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LINDA RUSALEPP

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Department of Botany, Institute of Ecology and Earth Sciences, Faculty of Science and Technology, University of Tartu, Estonia

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by Roman numerals:

I **Rusalepp, L.**, Lutter, R., Hepner, H., Kaasik, A., Tullus, A., **2021**. Secondary metabolites in leaves of hybrid aspen are affected by the competitive status and early thinning in dense coppices, *Annals of Forest Science*, 78, 1. https://doi.org/10.1007/s13595-020-01014-3.

II Tullus, A., **Rusalepp, L.**, Lutter, R., Rosenvald, K., Kaasik, A., Rytter, L., Kontunen-Soppela, S., Oksanen, E., **2021**. Climate and competitive status modulate the variation in secondary metabolites more in leaves than in fine roots of *Betula pendula*, *Frontiers in Plant Science*, 12, 2746. https://doi.org/10.3389/fpls.2021.746165.

III Kharel, B., **Rusalepp, L.**, Bhattarai, B., Kaasik, A., Kupper, P., Lutter, R., Mänd, P., Rohula-Okunev, G., Rosenvald, K., Tullus, A., **2023**. Effects of air humidity and soil moisture on secondary metabolites in the leaves and roots of *Betula pendula* of different competitive status, *Oecologia*, 202, 2, 193–210. https://doi.org/10.1007/s00442-023-05388-9.

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Author's contribution ('*' denotes a moderate contribution, '**' denotes a high contribution, '***' denotes a leading role).

	I	II	III
Original idea	***	**	*
Study design	**	**	**
Data collection	***	***	***
Analysis and interpretation	***	**	*
Manuscript writing	***	**	*

LIST OF ABBREVIATIONS

CHS	chalcone synthase
CNB	carbon-nutrient balance hypothesis
CP	canopy position
CS	competitive status
CT	condensed tannins
DHC	dihydrochalcone
DOM	competitively dominant trees
DW	dry weight
FAHM	free air humidity manipulation experiment
FLA	flavonoid
GDB	growth-differentiation balance hypothesis
HBA	hydroxybenzoic acids
HC	hydroxycinnamic acid derivative, hydroxycinnamate
HCH	6-hydroxy-2-cyclohexen-on-oyl
HPLC-MS	high performance liquid chromatography-mass spectrometry
LWA	leaf weight to area ratio ($g \cdot m^{-2}$)
MAT	mean annual temperature (°C)
PA	proanthocyanidin
PAL	phenylalanine ammonia lyase
SA	salicylic alcohol derivative, salicylate
SDU	standard deviation unit
SLA	specific leaf area ($cm^2 \cdot g^{-1}$)
SM	specialised/secondary metabolite
SUP	competitively suppressed trees
UV	ultraviolet

1. INTRODUCTION

Plants produce a wide array of metabolites that can be divided into primary and specialised metabolites. Primary metabolites are necessary for growth and metabolic functioning and include amino acids, carbohydrates, fats, proteins, and nucleic acids – all of which are necessary for the survival and well-being of the organism. All living organisms, not only plants, share the metabolic pathways used to create and use these compounds.

In contrast, specialised metabolites (SMs) increase the species' fitness for survival but are not essential for life. SMs can be classified into four major classes – terpenoids, phenolic compounds, alkaloids, and glucosinolates – and they have incredibly diverse structural features that play a variety of ecological roles. Although SMs were initially considered plant waste products (hence the older term "secondary metabolites"), it is now widely accepted that they possess numerous beneficial qualities, such as absorbing ultraviolet (UV) radiation, mitigating abiotic stresses (heat, drought, salinity, nutrient deficiency), deterring pathogen and herbivore attacks, and attracting pollinators. Plant SMs can also improve the sustainability of ecosystems (both natural and artificial) and impact nutrient cycling in the soil.

SMs are produced via different metabolic pathways than primary metabolites, and these pathways are specific to a given family or genus. Therefore, specific compounds or groups of SMs have been used as markers for botanical classification (chemotaxonomy) to help with systematic determination. However, although primary metabolites and SMs are produced via different pathways, distinguishing between them can be difficult, as the primary metabolism produces the molecules that serve as the building blocks for specialised metabolite pathways.

1.1. Growth-defence trade-off theories

Both primary and specialised (secondary) metabolisms use the same molecules as a starting point – a fact that has given rise to many theories attempting to predict the resource allocation patterns between primary and specialised metabolisms. Classical theories explaining the allocation patterns between growth and defence are founded on the existence of a finite supply of resources that can be allocated to the production of defensive compounds or growth (Bryant *et al.*, 1983, Herms and Mattson, 1992). According to these theories, the synthesis of chemical defences utilises photoassimilated carbon and other resources, such as water, mineral nutrients, and metabolic precursors, that would otherwise be devoted to producing growth-related metabolites. As a result, growth and defence are constrained by the availability of resources. The trade-offs between growth and defence within a species are typically examined in the context of the carbon-nutrient balance hypothesis (CNB) (Bryant *et al.*, 1983) and the growth-differentiation balance hypothesis (GDB) (Herms and Mattson, 1992). While the CNB claims that growth is limited by carbon or nitrogen deficiency, the GDB suggests that this relationship between growth and defence should only be apparent in abundant resources – or, in other words, when plant growth is carbon limited.

However, although they are widely used to explain the antagonistic relationship between growth and defence in natural and artificial ecosystems, these classical frameworks are often contested because they provide a relatively simplistic and one-dimensional view of the subject, preventing full comprehension of how plants adjust their phenotype in response to a variety of environmental challenges (Sestari and Campos, 2022). Classical theories mainly focus on mineral nutrition and light conditions, but plants are affected by many more environmental variables that are known to induce chemical defences. In addition to abiotic factors, biotic factors, such as competition with neighbouring plants, also affect a plant's ability to obtain resources, which may prove extremely important in the growth-defence trade-off (Matyssek *et al.*, 2005).

1.2. Biosynthesis and physiological function of phenolic compounds

Changes in SM contents are not stress specific, but the production of the same class of metabolites can be up- or down-regulated in response to various environmental factors. The complexity of metabolic pathways makes predicting the outcomes of studies on SM challenging. Outside of growth chambers, plants are exposed to more than one changing environmental factor at a time, as well as biotic factors that are often lacking in laboratory environments. Therefore, the changes in plant specialised metabolism are better studied in field experiments in which all factors, both abiotic (moisture, temperature, nutrient availability, pH, salinity, irradiation) and biotic (plant competition, pathogens, insects, herbivores), influence the plant simultaneously.

This thesis focuses on phenolic compounds. These compounds are produced in the phenylpropanoid pathway (Figure 1), which uses the aromatic amino acid phenylalanine (from the shikimate pathway) as a starting point and produces a wide array of derivatives with 1–3 or more aromatic rings. The key enzymes regulating the biosynthesis of phenylpropanoids are phenylalanine ammonia lyase (PAL), which catalyses the conversion of phenylalanine to *trans*-cinnamic acid, and chalcone synthase (CHS), which catalyses the conversion of 4-coumaroyl-CoA and malonyl-CoA to naringenin chalcone – a 15-carbon intermediate from which all flavonoids are derived (Vogt, 2010).

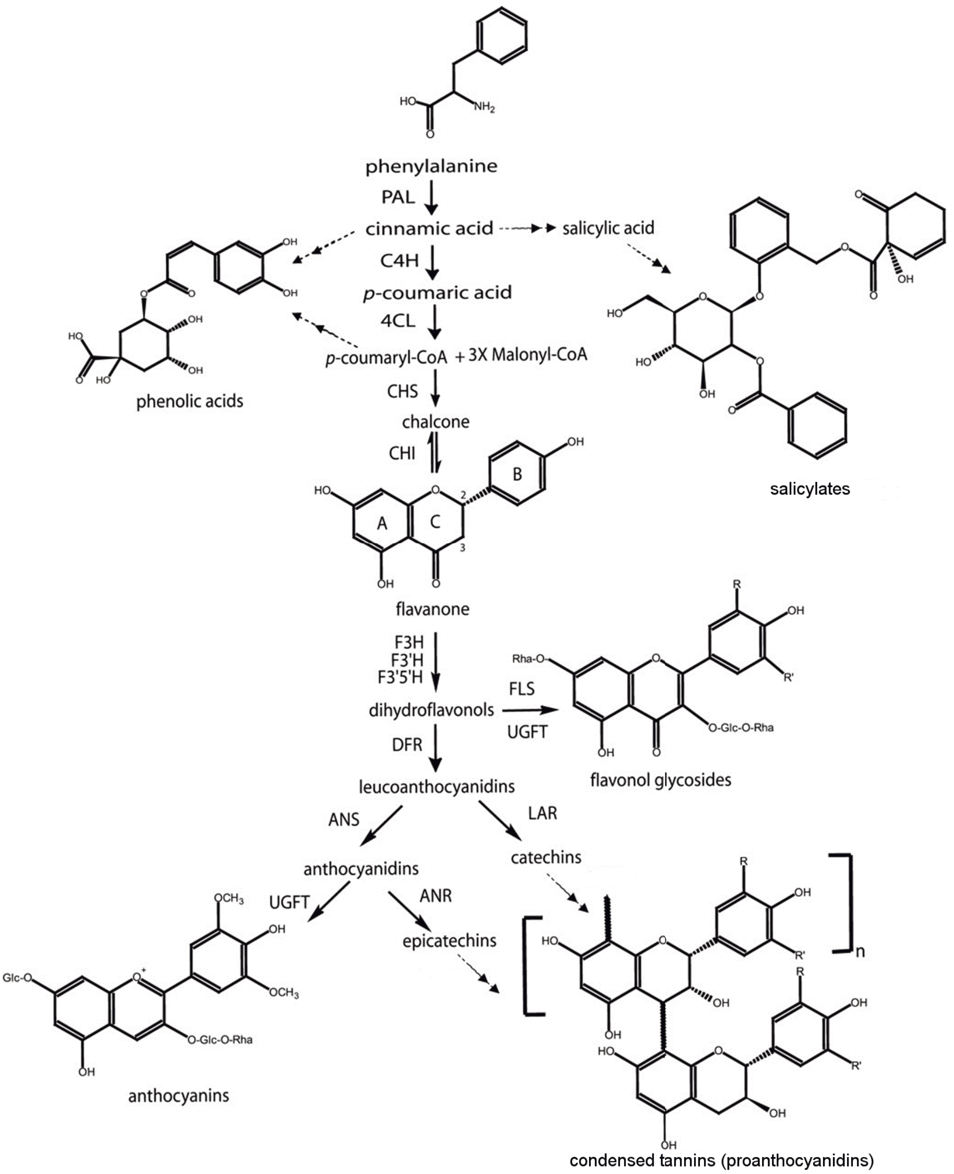

Figure 1. Phenylpropanoid pathway leading to production of phenolic acids, salicylates, flavonoids, anthocyanins, and condensed tannins. Chlorogenic acid is presented as an example of phenolic acids and tremulacin as salicylates. The flavanone illustrates the basic 15-carbon flavonoid structure with three rings (A–C). C2-C3 carbons are labeled. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; DFR, dihydroflavonol reductase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, UDP-Glc flavonoid glucosyltransferase. (With modifications from Mellway *et al.*, 2009)

Flavonoids

Flavonoids (FLA) are among the most prevalent phenolic SMs and can be further classified into many subgroups, including flavonols (e.g., quercetin, kaempferol, myricetin), flavanols (e.g., catechin, gallocatechin), condensed tannins (proanthocyanidins), and anthocyanidins (e.g., cyanidin, delphinidin). They provide a range of protective properties against abiotic and biotic stresses, including DNA alkylation, membrane stabilisation, reactive oxygen species scavenging, enzyme inhibition, and drought stress (Lavola, 1998, Kotilainen *et al.*, 2008, Morales *et al.*, 2010, Julkunen-Tiitto *et al.*, 2015, Nissinen *et al.*, 2017, Ahmed *et al.*, 2021, Qaderi *et al.*, 2023). Flavonoid synthesis can be induced under various stressful environmental conditions, including high irradiance, abnormal temperatures, and water stress.

Flavonoids are acknowledged as UV-absorbing compounds, though they are not the only class of metabolites to absorb UV radiation – all phenylpropanoids absorb the UV range of the solar spectrum (some more and some less). However, the role of flavonoids in attenuating the harmful effect of UV-B radiation is disputed, as their absorption maximum (320–380 nm) does not fall in the UV-B region (280–315 nm). In comparison to flavonoids, hydroxycinnamic acids have a higher molar extinction coefficient over the range of UV-B (Kaeswurm *et al.*, 2021). Nevertheless, the synthesis of flavonoids is preferred over the synthesis of hydroxycinnamates (Martinez *et al.*, 2016), leading to the belief that the main function of flavonoids is not UV-B absorption but antioxidant capability (Brunetti *et al.*, 2018).

Flavonoids have demonstrated antioxidant properties through several biochemical mechanisms, including the prevalent ability to scavenge free radicals either by electron delocalisation or hydrogen bond donation. Structural features, such as an ortho-dihydroxylated (catechol) B-ring and an unsaturated C2-C3 (see flavanone in Figure 1) bond in conjugation with a 4-keto group in the C-ring, enable the delocalisation of electrons from the B-ring, allowing hydroxyl groups at positions C3 and C5 to form hydrogen bonds with the keto group (Bors *et al.*, 1990). Non-flavonoid phenolic compounds with the same catechol-type structure, such as caffeic acid (hydroxycinnamate), may also act as antioxidants (Zang *et al.*, 2003). The number of hydroxyl groups in a phenolic compound has a strong, positive influence on the radical scavenging activity (Kongpichitchoke *et al.*, 2015, Platzer *et al.*, 2022). Flavonoids are present in plants mainly in glycosylated form to increase their solubility and membrane permeability and prevent the highly reactive hydroxyl groups from autoxidation, but *O*-glycosylation in the C3 and C5 positions decreases flavonoids' antioxidant potential (Agati *et al.*, 2012, Le Roy *et al.*, 2016, Platzer *et al.*, 2022).

In addition to their antioxidant properties, flavonoids can chelate transition metal ions, such as Fe(II) and Cu(II), and terminate free-radical-generating reactions. The chelating capacity of polyphenols is mostly dependent on adjacent hydroxyl groups in the B-ring (Chobot and Hadacek, 2011, Jomová *et al.*, 2019).

Catechins and condensed tannins (proanthocyanidins)

Condensed tannins (CT; also known as proanthocyanidins) are the oligomeric and polymeric end products of the flavonoid biosynthetic pathway (Dixon *et al.*, 2005). Found in the leaves, flowers, fruits, seeds, bark, and roots of many plant species, CTs are primarily composed of the monomeric flavan-3-ols catechin and/or epicatechin. They are the second most abundant biopolymer after lignin, but in contrast to that of cellulose, hemicellulose, and lignin, the polymerisation of flavanols is a nonenzymatic process (Dixon and Sarnala, 2020). CTs' major role in plants is to deter herbivores and insect pests and act as antimicrobial and antifungal defences (Henriksson *et al.*, 2003, Dixon *et al.*, 2005, Ullah *et al.*, 2017, 2019, Hammerbacher *et al.*, 2019). Plants growing in more challenging environments have been found to accumulate more tannins than plants growing in favourable conditions (Bussotti *et al.*, 1998, Top *et al.*, 2017), despite the relatively high metabolic cost of tannins compared to, for example, cellulose (Lewis and Yamamoto, 1989) and flavonoids (Kanehisa and Goto, 2000).

Similar to flavonoids, CTs' antioxidant activity is determined not only by the concentration but also by the structure of the CTs, such as the number and position of hydroxyl groups in the B-ring, the degree of polymerisation, and the stereochemistry of the C2-C3 link (Kraus *et al.*, 2003b, Top *et al.*, 2017). An increasing degree of polymerisation also increases the stability of CT and metal ion chelates (Yoneda and Nakatsubo, 1998).

CTs form a large portion of foliar dry weight (15–25%, although up to 40% has been recorded) (reviewed in Kraus *et al.*, 2003a). Due to their low rate of resorption during leaf senescence, tannins may undergo further concentration in senesced tissues, thereby constituting a large portion of leaf carbon input to soil (Tharayil *et al.*, 2011, Paaso *et al.*, 2017). Roots also contain CTs, but the proportion of root CTs compared to leaf CTs is small, and the concentrations of CTs in the roots and leaves are unrelated (Dettlaff *et al.*, 2018). CTs often inhibit leaf-litter decomposition (Kraus *et al.*, 2003a); their protein binding ability can inhibit or enhance enzymatic activity in the soil depending on CT concentration, decelerating or accelerating the decomposition of soil organic matter and promoting humus formation (Adamczyk *et al.*, 2017). Further, CTs' ability to bind proteins and organic nitrogen can enhance nitrogen retention and recycling in the soil (Dixon and Sarnala, 2020). In addition, CTs play an important role in enhancing the bioavailability of some micronutrient transition metal ions, such as Mn(II) and Fe(III), through complexation, and they can also reduce the potential toxicity of Al(III) (Kraus *et al.*, 2003a).

Phenolic acids

Phenolic acids are another large group of compounds from the phenylpropanoid pathway. They can be divided into hydroxybenzoic acids (HBA) (*p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids) and hydroxycinnamic acids (HC) (ferulic, caffeic, *p*-coumaric, and sinapic acids). Despite their involvement in a variety of stress reactions, HC production and protective effects *in vivo* are largely unknown. HC synthesis can be induced by wounding (Dixon and Paiva, 1995, Torres-Contreras *et al.*, 2014), herbivory (Kundu and Vadassery, 2019), UV-B radiation (Burchard *et al.*, 2000, Tegelberg and Julkunen-Tiitto, 2001, Mondolot *et al.*, 2006), and high salinity (Martinez *et al.*, 2016). In contrast, high temperature stress can down-regulate the synthesis of HCs (Martinez *et al.*, 2016).

The most abundant phenolic acid in plants is hydroxycinnamate chlorogenic acid – a combination of caffeic and quinic acids. Chlorogenic acids are potent antioxidants (Zang *et al.*, 2003) that have shown fungicidal properties (Martínez *et al.*, 2017). Polymerised hydroxycinnamoyl alcohol derivatives also contribute to the synthesis of one of the most well-known biopolymers, lignin (Vogt, 2010). Lignin composes a significant part of plant secondary cell walls, giving the supporting and water-conducting structures rigidity and hydrophobicity. Chlorogenic acids have been regarded as intermediates of lignin biosynthesis because of their structural resemblance to lignin monomers and also because they can be converted back to hydroxycinnamoyl alcohols and transported by the phloem sap from older to younger leaves, where they are needed for lignification (Dixon and Paiva, 1995, Mondolot *et al.*, 2006, Volpi e Silva *et al.*, 2019). The inverse kinetics of chlorogenic acids and lignin accumulation in various tissues of some plant species support the concept of chlorogenic acids' being an intermediate in lignin biosynthesis (Shen *et al.*, 2009, Volpi e Silva *et al.*, 2019 and citations therein).

Although the biosynthesis of HCs is induced by UV-B radiation – similarly to flavonoids (Tegelberg and Julkunen-Tiitto, 2001) – protection against harmful UV-B is likely not HCs' main function. While HCs have been shown to play an important role as a constitutive defence against UV-B radiation at an early stage of leaf development, as they have been present in the leaf since bud break, their importance in UV-B screening decreases with the increase in flavonoid synthesis (Burchard *et al.*, 2000, Mondolot *et al.*, 2006). Another reason for the decrease in HC content could be lignin synthesis in the subsequent stages of plant development (Shen *et al.*, 2009).

Little is known about the biosynthesis of HBAs, but research has confirmed that HAs are derived from the shikimate pathway and are not connected with phenylpropanoids (Vogt, 2010). Gallic acid and protocatechuic acid are considered precursors for the biosynthesis of hydrolysable tannins. Like HCs, HBAs are more abundant in young, expanding leaves, and they are similarly continuously transformed into insoluble cell-wall-bound phenolic compounds during the vegetation period (Ossipov *et al.*, 1997, Salminen *et al.*, 2002, Tuominen and Salminen, 2017). HBAs' main function seems to be deterring herbivores by decreasing the nutritive value of young leaves and making them unpalatable for leaf-feeding larvae (Salminen *et al.*, 1999). HBA content is not induced by UV radiation (Kotilainen *et al.*, 2008).

Salicylates

Salicylates (SA) are low-molecular-weight phenolic glycosides characteristic of the genera *Salix* and *Populus* from the family *Salicaceae*. Salicylates stem from the same phenylpropanoid pathway as flavonoids and phenolic acids. While the biosynthetic pathway leading to the simplest salicylate, salicin (2-*O*-glucoside of salicyl alcohol), has been studied to some degree, the pathways leading to more complex salicylates incorporating the 6-hydroxy-2-cyclohexen-on-oyl (HCH) moiety, like acetylsalicortin and tremulacin, are still poorly understood, and no specific enzyme involved in the synthesis has been established. *Trans*-cinnamic and benzoic acids are thought to be the precursors of salicin synthesis in plants (Ruuhola and Julkunen-Tiitto, 2003, Babst *et al.*, 2010), and cinnamic acid could be a precursor for the HCH moiety (Babst *et al.*, 2010).

Both abiotic and biotic stresses can influence the levels of SA, including wounding, ozone, UV radiation, and temperature (León *et al.*, 2001, Glauser *et al.*, 2008, Häikiö *et al.*, 2008, Khan and Khan, 2013, Jacobo-Velázquez *et al.*, 2015, Julkunen-Tiitto *et al.*, 2015, Nissinen *et al.*, 2017). Similarly to condensed tannins, salicylates act as antifeedants and defensive compounds on generalist herbivores (Hemming and Lindroth, 1995, Chen *et al.*, 2009 and citation therein). Their toxicity is believed to be caused by the degradation products of higher-molecular-weight salicylates, such as salicortin, tremulacin, and acetylsalicortin. Upon tissue rupture, higher-molecular-weight salicylates are enzymatically degraded to simpler salicin and a strong toxic feeding deterrent, HCH, which is further degraded to toxic catechol (Clausen *et al.*, 1989, Ruuhola and Julkunen-Tiitto, 2003, Babst *et al.*, 2010). The further degradation of salicin is slow. Both HCH and catechol have been shown not only to reduce the growth rate of generalist herbivores (Clausen *et al.*, 1989, Hemming and Lindroth, 1995, Ruuhola *et al.*, 2001b, Boeckler *et al.*, 2016) but also, on some occasions, to inhibit the growth of rust fungi (Sivadasan *et al.*, 2018). Although SA concentration does not often influence the extent of rust infection, rust can systematically induce salicortin and other phenolics in the plant (Hakulinen, 1998). Additionally, some specialist herbivores can utilise the glucose moiety of salicylates as a source of energy and produce defensive compounds simultaneously (reviewed in Opitz and Müller, 2009).

1.3. Specialised metabolite responses to environmental drivers

Temperature

Most previous research has shown that warming decreases the production of total phenolic compounds in the leaves of *Betula*, *Populus*, and *Salix* spp. (Kuokkanen *et al.*, 2001, Kosonen *et al.*, 2012, Nybakken *et al.*, 2012, Sobuj *et al.*, 2021). However, evidence of elevated temperatures' significantly increasing the production of phenolic compounds in *Betula pendula* is conflicted (Poeydebat *et al.*, 2021).

Elevated temperature has also been shown to reduce the concentration of catechin but not CTs in *B. pendula* leaves (Kuokkanen *et al.*, 2001), as well as the concentrations of both catechin and CTs in *Salix myrsinifolia* leaves (Nybakken *et al.*, 2012) and *P. tremula* leaves and roots (Nissinen *et al.*, 2017, Li *et al.*, 2021).

Similarly, the content of SA has been found to decrease in *Populus* spp. stems, bark, and leaves grown under elevated temperatures (Kosonen *et al.*, 2012, Sivadasan *et al.*, 2018, Sobuj *et al.*, 2021), while no temperature effect was observed in the roots (Li *et al.*, 2021). Nevertheless, increases in the contents of some individual SAs, including salicin and HCH-salicortin, were observed in the leaves of *Populus tremula* × *P. tremuloides* and *S. myrsinifolia* (Kosonen *et al.*, 2012, Nybakken *et al.*, 2012). An increased level of salicin – a degradation product of higher-molecular-weight SAs – might indicate an enhanced turnover rate of SAs under elevated temperatures (Kosonen *et al.*, 2012), although the turnover rate of salicylates is considered to be rather slow (Ruuhola and Julkunen-Tiitto, 2003). Further adding to the conflicting evidence, a field experiment by Nissinen *et al.* (2017) found that not only the concentrations of several individual salicylates increased with temperature rise, but also the total SA content in *P. tremula*.

Irradiance

Many studies have shown that increased UV radiation induces plant flavonoid synthesis (Lavola *et al.*, 1997, Lavola, 1998, Tegelberg and Julkunen-Tiitto, 2001, Morales *et al.*, 2011, Kim *et al.*, 2012, Nissinen *et al.*, 2017, Thitz *et al.*, 2021) and, consequently, that the exclusion of UV reduces the synthesis of flavonoids (Kotilainen *et al.*, 2008, Morales *et al.*, 2010). However, some studies have found that elevated UV radiation had no effect on flavonoid content (Sivadasan *et al.*, 2018, Sobuj *et al.*, 2021). In addition to UV radiation, the increased intensity of visible light has been shown to induce the synthesis of flavonoids (Agati *et al.*, 2011).

The effect of UV radiation on CT production is still unclear. Holopainen *et al.* (2018) claimed that UV radiation has no effect on tannin accumulation in Northern Hemisphere forest trees. Although examples of increased CT synthesis under elevated UV radiation or improved light conditions exist (Lavola, 1998, Osier and Lindroth, 2006, Thitz *et al.*, 2021), along with evidence of reduced CT synthesis in response to shading (Henriksson et al., 2003), elevated UV radiation has also been found to have no effect (Nissinen *et al.*, 2017) or a negative effect on CT production (Jaakola *et al.*, 2004). Adams *et al.* (2009) found that the concentration of hydrolysable tannins and CTs in various tree species did not differ on a latitudinal gradient as predicted by the UV-protectant hypothesis. Thus, CTs are generally not considered to play a photoprotective role (Jaakola *et al.*, 2004, Thitz *et al.*, 2021). Furthermore, Kao *et al.* (2002) found that CTs are not located in the upper epidermis in *P. tremuloides* leaves, where most UV filtering occurs, but in the lower epidermis and parenchyma cells.

The response of SA to elevated UV radiation appears to be species specific and, in general, negligible (Holopainen *et al.*, 2018), and the total content of SA seems to be unaffected by enhanced UV-B radiation (Tegelberg and Julkunen-

Tiitto, 2001, Warren *et al.*, 2003, Turtola *et al.*, 2005, Nybakken *et al.*, 2012, Sivadasan *et al.*, 2018). However, the content of some individual SAs, mainly salicortin and its derivatives, increased in some species, including *Populus trichocarpa* (Warren *et al.*, 2003) and *Salix myrsinites* L. × *S. myrsinifolia* Salisb., but decreased in *S. myrsinifolia* (Turtola *et al.*, 2005). Tegelberg and Julkunen-Tiitto (2001), who also studied *S. myrsinifolia*, found no effect of enhanced UV-B irradiation on salicortin, but saw a decrease in salicin content.

Wounding

Wounding has been shown to up-regulate the genes related to flavonoid synthesis in *Populus euramericana* Guinier (Kim *et al.*, 2012). It also increases the total phenolic content in *Solanum tuberosum* L., specifically chlorogenic acid, which can be used for lignification (Torres-Contreras *et al.*, 2014).

Wounding stress, which can be considered both an abiotic (e.g., wind damage) and biotic stress (Jacobo-Velázquez *et al.*, 2015), has been shown to increase the synthesis of CTs at the expense of salicylates in *P. tremuloides* Michx. (Kao *et al.*, 2002). However, Bennett *et al.* (2020) and Donaldson and Lindroth (2008) showed the opposite effect for the same species: decreased CT levels and increased SAs upon wounding. Thus, tree genotype can possess a stronger influence on foliar chemistry than environmental conditions (Bennett *et al.*, 2020).

Mechanical wounding, or wounding by herbivory, has also been shown to increase the synthesis of salicortin and tremulacin systemically in the leaves of both *Populus* and *Salix* species (Clausen *et al.*, 1989, Ruuhola *et al.*, 2001a, Donaldson and Lindroth, 2008). However, it induced a decrease in the contents of salicin, tremulacin, and tremuloidin in *P. tremuloides* roots upon defoliation (Li *et al.*, 2021). The increase in salicylates was significant in undamaged expanding and undamaged young leaves, but not in older or damaged leaves; only higher levels of degradation products were observed in damaged leaves. However, Julkunen-Tiitto *et al.* (1995) found the opposite was true for *S. myrsinifolia:* they detected an increase in salicortin levels in damaged leaves but saw no systemic reaction.

Biotic stresses

Unlike abiotic stresses, biotic stresses seem always to increase the synthesis of CTs (Mannino *et al.*, 2021). For example, a fungal infection in *Picea abies* (Hammerbacher *et al.*, 2019) and a rust fungus in *Populus nigra* L. (Ullah *et al.*, 2017, 2019) and *Salix* spp. (Hakulinen, 1998) induced the synthesis of catechin and CTs, which subsequently inhibit spore germination and hyphal growth (Ullah *et al.*, 2017, 2019).

Competition for resources is considered one of the most significant biotic stress factors, but little is known about its effect on plant specialised metabolism and the physiological processes underlying the response of trees to competition (Pommerening and Sánchez Meador, 2018). Based on the distribution of resources (light, nutrients, water, etc.), neighbouring trees can be differentiated either as competitively suppressed trees with impaired growth or as dominant trees with above-average growth. Competition can be intraspecific between neighbouring trees relying on the same resources but also within individual trees, where light, wind, air humidity, and temperature conditions vary both on a vertical axis between the upper and lower canopy and on a horizontal axis in leaves closer to or further away from the stem (Lämke and Unsicker, 2018). The allocation of nutrients also differs between lower- and upper-canopy leaves, being higher in the upper canopy (Tullus *et al.*, 2021, Kharel *et al.*, 2023). In silviculture, thinning is one of the major practises for reducing competition and reallocating resources among remaining trees (Long *et al.*, 2004, Forrester, 2019). Early-stage thinning is preferred for fast-growing northern deciduous species, such as hybrid aspen (Tullus *et al.*, 2012, Rytter, 2013).

However, the growth-defence trade-off has rarely been considered when evaluating the sustainability of different forest management practises. Combining metabolomics with other molecular or physiological techniques and the current knowledge on a species can provide a better understanding of plant stress responses. Further, the effect of competition on specialised metabolite profiles has not been studied sufficiently.

1.4. Ecometabolomics

The use of advanced analytical techniques to study low-molecular-weight metabolites in parts of or the entire living organism is termed “metabolomics” (Lin *et al.*, 2006). Metabolomics can be divided into targeted metabolomics and non-targeted metabolomics. Targeted metabolomics focuses on identifying and quantifying known sets of metabolites, while non-targeted metabolomics aims to provide a comprehensive metabolome, including unknown compounds (Zhao *et al.*, 2013). When non-targeted metabolomics is deployed to examine metabolome changes in response to a combination of stress factors, it is called “environmental metabolomics”, or “ecometabolomics”. Using this non-targeted analysis to study connections between changes in metabolite profiles and environmental conditions can lead to novel and unanticipated results and can answer questions that were not yet posed. Unlike measuring the physical characteristics of a plant or making observations, metabolomics can provide insight into the mechanisms of the stress reaction regarding changes in primary and specialised metabolites (Sardans *et al.*, 2020).

Metabolomics focuses on the compounds whose concentrations have changed in response to stress, irrespective of their relative content in the sample, and ideally, the metabolites are not grouped based on their chemical structure to avoid bias. Some of the aims of ecometabolomics include identifying the most significant biochemical differences between two or more treatment groups and discriminating between the metabolic profiles of environmentally stressed and healthy control organisms (Lin *et al.*, 2006).

Liquid chromatography-mass spectrometry (LC-MS) is the most used analytical technique for plant metabolomics studies due to its comprehensiveness, selectivity, sensitivity, relatively low cost, and excellent ability to separate and identify semipolar metabolites (which most plant specialised metabolites are) (Zhao *et al.*, 2013, Sardans *et al.*, 2020). LC-MS requires less sample preparation compared to gas chromatography, where derivatisation is often demanded. Additionally, LC-MS is particularly significant for studying numerous metabolic pathways simultaneously. The power of LC-MS can be further enhanced by collision-induced dissociation of the compounds, or fragmentation capacity, which can provide more information about the structural properties of metabolites and thus help identify the compounds (Sardans *et al.*, 2020).

Although ecometabolomics has gained popularity over the past decades, studies conducted in the wild, which are affected by a combination of environmental conditions and biotic interactions, are still lacking (Sardans *et al.*, 2020). However, studying metabolic shifts in isolation cannot provide a definitive understanding of biological processes. In addition, laboratory experiments are often conducted on model organisms, which could prove difficult with environmentally or economically relevant organisms. Furthermore, many metabolic shifts in response to biological stresses are indirect effects, and the observed metabolic shifts are not necessarily directly connected with the original stress factor (Bundy *et al.*, 2009).

1.5. Aims of the thesis

As stated above, most studies on how stress factors affect plants' specialised metabolism are either conducted in laboratory conditions or on model species. The goal of this thesis is to provide new knowledge of the impact of intraspecific and within-individual tree competition and environmental conditions on the specialised metabolite profile of two economically important tree species – hybrid aspen and silver birch – growing in field conditions. The thesis aims to identify specific metabolites that could indicate plant stress levels, to examine shifts in the metabolome, and to elucidate whether any growth limitations exist in connection with the production of phenolic specialised metabolites.

The specific aims of the thesis were as follows:

- To analyse the effect of early thinning on the specialised metabolite profile of competitively dominant and suppressed vegetatively regenerated hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) leaves (**I**).
- To analyse the effect of competitive status and canopy position on the specialised metabolite profile of silver birch (*Betula pendula* Roth.) leaves and fine roots (**II, III**).

The hypotheses were the following:

1) Thinning of vegetatively regenerated hybrid aspen stands decreases specialised metabolism in the remaining trees and diminishes the difference between dominant and suppressed trees' specialised metabolite profiles (**I**).
2) The specialised metabolite content is higher in plants or plant parts exposed to greater stress caused by environmental factors (temperature, air humidity, light conditions, nutrient availability) (**I–III**).
3) The difference in metabolite profiles within an individual tree's canopy is similar to the difference between dominant and suppressed neighbouring trees (**II**, **III**).

2. MATERIAL AND METHODS

2.1. Description of study sites

Three separate experiments were conducted on two early-successional, fast-growing deciduous tree species: hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) (**I**) and silver birch (*Betula pendula* Roth) (**II, III**).

Coppice thinning experiment (I)

The first study was conducted at a hybrid aspen regeneration experimental site in southern Estonia (Nõgiaru). The site was established in spring 2014 in a vegetatively regenerated hybrid aspen stand that had emerged from root and stump sprouts after clearcutting a 14-year-old hybrid aspen plantation. The stand comprised 14 clones in a random mixture originating from the same breeding programme in Finland, and the plant material improvement level and origin were similar (Tullus *et al.*, 2007). The initial density of the hybrid aspen coppice stand was 94,000 trees ha^{-1}, and this did not vary significantly across the 2-ha study area (Hepner *et al.*, 2020). The thinning trial of the stand began after the second growing season (early spring 2016). Areas with two thinning intensities were created, while some areas were kept as unmanaged control (Figure 2). Thinning was performed systematically using (i) the corridor method (C), by which 2-m-wide corridors were cut, leaving 1-m-wide uncut strips of trees, and the initial density was reduced by about two thirds to 23,000 trees ha^{-1}. Additionally, thinning was performed using (ii) the cross-corridor method (CC), by which the 2-m-wide corridors were cut in two perpendicular directions, leaving 1 × 1-m uncut patches of trees, and the initial density was reduced by about eight ninths to 9,000 trees ha^{-1}. All treatments were randomly distributed across the experimental area in three 30 × 40-m-sized replications. Each replication contained three circle plots with a size of 12.6 m^2 (radius = 2 m) in the control and 28.3 m^2 (radius = 3 m) in the corridor and cross-corridor treatments.

Leaf sampling was performed in mid-July 2017. Two model trees with different competitive statuses (a dominant and a suppressed tree) were chosen next to each circle plot. The model trees were selected so that the stem diameter of the dominant tree was greater than the upper diameter quartile in the given sample plot, and the diameter of the suppressed tree was less than the lower diameter quartile. The model trees were harvested, and the basic growth characteristics were measured in the field. All leaves were separated from the model trees, and fresh weight was determined. Twenty sample leaves were randomly selected across the crown of each model tree, weighed, and dried to a constant weight at 65 °C. Subsequently, the leaves were weighed to the nearest 0.01 g, and single leaf blade area was measured with the software package WinFolia (Regent Instruments Canada, Inc.) to estimate the mean leaf weight per area (LWA, g·m^{-2}).

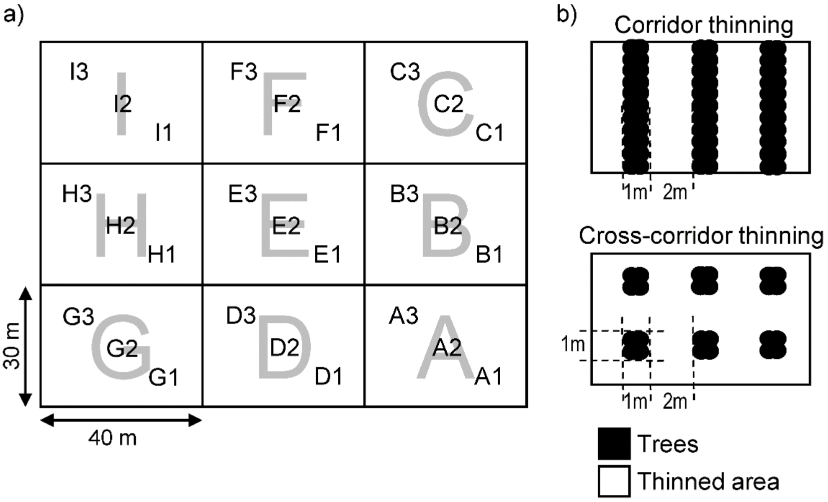

Figure 2. a) Design of the experimental area. Treatment replications are indicated with capital letters (A, F, H – unmanaged control; B, D, I – corridor thinning; C, E, G – cross-corridor thinning). Numbers 1, 2, and 3 indicate the circle plots in each replication. b) Graphic illustration of the applied thinning treatments (Rusalepp *et al.*, 2021)

Latitudinal study (II)

A second latitudinal study was conducted on young (6–9-year-old) silver birch plantations that had reached canopy closure and entered an intensive competitive growth phase. The six study sites were distributed along a 1,400-km latitudinal gradient (56–67° N) in northern Europe (Fennoscandia), where long-term mean annual temperature (MAT) ranges from −1 to 8 °C.

At each site, five pairs of trees were sampled in July 2018, and their basic growth characteristics were measured. Each pair included a competitively dominant and a competitively suppressed neighbouring tree; the distance between the two individuals varied from 1.2–3.5 m. The model trees were selected so that the height of the dominant tree exceeded the average height of its closest neighbouring trees by at least 1 m, and the height of the suppressed tree was at least 1 m less than the average height of its closest neighbours. From each model tree (10 trees from each site, 60 trees total), two composite leaf samples and one composite root sample were collected.

The leaves were collected from the upper and lower quarters of the south-facing side of the living crown. From both crown layers, a branch was cut with a 9.6-m extendable pole cutter (Ergo-Schnitt, Germany), and 35–40 mature leaves without visible damage (to avoid local damage-induced defence) were collected. Fresh leaf blade area was measured with the software package WinFolia (Regent Instruments Canada, Inc.). Thereafter, the leaves were dried to a constant weight at 60 °C and weighed to the nearest 0.01 g to estimate the mean specific leaf area (SLA, $cm^2 \cdot g^{-1}$).

Three to four 10–20 cm root fragments were extracted near each model tree stem (after visual confirmation that the root initiated from the given stem) from the 0–20-cm topsoil layer and preserved in a portable cooler during transportation to the laboratory. Fine roots (diameter < 2 mm) were separated in the laboratory from each root sample and dried to a constant weight at 40 °C for further analysis.

Humidity manipulation experiment (FAHM) (III)

The third study was performed at the free air humidity manipulation (FAHM) experimental site in Rõka, Järvselja Experimental Forest District in southeastern Estonia. The experimental area was established in 2006–2007 and covers 2.7 ha, containing nine 14 × 14-m experimental plots arranged in two rows and surrounded by a buffer plantation of hybrid aspen (Kupper *et al.*, 2011). The current study focused on silver birch trees, planted for the second FAHM study period (FAHM2) in spring 2013 in one-half of each experimental plot. The micro-propagated 2-month-old birches were planted in a 1 × 1-m arrangement. Out of the nine plots, three are control plots with ambient conditions, three are elevated relative air humidity (H) plots, and three are irrigation plots (Figure 3). The elevated relative air humidity plots were provided with a computer-controlled system of mist emitters and air blowers emitting water droplets of ~10 μm in size, and the relative air humidity inside the plots was increased by ca. 5% as a long-term seasonal average when humidification was switched on (Kupper *et al.*, 2011). Humidification was switched on only during the daytime and at ambient relative air humidity < 75%; hence, the overall seasonal average difference in relative air humidity between the control plots and elevated relative air humidity plots (also including times when humidification was not applied) was lessened. In the irrigation plots, soil was irrigated using perforated 13-mm-diameter irrigation hoses (Alfred Kärcher SE & Co. KG) situated on the ground between the tree lines (200 m of hose in each plot). The maximum water supply for a plot was 200 litres per hour. For irrigation, well water was used. In 2019, during the growing period (from May to September), 20% (78.3 mm total) precipitation was added to the ambient weekly precipitation via irrigation at the beginning of the following week to simulate the projected precipitation increase by 2100 in northern Europe (Scoccimarro *et al.*, 2015).

Root and leaf samples from silver birches were collected during the eighth growing season in mid-July 2019. The competitively dominant and suppressed tree pairs were selected randomly for sampling. Whether a tree was dominant or suppressed was determined based on the height difference between its closest neighbouring trees. On average, dominant trees were about 0.8 m taller and suppressed trees were about 0.8 m shorter than their neighbours. Two tree pairs were sampled from each plot, resulting in a total of 36 (18 dominant and 18 suppressed trees) trees. Leaves were collected from the south-facing side of the upper and the lower quarter of the living crown; 30–35 fully-grown leaves without visible damage were collected from both locations.

To determine the root specialised metabolite content, root samples (three to four root fragments originating from the root system of a sample tree) were excavated near the stem. Fine roots (diameter < 2 mm) were separated from the collected root samples and dried at 40 °C for the subsequent chemical analysis.

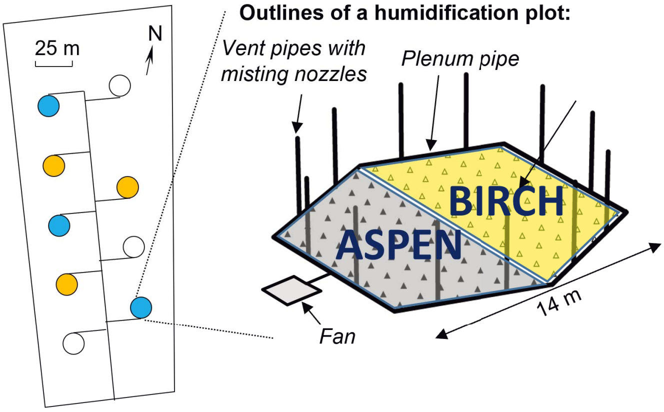

Figure 3. FAHM experimental design. Blue circles – air humidification plots; yellow circles – soil irrigation plots; white circles – control plots. (With modifications from Rosenvald *et al.*, 2021 Supplementary Fig 1.)

2.2. HPLC-MS

The sampled leaves were ground prior to the chemical analysis. The extracts were chromatographically analysed using a 1290 Infinity system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6450 Q-TOF mass spectrometer equipped with a Jetstream ESI source. The dried, ground plant material was extracted with an aqueous methanol solution of varying concentrations with a w/v ratio of 1:20 for leaves and 1:10 for roots for 24 hours in darkness at room temperature. An ultrasonic bath was used at the beginning of the extraction to improve the extraction yield. Subsequently, the extracts were centrifuged, and the supernatants were collected and then centrifuged again (**II**, **III**) or filtered through a syringe filter (**I**). Last, the supernatant was collected in a glass vial and subjected to a Zorbax 300SB-C18 column (2.1 × 150 mm; 5 µm; Agilent Technologies) kept at 40 °C. For the elution of the samples, a gradient programme of 0.1% formic acid in water (A) and acetonitrile (B) was used. Data acquisition and initial data processing were conducted using the MassHunter software (Agilent Technologies). Compounds were identified by comparing the m/z value, retention time, UV spectra, and MS^2 fragmentation patterns with standards, with data from the literature, or with the METLIN database (Agilent Technologies). The analysis were carried out in the Division of Food Hygiene and Safety of the Estonian University of Life Sciences.

2.3. Foliar nutrient analysis

Leaf total nitrogen content ([N], %) was analysed with the Kjeldahl method using a Kjeltec Auto 1030 Analyzer (Foss Tecator AB, Höganäs, Sweden). Phosphorus ([P], %) was determined spectrophotometrically *via* Kjeldahl acid digestion using a FIAstar 5000 Analyzer (FOSS Tecator AB), and potassium ([K], %) was determined flame-photometrically using the Sherwood Model 425 Flame Photometer in the Laboratory of Plant Biochemistry at the Estonian University of Life Sciences.

2.4. Data analysis

The extracted ion chromatogram (EIC) peak areas were transformed into standard deviation units (SDU, also z-score) in papers **II** and **III**. In paper **I**, the external calibration graph method was used to calculate the concentrations of compounds using commercial standards (salicin, catechin, chlorogenic acid, procyanidin B1, kaempferol-3-*O*-glucoside, rutin, myricetin, and phloridzin purchased from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

The effects of treatments on specialised metabolite content were analysed with a linear mixed model (LMM). Competitive status (**I**, **II**, **III**), canopy position (**II**, **III**), thinning intensity (**I**), and humidity treatment (**III**) were the fixed factors in the corresponding papers. Tree pair, sample plot, and tree were added as random factors where applicable. The average height of the dominant trees sampled at each site was added as a covariate in the latitudinal study (**III**) to capture confounding variation arising from differences in soil properties and sample tree age. The LMM analysis presented in papers was performed with the *lmer* function in the *lme4* package in the R Statistics software (R Core Team). Additional statistical analysis for this thesis was conducted using general linear models (GLM) in the software STATISTICA (StatSoft Inc., 2005, version 7.1).

The normality of model residuals was checked using residual histograms and Q-Q plots. The data was *log*- or square-root transformed when necessary. A significance level of $\alpha = 0.05$ was used to reject the null hypothesis after statistical tests.

3. RESULTS AND DISCUSSION

3.1. Higher irradiance increased flavonoid synthesis (I–III)

Many factors could have impacted the specialised metabolite content of plants by modifying the light conditions, such as the competitive status of the tree (**I**, **II**, **III**), the canopy position of the leaves (**II**, **III**), thinning of young stands (**I**), and the latitudinal gradient (**II**), which correlates with the length of the vegetation period and light spectral composition. Light availability is considered a dominant factor in plant competition (Poorter *et al.*, 2019) and is also a key factor influencing the production of specialised metabolites throughout the canopy (Downum *et al.*, 2001). The leaves in the upper canopy receive more light and have better light-harvesting abilities than those in the lower canopy.

The results from the field experiments showed that higher light intensity promoted flavonoid (FLA) production. FLA content was higher in upper canopy leaves than in lower canopy leaves in both silver birch and hybrid aspen (**I**, **II**, **III**). FLA was also higher in competitively advantaged, larger trees than their suppressed neighbours (**I**, **II**). The content of FLA in silver birch did depend on the competitive status of the tree in colder climates, but no difference was found in warmer climate conditions (**II**), including the FAHM experiment in Estonia (**III**). The content of FLA and hydroxycinnamates (HC) also increased towards lower latitudes, resulting in a longer growth period (**II**). Higher levels of FLA and HC in dominant (taller) trees, upper canopy leaves, and southern sites all indicate a greater need to protect against higher levels of irradiance (Agati *et al.*, 2012, 2020).

As demonstrated in paper **I**, modified light conditions resulted from two thinning treatments. After thinning, an increase in FLA and HC was observed only in suppressed trees. FLA and HC are essential in shielding plants from UV radiation (Burchard *et al.*, 2000, Groenbaek *et al.*, 2019, Del Valle *et al.*, 2020). In suppressed trees, the concentrations of FLA and HC increased correspondingly to the extent of thinning, reaching a comparable level to that of dominant trees. This indicated that the light conditions had improved overall and supported the association between UV radiation and FLA and HC biosynthesis (Lavola, 1998, Kotilainen *et al.*, 2008, Morales *et al.*, 2010, Nissinen *et al.*, 2017).

Dihydrochalcones (DHC) also belong to the class of flavonoids, but due to their open-chain structure, DHCs display a strong antioxidant potential despite their low concentrations in plants (Nakamura *et al.*, 2003, Dugé de Bernonville *et al.*, 2010, Ibdah *et al.*, 2018, Li *et al.*, 2018). Although the absorbance spectrum of DHCs (Minsat *et al.*, 2021) is similar to those of major flavonoids, including quercetin, myricetin, and kaempferol, DHC's biosynthesis does not seem to be induced by UV radiation. The contents of DHC remained low in dominant trees throughout the thinning experiment, while in suppressed trees, the amount of DHC increased manifold. This finding supports the photodamage hypothesis by Close and McArthur (2002), who suggested that the flavonoid content in plants in some environmental conditions is not low because of limited resources (like photosynthetically active radiation), but because flavonoids are not required. Although the hypothesis aimed to show that plants do not produce flavonoids to defend themselves against herbivory but to protect themselves from photodamage, Close and McArthur (2002) also stated that flavonoids mitigate photodamage by acting as antioxidants, not by thermal energy dissipation. This implies that the FLA, HC, and DHC content in suppressed trees increased considerably because of oxidative stress induced by excess light. The oxidative pressure for dominant trees did not change with the thinning treatments.

3.2. Thinning induced a systemic long-term increase in salicylate production in hybrid aspen (I)

With a significant impact on the total SM content, salicylates (SA) were the most prevalent class of metabolites in the examined hybrid aspen leaf samples (**I**). The total SA content was low and remained so in the dominant trees through all treatment levels (ca 2% foliar dry weight [DW]), while the content of SA in suppressed trees (ca 5–8% DW) was higher in the unthinned control group (Fig. 2 in **I**) and increased further with the extent of thinning, reaching levels four times higher than that in dominant trees.

Early thinning of a young, root-connected clonal aspen coppice stand could be seen as wounding or severe defoliation, as the roots of the cut trees likely remained alive (Baret and DesRochers, 2011). In a broader sense, thinning, wounding, and defoliation all result in reduced photosynthetic capacity and decreased carbon assimilation. Similar changes in phenolic accumulation were seen in a comparable shoot thinning and leaf removal study on *Vitis vinifera* (Torres *et al.*, 2021), but no such studies have been conducted on *Populus* or other forest tree species.

Defoliation is known to have a strong, negative long-term effect on the growth characteristics of *Populus* (Helbig *et al.*, 2021). Though a short-term increase in SA content in response to wounding and defoliation has been shown in *Salix* and *Populus* spp. (Clausen *et al.*, 1989, Ruuhola *et al.*, 2001a, Donaldson and Lindroth, 2008), long-term induced responses in foliar chemistry are still poorly understood (Osier and Lindroth, 2004). The SM profile of *Populus* is highly dependent on the genotype (Osier and Lindroth, 2004, 2006, Donaldson *et al.*, 2006, Bandau *et al.*, 2015, Hamanishi *et al.*, 2015, Eisenring *et al.*, 2023), and the genotype effect on SM can, at times, exceed the effect of the environment (Bennett *et al.*, 2020). However, the sampled trees in the thinning experiment belonged to different genotypes, and information on the specific genotype of each tree is missing. However, a significant long-term induction of salicylates was seen the year following the thinning.

Osier and Lindroth's (2004) 1-year-long defoliation experiment with *P. tremuloides* resembles our study. Osier and Lindroth (2004) found the content of SA to be strongly determined by the genotype, as well as the content of CTs. They argued that defoliation should increase the C:N ratio, as defoliation removes more mineral nutrients than carbon. Thus, the level of specialised metabolism should increase in the year following defoliation. However, no difference was observed in foliar [N] between the control and defoliated trees (Osier and Lindroth, 2004). In our thinning experiment (**I**), in which the stem and foliage nitrogen reserves were also removed, a decrease in foliar [N] occurred in both dominant and suppressed trees, whereas [P] decreased only in suppressed trees. At the same time, a significant increase in SA content was observed in the suppressed trees. This result coincides with findings in fast-growing African savanna tree species (Bryant *et al.*, 1991) and silver birch (Kaitaniemi *et al.*, 1998), where upon severe defoliation, a decrease in foliar nutrients was accompanied by increased total phenolics and CT content. A similar increase in phenolics was observed in the winter-deciduous tree species *Nothofagus pumilio,* with the difference that CT levels also dropped with leaf [N] (Piper *et al.*, 2015). This negative correlation between leaf nutrients and SM content aligns with the CNB hypothesis (Bryant *et al.*, 1983). In a defoliation treatment, the tree can theoretically regrow the leaves, either during the same vegetation period or the following year. In contrast, in a thinning experiment, the photosynthesising leaf biomass cannot be easily replaced, and the maintenance costs for the root system remain the same. Hence, thinning can have a greater and longer-lasting impact on tree growth and specialised metabolite profiles than short-term defoliation.

However, in contrast to the findings of study **I**, Osier and Lindroth (2004) found that defoliation significantly increased the foliar content of condensed tannins (CTs). Conversely, Donaldson and Lindroth (2008) observed no wound-induced long-term increase in SA or CT content in *P. tremuloides*. *Populus* genotypes can be categorised as high- or low-CT genotypes according to their relative investment in CTs (Bandau *et al.*, 2015). Low-CT genotypes have been shown to accumulate higher levels of SA and *vice versa*: high-CT genotypes accumulate lower levels of SA (Kao *et al.*, 2002, Donaldson and Lindroth, 2008, Mellway *et al.*, 2009, Kosonen *et al.*, 2012, Decker *et al.*, 2017, Bennett *et al.*, 2020). However, the content of CTs in hybrid aspen leaves (**I**) was not measured with a conventional method, such as the acid-butanol assay (Hagerman, 1988). Instead, the content of proanthocyanidins (shorter chain CTs) extracted along with other phenolic compounds was measured with HPLC-MS. This method does not provide the exact content of CTs in the sample. Additionally, the information about sample tree genotypes was absent (**I**). In high-CT genotype trees, biotic stress factors seem more likely to impact the content of CTs, as Osier and Lindroth (2004) might have observed, and in low-CT genotypes, the content of SA is likely to change. Considering that only slight changes in catechin and proanthocyanidin content were seen in both dominant and suppressed trees with thinning, while significant changes in SA content were seen only in suppressed trees, it can be assumed that the hybrid aspen in the study **I** were primarily low-CT genotypes. Thus, the specialised metabolism response to loss of leaf area may depend on the tree's genotype and result in increased SA or CT content.

Both of these metabolite classes serve a similar herbivore deterrent and anti-feedant purpose in *Populus* spp. Although the efficacy of CTs in deterring herbivores is disputable, evidence exists that tannins help retain nitrogen in the soil and hence may support tree recovery from defoliation (Madritch and Lindroth, 2015). The metabolic cost of SA and CT is above average among specialised metabolites and is strongly negatively correlated with growth rate (Ruuhola and Julkunen-Tiitto, 2003, Osier and Lindroth, 2006). While the maintenance cost of CTs is also high (Donaldson *et al.*, 2006), the maintenance cost and turnover rate of SA are considered to be low (Ruuhola and Julkunen-Tiitto, 2003). Salicylates are structurally simpler than CTs but more highly toxic defence compounds (Decker *et al.*, 2017). Since growth rate is negatively correlated with the production of both SA and CT, but SAs are more toxic and metabolically cheaper to keep, growing low-CT genotype aspens for biomass production rather than high-CT genotypes may be economically feasible. This conclusion is backed by work from Bandau *et al.* (2015), who found larger total leaf area, greater root mass, elevated foliar [C], and higher biomass accumulation in the low-CT genotype *P. tremula*.

The total contents of different compound groups in competitively advantaged (dominant) trees were not influenced by the thinning treatment, suggesting that at least for UV-induced compounds like FLA and HC, the optimal concentrations had already been reached. The observed lower total content of specialised metabolites in dominant trees compared to that of suppressed trees (**I**) supported the growth-differentiation balance hypothesis, which states that in a resource-rich environment, assimilated carbon is more readily allocated to growth, not the production of defence metabolites (Herms and Mattson, 1992). The experiment site of the thinning treatment had a high nutrient pool and good water retention properties (Lutter *et al.*, 2017). Thus, the dominant trees were growing in a high-resource environment, and the growth of the suppressed trees was limited by the shading of neighbouring trees rather than nutrient and water shortages. Although the total SM content in suppressed trees was two times higher than that in dominant trees, it did not result in significantly lower growth of the trees in thinning treatments, which also indicated an abundant nutrient pool in the soil.

Although it was hypothesised (**1**) that the decreased level of competition stress created by thinning would result in lower foliar SM content, such findings were not obtained. Instead, I found confirmation for the assumption that metabolite content is higher in plants withstanding greater stress (**2**). However, this stress was not caused by environmental factors but by the thinning of the vegetatively regenerated hybrid aspen stand. The content of specialised metabolites did not decrease in either the dominant or suppressed residual trees. Conversely, suppressed tree SM content increased with the extent of thinning, while dominant trees remained indifferent to the changes.

3.3. Intraspecific and within-tree competition drives the production of phenolic acids and condensed tannins in silver birch (II, III)

The specialised metabolite profiles of hybrid aspen and silver birch are highly genotypically variable; thus, identifying any one specific compound that would indicate a higher stress level for plants is challenging. Although studies **II** and **III** were both conducted on silver birch, the effect of intraspecific competition on foliar phenolic profiles varied (Table 1). Besides the light-induced increase in flavonoids covered in Subsection 3.1, changes in hydroxybenzoic acids (HBA, comprising protocatechuic acid hexoside isomers and galloyl-glucose isomers) and CT content (comprising (epi)catechin, (epi)gallocatechin and procyanidin dimers) were noticed. The CT content was affected by environmental conditions less than HBA was. HBAs and CTs are similar in the sense that they are both precursors of tannin biosynthesis. However, while HBAs are the precursors for hydrolysable tannins (gallotannins and ellagitannins), catechin is used to produce condensed tannins (syn. proanthocyanidins). HBAs are structurally simpler compounds than catechins. Both originate from the shikimate pathway, as do all phenolic compounds, but HBAs do not enter the phenylpropanoid pathway, in which polymeric condensed tannins are the end products. Thus, HBAs do not compete for the limited amount of phenylalanine necessary for the biosynthesis of condensed tannins, lignins, and flavonoids.

Table 1. The impact of competitive status, canopy position, and mean annual temperature on hydroxybenzoic acids and condensed tannins content, as well as the specific leaf area of birch leaves in the latitudinal (**II**) and FAHM studies (**III**). Higher-value occurrences are marked in the table.

	Latitudinal study Tullus *et al.* 2021 (**II**)			FAHM Kharel *et al.* 2023 (**III**)	
Parameter	MAT	CS	CP	CS	CP
HBA	Cold	SUP	Low	n.s.	Up
CT	Warm	n.s.	n.s.	SUP*	Low
SLA	Cold	SUP	Low	SUP	Low

n.s. – not significant; MAT – mean annual temperature; CS – competitive status; DOM – dominant trees; SUP – suppressed trees; SLA – specific leaf area; CP – canopy position; Up – upper canopy; Low – lower canopy.

* – significant only in irrigation plots.

Significantly higher foliar content of HBA was observed in competitively suppressed silver birch trees in the latitudinal study, but the CT content was unaffected (Fig. 4H in **II**). The foliar CT content in the FAHM study, however, was found to be higher in competitively suppressed birch trees, while the content of HBA was unaffected (Fig. 3H in **III)**. No pronounced effect of the FAHM treatment on the foliar phenolic content was observed. However, foliar HBA content was strongly negatively correlated with the sampling height of the leaves across all sites, irrespective of the trees' competitive status (Table 2 in **II**).

The effect of canopy position on HBA and CT content was different between the two studies. In the latitudinal study, the content of HBA was higher in the lower canopy (Fig. 4H in **II**), while in the FAHM study, it was significantly higher in the upper canopy leaves (Fig. 3C in **III**). The canopy position did not impact the foliar CT content in the latitudinal study, whereas in the FAHM study, a higher content of CTs was observed in the lower canopy (Fig. 3A in **III**).

The HBA content decreased significantly with longer vegetation periods and warmer climatic conditions (Table 2 in **II**). Conversely, the foliar CT content increased with mean annual temperature (MAT) ($r = 0.52, p < 0.001$; **II**). Like HBA, specific leaf area (SLA) was higher in suppressed trees (**I**, **II**, **III**) and in the lower canopy (**II**, **III**), and it decreased significantly in warmer climatic conditions (Fig. 5C in **II**). These observed variations in SLA agreed with previous knowledge that leaf morphology, especially SLA, is strongly affected by the light conditions both within the tree and intraspecifically (Poorter *et al.*, 2019). SLA was the best predictor for foliar HBA content in the latitudinal study ($r = 0.74$, $p < 0.001$, **II**), while in the FAHM study, the correlation between SLA and HBA was negative but weak ($r = –0.38$ $p < 0.01$, **III**).

In the latitudinal study, the sampled trees' height ranged from about 2–12 metres, whereas in the FAHM study, the suppressed trees were, on average, 2.9 metres, and the dominant trees were 4.9 metres tall. However, the trees in the two studies were of comparable age (6–9 years in **II**, 8 years in **III**) and had reached canopy closure. The variation in tree height, concurrent sampling height, and variance in light conditions in the FAHM study were likely too small to ascertain any consistent effect of competition on phenolic specialised metabolites. High within-site variability in leaf traits, including SLA, is well established (Gong and Gao, 2019). This could explain why SLA was not as good a predictor for foliar HBA content in the FAHM study as in the latitudinal study. Furthermore, in the FAHM study, the 14 × 14 m experimental plots were surrounded by buffer zone hybrid aspen trees planted in 2006. The buffer zone trees were more than twice as tall (> 10 m) as the sampled silver birches. In addition, the birch trees (mean height 3.9 m) in the experimental plots were partly shaded by the hybrid aspen (mean height 5.4 m) growing on the southern and western sides of the experimental plots (Figure 3) (Tullus *et al.*, 2020). Thus, all silver birch trees could be classified as suppressed trees compared to the average stand height on the FAHM site (including the buffer zone). Therefore, the inconsistent impact of competitive status on silver birch SM between the two studies is expected.

Regardless of the varying impact of competition on foliar HBA and CT content between the two studies, the contents of HBA and CT showed a negative correlation in both studies ($r = –0.41$, $p < 0.001$, **II**; $r = –0.45$, $p < 0.001$, **III**). This coincided with previous findings of a negative correlation between HBA and CT contents (Salminen *et al.*, 2004, Vanhakylä and Salminen, 2023). HBA content and SLA are known to be higher in young, developing leaves, and the values decrease continuously during leaf maturation (Salminen *et al.*, 2001, 2004, Campioli *et al.*, 2009, Poorter *et al.*, 2009, Vanhakylä and Salminen, 2023). The content of condensed tannins, however, is known to reach maximum values before senescence (Salminen *et al.*, 2004, Vanhakylä and Salminen, 2023). Shade leaves, competitively suppressed trees, and the trees growing at northern sites with higher SLA and HBA values similarly receive less light. Higher SLA and HBA in mature leaves indicate the leaves' lower photosynthetic potential and, as a result, a lower accumulated carbon amount per leaf area that could be used for leaf biomass growth, as well as a lower need to reinforce the leaf. Increase in birch leaf dry weight has been shown to follow the increase in ellagitannin content – the polymerisation product of HBAs – closely, suggesting that insoluble ellagitannins are involved in leaf thickening (Salminen *et al.*, 2001, Tuominen and Salminen, 2017).

When higher HBA content is likely the result of a lower carbon assimilation rate due to suboptimal light conditions and temperature, higher values of CTs could indicate a deficient nutrient or water supply for plant growth. A higher CT content was seen in southern sites (**II**), where the growth period is longer and light intensity is higher, but N is less available. According to the GDB, excess C is diverted to specialised metabolite production under low nutrient supply (Herms and Mattson, 1992). Despite the high metabolic cost (Lewis and Yamamoto, 1989), increased concentrations of tannins are commonly found in lower canopy and suppressed trees (i.e., more stressful conditions) (Borzak *et al.*, 2017). CT production can be viewed as a method for utilising excess energy and assimilated carbon to diminish damage to the photosynthetic apparatus with piling assimilates when growth is constrained by nutrient limitations (Prescott *et al.*, 2020).

In our study **II**, the effect of latitudinal gradient on HBA and CT content diverged from the results of Adams *et al.* (2009), who found no latitudinal difference in the content of hydrolysable or condensed tannins in four deciduous tree species (*Acer rubrum*, *Fagus grandifolia*, *Quercus alba*, *Liquidambar styraciflua*). They also collected the leaves at phenologically analogous stages. Our samples from three southern sites were collected two weeks earlier than those from the three northern sites, which was, however, likely insufficient to offset fully the differences in the phenological stages between the sites. Considering the seasonal variation in HBA and CT content (Vanhakylä and Salminen, 2023), identifying the direct impact of MAT on the content of these metabolites is difficult.

Changes in the light-inducible FLA and HC content were similar between dominant and suppressed neighbouring trees and between lower and upper canopy leaves within a tree, as suggested by our third hypothesis. For both SM classes, the contents were higher in the upper canopy and competitively dominant trees. However, the HBA content showed the opposite response: leaves shaded by neighbouring trees or by upper canopy leaves had higher HBA content, although these results were not consistent between the two silver birch studies. Changes in metabolite profiles were also not a universal response in all SM classes.

3.4. Silver birch root phenolic content is lower in stressful conditions (II, III)

While one specific stress-related compound was not detected in the leaves, some compounds showed significant results in the phenolic profile of silver birch fine roots. A root SM tentatively identified as catechin *O*-pentoside (Liimatainen, 2013), belonging to the group of flavan-3-ols, showed the most distinct response to more than one factor, although the responses differed between studies. In the FAHM study, catechin *O*-pentoside content was significantly higher in the fine roots of competitively dominant trees ($p < 0.01$, **III**), while in the latitudinal study, the effect of competitive status was only visible in three northern sampling sites, where the content was higher in competitively suppressed trees ($p < 0.01$, **II**). The content of catechin *O*-pentoside tended to decrease with MAT's increase (Table 2), and the decrease was statistically significant for suppressed trees ($r = -0.54$, $p = 0.002$). The content of this compound also differed noticeably between the FAHM treatments (Suppl. material, Fig. S3 in **III**, compound FLA6), being higher in control plots than in the air humidification or soil irrigation plots.

In the FAHM study, total root SM content was higher in the control plots compared to the treatment plots (Fig. 4C in **III**). The FAHM control plots were distinguished in the ordination biplot (Fig. 7B in **III**). Most metabolite's contents increased in the FAHM control plots, suggesting that FAHM treatments (air humidification and soil irrigation) significantly affected silver birch fine root specialised metabolites. The fine root biomass in July 2019 was considerably higher in the treatment plots than the control plots (K. Rosenvald, unpublished), which aligned with previous knowledge from the same experimental site (Rosenvald *et al.*, 2014). Previous work has shown that silver birch produces thinner and longer root tips in humidification treatments (Parts *et al.*, 2013). An irrigation treatment conducted on another economically important forest tree species, *Pinus sylvestris* (Herzog *et al.*, 2014), yielded similar results to ours. Herzog *et al.* (2014) found that the root biomass increased during irrigation, and they also detected the elongation of fine roots. Although thinner roots had higher lignin content in a study across multiple tree species (Xia *et al.*, 2021), irrigation did not affect the phenolics and lignin content in *P. sylvestris* roots (Herzog *et al.*, 2014).

In the latitudinal study, the content of total root SM tended to be lower in the northern sites, though the difference was statistically insignificant (Fig. 6A in **II**). The root biomass was not assessed (**II**), but the root biomass fraction of the entire tree biomass is thought to increase from south to north (Ostonen *et al.*, 2017, Tenkanen *et al.*, 2021). Further, the percentage of absorptive roots is known to be higher at low MAT as well (Zadworny *et al.*, 2016). In accordance with our findings, the phenolic content in *P. sylvestris* fine roots was found to be lower in colder climates (Zadworny *et al.*, 2017). The lower content of root SM and, presumably, higher root biomass were consistent between the two silver birch studies (**II**, **III**). No considerable difference was seen in the total root SM content between suppressed and dominant trees (**II, III**).

One of the main compound classes in fine roots was HBA. Although the increase of the total root SM with MAT was statistically insignificant, a strong MAT effect was observed: HBA content increased in warmer climates (Table 2; Fig. 6 in **II**). Further, HBA content was significantly higher in the control plots compared to the air humidification plots in the FAHM treatment (Fig. 4A in **III**). The data suggest that the predicted relationship between fine root biomass growth and a decrease in HBA content in silver birch could be the same as in the leaves (Salminen *et al.*, 2001, Tuominen and Salminen, 2017). Moreover, the lower content of unbound coumaroylquinic acid (a hydroxycinnamate) in colder climates (Table 2; **II**) could be considered indirect evidence of root lignification. Most phenolic acids partake in plant cell wall strengthening in some way. While HBA content would decrease continuously during fine root growth, as it would be utilised for insoluble hydrolysable tannins biosynthesis, hydroxycinnamates are used for synthesising lignins. Changes in these compounds were not assessed because neither higher-molecular-weight hydrolysable tannins nor lignin can be extracted by maceration in an aqueous methanol solution. Nevertheless, it could be hypothesised that the lignification rate was higher in silver birch trees growing at northern sites (**II**) because of their lower HBA and HC content, which could have been used for lignin synthesis.

Hydrophobic lignin limits water loss from root tissues while simultaneously facilitating water and nutrient transportation over longer distances, strengthening the root, and promoting soil penetration (Barros *et al.*, 2015, Pereira *et al.*, 2018). Thinner and longer short and fine roots in colder climates (Ostonen *et al.*, 2017) would thus benefit from a higher level of lignification. Furthermore, the turnover rate of fine roots is slower in the cold north (Yuan and Chen, 2010, Finér *et al.*, 2011, Zadworny *et al.*, 2017), and a longer lifespan may require increasing the structural strength of the roots.

Higher fine-root biomass in wetter conditions (**III**) may result from changes in the ectomycorrhizal fungal community. Mostly hydrophilic morphotypes dominating in humid conditions (Parts *et al.*, 2013) are less efficient in nutrient and water transport (Hobbie and Agerer, 2010). Hence, higher fine-root biomass is needed to ensure sufficient nutrient uptake. In colder climates, soil nitrogen and water availability are the main growth-limiting factors (Bergh *et al.*, 1999, De Frenne *et al.*, 2013). Increased allocation of carbon to root biomass growth in colder climates (Ostonen *et al.*, 2017, Tenkanen *et al.*, 2021) and wetter environments (Herzog *et al.*, 2014, Rosenvald *et al.*, 2014) reflects the increased significance of below-ground processes in limited-resource conditions (Berendse and Möller, 2009). Producing relatively more fine roots, having an extensive rooting strategy, and undergoing lignification all seem to be common adaptations for coping with decreased N availability and turnover rates in rough climatic conditions (Ostonen *et al.*, 2007, Valverde-Barrantes *et al.*, 2015).

Table 2. Heatmap of silver birch fine root specialised metabolites in relation to mean annual temperature (different sampling sites) (**II**). Values are given in standard deviation units (SDU).

Root metabolites (Tullus *et al*. 2021)			North				South		
		Site MAT	KO −1.0	LO 2.6	BJ 3.4	NY 6.3	LA 6.1	SV 7.9	
Metabolite name	class	mz@r.t.\ mean H	4.9	7.9	8.3	3.9	7.1	10.5	
Protocatechuic acid hexoside 1	HBA	315.0722@2.3	–0.23	–0.43	0.06	–0.93	0.38	1.15	*
Protocatechuic acid pentosyl hexoside	HBA	447.1144@3.2	–0.75	–0.80	–0.10	–0.59	0.63	1.61	***
Protocatechuic acid rhamnosyl hexoside	HBA	461.1301@3.8	–0.48	–0.67	–0.21	–0.69	0.24	1.82	***
Syringic acid glucoside	HBA	359.0984@3.4	0.22	0.01	–0.13	0.59	–0.43	–0.26	
Syringic acid sulfoglucopyranoside	HBA	439.0552@2.6	–0.04	0.38	0.08	–1.09	–0.41	1.08	
Syringic acid sulforhamnopyranoside	HBA	423.0603@10.6	–0.26	0.15	–0.50	–0.30	–0.17	1.09	*
Vanillic acid sulfoglucopyranoside	HBA	409.0446@2.1	–0.16	0.08	–0.04	–1.03	0.46	0.69	
Coumaroylquinic acid	HC	337.0929@9.1	–0.46	–0.47	–0.44	0.71	0.25	0.42	**
C-Glucosyl trihydroxyflavanone	FLA	433.1140@12.6	–0.39	–0.04	–0.21	–0.72	0.87	0.47	
C-Glucosyl tetrahydroxyflavanone	FLA	449.1089@6.4	–0.80	0.09	–0.13	–0.57	0.87	0.54	**
Catechin C-glucuronide	FLA	465.1038@3.7	0.09	0.29	–0.12	0.05	0.17	–0.48	
Catechin *O*-pentoside	FLA	421.1140@5.3	0.86	–0.14	0.20	0.57	–0.82	–0.67	***
Catechin	FLA	289.0718@5.5	1.25	–0.43	–0.34	–0.18	–0.70	0.41	*
B-type PA dimer	FLA	577.1351@4.9	0.67	–0.05	–0.33	–0.73	–0.38	0.83	
B-type PA trimer	FLA	865.1985@5.6	0.64	0.85	–0.17	–0.69	–0.72	0.09	**
B-type PA tetramer	FLA	1153.2619@7.5	0.62	0.75	–0.12	–0.53	–0.77	0.06	**
A-type PA dimer	FLA	575.1195@2.3	–0.66	–0.29	0.01	–0.56	0.38	1.12	***
A-type PA dimer	FLA	575.1195@3.0	–0.62	–0.12	0.06	–0.65	0.56	0.78	**
A-type PA trimer	FLA	863.1829@3.1	–0.64	–0.11	0.24	–0.51	0.40	0.61	*

KO – Kolari; LO – Lövånger; BJ – Bjästa; NY – Nyköping; LA – Länghem; SV – Svalöv; MAT – mean annual temperature (°C); mean H – mean height of the trees at the sampling site (m); mz@r.t. – mass to charge ratio and retention time (min) of the compound; HBA – hydroxybenzoic acids; HC – hydroxycinnamates; FLA – flavonoids; PA – proanthocyanidin

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

According to hypothesis (**2**), it was expected to see higher SM content in the roots of trees exposed to greater environmental stress (i.e., wet and cold conditions). However, the results obtained from silver birch root SM analysis did not confirm this. Contrary to the GDB hypothesis (Herms and Mattson, 1992), the carbon allocation to SM did not increase in more stressful conditions. Instead, changes in fine-root biomass were seen, which promoted nutrient and water acquisition from the soil. The higher content of root SMs in warmer climates (**II**) could mean that excess photosynthates were discarded through the roots as exudates (Prescott *et al.*, 2020).

3.5. Limitations and practical applications

Besides environmental conditions, many intrinsic factors, including genetics (Osier and Lindroth, 2004, 2006, Donaldson *et al.*, 2006, Bandau *et al.*, 2015, Hamanishi *et al.*, 2015, Bennett *et al.*, 2020, Eisenring *et al.*, 2023) and age (Smith *et al.*, 2011, Couture *et al.*, 2014), affect plant specialised metabolism. Our study was conducted on a vegetatively regenerated hybrid aspen stand. The initial plantation consisted of various, randomly planted genotypes; therefore, the specific genotypes of individual trees could not be traced. Suppressed and dominant trees were chosen as close pairs to increase the likelihood that they descended from the same parent tree and belonged to the same genotype. Additionally, the sampling area was added to the statistical model as a random factor to avoid spatial autocorrelation. Regardless of possible random variation caused by genotype or microsite, the trees reacted differently to thinning treatments depending on their competitive status. In study **II**, the silver birch age was relatively invariant across all sites, but some impact of tree height on SM was still perceivable. Therefore, the dominant trees' average height was included in the statistical data analysis to mitigate possible age- and site-related confounding effects. Different silver birch genotypes were present in the six plantations (**II**), but since none of them was present at every site, testing the genotype × environment interaction was unfeasible.

The extraction method used for SM analysis is like the saying "A jack of all trades is a master of none, but oftentimes better than a master of one". Maceration with an aqueous methanolic solution is suitable for extracting a number of compounds from different chemical classes, but it lacks the ability to extract higher-molecular-weight condensed and hydrolysable tannins. The largest CT identified in this thesis was a B-type proanthocyanidin tetramer. Hydrolysable tannins were not analysed, although their precursors (HBA) were detected. Future studies should consider whether tannin analysis is of importance and make changes in the extraction method accordingly. The precise content of specialised metabolites was not determined in the silver birch studies due to the large number of compounds. This prevents comparing the concentrations with results from other studies but does not affect studying the changes in SM profiles. Using gallic acid as a standard for rough content estimation could be considered, however.

The findings of this thesis can be implemented in climate-adapted forestry, including stand density and composition management. Regarding the hybrid aspen thinning study (**I**), less intensive early thinning or corridor thinning followed by traditional selective thinning are suggested. A less-drastic reduction in photosynthetic leaf area would be more effective in redistributing resources during the early management of coppice forests. Although the increased SA content in suppressed trees increases pathogen and herbivore resistance, SM biosynthesis is an additional metabolic cost that occurs at the expense of growth. However, further studies are required to clarify the duration of such an impact over a longer time period following thinning, as well as for other species besides hybrid aspen.

The possible negative correlation between root HBA content and fine-root biomass also needs further investigation. Lower HBA content could be indirect evidence of a higher fine-root biomass share and more stressful growing conditions. Root SM analysis is less time-consuming than assessing biomass because of the small sample size needed for chemical analysis – typically less than a gram. Although the instrumentation necessary for liquid-chromatographic HBA determination is more sophisticated and the analysis is more expensive than a set of balances, the high throughput of HPLC-MS could significantly reduce the workload. Like roots, foliar HBA and CT content could be indicators of stressful conditions (i.e., nutrient or light availability). Higher HBA:CT ratios could be indicative of situations in which photosynthesis is limited, while lower HBA:CT ratios might show lower nutrient availability. As the ratio changes during leaf development, this could indicate leaf developmental stage, as long as the trees are growing similar resource conditions.

4. CONCLUSIONS

The aim of this thesis was to investigate the impact of competition and environmental conditions on the specialised metabolism (SM) of hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) and silver birch (*Betula pendula* Roth.) trees growing in field conditions. The studies were linked by estimating intraspecific competition on SM between neighbouring trees. Suppressed and dominant trees' metabolisms were analysed separately to determine how environmental conditions affect trees of different competitive statuses. In the silver birch studies, within-tree competition between the upper canopy and lower canopy was also included. The thesis also aimed to identify any specific metabolite or metabolite class related to greater stress and/or growth limitations. This thesis thereby attempts to contribute to ecometabolomic studies conducted in the wild, where trees are affected by a combination of biotic and abiotic factors, unlike experiments carried out in growth chambers.

The flavonoid content of silver birch and hybrid aspen leaves was higher in the upper canopy, regardless of the tree size, and in competitively dominant trees. We expected to observe higher metabolite content in plants or plant parts exposed to greater environmental stress – in this case, light availability. We hypothesised (**3**) that the difference in metabolite profiles within an individual tree's canopy would be similar to the difference between dominant and suppressed neighbouring trees. In both cases, the availability of light was constrained by shading. Light availability as the main driver of flavonoid biosynthesis aligns with previous knowledge (Agati *et al.*, 2011, Kim *et al.*, 2012, Nissinen *et al.*, 2017, Thitz *et al.*, 2021).

In the hybrid aspen thinning study (**I**), we expected a general decrease in SM content in both dominant and suppressed residual trees because of the diminished competition for resources, such as light and mineral nutrients. However, thinning did not affect the SM content of dominant residual trees, and the SM content of suppressed trees increased significantly. This finding implies that the dominant trees were growing in a high-resource environment, irrespective of the stand density. Increased salicylate content in the suppressed trees improved their resistance to herbivory and pathogens, but also indicated an elevated stress level. Although the first hypothesis regarding thinning and reducing the SM content of residual trees was not supported, higher SM content in suppressed residual trees is in line with the second hypothesis about finding higher metabolite content in more stressed plants. The stress caused by thinning likely outweighed the increase in photosynthetically active radiation (and, likely, nutrient) availability.

No explicit conclusion could be drawn about the effect of competition on silver birch SM profiles other than flavonoids. Foliar hydroxybenzoic acids (HBA) content was higher in suppressed trees, in the lower canopy, and in colder climates in the latitudinal study (**II**), but similar differentiation between competitive statuses was not observed in the FAHM study's trees (**III**). This was likely due to the large height difference between the sampled and buffer-zone trees. In consequence, the silver birch trees in study **III** were likely all suppressed trees, and the findings should be interpreted accordingly. Nevertheless, foliar HBA and condensed tannins (CT) content were negatively correlated in both studies. The proportion of these compounds could serve as an indicator of leaf developmental stage as well as resource availability. HBA content is known to be higher in young leaves (Salminen *et al.*, 2001, 2004, Campioli *et al.*, 2009, Poorter *et al.*, 2009, Vanhakylä and Salminen, 2023), while CTs accumulate over the growth period (Salminen *et al.*, 2004, Vanhakylä and Salminen, 2023). Higher HBA content could indicate impaired growth, for example, in cold or low-light conditions, whereas higher CT content could mean lower mineral nutrient or water availability in a situation where photosynthesis is not constrained (Prescott *et al.*, 2020). HBA was a good predictor of stressful conditions in silver birch leaves as well as roots. In contrast to the leaves, HBA content in roots was lower in colder climates (**II**) and wet conditions (**III**).

The effect of competitive status and canopy position can vary depending on the study's scale. The differences observed in colder climates diminished in warmer climates, and vice versa. The phenolic metabolite profiles illustrated the different needs for protection against irradiance possessed by suppressed and dominant trees, as well as illustrating different resource supply. Competitively suppressed trees were more susceptible to different environmental drivers than dominant trees were. These suppressed trees with lower leaf area and biomass had a smaller buffering capacity for changing and disadvantageous environmental conditions. In study **I**, thinning had no effect on dominant trees' SM, but suppressed trees responded strongly to diminished photosynthesising leaf area and an increased relative amount of root mass requiring maintenance. Suppressed trees also reacted more strongly to changes in MAT. The effect of competitive status was most noticeable in colder climates (MAT < 4 °C) and was not observed at lower latitudes.

This thesis shows how extensive thinning, mean annual temperature, and a tree's competitive status can affect the phenolic SM content in hybrid aspen and silver birch leaves and silver birch fine roots. The effect of competition on foliar and root SM profiles of silver birch growing across a latitudinal gradient was studied for the first time. Future SM studies on trees should distinguish between dominant and suppressed trees, as the metabolic response strength can vary considerably between competitively different neighbours. Forest management practises should consider the long-lasting effects of extensive thinning on suppressed residual tree SM profiles, as well as the fact that SM synthesis is an additional cost at the expense of tree growth.

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SUMMARY IN ESTONIAN

Keskkonnategurite ja konkurentsi mõju hübriidhaava ja arukase fenoolsete metaboliitide profiilile

Taimed on paiksed organismid, mille tõttu ei ole neil võimalik ebasoodsate tingimuste eest ära minna. Selle asemel on taimedel kujunenud erinevad kaitsestrateegiad herbivooride, putukkahjurite, seenpatogeenide jt tõrjeks, aga ka näiteks tolmeldajate ligimeelitamiseks. Samuti on kujunenud kaitsemehhanismid taime kahjustavate keskkonnatingimustega toime tulemiseks. Lisaks anatoomilistele kohastumustele toodavad taimed suurel arvul erinevaid kaitseaineid, mis aitavad eelpool nimetatud teguritega paremini hakkama saada. Selle töö fookuses on fenoolsed spetsialiseerunud metaboliidid (SM, varem kutsutud ka sekundaarseteks metaboliitideks). Fenoolsete metaboliitide hulka kuulub palju ühendeid, mis tavainimesele tuttavad võiksid olla – näiteks head antioksüdandid flavonoidid, salitsülaadid, millel põhineb tuntud ravim aspiriin ehk atsetüülsalitsüülhape, ning tanniinid, mis annavad mustale teele ja punasele veinile neile omase mõrkja maitse.

Enamus taimede kaitseainete ja stressitegurite vaheliste seoste uurimustest on tehtud kasvukambrites või mudelorganismidel, kuid selliselt saadud teadmisi on keeruline praktikas kasutada, sest looduses esinevad erinevad keskkonnamõjud korraga. Keskkonnatingimuste koosmõjud on tihti etteennustamatud. Samuti sõltub kaitseainete profiil taime vanusest ning kasvukambrisse suuri puid reeglina ei mahuta. Käesolevas doktoritöös uuritakse, kuidas keskkonnatingimused, puudevaheline ja võrasisene konkurents ning metsanduslikud praktikad (harvendusraie tüübid) mõjutavad vabas looduses kasvavate hübriidhaava (*Populus tremula* L. × *P. tremuloides* Michx.) ja arukase (*Betula pendula* Roth.) lehtedes ning juurtes leiduvate fenoolsete ühendite sisaldust. Töö käigus analüüsiti valgustingimuste ja harvendusraie mõju hübriidhaava SM sisaldusele. Arukasel uuriti geograafilise laiuskraadi ja sellest tulenevate keskkonnatingimuste muutuste mõju lehtedele ja juurtele sõltuvalt puudevahelisest konkurentsist (võrreldi kasvus allajäänud või domineerivaid puid). Lisaks vaadeldi suurenenud õhu- ja mullaniiskuse mõju arukase SM sisaldusele õhuniisutuse manipulatsiooni (FAHM) tingimustes. Täiendavaks eesmärgiks oli leida konkreetseid ühendeid, mis võiks viidata taimi mõjutavatele erinevatele stressifaktoritele ja -tasemele.

Tehnoloogia arengu tõttu on taimede keemilise koostise määramine muutunud üha lihtsamaks ja ka odavamaks. Metaboliitide mitte-sihitud analüüs võimaldab leida ootamatuid seoseid ja vastuseid küsimustele, mida otseselt ei püstitatud. Käesolevas töös on kasutatud sihitud ja mitte-sihitud analüüsi vahepealset varianti, kus ei määratud konkreetsete ainete sisaldust, kuid ei uuritud ka täit metaboloomi. Rõhk oli pandud fenoolsetele ühenditele, aga mitte ühelegi spetsiifilisele ainele. Metaboliitide analüüsimiseks taimekudedes need kuivatati kuivatuskapis, seejärel peenestati ning ekstraheeriti metanooli vesilahusega. Saadud ekstrakte analüüsiti vedelikkromatograaf-massispektromeetril (HPLC-MS). Hübriidhaava katses määrati standardainetega ühendite massikontsentratsioonid. Arukase katsetes piirduti kromatograafilise piigi pindala määramisega ning statistilistes analüüsides kasutati standardiseeritud väärtusi. Tihti ei olegi oluline teada täpset massikontsentratsiooni, sest see sõltub väga palju kasvukeskkonnast, puu vanusest, kemotüübist ja ka genotüübist. Täpsest kontsentratsioonist olulisem võib olla nende ainete sisalduse dünaamika.

Tulemustest selgus, et kasvus allajäänud ja domineerivate hübriidhaava ning arukase lehtede flavonoidide sisaldus oli erinev. Kasvus domineerivate puude flavonoidide sisaldus oli suurem kui kasvus allajäänud puudel, mis oli ootuspärane, sest suuremate puude jaoks on valgustingimused paremad. Võrasisene konkurents tingis sarnased muutused arukase lehtede flavonoidide profiilis – võra alumistes lehtedes oli flavonoidide sisaldus madalam kui võra ülemistes lehtedes. Flavonoidide sisaldus sõltus ka aasta keskmisest temperatuurist, ning oli suurem lõunapoolsetel aladel kasvavate arukaskede lehtedes. On üldteada, et valgustingimused mõjutavad flavonoidide sünteesi taimedes.

Vastupidiselt flavonoididele oli hüdroksübensoaatide (HBA) sisaldus suurem arukase võra alumistes lehtedes, kasvus allajäänud puudes ning põhjapoolsematel aladel. HBA sisaldus on teadaolevalt kõrgem noortes lehtedes. Lehe arengu käigus sünteesitakse HBA-st rakukestaga seotud hüdrolüüsuvaid tanniine, mis aitavad lehte tugevdada. Kondendseerunud tanniinid (CT) on funktsioonilt HBA-le sarnased, kuid struktuurilt keerulisemad ja ainevahetuslikult kulukamad toota. CT-d on fenüülpropanoidse ainevahetusraja lõpp-produktid ning erinevalt HBA-st nende sisaldus taimekudedes kasvab kasvuperioodi jooksul. Arukase lehtede CT sisaldus sõltus käesolevas töös keskkonnatingimustest vähem kui HBA sisaldus. Lehtede HBA ja CT sisaldused olid negatiivses korrelatsioonis. HBA ja CT vahelise suhte muutused võivad olla heaks indikaatoriks kasvuks vajalike ressursside kättesaadavuse kohta. Kõrgem HBA sisaldus võib viidata lehe nooremale arengufaasile või fotosünteesi pärssivatele kasvutingimustele, samas kui kõrgem CT sisaldus on omane vanematele lehtedele ning võib olla märk toitainete puudusest. Arvatakse, et CT tootmine võib olla üheks võimaluseks üleliigsest assimileeritud süsinikust vabanemiseks, mis aitab vältida lehtede valguskahjustusi. Kui arukase lehtedes oli HBA sisaldus suurem põhjapoolsetel aladel, siis juurte HBA sisaldus oli vastupidises seoses. Arukase peenjuurte HBA sisaldus oli suurem just lõunapool ning FAHM katsealal kontrollringides kasvanud puudes võrreldse niisutusringides kasvanud puudega. Seega võib HBA sisaldus olla sobilik stressiindikaator nii lehtedes kui juurtes. Lehtede ja juurte HBA sisalduse muutused arukasel viitavad suurenenud stressile jahedamates, niiskemates ja varjulisemates kasvutingimustes.

Hübriidhaava harvenduskatses eeldati, et vähenenud konkurents valgusele ja toitainetele toob kaasa lehtede madalama fenoolsete ühendite sisalduse. Saadud tulemused näitasid vastupidist – kasvus allajäänud hübriidhaabade SM sisaldus tõusis umbes neli korda, samas kui kasvus domineerivate puude SM sisaldusele ei avaldanud harvendus mingit mõju. Suurenenud flavonoidide sisaldus väiksemates puudes iseloomustab harvendusjärgset valgustingimuste paranemist. Kõrgem salitsülaatide sisaldus parandab väiksemate puude kaitsevõimet patogeenide ja herbivooride vastu, kuid samas on nende ainete tootmine puule kulukas ning võib pärssida puu edasist kasvukiirust. Intensiivse harvendamise negatiivne mõju osutus suuremaks paranenud valgustingimuste ja toitainete kättesaadavuse positiivsest mõjust.

Doktoritööst selgub, et puude konkurentsistaatuse ja võrapositsiooni mõju fenoolsete metaboliitide sisaldusele sõltub uuringu mastaabist. Külmemas kliimas esinevad seosed ei pruugi olla olulised soojemas kliimas ja vastupidi. Fenoolsete ühendite sisaldus lehtedes ja juurtes näitab erinevat kaitsevajadust aga ka erinevat ressursside kättesaadavust. Kasvus allajäänud puud olid keskkonnatingimuste muutustele tundlikumad kui domineerivad puud. Väiksematel puudel on väiksem lehepind ja biomass, mille tõttu on selliste puude puhverdusvõime ebasoodsate olude suhtes väiksem. Hübriidhaava harvenduskatses toimusid muutused ainult väiksemate puude metaboliitide profiilis. Kasvus allajäänud arukased reageerisid tugevamalt aasta keskmise temperatuuri muutumisele. Konkurentsistaatuse mõju SM profiilile oli kõige tuntavam just külmemas kliimas, kus aasta keskmine temperatuur on alla 4 °C. Eelnevat arvesse võttes tuleks edaspidi sarnastes uurimustes taimede SM sisaldust määrates lähtuda nende konkurentsistaatusest.

Käesolevast doktoritööst selgub, kuidas ulatuslik harvendusraie, aasta keskmine temperatuur ja puude konkurentsivõime mõjutavad fenoolsete ühendite sisaldust hübriidhaaval ja arukasel. Töö tulemusi saab rakendada metsanduses – puistu tiheduse ja koosseisu kujundamisel. Vähemintensiivne varane harvendusraie on soovituslik, et pikemas perspektiivis puistu kasvupotentsiaali efektiivsemalt ära kasutada. Harvenduse pikaajaline mõju SM sisaldusele lehtedes ja juurtes vajab edasist uurimist.

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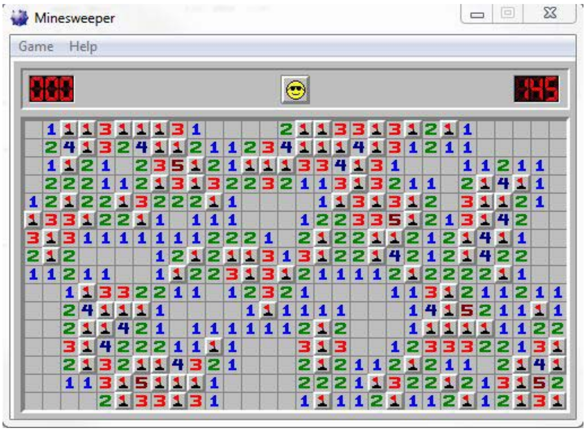

PUBLICATIONS

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List of publications

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Conference presentations

2018 34th Annual Meeting of the International Society of Chemical Ecology, Budapest, Hungary, poster "*Hypericum* spp. and humidity: which is better, drier or wetter?"

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