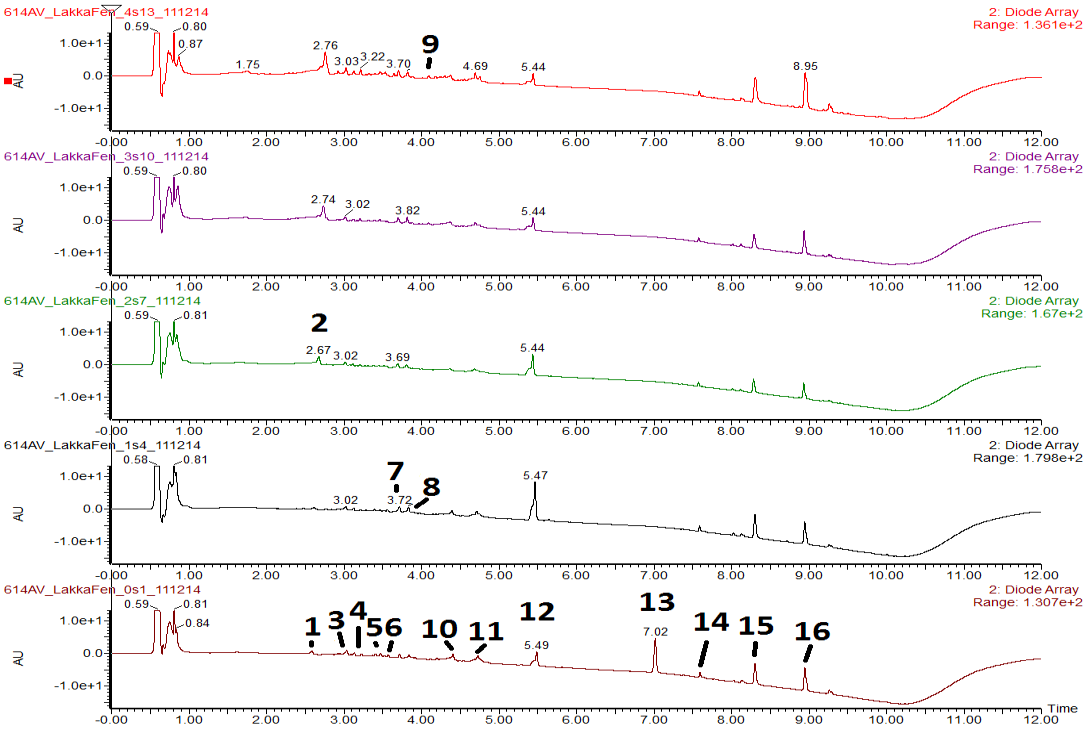

Figure A- 8. LC-DAD –data of KAS 371/2. From bottom to top: days 0, 4, 7, 11, and 14. Peak numbers correspond to those found in Appendix 3.

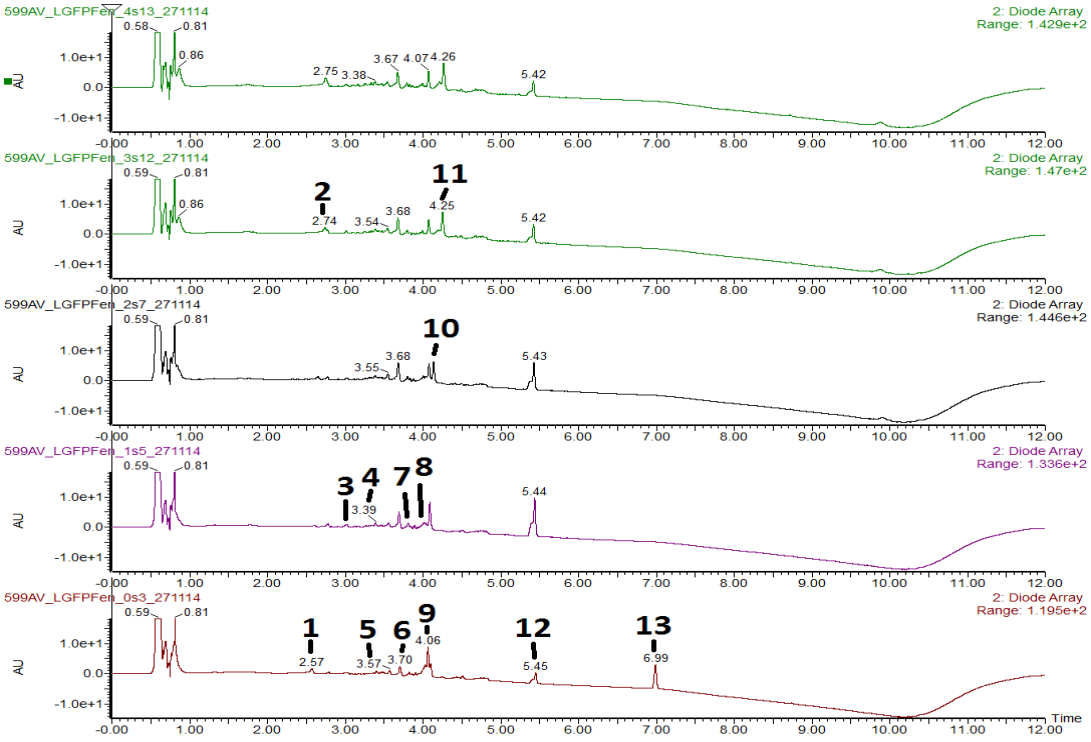

Figure A- 9. LC-DAD –data of KAS 371/2 GFP. From bottom to top: days 0, 4, 7, 11, and 14. Peak numbers correspond to those found in Appendix 3.

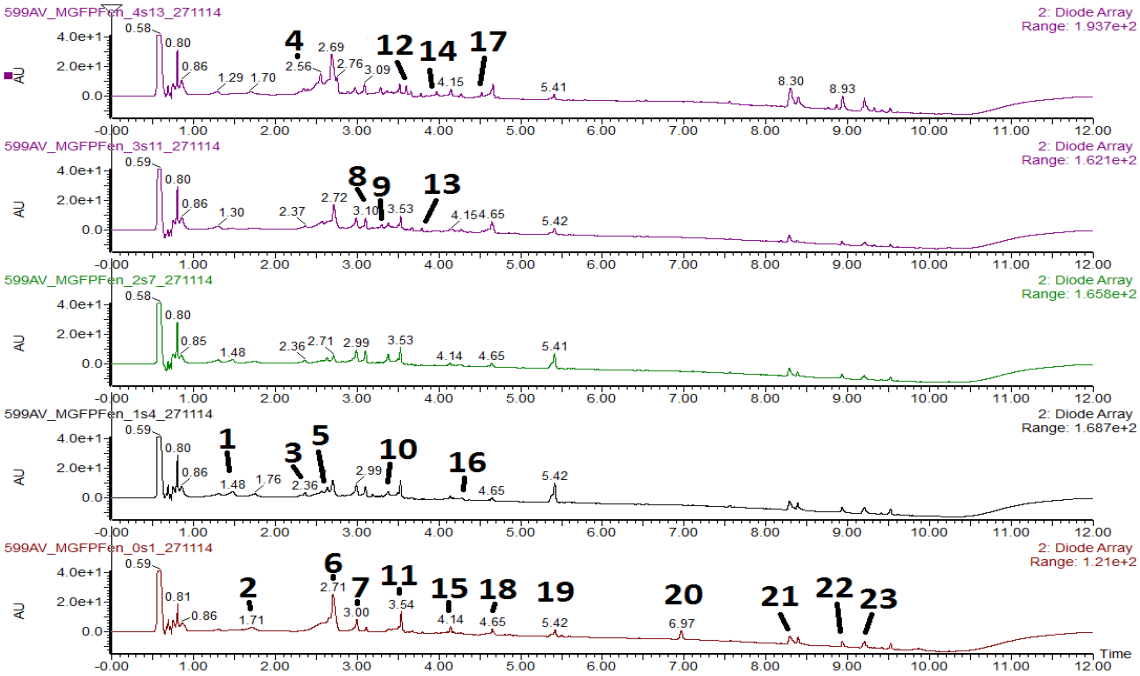

Figure A- 10. LC-DAD –data of KAS 341/8 GFP. From bottom to top: days 0, 4, 7, 11, and 14. Peak numbers correspond to those found in Appendix 3.

LC-DAD –data of the elicitations

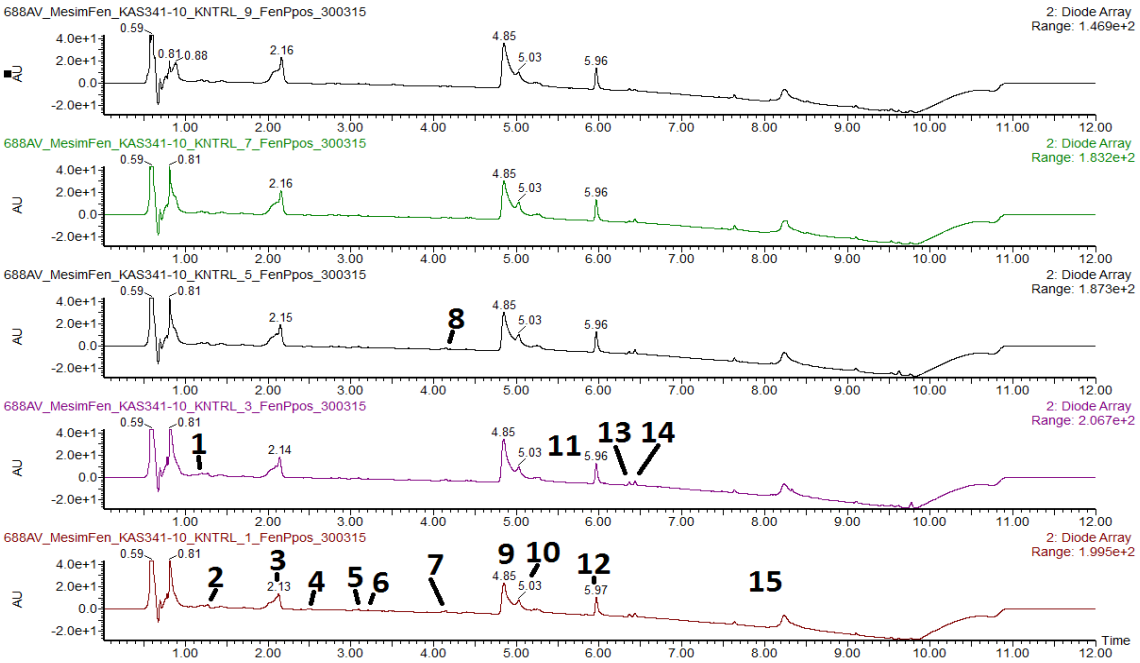

Figure A- 11. LC-DAD –data of KAS 341/10 control. From bottom to top: 7th -12th day of cultivation. Peak 9 is the internal standard, morin. Peak numbers correspond to those found in Appendix 3.

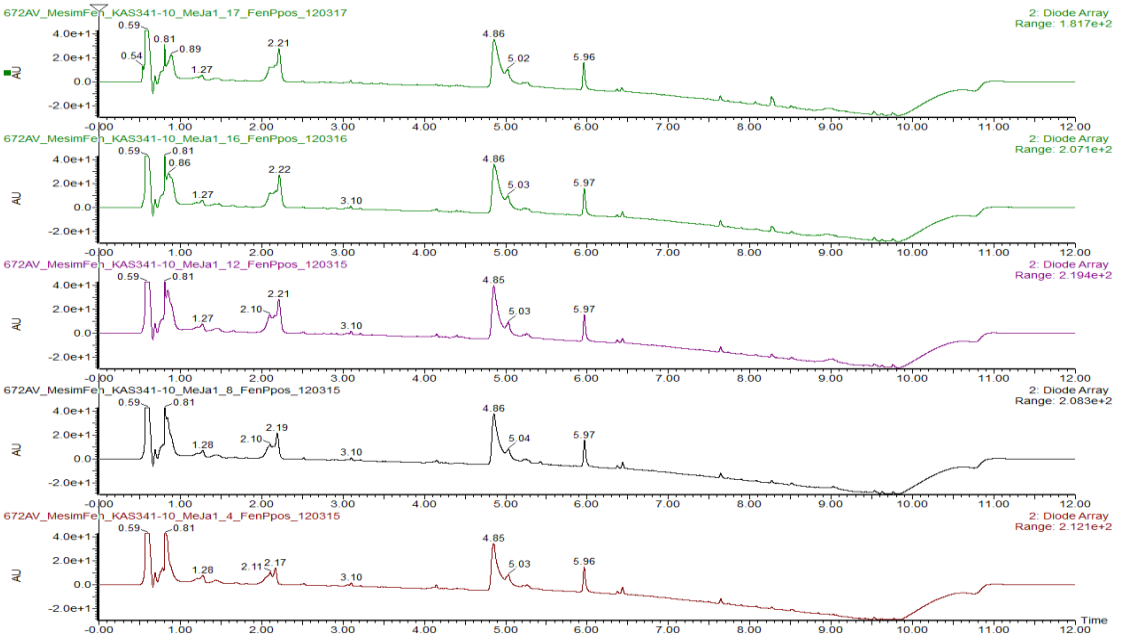

Figure A- 12. LC-DAD –data of MeJa 22.4 mg L^{-1} elicitation. From bottom to top: after the addition of elicitor, and 24h, 48h, 72h, and 96 hours from the addition.

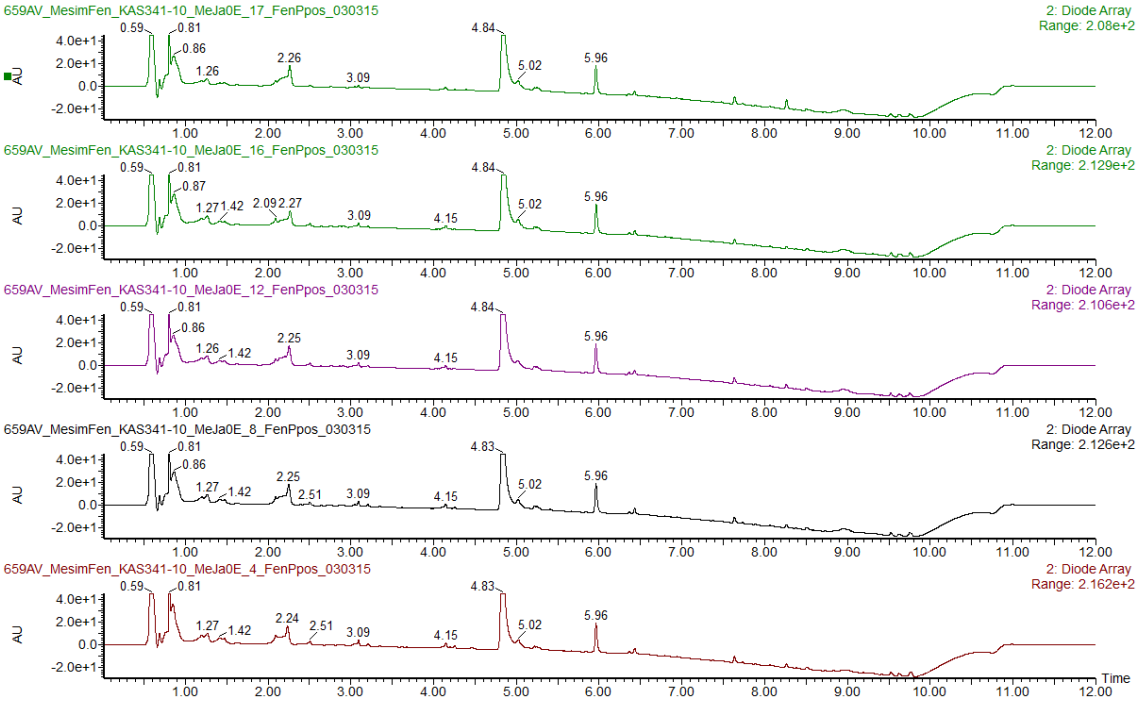

Figure A- 13. LC-DAD –data of MeJa 11.2 mg L^{-1} elicitation. From bottom to top: after the addition of elicitor, and 24h, 48h, 72h, and 96 hours from the addition.

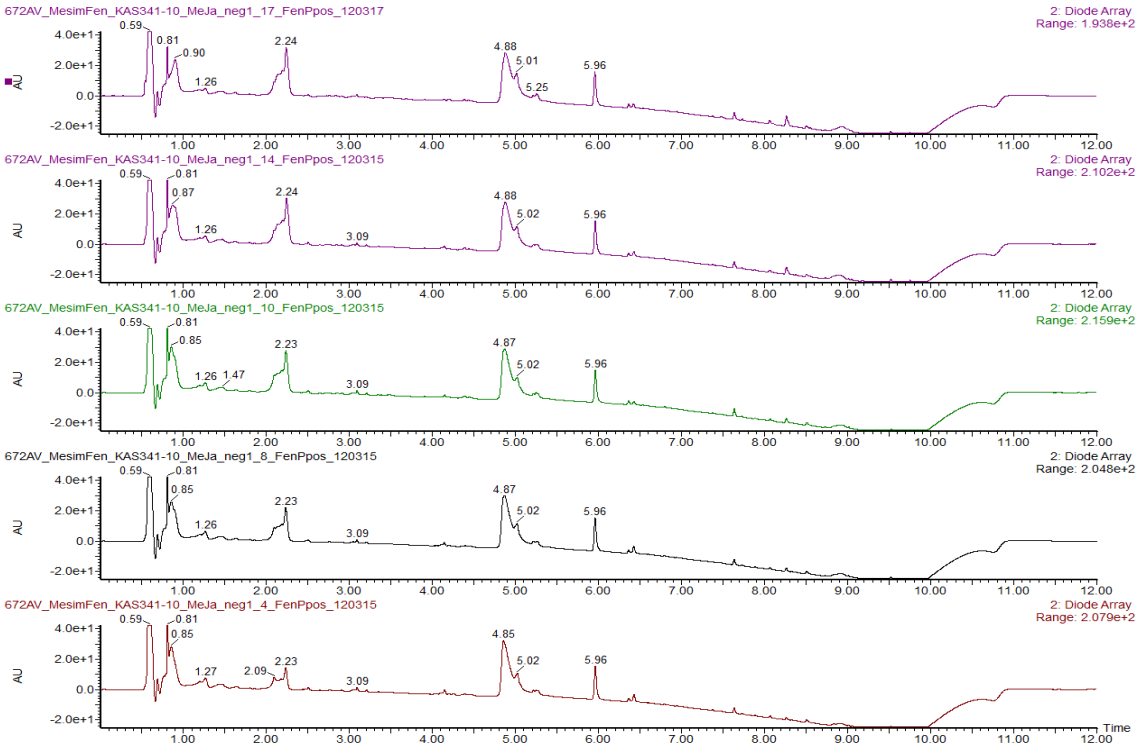

Figure A- 14. LC-DAD –data of MeJa 2.24 mg L^{-1} elicitation. From bottom to top: after the addition of elicitor, and 24h, 48h, 72h, and 96 hours from the addition.

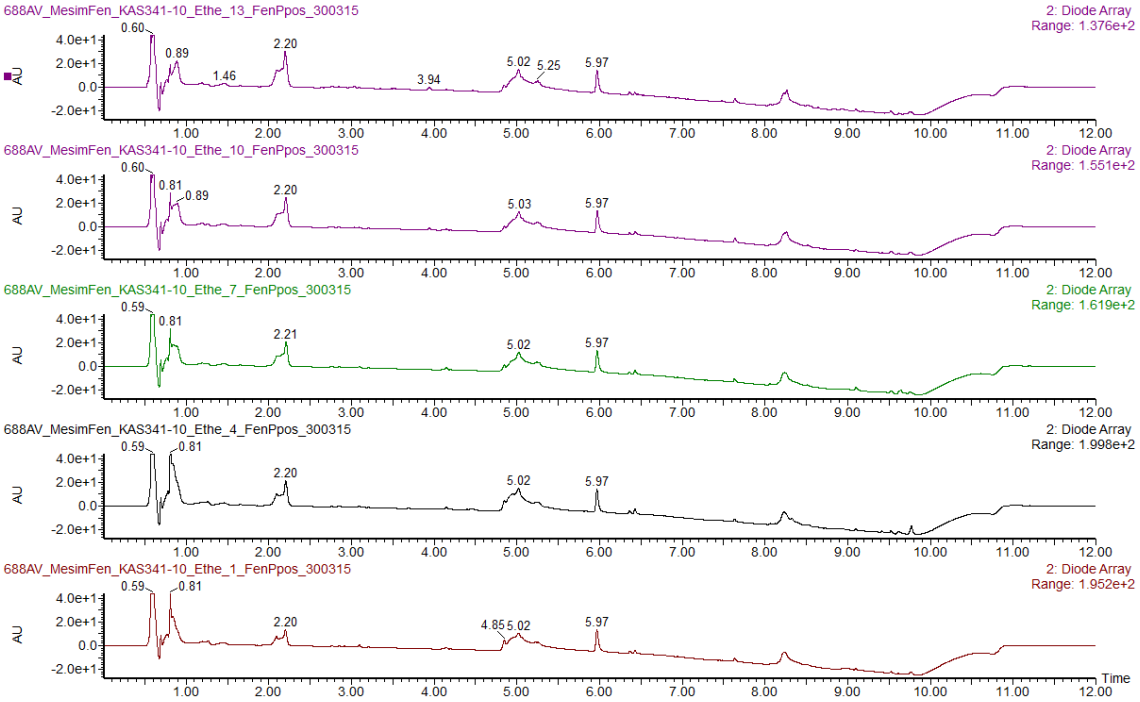

Figure A- 15. LC-DAD –data of ethephon 200 mg L^{-1} elicitation. From bottom to top: after the addition of elicitor, and 24h, 48h, 72h, and 96 hours from the addition.

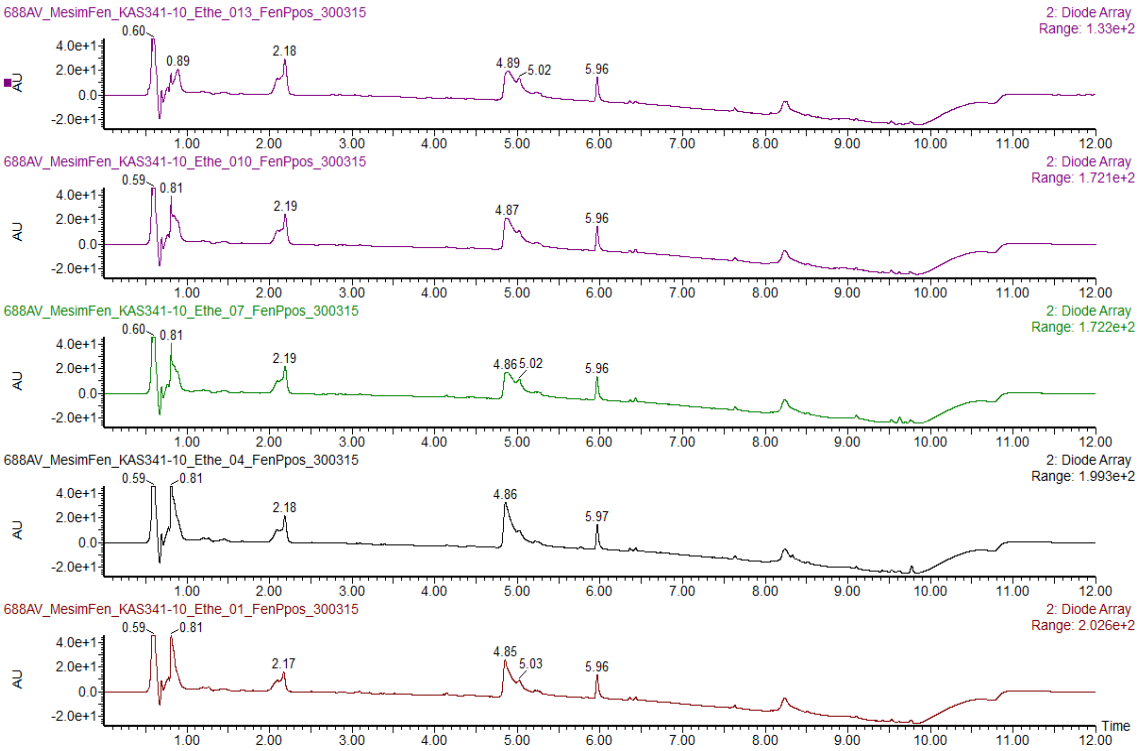

Figure A- 16. LC-DAD –data of ethephon 100 mg L^{-1} elicitation. From bottom to top: after the addition of elicitor, and 24h, 48h, 72h, and 96 hours from the addition.

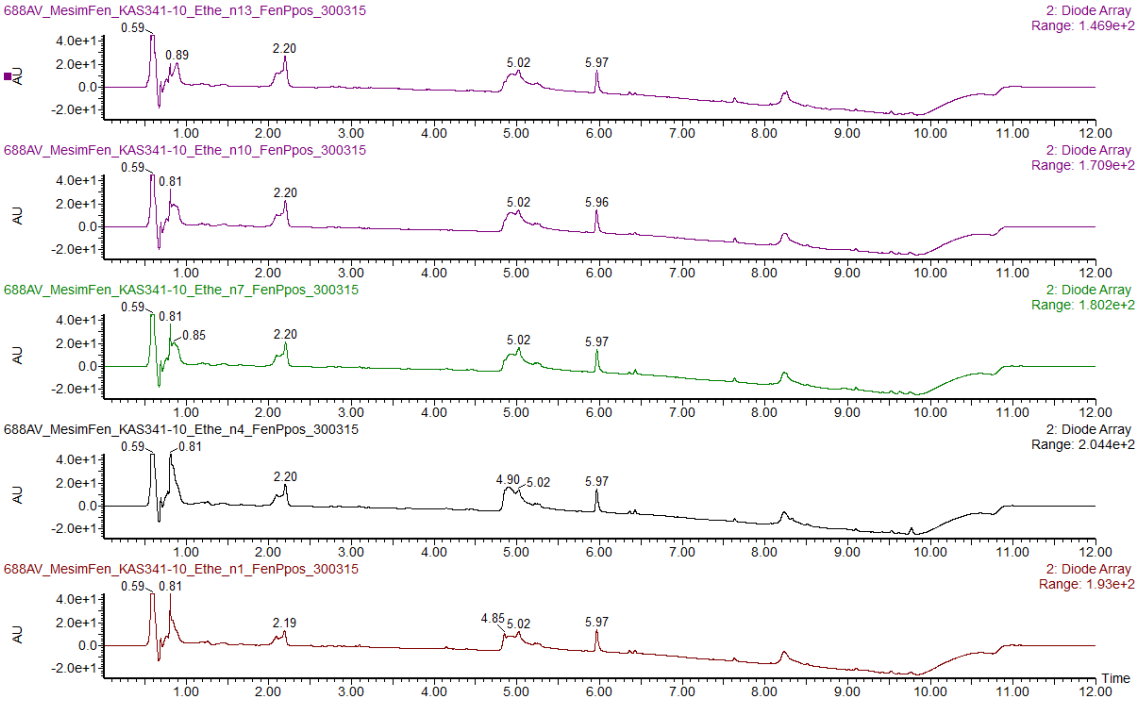

Figure A- 17. LC-DAD –data of ethephon 20 mg L^{-1} elicitation. From bottom to top: after the addition of elicitor, and 24h, 48h, 72h, and 96 hours from the addition.