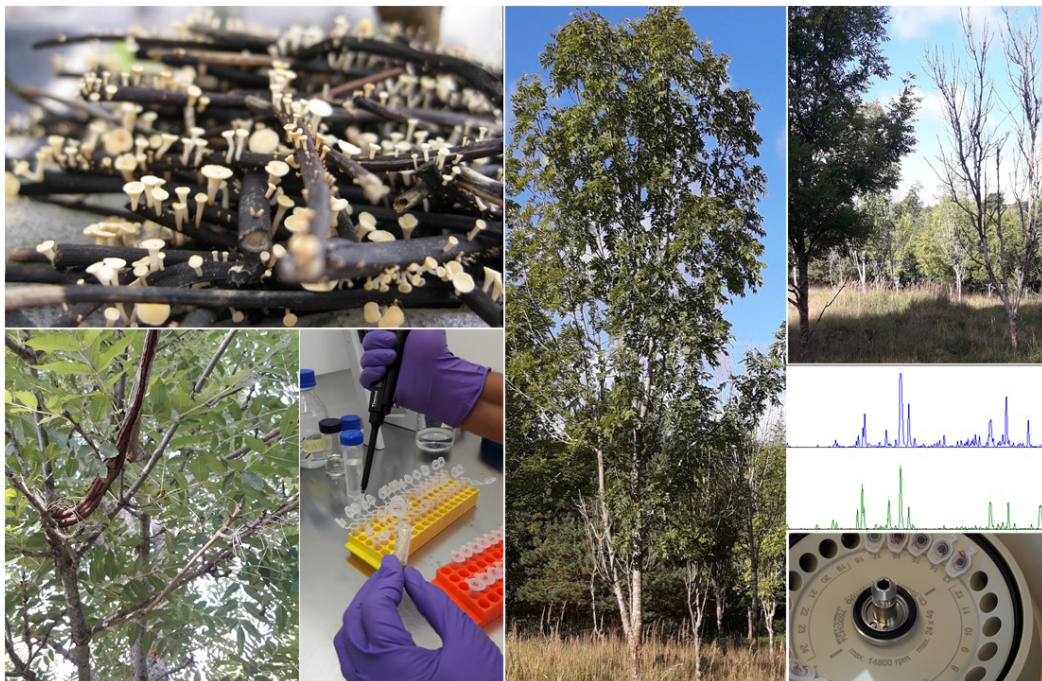


# Investigation of constitutive phloem phenolics in European ash (*Fraxinus excelsior*) with different phenotypic susceptibility to ash dieback.

*Undersökning av konstitutiva fenoler i floem från ask (*Fraxinus excelsior*) med olika mottaglighet för askskottsjuka*

Hjalmar Holm



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## 1.1 Abstract

European Ash (*Fraxinus excelsior*) is an important species for biodiversity throughout Europe. The species is critically threatened due to an alien invasive fungus, *Hymenoscyphus fraxineus*. Some individual *F. excelsior* trees however show better resistance to *H. fraxineus* giving hope that the population can be saved. The underlying mechanisms associated with this resistance however are still not clear. In earlier work, chemotypes could be clearly distinguished between susceptible and resistant ash trees.

The aim of this thesis is to investigate the quantitative and qualitative differences in ash constitutive phenolics that may in part explain the observed resistance in some ash trees. Ash trees of known susceptibility to *H. fraxineus* from five different European countries were sampled for stem phloem tissue and phenolics were extracted in a butylated hydroxyanisole methanol solution. Chemical analysis using Time-of-Flight Mass spectrometry reveals significant differences in levels of several phenolic compounds, foremost the coumarin fraxin and the secoiridoid ligustroside, which is more prevalent in trees resistant to *H. fraxineus* than in susceptible ones. Fraxin is also more abundant in the resistant natural host of *H. fraxineus*, *F. mandshurica*, than in susceptible species, such as *F. excelsior*. This study may help in advancing breeding efforts by identifying potential biomarkers that are associated with resistance.

*Keywords:* ash, *Fraxinus excelsior*, dieback, *Hymenoscyphus fraxineus*, phenolics, secondary metabolites

## Sammanfattning

Asken (*Fraxinus excelsior*) är ett viktigt träd för biodiversitet inom stora delar av Europa. Arten är nu hotad av en invasiv sjukdomsbringande svamp, *Hymenoscyphus fraxineus*. Vissa askträdsindivider visar på en bättre resistens mot *H. fraxineus*, vilket inger hopp för att kunna rädda arten. De underliggande mekanismerna för denna resistans är emellertid inte fullt ut förstådd än, men i tidigare arbeten har man kunnat särskilja kemotyper mellan mottagliga och resistanta askar.

Syftet med denna studie är att undersöka de kvantitativa och kvalitativa skillnaderna i sammansättningen av askens konstitutiva fenoler, vilka till dels förmodas kunna förklara den observerade resistansen hos vissa askträd. Floemprover togs från kvistar på askar av känd känslighetsgrad till *H. Fraxineus* från fem europeiska länder och fenoler extraherades i en butylerad hydroxyanosol-metanol-lösning. Kemisk analys med hjälp av tidsflykts-masspektrometer (en. Time-of-Flight) visade på signifikanta skillnader i nivåer av flertalet fenoler, företrädesvis kumarinen fraxin och secoiridoiden ligustrosid, som finns i betydligt större mängd i askar resistanta mot *H. Fraxineus* än i mer mottagliga artfränder. Fraxin förekommer också i högre nivåer i patogenen *H. Fraxineus*' naturliga, och resistanta, värdart, manchurisk ask, *F. mandshurica*, än i mottagliga arter, såsom *F. excelsior*. I och med denna upptäckt ter sig försöken att genom förädlingsarbete för resistans rädda asken som ekologiskt och ekonomiskt funktionell art som mer hoppfulla.

*Nyckelord:* ask, *Fraxinus excelsior*, askskottsjuka, *Hymenoscyphus fraxineus*, fenoler, försvarsämnen

# Table of contents

<b>Abbreviations</b>	<b>7</b>
<b>1 Introduction</b>	<b>9</b>
1.1 Ash as an important species for biodiversity, culture and economy	9
1.2 Invasion history	12
1.3 Biology of <i>Hymenoscyphus fraxineus</i>	13
1.4 Disease control	16
1.5 Resistance as a genotypical trait	17
1.6 True resistance, or disease escape?	18
1.7 Constitutive phenolic compounds as a possible trait of resistance	19
<b>2 Aim</b>	<b>22</b>
<b>3 Materials and Methods</b>	<b>23</b>
3.1 Plant Material	23
3.2 On the choices of stem phloem over leaf material	24
3.3 Phenolics extraction	24
3.4 Chemical analysis	25
3.4.1 Metabolomics on phenolic compounds in ash tree phloem extracts by LC-MS/Q-TOF	25
3.5 Statistical analysis	26
<b>4 Result</b>	<b>27</b>
<b>5 Discussion</b>	<b>39</b>
5.1 On the findings	39
5.1.1 Compounds found	39
5.1.2 Principal components analysis	41
5.2 Parallels and comparisons with earlier studies etc.	41
5.3 Limitations	44

5.4	Considering future research	45
5.5	Conclusions	46
	<b>References</b>	<b>47</b>
	<b>Acknowledgements</b>	<b>59</b>
	<b>Appendix 1</b>	<b>60</b>



## Abbreviations

ADB	Ash dieback
DNA	deoxyribonucleic acid
EAB	Emerald Ash Borer:
FT-IR	Fourier transform infrared
HPLC	High Pressure Liquid Chromatography
m/z	Mass per charge ratio
OTU	Operational taxonomic unit (e.g. species)
SIMCA	Soft Independent Modelling of Class Analogies



## 2 Introduction

### 2.1 Ash as an important species for biodiversity, culture and economy

European ash, also known as Common Ash (*Fraxinus excelsior*), is severely declining throughout most of its European range (figure 1); from France and Britain (Webber and Hendry, 2012; Husson *et al.*, 2011) in the west to Romania, Belarus and western Russia (Chira *et al.*, 2017; Musolin *et al.*, 2017) in the east, and from Norway (Timmermann *et al.*, 2017) in the north to central Italy in the south (Luchi *et al.*, 2016). The epidemic, known as ash dieback (ADB), is thought to have started around the early 1990's and was first observed in Poland (Kowalski, 2006) but is now affecting most European countries. There is concern about the very survival of the ash as such, and hence European Ash is updated as Near Threatened (NT) on the IUCN Red List (Khela and Oldfield, 2018) and as Endangered (EN) on the Swedish national red list (Artdatabanken, 2015). The mortality is high, for example Vacek *et al.* (2015) described a mortality of 2,7 % per year in trials in the Czech Republic, while Löhmus and Runnel (2014) observed a close to 15 % mortality per year in a case study in Estonia. An average annual mortality of around 9 % is observed in Lithuania (Pliūra *et al.*, 2017), and 7 % in Sweden (Stener, 2018). The natural regeneration is also severely threatened; ash is being replaced by other species, such as birch (*Betula spp.*) and grey alder (*Alnus incana*) after clear cuts of previous ash stands due to extraordinary poor performance of ash seedlings under infection pressure of *H. fraxineus* (Lygis *et al.*, 2014).

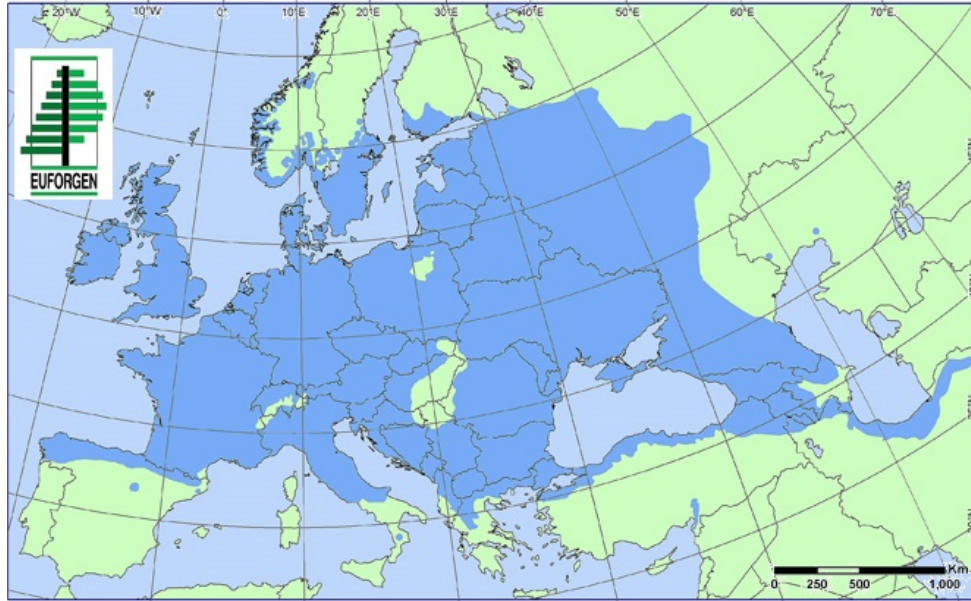


Figure 1. The distribution of *Fraxinus excelsior*, EUFORGEN 2009, [www.euforgen.org](http://www.euforgen.org).

Ash is considered a keystone species, i.e. it is important to many other species, and has a proportionately high importance for biodiversity (Pautasso *et al.*, 2013a) even in countries where it is rare, such as Sweden (Skogsdata, 2017), where 483 species were found to be associated with ash, and of them 112 had ash as preferred host and 52 were entirely dependent upon ash. In all at least 115 species are at high risk of co-extinction in Sweden, if ash functionally disappears, and additionally 111 species are at an intermediate extinction risk (Hultberg *et al.*, 2019). Corresponding data for the UK is at least 955 species associated with ash (Mitchell *et al.*, 2017). These figures are likely to be gross underestimates, as for example microfungi are comparatively little studied and poorly understood. In a study of the mycobiome of ash in Sweden (Agostinelli, 2018), the microfungi composition was qualitatively partly different between highly susceptible and more resistant trees, suggesting that breeding towards resistance to *H. fraxineus* will likely disfavour microfungi associated with susceptible trees. Susceptible trees also typically had fewer symbiotroph and relatively more patho- and saprotroph OTUs (operational taxonomic units, that is presumably species) than intermediate and resistant trees. However, only 40 % of the OTUs were assigned a trophic level (1660 of these exclusive to ash); a clear indication of our poor knowledge in that field (Agostinelli, 2018).



Figure 2. A healthy, resistant ash surrounded by many susceptible ash trees at the Snogeholm seed orchard. Photo: Hjalmar Holm.

Many species of epiphytic lichens that are specialized on old trees rely on *F. excelsior* as their preferred substrate (Marmor *et al.*, 2017), and are already under threat due to habitat fragmentation and loss, and over all low population sizes. The rapid loss of host trees may well contribute to regional extinction, due to their inherent slow recolonization rates (Lõhmus and Runnel, 2014). In an Estonian study, *F. excelsior* was found to be the tree species with most red-listed epiphytic lichens, and even though most of them could live on other species (*Quercus robur*, *Acer platanoides*, *Ulmus glabra*,

and potentially *Populus tremula*) these are regionally not common enough to fully compensate as habitat (Marmor *et al.*, 2017). This is in line with Hultberg *et al.* (2019), who found the main alternative host for ash-associated to be *Ulmus spp.*, but since that tree is rare due to Dutch elm disease, conservation plans cannot depend upon it. *Quercus spp.*, *Fagus sylvatica*, *Populus tremula* and *Acer spp.* are capable of supporting > 95 % of the ash-associated species in Sweden; however, they are all quite rare, (together < 4 % of standing timber volume in Sweden) (Skogsdata, 2017).

There has been doubt whether the ash dieback is a net threat to biodiversity, as there can be positive effects as well, especially if management is influenced by conservational goals rather than mainstream forestry goals. This includes a rapid increase of much needed dead wood and open glades in European forests (Heilmann-Clausen, Bruun and Ejrnæs, 2013). However, as Pautasso *et al.* (2013b) point out, these effects will be only temporal (as ash recruitment is affected) and Europe has already few tree species compared to Asia and North America, *F. excelsior* mainly occurs shattered in the landscape and glades will shortly be filled by other tree species, which typically form a denser canopy, yielding a darker understory. In all, the ash dieback epidemic is likely to result in less diverse forests (Pautasso *et al.*, 2013b), agricultural margins and urban nature (Pautasso *et al.*, 2013a).

Ash is an important species even outside forests, especially in margins in the agricultural landscape (Orłowski and Nowak, 2007). Pollarding of ash trees was once a widespread practice in Europe (Petit and Watkins, 2003) and pollarded ashes are important for epiphyte richness in western Norway (Moe and Botnen, 1997). Predominantly old pollarded ash trees are still present in several countries, being features of past agricultural practices and considered important bearers of culture and biodiversity (Anon, 2007; Jansson *et al.*, 2017; Sebek *et al.*, 2013).

## 2.2 Invasion history

The overshadowing cause of this widespread population decline is *Hymenoscyphus fraxineus* (formerly *Hymenoscyphus pseudoalbidus* (Baral, Queloz and Hosoya, 2014)), also called *Chalara fraxinea* primarily for its asexual anamorphic stage (Kowalski, 2006; Kowalski and Holdenrieder, 2009; Queloz *et al.*, 2011). The fungus originates from Eastern Asia, where it behaves primarily as an endophyte and saprophyte of leaves of East Asian

species of the *Fraxinus* genus, such as Manchurian Ash (*F. mandshurica*) and Chinese Ash (*Fraxinus chinensis*) (Gross and Han, 2015). Thus, it is not pathogenic to native *Fraxinus* species within its native range (Zhao *et al.*, 2012; Cleary *et al.*, 2016). In Europe, *H. fraxineus* is severely pathogenic on at least *Fraxinus excelsior* and *F. angustifolia* (Kádasi-Horáková *et al.*, 2017), with which it lacks co-evolutionary history, which is thought to be true also for *F. ornus*, on which *H. fraxineus* can complete its life cycle, but only seem to cause relatively mild symptoms (Kirisits, 2017). Its infectious capacity and rapid spread have now made it quite famous in terms of invasive alien species worldwide. At the moment it is confined to Europe and Asia but poses a risk to other ash around the world especially in North America if it were to ever establish there. The natural range of the native *Fraxinus* host (and presumptively the fungus itself) is between E100° and E146°, and between N30° and N53°, encompassing Russian Far East, northern Japan, northern Korea, and most of China (Yihong, 1995).

### 2.3 Biology of *Hymenoscyphus fraxineus*

In its native range *H. fraxineus* has a similar ecological role as *H. albidus*, a closely related species native to Europe which also grows in *Fraxinus* species but is not pathogenic to *F. excelsior* (Baral and Bemann, 2014; Kowalski, Bilański and Holdenrieder, 2015; Husson *et al.*, 2011). These days, *H. albidus* has been mainly replaced by *H. fraxineus* (McKinney *et al.*, 2012a), because they occupy the same ecological niche and because *H. fraxineus* is more competitive than *H. albidus* (Kirisits, Dämpfle and Kräutler, 2013; Hietala *et al.*, 2018). Morphologically they are so similar that one needs microscopy to discern the one from the other (Baral and Bemann, 2014). The life cycle of both *H.* species is centred around the decomposition of senesced leaves, i.e. leaf litter on the forest floor.

*Hymenoscyphus fraxineus* infects ash leaves via ascospores during the summer and decomposes leaves after they have fallen in the autumn (Baral and Bemann, 2014), taking advantage of being already established in the leaf before senescence. During the following growth season, the fungus forms a black pseudosclerotial plate (see figure 5.) on the leaf rachis, by which time the leaf blades are disjoint, and white apothecia are formed in

mid-summer which produce and disperse ascospores (Kowalski and Holdenrieder, 2009; Baral and Bemmman, 2014).



*Figure 3.* A newly infected leaflet. Notice the partial discoloration. Photo: Hjalmar Holm.

The ascospores adhere to the surface of ash leaves, which they are able to penetrate after germination by forming an appressorium at the tip of their germination tube, possibly supported by improving its adherence to the leaf surface by extracellular mucilage (Cleary, Daniel and Stenlid, 2013b). Given the observed sporulation peak in mid to late summer (Hietala *et al.*, 2018), it is speculated that *H. fraxineus* in its natural range (and natural hosts) has an endophytic or dormant stage until leaf senescence (Cleary *et al.*, 2016), but on susceptible species such as *F. excelsior*, *H. fraxineus* grows directly (Hietala *et al.*, 2013), and causes necrosis in the leaf (figure 3) and thereby acting as a pathogen. It is also capable of spreading down the rachises to



the stem phloem and xylem (Schumacher, Kehr and Leonhard, 2009), causing necrosis, cankers (figure 4), and on small to medium sized stems and branches girdling, causing eventual wilting of shoots and dieback of the crown (Kowalski and Holdenrieder, 2009; Cleary, Daniel and Stenlid, 2013b; Bakys *et al.*, 2009). The fungus even infects the seeds to a certain extent (Cleary *et al.*, 2013a), and the base of the trees causing basal lesions, in a French study 33 % of inspected trees were affected this way (Husson *et al.*, 2012). The basal lesions are visually similar to *Phytophthora*-caused lesions, but Husson *et al.* (2012) found no *Phytophthora* on such lesions. *Armillaria* was however common in that same study, but only in combination with *H. fraxineus*, leading the authors to the conclusion that *Armillaria* plays a minor role in the dieback and attacks mainly trees already weakened by *H. fraxineus* (Husson *et al.*, 2012). The significance of basal lesions for the *H. fraxineus* pathology is a topic of ongoing research (Marçais *et al.*, 2017).



Figure 4. Stem lesions, dead twigs and dead rachises on a resistant tree. Indicated by arrows. All *F. excelsior* trees can get infected by *H. fraxineus*, but some, like this one, can successfully resist the pathogen and compensate for the loss. Photo: Hjalmar Holm

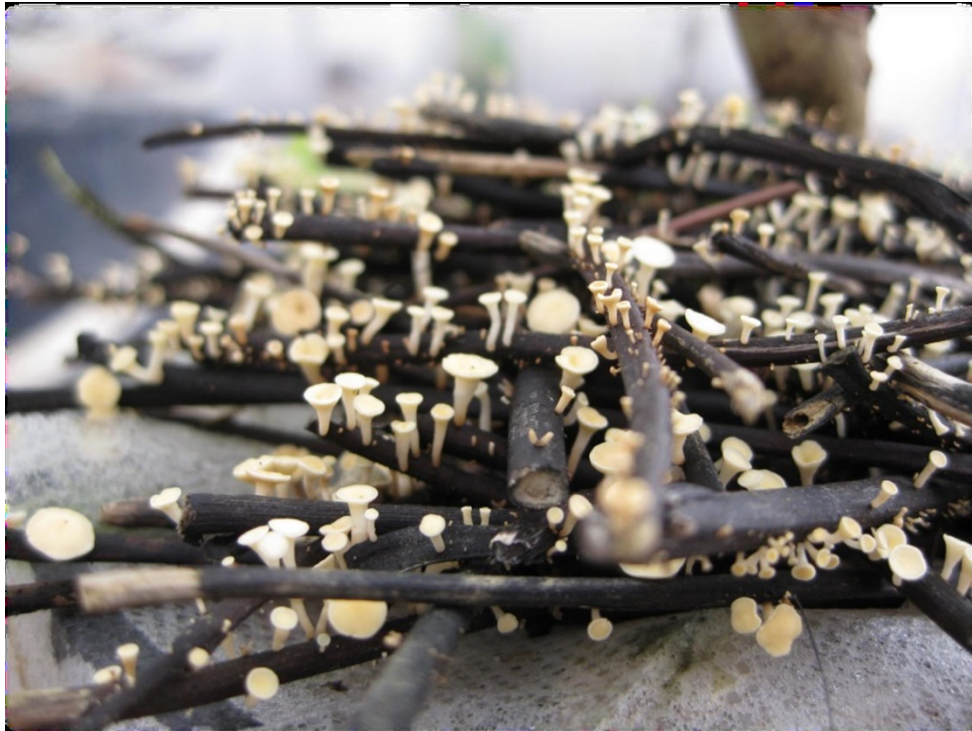


Figure 5. Fruiting bodies of *Hymenoscyphus fraxineus* on last-year ash leaf rachises (with the leaflets detached) with the typical black pseudosclerotial plate produced by *H. fraxineus*. Photo: M. Cleary, Hietala & Solheim 2011, EPPO Bulletin.

## 2.4 Disease control

There has been quite varied opinion and research on how to control the disease. This has included assessments of the possible prolonging individual tree lifespans by pruning (Marciulyniene *et al.*, 2017). In that study, the spread of the fungus in infected trees can be pathologically pruned away by cutting the branches at least 30 cm from visible edge of the stem lesion. The effect of pollarding has also been assessed (Bengtsson, Stenström and Finnsberg, 2013), but the effect of continuing pollard practices has not shown to alter infection rates or disease severity (Bengtsson, 2016). The possibility to heat treat seedlings is promising (Hauptman *et al.*, 2013), as *H. fraxineus* has optimal growth between c. 15° to 25 C° and temperatures of 36° C and higher are lethal for the pathogen. This may well limit the disease severity in the warmest regions of ash distribution (Grosdidier, loos and Marçais, 2018). Chemical treatment has also been tried and potassium phosphite appeared to limit the growth of *H. fraxineus*, at least *in vitro*

(Tkaczyk *et al.*, 2017). While some of these findings may be feasible in urban locations, special protected sites and nurseries, it is doubtful that any of them provide a long-term sustainable solution for ash as a forest tree in most of its distribution range. More promising in this regard is the discovery of genetic resistance against the pathogen (Lobo *et al.*, 2014; Stener, 2018).

## 2.5 Resistance as a genotypical trait

There is high genotypic variation in disease susceptibility to *H. fraxineus*, and one can often find relatively healthy trees growing next to severely diseased trees, see for example Stener (2018). This variation in susceptibility is to a significant degree genetically correlated. While virtually no genotypes are completely resistant (all of them are affected to some degree), a small portion of the genotypes remain relatively healthy even when exposed to high infection pressure. In clonal seed orchards and progeny trials, this is demonstrated to be an inheritable trait (Pliūra *et al.*, 2011; McKinney *et al.*, 2011; Lobo *et al.*, 2014; Stener, 2018, 2013), but a recent (Wohlmuth, Heinze and Essl, 2018) study found no correlation between damage severity of parent trees and that of their offspring in naturally regenerated stands, suggesting that there is currently no significant *in situ* natural selection for highly resistant ash trees, at least not in those stands. However, this can in part be due to absence of trees with sufficiently high resistance in those sites, and to the low detection rate of parent trees of saplings, making heredity difficult to assess. While Pliūra *et al.* (2011) also found significant variation in susceptibility between provenances, the Lithuanian trees generally had higher vitality after exposure to *H. fraxineus* than ash trees originating from other European countries. Other studies that confirm genotypic resistance on an individual level, have found weak or no difference in resistance between stands (Stener, 2018; Wohlmuth, Heinze and Essl, 2018). There is no evidence that the variation in provenance resistance mentioned above stems from a decreased virulence of *H. fraxineus* in its older populations in Europe, as the virulence is found to be virtually the same in Lithuania (among the first countries where pathogen outbreak occurred) and Switzerland (a more recently established population) (Lygis *et al.*, 2017); a finding that is reinforced by a genetic study of the pathogen in those same countries two years prior (Burokiene *et al.*, 2015). The genetic variation in both sub-populations and the metapopulation is relatively high, albeit with few alleles, and similar in the two distant parts of the range of the pathogen, which can be explained

by natural wind dispersal of the genetic variation present in the early invasion stages in Poland and Lithuania and sexual rather than clonal reproduction (Burokiene *et al.*, 2015). This largely confirms an earlier study with isolates from Finland, Estonia and Latvia, which revealed a surprisingly large genetic variation (e.g. with 14 haplotypes out of 32 isolates) compared to other alien invasive pathogens (Rytönen *et al.*, 2011). According to theories of co-evolution, there is a pressure towards optimal virulence (Dybdahl and Storer, 2003), so that trait doesn't affect the overall fitness of the parasite too negatively; obviously it does not promote pathogen fitness to drive the host extinct, and a lowered virulence would be beneficial for the pathogen in this case. However, no such trend can be seen for *H. fraxineus* in Europe as of this date. *F. excelsior* is wind pollinated and has a complicated gender system, with individual flowers, inflorescences and trees on a continuum from male to female. In practice, *F. excelsior* trees tend to lean heavily towards either male or female gender, and even though there is a significant proportion of true hermaphrodite trees, pollen from male flowers is more vital than pollen from hermaphrodite ditto and combined with the potential for sexual competition to the ovary, which reinforces the dioecious tendency (Tal, 2006; Wallander, 2001), probably limiting self-pollination and increasing genetic recombination. Even in relatively isolated stands the proportion of saplings with parent trees from outside the stands can be high, suggesting a range of at least a few hundred meters of pollen and seed dispersal (Wohlmuth, Heinze and Essl, 2018; Beatty *et al.*, 2015). This is hopeful for potential ash recolonization in the future.

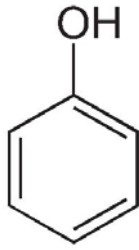
## 2.6 True resistance, or disease escape?

The resistance to ash dieback seems to be partly phenologically induced; Nielsen *et al.* (2017) found that seedlings which had begun flushing when inoculated with *H. fraxineus* developed less severe symptoms than seedlings which had not begun flushing when inoculated. This adds to the observations from the trials described by McKinney *et al.* (2011), who like Stener (2018) also found a correlation between early leaf senescence and dieback resistance. It has therefore been speculated that the lower susceptibility might be due to disease escape (McKinney *et al.*, 2011), but this is contradicted by both a Norwegian (Hietala *et al.*, 2013) study of *H. fraxineus* sporulation in an infected ash stand, where both spore spread in the stand and *H. fraxineus* DNA (of both mycelia and spore origin, presumptively) inside

ash leaves reached a high plateau well before leaf senescence, and a Danish study (McKinney *et al.*, 2012b), where resistant genotypes developed shorter lesions after *H. fraxineus* inoculation than did susceptible genotypes; an indication that an active defence is limiting the growth of the pathogen. Additionally, resistant genotypes show higher tolerance to viridiol (Cleary *et al.*, 2014), a long known fungal phytotoxin (Jones, Lanini and Hancock, 1988), proven to be produced also by *H. fraxineus* and causing discolouring and necrosis on ash leaves (Andersson *et al.*, 2010). Still, the mechanisms behind phenological and genotypical resistance remain largely unknown.

## 2.7 Constitutive phenolic compounds as a possible trait of resistance

Phenolics are long-known to be part of tree defence against pathogen fungi (Kemp and Burden, 1986), and *Fraxinus* species, such as *F. excelsior*, have a wide range of bioactive phenolic compounds (Kostova and Iossifova, 2007). Defence chemicals, such as antifungal phytochemicals, can be produced both constitutively, that is, prior to infection, and induced in response to damage to the tissue either by mechanical (abiotic) means or by a biotic agent. Some responses therefore may be entirely non-specific but in some cases, defences can be pathogen specific (Nicholson and Wood, 2001; Lattanzio, Lattanzio and Cardinali, 2006). Furthermore, defences are present in different parts of the tree: leaf and phloem are probably the most relevant parts concerning *H. fraxineus*, as these are the tissues primarily affected by this fungus (Cleary, Daniel and Stenlid, 2013b). Phloem, and to some extent xylem, defence chemistry in *Fraxinus* species is subject to much research in response to Emerald Ash Borer (EAB, *Agrilus planipennis*), an invasive pest on *Fraxinus* species in North America (Eyles *et al.*, 2007; Villari *et al.*, 2016). The native range and hosts of EAB are similar to *H. fraxineus*, namely East Asia and *F. mandshurica* and *F. chinensis* (Liu *et al.*, 2003), in its native range in east Asia which make them particularly interesting to study. Thus, research on defensive phytochemicals and their roles in *F. mandshurica* can be of value in search of understanding resistance to *H. fraxineus* in European *Fraxinus* species as well.



*Figure 6.* The chemical structure of the phenol, the simplest of the phenolics. A phenolic is a compound with a hydroxyl group bonded directly to an aromatic hydrocarbon group. Source: NEUROtiker [Public domain], Wikimedia Commons, 2007.

In a recent study, Villari *et al.* (2018) successfully demonstrated that of using a chemical finger-printing technique involving Fourier transform infrared (FT-IR) spectroscopy could identify resistant phenotypes. In that study, spectroscopic analysis of phenolic extracts from phloem samples of uninfected trees representing different disease phenotypes (i.e. highly susceptible and highly resistant to *H. fraxineus*) across Europe, combined with chemometric statistics (employing soft independent modelling of class analogy; SIMCA) on chemical fingerprints (spectra of all metabolites), they were able to accurately discriminate between resistant and susceptible genotypes, see figure 6.

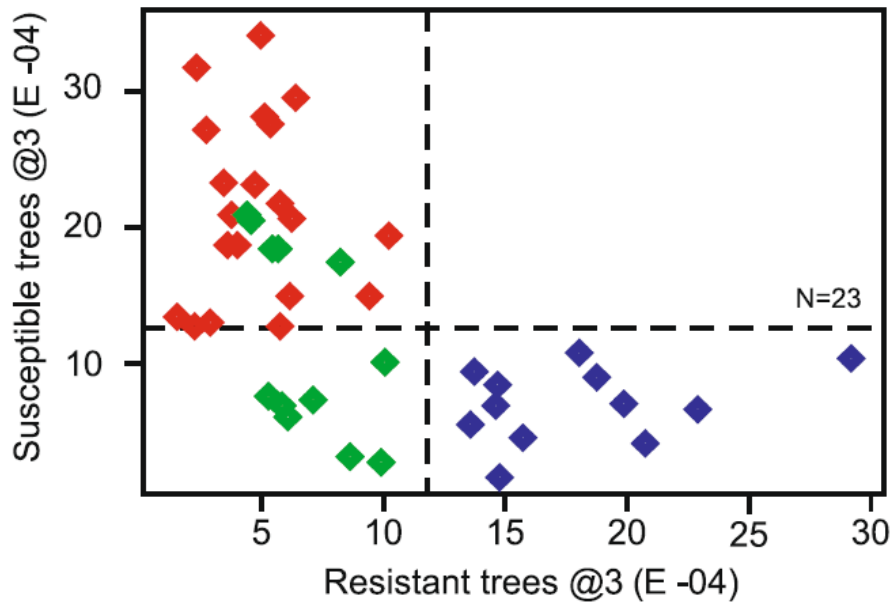


Figure 7. SIMCA Coomans plot of the samples used to verify the 3-factor model used in Villari *et al.* (2018). The distance between the sample is relative and dimension free. Notice how the resistant trees (red dots) are separated from the susceptible trees (blue dots) by the the critical sample residual thresholds, indicated by dashed lines. The green dots indicate intermediate trees, which were not used in this study. Figure replicated by kind permission of the authors (Villari *et al.* 2018).

This result strongly supports the idea that resistance is linked to constitutive phenolic composition (Villari *et al.*, 2018). While the FT-IR phenotyping technique has shown large promise to be able to identify resistant trees in the field based on their known chemical spectra associated to that phenotype, it is still unclear what the chemicals are that are contributing to their resistance. Thus, knowing how individual constitutive phenolics differ qualitatively and quantitatively between susceptible and resistant phenotypes used in that study, would provide a deep insight to the mechanisms of defence leading to increased resistance to this deadly pathogen and would help to advance efforts towards breeding for resistance (e.g. through marker assisted selection) to prevent European ash from becoming functionally extinct (Gross *et al.*, 2014; Stener, 2018; Pautasso *et al.*, 2013a).

## 3 Aim

The aim of this study is to investigate the qualitative and quantitative differences in constitutive phenolics between genotypes which are resistant and susceptible to *H. fraxineus*.

### 3.1.1 Hypotheses

I have two hypotheses: Between the resistant and susceptible phenotypes:  
1. there is no qualitative difference in secondary metabolites, and 2. there are quantitative differences in secondary metabolites.



*Figure 8.* Ash trees displaying different level of damage. The more resistant ones have grown considerably higher since the outbreak of dieback than have the more susceptible ones. Originally trees were standing much denser, but many trees have died and has since been removed. Photo: Hjalmar Holm.



## 4 Materials and Methods

### 4.1 Plant Material

The material used in this study was based on earlier work that used advanced phenotyping through a chemical fingerprinting technique to discriminate between susceptible and resistant phenotypes.

Based on the earlier work from Villari *et al.* (2018), stem phloem and leaf tissue were collected from trees having known susceptibility towards *H. fraxineus* from genetic trials that had been periodically assessed during several years in six different countries: Sweden, Denmark, Lithuania, Germany, France and Austria. The locations (field trials) were originally established to test ash provenance (Enderle *et al.*, 2013) or progeny (Muñoz *et al.*, 2016; Pliūra *et al.*, 2011, 2014; Pliūra and Baliuckas, 2007) or established as clonal seed orchards (Kirisits and Freinschlag, 2012; McKinney *et al.*, 2011; Stener, 2013; Heinze *et al.*, 2017), see Table 1. The assessments and subsequent collection of genotypes to be included in the advanced phenotyping study were based on previous assessments of disease severity as the extent of crown damage. In total, 76 genotypes were selected representing families with high or low susceptibility. All tissue samples collected were frozen on site and kept at a temperature below -20 C° until processing. For further details on the collection process and the processing of the original samples, see Villari *et al.* (2018). There remained some sample material unused from the study by Villari *et al.* (2018). Of those, 20 were classified as highly resistant and 13 as susceptible, and this subset of samples from Villari *et al.* (2018) was used for further chemical analysis described below.

Table 1. *Samples from Villari et al. 2018 used in this study.*

Country	Location	Type of trial	Sampling date	Resistant genotypes used in this study and in Villari <i>et al.</i> (2018), in parenthesis.	Susceptible genotypes used in this study and in Villari <i>et al.</i> (2018), in parenthesis.
Austria	Feldkirchen an der Donau	Clonal seed orchard	9 <sup>th</sup> of June	6 (7)	7 (7)
Denmark	Tuse næs	Clonal seed orchard	2 <sup>nd</sup> and 4 <sup>th</sup> of June	1 (3)	2 (3)
France	Devecey	Provenance and progeny trial	18 <sup>th</sup> of June	3 (7)	4 (7)
Germany	Weisweil	Provenance trial	19 <sup>th</sup> of May	0 (5)	0 (5)
Lithuania	Sasnavas	Clonal collection	2 <sup>nd</sup> of June	3 (5)	0 (4)
Sweden	Snogeholm	Clonal seed orchard	28 <sup>th</sup> of May	7 (7)	0 (4)
Total number of genotypes				20 (34)	13 (30)

## 4.2 On the choices of stem phloem over leaf material

I had the option of using either stem phloem or leaf samples in my study, but since leaf chemotype has been shown to be heavily affected by geography (Villari *et al.*, 2018), I opted at using phloem tissue for extracting phenolics.

## 4.3 Phenolics extraction

The 33 phloem tissue samples were ground to powder with a mortar and pestle, constantly kept frozen with liquid nitrogen to avoid warming and oxidation that would follow with higher temperatures, that could result in the breakdown of the chemical components. Ground phloem tissue was weighed to approximately 100 mg in cold eppendorf tubes. Thereafter the protocol 1. was followed: Butylated hydroxyanisole (BHA) was added to high

performance liquid chromatography (HPLC) grade methanol to a concentration of 0.5mg/mL. The extraction was made by adding 500  $\mu$ L of BHA-HPLC-methanol solution in each sample, after which the tubes were stored at 4 °C in the dark for 48 hours, and then centrifuged at 12,000 $\times$ g for 5 minutes, separating the solids from the rest of the material to the resulting pellet. The supernatant was transferred to microcentrifuge tubes. This process, from the addition of the BHA to and including transferring the supernatant to microcentrifuge tubes, was done twice and the supernatants for each sample were pooled and stored in - 20 °C until further analysis. This protocol is similar to the methods in Villari *et al.* (2018) and Cipollini *et al.* (2011). Approximately 500  $\mu$ L per tube of solvent phloem sample was sent for further analysis.

## 4.4 Chemical analysis

Chemical analysis of samples was done at The Faculty of Science, of Copenhagen University, using a Liquid Chromatography-Mass -Time-of-Flight spectrometer (LC-MS/Q-TOF), which enables compound identification and quantification. Identification was based on detected fragmentation patterns and mass per charge (m/z) for each individual compound and lists of known compounds.

### 4.4.1 Metabolomics on phenolic compounds in ash tree phloem extracts by LC-MS/Q-TOF

Samples were diluted 10-fold with milliQ grade water prior to analysis by LC-MS. In order to identify potential differences and products in the plant extracts they were subjected to metabolomics analysis by LC-MS/Q-TOF. Chromatography was performed on a Dionex UltiMate® 3000 Quaternary Rapid Separation UHPLC+ focused system (Thermo Fisher Scientific, Germering, Germany). Separation was achieved on a Kinetex 1.7u XB-C18 column (100 x 2.1 mm, 1.7  $\mu$ m, 100 Å, Phenomenex, Torrance, CA, USA). Formic acid in water (0.05%, v/v) and acetonitrile (with 0.05% formic acid, v/v) were employed as mobile phases A and B, respectively. Gradient conditions: 0.0-2.0 min, 2% B; 2.0-35.0 min, 2-30% B; 35.0-40.0 min 30-40% B, 40.0-47.0 min 40-60% B, 47.0-50.0 min 60-100% B, 50.0-54.0 min 100%

B, 54.0-55.0 min 100-2% B and 55.0-60.0 2% B. The mobile phase flow rate was 300  $\mu\text{l min}^{-1}$ . The column temperature was maintained at 30°C. Four wavelengths (250 nm, 260 nm, 275 nm and 290 nm) were monitored by a UV-VIS detector. The liquid chromatography was coupled to a Compact microTOF-Q mass spectrometer (Bruker, Bremen, Germany) equipped with an electrospray ion source (ESI) operated in positive ionization mode. The ion spray voltage was maintained at -3900 V. Dry temperature was set to 250°C and dry gas flow was set to 8 L  $\text{min}^{-1}$ . Nebulizing gas was set to 2.5 bar and collision energy to 15 eV. Nitrogen was used as dry gas, nebulizing gas and collision gas. Mass spectra were acquired in the range from 50-1000  $m/z$  for MS and 200-800  $m/z$  for MS/MS.  $\text{Na}^+$ -formate clusters were used as calibrant and injected at the beginning of each run. All files were automatically calibrated based on the compound spectra collected from the  $\text{Na}^+$ -formate clusters. Quality control samples (QC) consisting of a mix of equal aliquots of each individual sample were used to monitor technical variation throughout the run sequence. OC samples were injected 5x at the beginning of the sequence to prime the system and after every sixth sample throughout the run sequence. Data acquired from QC samples was used for data normalization.

Detected compounds not identified as previously known were assigned provisional names based on the ordinal of their retention times and the designation NI (Not Identified). Thus, the first detected unknown compound was named NI 01, the second NI 02, etc.

## 4.5 Statistical analysis

Data obtained for phenolics were tested for normality of distribution. Data with normal distribution was tested with Student's t-test. Data not found to be normally distributed were instead subjected to a non-parametric test; Mann-Whitney's U-test. Table 3 displays which test was used for which data. The data processing was made in Minitab 18 (Minitab, LLC).

To further investigate how different resistant and susceptible phenotypes were from each other, I conducted a principal components analysis (PCA), which reduces the dimensions in the data set to the two dimensions (in a two-dimensional PCA, which was used in this study) that explains the observed variation in the data the most. My assumption was that the analysis would differentiate between susceptible and resistant phenotypes.

## 5 Result

Chemical analysis of all samples revealed a total of 51 methanol soluble compounds (putatively phenolics), of which 16 were identified. The relations between detected compounds, retention time and ion intensity at each m/z (mass per charge ratio) spectrum were visualized in chromatograms for individual samples, see figure 9 for an example. The ion intensity at each m/z spectrum reflects the amount of each compound, which could be compared between compounds by comparing the peak areas of the peaks in the chromatograms. The dominant compounds thus identified were fraxin and ligustroside, with on average 19 and 15 % of the total peak area, see figure 10 and table 2.

Nine compounds were detected at intermediate levels (with an average above 2 % of total peak area), together comprising 37 % of the remaining total peak area. In order of descending magnitude, they included esculin, NI15, pinoresinol glucoside, NI 12, a possible oleuropein derivative (NI 32), NI 36, tyrosol hexoside, oleuropein and NI 37.

Of the 11 major compounds in this data set, seven were found at significantly higher levels in resistant trees (p-values in parenthesis): fraxin (<0.001), ligustroside (0.025), NI 15 (<0.001), pinoresinol glucoside (0.027), NI 32 (0.004), NI 36 (0.001) and NI 37 (0.001). One was found at higher levels in susceptible trees; NI 12 (0.001), whereas the levels of esculin, tyrosol hexoside and oleuropein were found at similar levels in resistant and susceptible trees.

Resistant trees had both more and higher amounts of secondary metabolites than susceptible trees. Individual trees of the resistant group had significantly more detected phenolics than the susceptible (on average 45.65 compounds of 51, compared to 41.15 for susceptible trees, p 0,038). The total level of detected methanol solubles (presumably phenolics) as a class

was higher in resistant trees than in susceptible trees ( $p = 0,002$ ,  $t = 3,11$ ). Most of the individual phenolic compounds detected (27 of 51) were found at a significantly higher abundance in resistant trees, and only three were found at a lower abundance in resistant trees. See table 3.

Eight compounds, (NI 11, NI 12, fraxin, NI 15, NI 17, NI 20, NI 29 and ligustroside) showed significant differences between resistant and susceptible trees when tested with t-tests. Of those, two of them, NI 11 and NI 12, showed a higher level for susceptible than resistant trees, while six showed a higher level for resistant trees.

For compounds tested with non-parametric methods, NI 4, NI 7, NI 8, NI 9, NI 10, NI 14, NI 18, NI 19, pinoresinol dihexoside, NI 21, NI 23, calceolarioside A, pinoresinol glucoside, NI 26, NI 28, NI 30, NI 32, NI 33, NI 36 and NI 37 showed significant differences between susceptible and resistant trees. Of these, only NI 36 had higher levels for susceptible, while all other compounds with significant differences were found in higher levels in resistant trees.

All compounds were found in each group (resistant or susceptible), although in the susceptible group, NI 04 and NI 08 were present in only one and two samples, respectively, and compounds typically found at lower levels were more often missing in individuals of either group, than compounds found at higher levels.

A principal components analysis showed incomplete separation of the resistant and susceptible groups, see figure 11, but instead groups after country of origin, see figure 12.

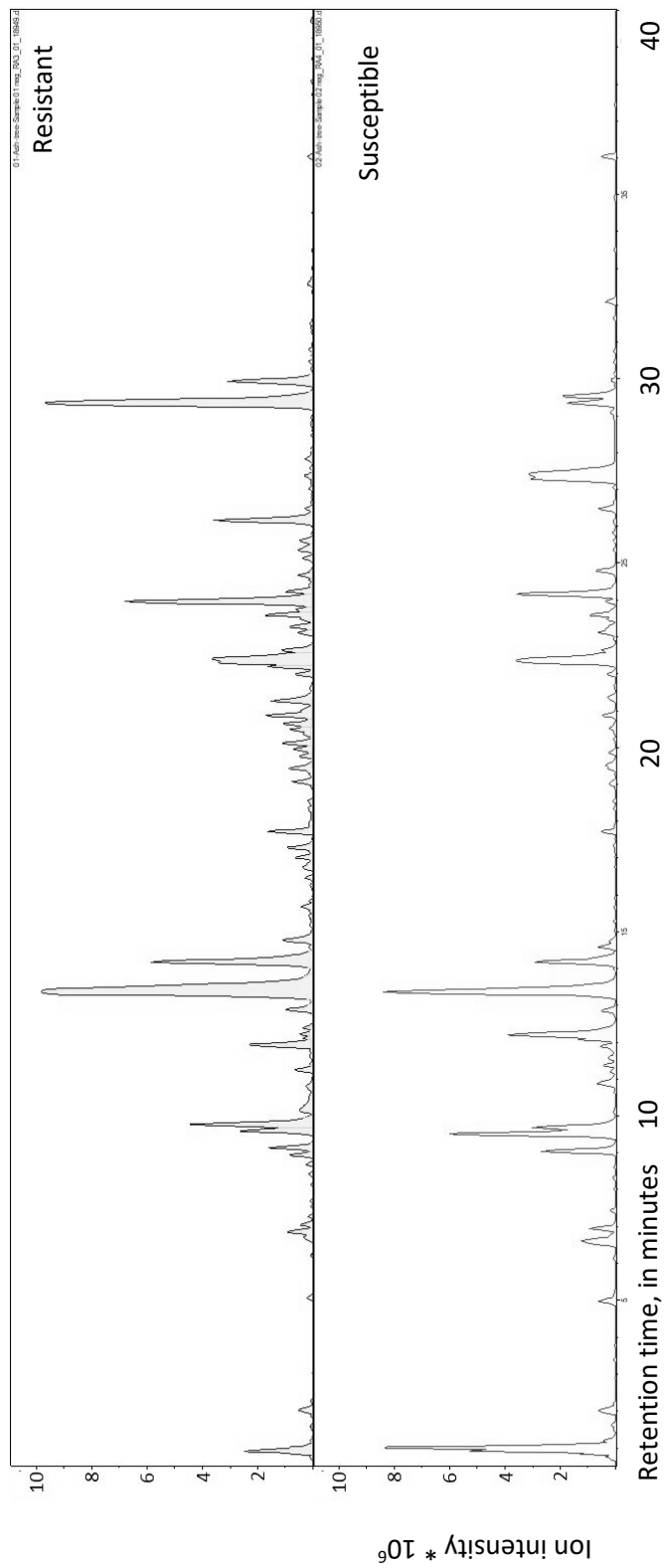


Figure 9. Reconstructed total ion current (TIC) chromatograms of two samples, representing two individual trees, one from a resistant tree (above, in purple) and one from a susceptible (below, in green). These chromatograms show the ion intensity at each point in time, where each time point in turn represents a mass spectrum each. Each major peak represents (at least) one compound but may contain several other compounds as well, though the compounds have been subjected to separation to avoid this, see subchapter 4.4. Notice how the upper, purple graph (representing a resistant tree) has higher amplitudes in general. Compare with figure 10.

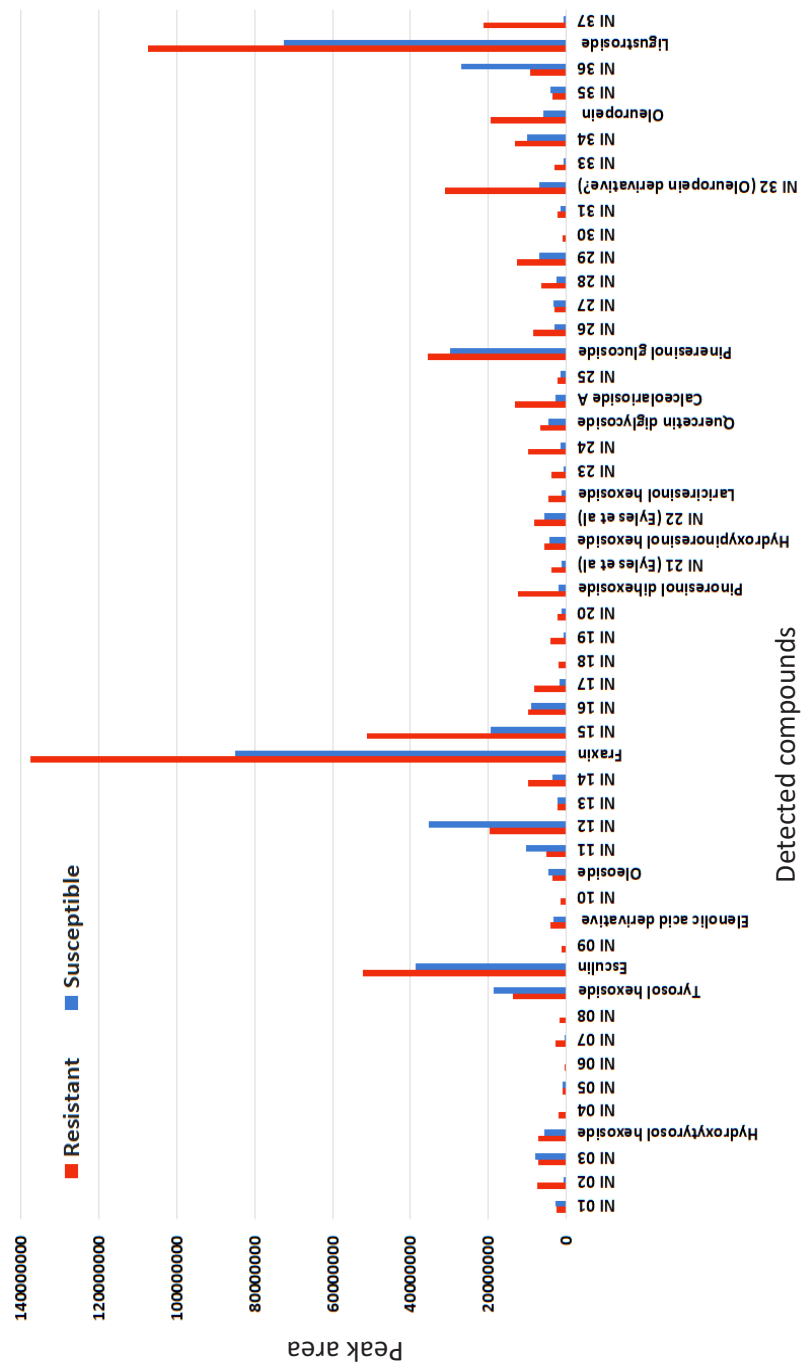


Figure 10. The average peak area for all detected compounds for resistant trees (red columns) and susceptible trees (blue columns). On the y-axis is the unitless peak area, which is calculated from the ion intensity of each compound detected and reflects concentrations of each compound. On the x-axis are all detected compounds in order by retention time.



Table 2. Phenolic compounds detected by LC-MS/Q-TOF from samples.

Compound name <sup>1</sup>	Retention time <sup>2</sup>	m/z <sup>3</sup>	Fragments <sup>4</sup>	Average peak area <sup>5</sup>	Relative peak area <sup>6</sup>
NI 01	5.1	329.0866	165, 109, 149	2626290	0.43%
NI 02	6.2	315.0712	152, 108, 165, 121	878453	0.15%
NI 03	6.7	345.1197	313, 151, 179, 123	7695371	1.27%
Hydroxytyrosol hexoside	7.0	315.1076	135, 153, 119, 101, 113, 161, 179	6519204	1.08%
NI 04	7.3	339.0711	177, 131	1198689	0.20%
NI 05	7.5	313.0917	151, 123	946533	0.16%
NI 06	8.1	407.1545	389, 313, 377, 377, 357, 161, 345, 183, 101	352631	0.06%
NI 07	8.4	407.1546	389, 377, 313, 377, 357, 161, 183, 345, 101	1941620	0.32%
NI 08	8.9	491.1702	329, 179, 119, 161, 101	1154410	0.19%
Tyrosol hexoside	9.1	299.1131	119, 101, 179, 113, 161, 149, 143, 137, 131	15633384	2.58%
Esculin	9.7	339.0720	177.0	46907054	7.75%
NI 09	11.3	399.0918	191, 176, 353	898260	0.15%
Elenolic acid derivative	11.6	565.1761	403, 179, 223, 265, 161, 119, 101, 265, 283	3710868	0.61%
NI 10	11.9	531.1340	207, 192	1054563	0.17%
Oleoside	12.1	389.1079	345, 183, 121, 209, 165, 119, 101	3900711	0.64%
NI 11	12.2	565.1767		7227594	1.19%
NI 12	12.2	403.1238		25944623	4.29%
NI 13	12.4	241.0712		2178238	0.36%
NI 14	12.9	369.0822	207, 192, 249	7274587	1.20%
Fraxin	13.4	369.0825	207, 192, 354	116855858	19.32%
NI 15	14.2	221.0455	206, 191	38711100	6.40%

Compound name <sup>1</sup>	Retention time <sup>2</sup>	m/z <sup>3</sup>	Fragments <sup>4</sup>	Average peak area <sup>5</sup>	Relative peak area <sup>6</sup>
NI 16	14.3	369.0819	207	9578690	1.58%
NI 17	14.8	221.0453	206, 191	5615935	0.93%
NI 18	15.2	583.2027	375, 327, 195, 179	1352727	0.22%
NI 19	15.7	537.1963	375, 327, 357, 179, 195	2690658	0.44%
NI 20	17.4	581.1863	177, 373, 195, 162	1920972	0.32%
Pinoresinol dihexoside	17.7	681.2405	357, 519, 161	8239673	1.36%
NI 21 <sup>7</sup>	19.1	523.2168	361.0	2861068	0.47%
Hydroxypinoresinol hexoside	19.5	535.1817	373, 343, 211, 313	5186046	0.86%
NI 22 <sup>7</sup>	20.0	555.1715	151, 403, 223, 179, 537, 291, 323, 393, 361	7235161	1.20%
Lariciresinol hexoside	20.2	521.2015	329, 359, 161	3210555	0.53%
NI 23	20.4	565.1910	339, 327, 403, 207, 521, 161	2605491	0.43%
NI 24	20.7	507.1494	475, 341, 161, 179, 203, 323, 281, 195	6606684	1.09%
Quercetin diglycoside	20.9	609.1456	301, 179, 151, 271	5856452	0.97%
Calceolarioside A	21.3	477.1386	161, 179, 341, 203, 135, 315	9122024	1.51%
NI 25	22.0	593.1503	285, 116	1983559	0.33%
Pineresinol glucoside	22.4	519.1870	357, 151, 342, 161, 136	33242395	5.50%
NI 26	22.7	447.0921	284	6305739	1.04%
NI 27	23.2	593.1503	285, 387, 117	3152575	0.52%
NI 28	23.3	387.1439	181, 151	4872159	0.81%
NI 29	23.6	447.0925	285	10419710	1.72%
NI 30	23.8	477.1038	314, 357	631318	0.10%
NI 31	23.8	623.1618	315, 206, 117	1926396	0.32%

Compound name <sup>1</sup>	Retention time <sup>2</sup>	m/z <sup>3</sup>	Fragments <sup>4</sup>	Average peak area <sup>5</sup>	Relative peak area <sup>6</sup>
NI 32 <sup>8</sup>	24.0	539.1764	377, 291, 275, 101, 239, 179, 359, 419, 127	21581371	3.57%
NI 33	24.3	477.1034	314, 357, 271, 151	2036531	0.34%
NI 34	25.5	569.1872	403, 537, 151, 223, 179, 305, 375, 337	11981610	1.98%
Oleuropein	26.1	539.1757	377, 275, 307, 223, 179, 403, 149, 345	14178679	2.34%
NI 35	26.5	337.1069	322, 306	3765799	0.62%
NI 36	27.4	569.1871	223, 137, 385, 205, 265, 161, 179, 315, 101	16332756	2.70%
Ligustroside	29.4	523.1818	361, 291, 259, 101, 223, 127, 179,	93642604	15.48%
NI 37	30.0	553.1920	321, 391, 289, 101, 223, 179, 403	13198703	2.18%

<sup>1</sup>Putative name for identified phenolic compounds and provisional names for not identified (NI) compounds.

<sup>2</sup>The retention time in the LC-MS/Q-TOF spectrometry

<sup>3</sup>Mass per charge ratio

<sup>4</sup>Fragments m/z in order of decreasing abundance

<sup>5</sup>The unitless peak area, averaged for all samples

<sup>6</sup>The share in per cent of the total peak area, averaged for all samples

<sup>7</sup>Identified in Eyles *et al.* (2007).

<sup>8</sup>A possible Oleuropein derivate?

Table 3. The relationship between susceptible and resistant trees in terms of peak area of detected compounds.

Compound name <sup>1</sup>	Method applied <sup>2</sup>	Difference <sup>3</sup>	Significance (p-value) <sup>4</sup>
NI 01	Student's t-test	-	
NI 02	Mann-Whitney	-	
NI 03	Student's t-test	-	
Hydroxytyrosol hexoside	Mann-Whitney	-	
	Mann-Whitney	R	<0.001
NI 05	Student's t-test	-	
NI 06	Mann-Whitney	-	
NI 07	Mann-Whitney	R	0,047
NI 08	Mann-Whitney	R	0.014, adjusted 0.005
Tyrosol hexoside	Mann-Whitney	-	
Esculin	Student's t-test	-	
NI 09	Mann-Whitney	R	<0.001, W 434
Elenolic acid derivative	Student's t-test	-	
	Mann-Whitney	R	<0.001, W435
Oleoside	Mann-Whitney	-	
	Student's t-test	S	0.001, t -3.54
NI 12	Student's t-test	S	< 0.001, t -3.85
NI 13	Student's t-test	-	
NI 14	Mann-Whitney	R	0.021, W 396
Fraxin	Student's t-test	R	<0.001, t 3.79
NI 15	Student's t-test	R	< 0.001 t 4.47

Compound name <sup>1</sup>	Method applied <sup>2</sup>	Difference <sup>3</sup>	Significance (p-value) <sup>4</sup>
NI 16	Student's t-test	-	
NI 17	Student's t-test	R	<0.001, t 4.44
NI 18	Mann-Whitney	R	0.003, W 415
NI 19	Mann-Whitney	R	0.002, W420
NI 20	Student's t-test	R	0.013, t 2.37
Pinoresinol dihexoside	Mann-Whitney	R	0.009, W405
NI 21 <sup>5</sup>	Mann-Whitney	R	0.041, W388
Hydroxypinoresinol hexoside	Student's t-test	-	
NI 22 <sup>5</sup>	Mann-Whitney	-	
Lariciresinol hexoside	Mann-Whitney	-	
NI 23	Mann-Whitney	R	0.002, W 419
NI 24	Mann-Whitney	-	
Quercetin diglycoside	Mann-Whitney	-	
Calceolarioside A	Mann-Whitney	R	0.010, W 401
NI 25	Mann-Whitney	-	
Pinoresinol glucoside	Mann-Whitney	R	0.027, W393
NI 26	Mann-Whitney	R	0.043, W387
NI 27	Mann-Whitney	-	
NI 28	Mann-Whitney	R	0.001, W427
NI 29	Student's t-test	R	0.008, t 2.57
NI 30	Mann-Whitney	R	0.036 W388
NI 31	Mann-Whitney	-	
NI 32 <sup>6</sup>	Mann-Whitney	R	0.004, W 412

Compound name <sup>1</sup>	Method applied <sup>2</sup>	Difference <sup>3</sup>	Significance (p-value) <sup>4</sup>
NI 33	Mann-Whitney	R	0.009, W 405
NI 34	Mann-Whitney	-	
Oleuropein	Mann-Whitney	-	
NI 35	Student's t-test	-	
NI 36	Mann-Whitney	S	0.001, W 257
Ligustroside	Student's t-test	R	0.025, t 2.04
NI 37	Mann-Whitney	R	0.001, W 417
Total detected methanol solubles <sup>7</sup>	Student's t-test	R	0.002, t 3.11

<sup>1</sup>Names as per table 2.

<sup>2</sup>The method chosen depended on the normality of data distribution. Student's t-test was used for data with normal distribution, and Mann-Whitney for data with non-normal distribution.

<sup>3</sup>The difference between resistant and susceptible genotypes. "R" denotes that the compound was found in higher amounts in resistant genotypes compared to susceptible ones, and "S" denotes that the opposite situation was found. No significant difference between resistant and susceptible trees is denoted by "-".

<sup>4</sup>The significance of the tests described with the p-values.

<sup>5</sup>Also detected in Eyles *et al.* (2007).

<sup>6</sup>A possible Oleuropein derivative?

<sup>7</sup>The total amount (in terms of peak area) of all detected phenolic compounds combined.

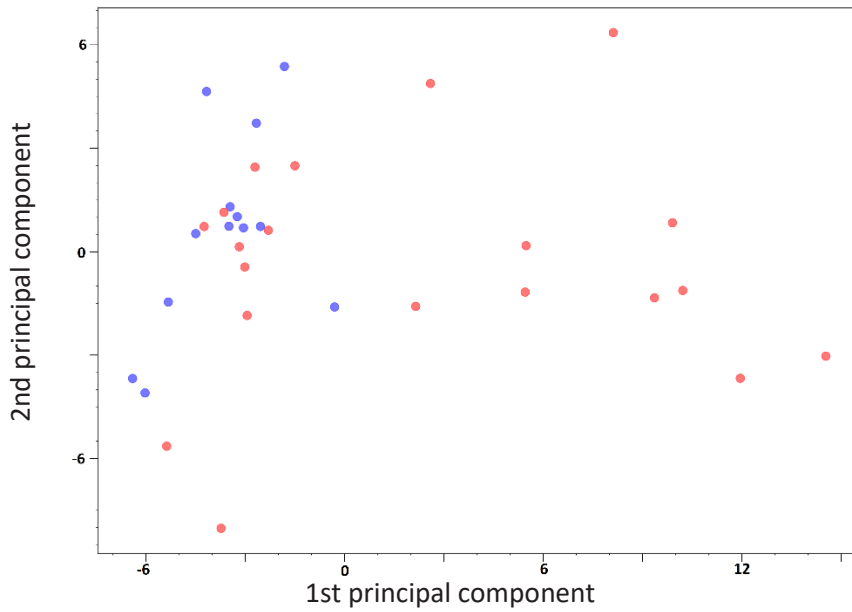


Figure 11. Principal component analysis plot with samples from the resistant trees indicated by red dots, and samples from susceptible trees by blue dots. The two groups overlap.

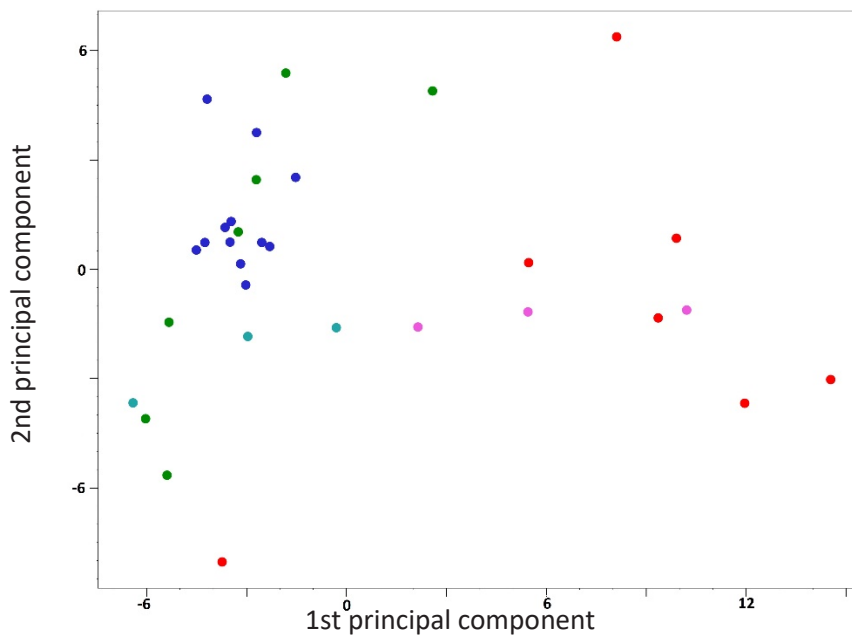


Figure 12. Principal components analysis plot with the same data as in figure 11 but with dots coloured according to country of origin of the corresponding samples. Blue = Austria, teal = Denmark, green = France, pink = Lithuania and red = Sweden

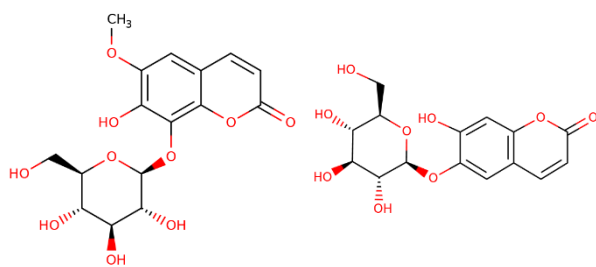


Figure 13. The chemical structure of fraxin (to the left) and esculin (to the right). Source: Toxnet, U.S. National Library of Medicine, 2019. <https://chem.nlm.nih.gov/chemidplus/>

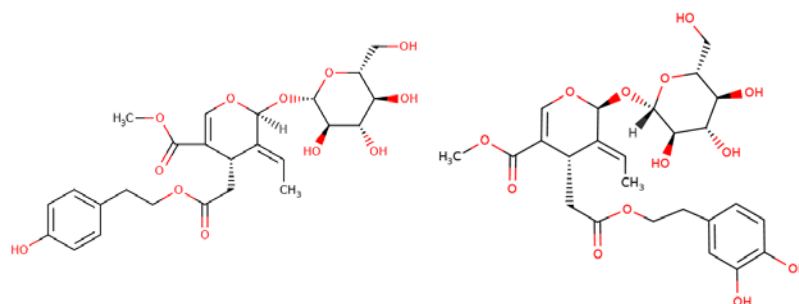


Figure 14. The chemical structure of ligustroside (to the left) and oleuropein (to the right). Source: Toxnet, U.S. National Library of Medicine, 2019. <https://chem.nlm.nih.gov/chemidplus/>

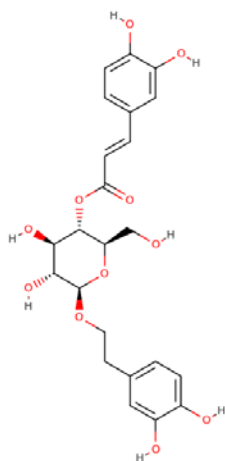


Figure 15. The chemical structure of calceolarioside a. Source: Toxnet, U.S. National Library of Medicine, 2019. <https://chem.nlm.nih.gov/chemidplus/>



## 6 Discussion

In this study I investigated the qualitative and quantitative differences in constitutive phenolics in different genotypes of *Fraxinus excelsior* which are resistant and susceptible to *Hymenoscyphus fraxineus* in order to improve our understanding of the mechanisms of genetic resistance to *H. fraxineus*.

### 6.1 On the findings

I found that the levels of fraxin and ligustroside, among several other phenolics, were significantly higher in resistant genotypes than in susceptible ones.

#### 6.1.1 Compounds found

Fraxin, a glucoside of fraxetin, and esculin, a glycoside of esculetin, described for example by Kostova and Iossifova (2007), are members of the coumarin group and are well known prevalent phytochemicals in stem phloem in *Fraxinus* species (Iossifova, Kostova and Evstatieva, 1997) (as well as in several other species, such as in *Aesculus hippocastanum* (Stanić, Jurišić and Brkić, 1999)), and are often discussed together in scientific literature. Also in this study both were found at high levels, but only fraxin was found at different levels in resistant and susceptible genotypes. Fraxin is of interest in modern day medicine e.g. for its ability to protect human cells from oxidative stress (Whang *et al.*, 2005) and both have been used in traditional medicine (Li *et al.*, 2005; Chen *et al.*, 2017). Coumarins are in general of great interest for medical research, but their function in the plants from which they originate is less studied (Martins Borges *et al.*, 2008). Iossifova *et al.* (1994) found the antimicrobial activity of both fraxin and esculin

to be limited, although their aglucones fraxetin and esculetin were found to be potent against bacteria (*Staphylococcus aureus* and *Escherichia coli*). Similarly, Mercer *et al.* (2013) found fraxetin and esculetin to have an antifungal effect against human dermatophytes, but their glucones were devoid of antifungal effect. Perhaps the presence of the glucones can still be important, for example for rapid synthetization of their respective aglucones through hydrolysis.

Ligustroside, also known as ligstroside, is described e.g. in Iossifova, Mikhova and Kostova (1993) and is a secoiridoid glucoside with antimicrobial activity (Iossifova *et al.*, 1994) and a direct precursor of oleuropein (see for example Soler-Riveras, Espín and Wichers (2000)), a prevalent compound in olives (Omar, 2010). Interestingly, the levels of oleuropein were similar in resistant and susceptible trees, whereas the levels of the precursory ligustroside differed significantly. It is thus possible that the role of ligustroside, if it plays a part in defence against *H. fraxineus*, is either direct or indirect, that is, that oleuropein may be the active part in defence but that the synthesis of sufficient levels of oleuropein to resist the pathogen during infection is dependent on a high reservoir of ligustroside.

I found four lignans, the largest group of identified compound in this study, but not one with great amplitude, which seems to be in accordance with earlier investigations (Kostova and Iossifova, 2007). This group is known to have antifungal (and antibacterial) activity, for example pinoresinol and lariciresinol (Céspedes *et al.*, 2006), which are related to all four lignans identified in this study.

The phenylethanoid glycoside calceolarioside A, (also known as desrhamnosyl acetoside) described for example by Kostova and Iossifova (2007), was also found at higher amounts in resistant than in susceptible trees, and it possesses antimicrobial activity (Shoyama, Matsumoto and Nishioka, 1986). Although found only at relatively low levels even, it may still contribute to resistance.

The remaining identified compounds (oleoside, tyrosol hexoside, hydroxytyrosol hexoside and quercetin diglycoside) were found at low levels in both resistant and susceptible trees with no significant differences

### 6.1.2 Principal components analysis

The principal components analysis (figures 11 and 12) did not show a complete separation between resistant and susceptible genotypes, but to some extent indicate a relationship between samples dependent on geography and phylogenetics. The Austrian samples grouped mid-left and the French and Danish forming a loose cluster around the Austrian, representing the Central European population and the Swedish and Lithuanian, representing the Eastern European population, are scattered and mostly to the right. This is in line with research on *F. excelsior* phylogenetics and post glacial recolonization routes, (Heuertz *et al.*, 2004). However, this relationship was not expected in the PCA, as phloem tissue from the same genotypes (albeit in a larger sample size) did not show strong links to geography, see Villari *et al.* (2018). This may be an effect of the fact that for Sweden and Lithuania, I had only resistant samples available, see table 1, and that the sample size is small.

## 6.2 Parallels and comparisons with earlier studies

Constitutive phenolic compounds have been a topic of high research interest for discovering the basis of resistance in different *Fraxinus* species towards the Emerald Ash Borer (EAB). Previous work has found that some lignans (e.g. pinoresinol dihexoside) are more abundant in *Fraxinus* species resistant to EAB (e.g. *F. mandshurica*) than in susceptible species (e.g. *F. excelsior*, *F. nigra*, *F. pennsylvanica*, *F. americana*) (Whitehill *et al.*, 2012). The lignans, which may well play a role in genetic resistance to EAB (Villari *et al.*, 2016) are also interesting for research on *H. fraxineus*, as species without a coevolutionary history with EAB also lacks the same for *H. fraxineus*, as mentioned earlier. Results from such EAB studies are helpful for comparisons in ash dieback studies, and indeed there has been some cross-over studies, for example Sollars *et al.* (2017) , who found evidence that iridoid glucosides may play a part in susceptibility to *H. fraxineus*, as higher abundance were found in more susceptible genotypes than in more resistant (Sollars *et al.*, 2017). It would thus be very interesting to know if the compounds NI 12, NI 12 and NI 36 in this study belong to the iridoid glucosides, but unfortunately, that is not revealed due to reference library limitations. The only identified iridoids in this study are secoiridoids, see table 4.

It is of interest to compare not only *F. excelsior* genotypes of different susceptibility, but also species of the *Fraxinus* genera with different susceptibility to *H. fraxineus*, especially to compare with the natural host of *H. fraxineus*, *F. mandshurica*. See table 4.

Whitehill *et al.* (2012) found similar levels of esculin in the susceptible *F. excelsior* and the resistant *F. mandshurica*, suggesting that esculin does not play a vital part in resistance, which is in line with these findings. Oleuropein was found in much higher levels in *F. mandshurica*, but in similar levels in resistant and susceptible genotypes in this study, so the higher amplitudes in *F. mandshurica* could be a coincidence. Cleary *et al.* (2014) found oleuropein to be downregulated in leaves of a resistant genotype when treated with viridiol, compared to untreated control, whereas demethyleuropein was up-regulated in another resistant genotype.

Interestingly, ligustroside were found at lower levels in *F. mandshurica* than in *F. excelsior* and two North American species (Eyles *et al.*, 2007; Whitehill *et al.*, 2012) while that compound is higher in resistant genotypes in this study. Cleary *et al.* (2014) found that the related compound demethyl-ligustroside was upregulated in leaves of a resistant genotype treated with viridiol compared to untreated control. There is thus contradictory evidence regarding if ligustroside confers resistance to *H. fraxineus*, but this could be worth investigating further.

Tyrosol hexoside was downregulated in a resistant genotype in Cleary *et al.* (2014) and in higher abundance in susceptible than in resistant species but had no significance in this study. It is thus at least unlikely that a higher amount of tyrosol hexoside confers resistance against *H. fraxineus*.

More interesting is that fraxin was found in much higher levels in *F. mandshurica* than in *F. excelsior*. Fraxin was also found in *F. mandshurica* but not in *F. pennsylvanica* or *F. americana* in both the Eyles *et al.* (2007) study and in the Whitehill *et al.* (2012) study. This is interesting as both *F. pennsylvanica* and *F. americana* are susceptible to *H. fraxineus*, albeit to perhaps a lower degree than *F. excelsior* (Gross and Sieber, 2016; Nielsen *et al.*, 2017).

Another compound of some interest is calceolarioside A, as it was found in higher amplitudes both in resistant and susceptible genotypes in this study, and in *F. mandshurica* and *F. excelsior* in the Whitehill *et al.* (2012) study.

Table 4. Comparisons between studies for identified compounds.

Peak number	Identified compounds in this study <sup>1</sup>	Significance in this study <sup>2</sup>	Differences in interspecific studies <sup>3</sup>	Regulated in response to viridiol in leaf <sup>4</sup>
<i>Simple phenolics</i>				
4	Hydroxytyrosol hexoside	-	R	
10	Tyrosol hexoside	-	S	↓R
<i>Coumarins</i>				
11	Esculin	-	-	
20	Fraxin	R	R	
<i>Secoiridoids</i>				
15	Oleoside	-	NA	
47	Oleuropein	-	R	↓R, ↑R <sup>6</sup>
50	Ligustroside	R	S	↑R <sup>6</sup>
<i>Lignans</i>				
27	Pinoresinol dihexoside	R	R <sup>5</sup>	
29	Hydroxypinoresinol dihexoside	-	NA	
37	Pinoresinol glucoside	R	R <sup>5</sup>	
31	Lariciresinol hexoside	-	NA	
<i>Flavonoids</i>				
34	Quercetin diglycoside	-	NA	↓R <sup>7</sup>
<i>Phenylethanoids</i>				
35	Calceolarioside A	R	R	

<sup>1</sup>Name

<sup>2</sup>The difference between resistant and susceptible genotypes. "R" denotes that the compound was found in higher amounts in resistant genotypes compared to susceptible ones, and "S" denotes that the opposite situation was found. No significant difference between resistant and susceptible trees is denoted by "-".

<sup>3</sup> The difference between resistant and susceptible *Fraxinus* species in Whitehill *et al.* (2012) and Eyles *et al.* (2007). Both studies include *F. mandshurica*, *F. pennsylvanica*, and *F. americana*, but only Whitehill *et al.* (2012) includes *F. excelsior*. "R" denotes that the compound was found in higher amounts in resistant genotypes compared to susceptible ones, and "S" denotes that the opposite situation was found. No significant difference between resistant and susceptible trees is denoted by "-". NA indicates that no comparison was possible due to lack of data.

<sup>4</sup> Comparison to Cleary *et al.* (2014). Change compared to untreated controls for susceptible and resistant genotypes treated with viridiol, a phytotoxin produced by *H. fraxineus*. ↑R indicates that in resistant genotypes, that compound was upregulated compared to untreated control. ↓R indicates downregulated compared to control. ↑S indicates upregulated in susceptible genotypes etc.

<sup>5</sup>Not including *F. excelsior* but only *F. mandshurica* and North American species.

<sup>6</sup> Demethylated counterpart. Cleary *et al.* (2014).

<sup>7</sup> Conjugation with a hexose–deoxyhexose disaccharide, Cleary *et al.* (2014).

Pinoresinol dihexoside was found in much higher amplitudes in *F. mandshurica* than in both *F. pennsylvanica* and *F. americana* in Eyles *et al.* (2007) and found in *F. mandshurica* but not detected at all in other *Fraxinus* species in Whitehill *et al.* (2012) while found at higher levels in resistant genotypes in this study. Though found at much lower levels than fraxin in this study and in the Whitehill *et al.* (2012) study, it was found in moderately higher levels in the Eyles *et al.* (2007) study and can putatively play a part in resistance.

It is worth noting that the levels of the same compounds in the same species differed between the Eyles *et al.* (2007) study and the Whitehill *et al.* (2012) study. This can be due to several reasons. Therefore I do not compare absolute levels between studies but only relative to other species in the same study, as the methodology is consistent between species within those studies but not necessarily between studies.

### 6.3 Limitations

In this study, I investigated only a subset of the samples included in the Villari *et al.* (2018) study, and the small sample size may lead to some limitations to widespread interpretations of the results.

Since there can be more than one compound with the same  $m/z$ , several compounds can potentially be found under the same peak in the chromatograms. The MS system used is however good at separating co-eluting compounds and the results are likely representative for the real circumstances. To be completely sure further steps of validation need to be taken, which however is beyond the resources available for this study. These results thus give a good indication on what could be in interest to investigate further rather than an absolute answer as to which secondary metabolites are the key to resistance.

Many of the compounds were not identified, which is normal for this kind of study (Christoph Crocoll, pers. comm.), but that is not to say that they are necessarily new discoveries. Indeed NI 21 and NI 22 were also detected in Eyles *et al.* (2007), where they were labelled as previously unidentified compounds (peak number 21 and 23), and it is likely that several other of the compounds have been found before in other studies. However, this is not a vastly explored field, lacking adequate standard chemical compounds libraries, which makes identification difficult. Standard libraries and methods that

exists are mostly developed for studies on compounds in humans and animals, (such as medical research) and studies on plant metabolomics rely on in-house resources to a large extent.

That the compounds in higher quantity were identified is however a sign of some robustness in the chemical analysis and compound identification. Based on the available information (retention time, mass to charge ratio, fragmentation patterns) it is possible to say something about unidentified compounds. Specifically, NI 32 is purportedly an oleuropein derivative.

Concentration of phenolic compounds can vary quite significantly over time and space even within the same species; it even varies over the course of the day within the very same tissue (Soengas *et al.*, 2018). It would thus be welcome with a larger study which can take this into account and compensate with more replicates, representing phenotypes.

Although different compounds look differently interesting it is important to keep in mind that natural defensive compounds are active not one at a time but several at once. Thus the activity of single compounds can be weak alone but have synergistic effects when combined with others with which they naturally cooccur, as demonstrated for the antifungal activity of lignans in Céspedes *et al.* (2006).

## 6.4 Considering future research

It would be of interest to validate these findings with a broader study, and to experimentally explore the specific effects on resistance of the identified phenolics which differed significantly between resistant and susceptible genotypes. If one or several compounds can be attributed to resistance, breeding on genetic ability to produce that or those phenolics in sufficient quantities could be targeted in the screening. The complete genome of *F. excelsior* has been sequenced (Sollars *et al.*, 2017) and a next step could be to find the genes that code for the synthesis of the respective phenolics, especially for the promising coumarin fraxin, and guide the use of transcriptomic markers.

Another topic worthy of further research might be to assess the possibility to use mycovirus as a biological control (Schoebel, Zoller and Rigling, 2014).

## 6.5 Conclusions

In this study I present indications that high synthesis of fraxin may be a key to resistance towards *H. fraxineus*. This makes efforts for the functional salvation of *F. excelsior* through genetic improvements and breeding programmes more hopeful by identifying potential chemical biomarkers associated with resistance.



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## Appendix 1

### Protocol 1.

1. Add 500  $\mu$ l of high-performance liquid chromatography (LC) grade methanol with 0.5 mg/mL butylated hydroxyanisole, to 100 mg of ground stem phloem.
2. Keep the sample over 48hr in the dark at 4°C.
3. Centrifuge (12,000 $\times$ g for 5 min) to remove solids, and transfer the supernatant to 1.5-ml microcentrifuge tube.
4. Repeat steps 1, 2 and 3 for the same sample.
5. Pool the extracts from the same sample and store it at -20°C.