#### **RESEARCH PAPER**



# Canditate metabolites for ash dieback tolerance in *Fraxinus* excelsior

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#### Abstract

Ash dieback, a forest epidemic caused by the invasive fungus *Hymenoscyphus fraxineus*, threatens ash trees throughout Europe. Within *Fraxinus excelsior* populations, a small proportion of genotypes show a low susceptibility to the pathogen. We compared the metabolomes from a cohort of low-susceptibility ash genotypes with a cohort of high-susceptibility ash genotypes. This revealed two significantly different chemotypes. A total of 64 candidate metabolites associated with reduced or increased susceptibility in the chemical families secoiridoids, coumarins, flavonoids, phenylethanoids, and lignans. Increased levels of two coumarins, fraxetin and esculetin, were strongly associated with reduced susceptibility to ash dieback. Both coumarins inhibited the growth of *H. fraxineus in vitro* when supplied at physiological concentrations, thereby validating their role as markers for low susceptibility to ash dieback. Similarly, fungal growth inhibition was observed when the methanolic bark extract of low-susceptibility ash genotypes was supplied. Our findings indicate the presence of constitutive chemical defense barriers against ash dieback in ash.

Keywords: Ash dieback, chemotype, coumarin, Fraxinus, Hymenoscyphus, secoiridoid, tolerance.

#### Introduction

In recent decades, European common ash (*Fraxinus excelsior*) has been severely affected by ash dieback (ADB) disease, caused by the invasive fungal pathogen *Hymenoscyphus fraxineus* (Baral *et al.*, 2014); it also infects narrow-leaved ash (*E angustifolia*) (Kirisits *et al.*, 2010). *Hymenoscyphus fraxineus* was introduced in Europe and was first described in *E excelsior* in Poland (Kowalski, 2006). Today, its distribution overlaps with most of the natural distribution of *E excelsior*, with the

exception of some areas in Southern and Western Europe. The pathogen infects the leaves of the host and translocates through the leaf rachis and petiole into the shoot, which leads to crown dieback. Over a number of years, this process results in crown loss and eventual death of ash trees (Gross *et al.*, 2014). Alternatively, conditions of high humidity and high infection pressure (Havrdová *et al.*, 2017) enable the pathogen to directly infect stem collars at ground level and

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cause the trees to collapse from the base (Chandelier et al., 2016; Enderle et al., 2017), perhaps through the direct infection of stem lenticels (Nemesio-Gorriz et al., 2019). The capacity of *H. fraxineus* to colonize living tissue in ash is the key to its success as a pathogen. High tree mortality rates are found in ash forests as a consequence of the action of the pathogen (Coker et al., 2019). Despite the high susceptibility of F. excelsior to H. fraxineus, a small proportion of genotypes in ash populations show a low susceptibility to the pathogen. These genotypes appear as healthy trees with negligible disease symptoms in environments with high disease pressure and high tree mortality. It is not clear if host defense to ADB relies on induced or constitutive defense mechanisms. Genetic studies have shown that low susceptibility to ADB is genetically heritable (Muñoz et al., 2016), polygenic in nature (Stocks et al., 2019), and is stable in trees which are vegetatively propagated (Douglas et al., 2017; Stener, 2018). After trees with low susceptibility are identified, they can be propagated with the aim of assembling collections as starting material for breeding populations (Plumb et al., 2019) and for vegetative propagation (Douglas et al., 2017). Low susceptibility to ADB is a polygenic trait that can be predicted using molecular markers (Stocks et al., 2019). Once validated, markers for low susceptibility can aid greatly in the identification of ash genotypes and would facilitate selection among progeny from controlled and natural crosses.

Metabolomics profiling is an ideal tool to identify and understand factors for differentiating between different susceptibility groups (Cleary et al., 2014; Sambles et al., 2017; Sollars et al., 2017), as it is a high-throughput technique and only requires small amounts of vegetative material. A metabolomics study in ash seedlings (Cleary et al., 2014) identified metabolites associated with genotypic groups with different susceptibility after treatment with a fungal elicitor. Another study on F. excelsior leaf material identified five secoiridoids associated with high susceptibility to ADB in a Danish population of F. excelsior (Sambles et al., 2017; Sollars et al., 2017). A recent study differentiated genotypes with different susceptibility to ADB using advanced spectroscopy on bark samples (Villari et al., 2018). Bark and leaf chemistry in ash show major quantitative and qualitative differences (Iossifova et al., 1997). Even though leaves are the most common initial infection point for H. fraxineus (Gross et al., 2014), the pathogen only causes substantial damage and dieback once it colonizes the phloem, making phloem a crucial tissue for defense. Even though previous studies have identified metabolites associated with groups of genotypes with different susceptibilities to ADB (Cleary et al., 2014; Sambles et al., 2017; Sollars et al., 2017), specific candidate markers for reduced susceptibility to ADB were not validated by testing their activity on H. fraxineus. Therefore, the present study aimed to identify specific metabolites that are constitutively present in F. excelsior which associate with reduced susceptibility to ADB, and to validate their biological effects on the pathogen in vitro. The analyses included a large number of genotypes with a broad genetic background, from which bark samples were taken in the absence of the pathogen. The final objective was to identify and validate biochemical markers associated with low susceptibility to ADB. These

markers may be useful as screening tools for plant material in breeding programs. This would save the significant amount of work, time, and resources associated with inoculation experiments and screenings for natural selection. At the same time, the study aimed to better understand the putative chemical defense mechanisms in *E excelsior* against *H. fraxineus*.

To improve the clarity of the manuscript, definitions for the following terms are given: (i) resistance—situation of complete immunity to the pathogen in the host where the pathogen is unable to infect the host; (ii) tolerance—situation where the host allows infection to a small extent but this infection does not spread and the general health and growth rate of the host are not affected; (iii) susceptibility—failure by the host to contain the spread of the pathogen once it has been infected; susceptibility leads to major disease symptoms and, in some cases, to the death of the host; and (iv) defense—combination of physiological processes that allow the host to respond to the infection of a pathogen, resulting in resistance, tolerance, or susceptibility.

#### Materials and methods

#### Plant material

Sets of *E* excelsior genotypes consisting of 19 low-susceptibility (LS) and 19 high-susceptibility (HS) genotypes to ADB were used for the metabolomics comparison. Scion wood from all the genotypes was grafted and grown under the same conditions in an unheated glasshouse in Dublin (Ireland). Current year shoots of all the genotypes, which had grown from the grafted plants, were used for the study. All shoots were healthy, with no evidence of dieback symptoms on leaves or stems. Details on the selection parameters of the HS and LS genotypes are given below.

#### High-susceptibility (HS) genotypes

Sixteen trees were selected in a 12-year-old European (RAP) provenance trial (https://cordis.europa.eu/project/rcn/58712/factsheet/en) of *F. excelsior* located in Slatta, Ireland. The trial consisted of 37 European provenances (three replicates per provenance each consisting of 36 trees) and border trees composed of one single lot of commercial Irish seedlings (common seed lot).

A further three trees were selected in a different provenance trial in the Manch Estate (Ireland). These trees belonged to two Irish provenances (Grantstown and Kilmacrennan) and one to the Danish seed orchard FP202 (https://static-curis.ku.dk/portal/files/21206956/final\_wp34.pdf).

In the 3 years before this study, both trials above were monitored by the Irish Department of Agriculture for ADB symptoms and for the presence of *H. fraxineus* [quantitative PCR (qPCR) test; Chandelier *et al.*, 2010)] and found to be negative throughout the first 2 years. *Hymenoscyphus fraxineus* was confirmed in the third year of the trials when highly symptomatic plants were selected as the HS genotypes.

The 19 HS genotypes were selected amongst 5235 trees examined (3939 in Slatta and 1296 in Manch Estate). The HS trees were identified by cutting down a branch within the crown canopy and carefully assessing the stems for evidence of current year infection. Trees in which shoot dieback was present in >75% of the crown shoots were categorized as highly susceptible because of the quantity of shoots with symptoms which developed during the first year of infection by the pathogen. Details of the HS genotypes are given in Supplementary Table S1 at *JXB* online.

#### Low-susceptibility (LS) genotypes

Nineteen trees were selected as healthy trees in a 14-year-old European (RAP) provenance trial of *E excelsior* (containing the same provenances as

the RAP trial in Ireland (see above; as well as 14 additional provenances from France). The trial is located in Stevoort, Belgium and established and monitored by the Research Institute for Nature and Forest (INBO).

In the year before this study (2012) started, the trial was monitored for ADB symptoms and the presence of *H. fraxineus* was confirmed. Subsequently, in the period 2013–2017, the symptoms of ADB were scored per tree annually. From 2013 to 2017, the percentage of dead trees as a result of ADB increased from 24.6% to 50.3%, confirming the high disease pressure in Stevoort. In 2017, the 19 LS trees were selected amongst 5508 examined.

The LS trees were selected as healthy trees on the basis of having <10% of shoot dieback in crowns and an absence of stem or collar infections. Details on the LS genotypes are given in Supplementary Table S1.

#### Sample preparation

Shoots from the current season of growth were cut in July from the grafted plants of each selected genotype; bark (including phloem) from internodes which were at least 5 cm long was stripped off, placed in 15 ml Falcon tubes, and immediately stored in liquid nitrogen until further use. Three samples per genotype were collected. Once in the laboratory, samples were homogenized using liquid nitrogen and porcelain mortars and pestles. Ground samples were freeze-dried and 50 mg of ground sample were used for extraction.

Each of the samples was extracted using 1500  $\mu$ l of methanol (MeOH) shaking in a vortex for 30 min. Samples were then centrifuged for 15 min and 1000  $\mu$ l of the supernatant was aspirated with a pipette. The supernatant was then run though a Macherey-Nagel Chromabond HR-X 1 ml/30 mg column to clean the sample.

## Metabolomics analysis, candidate metabolite identification, and data analysis

HPLC-HRMS/MS measurements were recorded on a Bruker Compact O-TOF instrument controlled by Bruker O-TOF control ver.4.0 with chromatographic separation done on an Agilent Infinity 1260 HPLC. The HPLC measurements were conducted on a Agilent Zorbax SB-C18 column (3.5 µm, 4.6×150 mm), with a constant flow rate of 0.5 ml  $min^{-1}$  at 35 °C, a binary solvent system of H<sub>2</sub>O+0.1% formic acid (A)/ MeCN+0.1% formic acid (B), and a linear gradient (%B): 0', 5%; 2', 5%; 10', 20%; 30', 40%; 36', 50%, 38', 95%; 47', 95%; 49', 5%; 50', 5%. The electron spray ionization (ESI) source parameters were as follows: negative polarity, a mass range of m/z 50–1300, end plate offset of 500 V, capillary voltage of 4500 V, nebulizer pressure at 1.8 bar, dry gas flow of 9.0 l min<sup>-1</sup>, and a dry gas temperature of 220 °C. For small molecule MS/MS measurements with negative ionization, the standard settings provided by Bruker were used as follows: auto MS/MS, a mass range of m/z 20–40 000 with three precursors. The high-resolution LC-MS raw spectra were first centroided by converting them to mzXML format using the MS Convert feature of ProteoWizard 3.0.18324. Data processing was subsequently carried out with R Studio v1.1.463 using the Bioconductor XCMS package v 3.4.2 (Smith et al., 2006; Tautenhahn et al., 2008; Benton et al., 2010), which contains algorithms for peak detection, peak deconvolution, peak alignment, and gap filling. The resulting peak list was uploaded into Metaboanalyst 4.0 (Chong et al., 2018), a web-based tool for metabolomics data processing, statistical analysis, and functional interpretation where statistical analysis and modeling were performed. Missing values were replaced using a (K-nearest neighbor) KNN missing value estimation. Data filtering was implemented by detecting and removing non-informative variables that are characterized by near-constant values throughout the experimental conditions by comparing their robust estimate interquartile ranges (IQRs). Data were autoscaled. Out of the 3020 mass features originally detected, 2991 were used for the principal least square discriminant analysis (PLS-DA).

For the identification of candidate metabolites, the individual mass features that contributed to the separation between the different classes were further characterized by applying a range of univariate and multivariate statistical tests to determine their importance including the Pls-da importance variables, *t*-test, and random forest. The presence of each feature of interest was confirmed in the raw data and the molecular formula was estimated using Bruker Compass Data Analysis 5.1. This information, along with the retention time, accurate mass, and MS/MS spectra were used to probe into the existing literature and databases. MS/MS spectra files were also centroided and imported into GNPS (Wang et al., 2016) for spectral matches and classical molecular networking. The dataset is available in the Dryad Digital Repository at doi:10.5061/dryad.7m0cfxpqv.

### Bioassay on the effect of the methanolic extracts of LS and HS aenotypes on the growth of H. fraxineus

The experiment was designed so that the methanolic extract from 15 g of fresh bark would be added to 15 ml of ash dust agar (ADA) medium to ensure that the fungus would grow in the presence of total metabolite concentrations equivalent to physiological bark concentrations. For the preparation of the extract, fresh shoots were collected from grafted plants of five HS genotypes (01B, 31A, 46D, 48C, and 56C) and five LS genotypes (37A, 39A, 39E, 45C, and 47A) from the same plants that were used for the metabolomics analysis. The selection of the genotypes was done based on material availability and a broad representation of the HS and LS groups in Fig. 1b. Current year shoots were harvested in July and brought into the lab, where an even mix of bark from each of the HS and LS genotypes was prepared by stripping off equal amounts of internodal sections manually; 15 g were weighed for each genotype group. The bark samples were then homogenized with liquid nitrogen using a porcelain mortar and a pestle. Ground samples were freeze-dried and then extracted by adding 20 ml of MeOH and shaking in a vortex for 30 min. The extract was then centrifuged for 15 min and the clear supernatant was decanted into a new 50 ml Falcon tube. The MeOH in the supernatant was then evaporated by applying nitrogen and, once evaporated, the extract was rediluted in 600 µl of MeOH.

Petri dishes (Ø 65mm) were filled with 5 ml of ADA (Botella et al., 2016) each and were marked on the bottom with two perpendicular lines crossing their center, forming four axes that would aid colony growth measurement. Three Petri dishes were used for each treatment. The treatments were: control ADA, ADA supplemented with 200 µl (4%) of MeOH, ADA supplemented with 200 µl of the methanolic extract of HS genotypes, and ADA supplemented with 200 µl of the methanolic extract of LS genotypes. In each case, MeOH and methanolic extracts were dispensed carefully in small drops around the edge of the Petri dish. Once prepared, Petri dishes were allowed to dry for 30 min to let the MeOH evaporate. Petiole sections of 5 mm were co-cultured and colonized by the H. fraxineus isolate Erk2D (Bengtsson et al., 2012), which was obtained from Dr Malin Elfstrand (Swedish University of Agricultural Sciences, Sweden). Colonized petiole sections were placed in the center of the dishes at the point where the two drawn lines intersected. Petri dishes were kept at room temperature in darkness over a period of 15 d, and a mark was made at the edge of the colonies on each of the four axes of the drawn lines. For each Petri dish, the mean value for each of the four axes was annotated, then the colony area was estimated using the formula  $\pi \times r^2$ . Colony area data were exported to Minitab17 (www.minitab.com), and ANOVA tests were performed to compare the mean colony area for each of the treatments and isolates.

#### Fungistatic effect of coumarins on H. fraxineus

Physiological concentrations of fraxetin and esculetin in extracts from LS and HS *F* excelsior genotypes were estimated. For the estimation, the molar concentration of the coumarins was calculated based on the integral ratio between the sample (100  $\mu$ l of bark extract from HS and LS genotypes) and the reference (1.30 mg of *p*-coumaric acid in 100  $\mu$ l of MeOH). The molar concentration was multiplied by the molar masses of fraxetin (208.169 g mol<sup>-1</sup>) and esculetin (178.14 g mol<sup>-1</sup>). The amount of esculetin was in the range of 39.6–67.0  $\mu$ g in HS samples and 92.4–211.9  $\mu$ g in LS samples. The amount of fraxetin was in the range of 43.0–73.0  $\mu$ g in HS samples and 100.6–230.6  $\mu$ g in LS samples. The average amounts of coumarins in the bark extracts were used to estimate the physiological concentration in fresh bark considering that 50 mg of dry



**Fig. 1.** Overview of the metabolomics comparison in methanolic bark extracts from *F. excelsior* genotypes with different susceptibilities to ash dieback. Two sets of samples were used, *F. excelsior* low susceptibility group (LS) (*n*=19), marked in blue, and *F. excelsior* high susceptibility group of (HS) (*n*=19), marked in red. (a) Representation of the results of the PCA analysis; (b) dendrogram representation of the comparison of the two groups (LS and S); (c) heatmap representing the relative abundance (log2) of 2982 mass features in each of the samples.

bark were extracted with 1500  $\mu l$  of MeOH and that the average water content of fresh ash bark is 42%:

$$\frac{\text{Coumarin } \mu \text{ gin } 100 \ \mu \text{ L}}{100 \ \mu \text{ L}} * \frac{1500}{50 \text{ mg}} * \frac{1000 \text{ mg}}{1 \text{ g}} * \frac{1 \text{ g fresh bark}}{(1 - 0.42) \text{ g dry bark}}$$
  
= Coumarin  $\mu$  gperg of fresh bark

The estimated physiological bark concentrations for esculetin were 53.3  $\mu$ g g<sup>-1</sup> and 152.2  $\mu$ g g<sup>-1</sup> for HS and LS genotypes, respectively. The estimated physiological bark concentrations for fraxetin were 58  $\mu$ g g<sup>-1</sup> and 165.6  $\mu$ g g<sup>-1</sup> for HS and LS genotypes, respectively. Three isolates of *H. fraxineus* (Erk2D, Ö3, and 21C) (Bengtsson *et al.*, 2012) were obtained from Dr Malin Elfstrand (Swedish University of Agricultural Sciences, Sweden) and subcultured in ADA (Botella *et al.*, 2016). Aliquots of 12 ml

of ADA were measured in a 15 ml Falcon tube, where ADA was supplemented with 100  $\mu$ l of DMSO (ADA control) or the corresponding LS and HS physiological concentrations of fraxetin (Sigma-Aldrich, 18224-50MG) and esculetin (Sigma-Aldrich, 246573-1G), which were pre-diluted in 100  $\mu$ l of DMSO. The final DMSO concentration in all media was kept at 0.0083% in order to avoid toxicity to the isolates. Falcon tubes were inverted gently several times to mix the contents and were then poured into Ø 65 mm Petri dishes, which had been previously marked in the bottom with two perpendicular lines crossing their center, forming four axes that would aid colony growth measurement. In each case, a 5 mm petiole piece colonized by the corresponding *H. fraxineus* isolate (see method above) was placed in the center of each of the Petri dishes at the point where the two drawn lines intersected. Three technical replicates per treatment and per isolate were made. Petri dishes were kept at room temperature in darkness over a period of 15 d and a mark was drawn at the edge of the colonies on each of the four axes of the drawn lines. For each Petri dish, the mean value for each of the four axes was annotated, then the colony area was estimated using the formula  $\pi \times r^2$ . Colony area data were exported to Minitab17 (www.minitab.com), and ANOVA tests were performed to compare the mean colony area for each of the treatments and isolates.

#### Results

#### HS and LS F. excelsior genotypes represent two different F. excelsior chemotypes

Statistical analysis of the metabolite profiles revealed two separate groups for *F. excelsior* corresponding to genotypes with a low susceptibility (LS) and those with a high susceptibility (HS) to ADB (Fig. 1). The PLS-DA revealed that PC1 accounted for 40.4% and PC2 accounted for 17.5% of the variance and contributed to the best separation of the groups. A 2D score plot was constructed and 95% confidence regions for each group marked, allowing clear discrimination of HS and LS genotypes based on their metabolic profiles. The two sample classes clustered separately and the HS group showed a higher variance (Fig. 1a). Furthermore, a dendrogram of the metabolic profiles of the analyzed genotypes showed two clearly separated subclusters corresponding to their level of susceptibility and not to their geographic origin, which is represented by the two first digits in each sample name (Fig. 1b). A heatmap representation of the relative levels for 2991 mass features for all the samples further supported a clear difference between HS and LS genotypes (Fig. 1c). Sections I and II of the map include mass features that are generally more abundant in either the HS or LS groups, respectively, while section III includes mass features with no association with either of the two groups.

# Candidate metabolites associated with HS or LS to H. fraxineus

Out of 2991 mass features which were selected after filtering and quality control, a total of 64 candidate metabolites were identified as being associated with either HS or LS genotypes as a result of multiple statistical tests. Twentyfive of the candidates were more abundant in HS genotypes and 39 were more abundant in LS genotypes. For 53 of the candidates, a compound identity was annotated with confidence based on their accurate mass, MS/MS fragmentation matched with databases, and UV spectra, while 11 candidates were not identified. MS/MS fragmentation patterns of these candidates can be found in Supplementary Fig. S1. Based on their chemical family, 21 secoiridoids, eight coumarins, six lignans, six phenylethanoids, five flavonoids, four cinnamic acid esters, two monolignols, one shikimate derivative, and citric acid were positively identified (Table 1). From these, nine metabolites had been reported previously as important chemical defenses in ash (Cleary et al., 2014; Sambles et al., 2017; Sollars et al., 2017). For each chemical family, details of the levels of the annotated metabolites in the HS and LS groups are presented in Supplementary Fig.

S2. In addition, a detailed heatmap representing the levels of the 64 candidate metabolites in all the genotypes in the study can be found in Supplementary Fig. S3.

# Fungistatic activity of the methanolic extract from bark samples taken from HS and LS genotypes

Our results show that, in terms of fungal growth, the largest colony area after 15 d was observed on control ADA medium (Fig. 2). The addition of 4% MeOH, even after letting it air-dry for 30 min, had a penalty on the growth of *H. fraxineus*. The addition of the methanolic extract from HS genotypes (in 4% MeOH) resulted in a similar colony area to the addition of 4% MeOH alone. The addition of the methanolic extract from LS genotypes (in 4% MeOH) completely inhibited the growth of *H. fraxineus* after 15 d, even though the fungus remained alive at the top of the inoculation plugs (Supplementary Fig. S4). A brownish coloration appeared in the medium for the Petri dishes that were supplemented with a bark methanolic extract. Supplementary Fig. S4 shows the Petri dishes at the end of the experiment (15 dpi).

# Fungistatic activity of physiological concentrations of fraxetin and esculetin on H. fraxineus

Among the 39 candidate metabolites that were identified associated with LS genotypes, the two coumarins fraxetin and esculetin ranked first and fifth in terms of coefficient score (Table 1). Due to the high relevance and commercial availability of these coumarins, they were selected for a bioassay to determine their effects at physiological concentrations on three isolates of *H. fraxineus*.

A significant reduction in the growth of three H. fraxineus isolates (Erk2D, 21c, and Ö3) was observed after supplementing ADA medium with either fraxetin or esculetin. After 15 d, the colony area for all three fungal isolates was significantly reduced by the addition of the coumarins to the medium, depending on concentration (Table 2). For all three isolates, coumarin concentrations corresponding to the level found in LS genotypes (EsLS and FrLS) significantly inhibited fungal growth. At the physiological concentration of fraxetin in HS genotypes (FrHS), fungal growth inhibition was significant for isolate Erk2D and nearly significant for the two fungal isolates 21c and O3 (Table 2). For isolates 21c and Ö3, esculetin showed a stronger fungistatic effect compared with fraxetin even though it showed a lower coefficient mean score in our results. Isolate 21c was the least affected by the addition of the coumarins. For isolates Erk2D and O3, addition of esculetin at the physiological concentration for LS genotypes (EsLS) significantly reduced the colony area compared with the physiological concentration for HS genotypes (EsHS). For fraxetin, this was only observed in isolate Erk2D. In all cases, the addition of coumarins to the medium caused the appearance of a brown coloration.

 Table 1. List of 64 candidate metabolites from bark extracts associated with LS and HS phenotypes of F. excelsior

Frame         Counterins         207.000         10.70         97.00         2.02-16         LS           Control 10         Boorloods         895.22         10.54         995.2         2.40E-15         LS           Unrown         Control 10         277.142         42.29         91.88         15.92E-11         LS           Exclusion         -         563.187         253.18         88.34         15.92E-11         LS           Exclusion         -         563.177.18         14.47         89.14         1.07E-10         LS           Unrown         -         667.171.48         14.14         88.18         3.09E-10         LS           Unrown         -         405.179         17.76         17.48         81.81         83.17-10         LS           Syntyments to thisrolglocolicity effect         Lipronic         11.32         81.48         81.98         6.02E-10         LS           Syntyments to thisrolglocolicity effect         Lipronic         11.47         81.27         82.66         LS           Appath/biologic D         Courrains         92.10         12.22         10.26         12.42         12.16         LS           Appath/biologic D         Courrains         92.10	Compound identity	Biochemical class	[M-H] <sup>-</sup> m/z	RT (min)	Coefficient mean score	P-value from t-test	More abundant in group
Example BSequenciation685.22715.4460.622.41.5LSUninovan C28164013 hoxosite-53.18725.3180.6453.11LSUninovan C28164013 hoxosite-53.18725.3180.6453.161LSUninovan-837.16445.9180.1440.77-10LSUninovanSencification13.32416.4480.663.31-11LSCalcostanciads BPernyethranicia477.13817.7580.414.61.64LSCalcostanciads BSencification90.94492.8380.896.00-00LSUninovan C2111000-40.31.6413.0282.450.4460.00LSUninovan C3110000Sencification40.31.4614.9282.450.4460.00LSUninovan homosciteUninovan homoscite-40.31.6614.7776.001.52Pacification BSencification50.719712.0274.037.16.03LSAnguettification BSencification50.719712.027.43.071.52CSPacification BSencification50.719712.027.43.071.52CSPacification BSencification50.719712.027.43.071.52CSPacification BSencification50.719712.027.43.07LSSPacification BSencification50.71977.160.17.107LSSPacification BSencification50.7197 <t< td=""><td>Fraxetin</td><td>Coumarins</td><td>207.029</td><td>15.75</td><td>97.76</td><td>2.25E-16</td><td>LS</td></t<>	Fraxetin	Coumarins	207.029	15.75	97.76	2.25E-16	LS
Ukingma-277.14242.3983.841.531.53BacularinaCournarina177.01814.4783.141.531.53BacularinaCournarina177.01817.708.1.413.00E.10L5UhingmaSenairidacia1133.30418.0.48.6.633.00E.10L5UhingmaParabara177.01817.708.1.144.494E.10L5UhingmaCalcalarinacia193.30418.0.48.6.633.00E.10L5UhingmaCalcalarinacia193.30418.0.48.6.633.00E.10L5UhingmaCalcalarinacia19.7.1817.7.18 <td< td=""><td>Excelside B</td><td>Secoiridoids</td><td>685.227</td><td>18.54</td><td>96.52</td><td>2.49E-15</td><td>LS</td></td<>	Excelside B	Secoiridoids	685.227	18.54	96.52	2.49E-15	LS
Uhencom-52.8.1723.3188.845.8.8.141.5.8Uhencom-37.16441.4786.141.076-101.5UhencomScontinkin11.3.2411.6486.643.3.16-101.5Calendarinscin RPhanylernancias477.13617.758.6.146.18.641.5Calendarinscin RScontinkin10.9.278.6.136.18.8-101.5UhencomScontinkin909.29427.838.5.936.069-101.5Syngarenan Inter-ofiguccatis-resistedUpramo7.7001.148.3.649.466-001.5Cance ador -keitodScontinkin909.29427.838.5.934.066-001.5Cance ador -keitodUpramo7.192.071.8.161.8.11.8.1ScontinkinScontinkin8.1489.199.2.459.46E-001.5Anguetificioxida DCancervira833.0971.4.177.9.22.9.161.5ScontinkinScontinkin830.0971.4.177.19.071.51.51.5ScontinkinScontinkin830.0971.4.177.19.071.51.51.5ScontinkinUpramo857.1031.1.677.19.071.51.51.5ScontinkinScontinkin830.091.0.177.19.071.51.51.5ScontinkinScontinkin830.091.0.171.0.161.51.51.5ScontinkinScontinkin830.091.0.17 <td>Unknown</td> <td>-</td> <td>277.142</td> <td>42.29</td> <td>93.86</td> <td>1.69E-13</td> <td>LS</td>	Unknown	-	277.142	42.29	93.86	1.69E-13	LS
EardetinCoursin's177.01114.4718.1410.17.10U.B.Uhrown-307.1544.3086.4430.06.10LSUhrownSecondrictis1133.24418.648.6330.151.01LSCalcocatorotof RPreyntemonits47.17317.768.64.14.34.67.10LSUhrownSecondrictis90.9442.288.56.86.096.10LSSyntagreesino betsdiglucoside-relatedUpront787.26015.148.36.54.096.20LSCanin acid-heitandCauraran221.14411.3224.460.406.06LSArguathiotastis ASecondrictis701.22218.028.14.42.366.66LSArguathiotastis ASecondrictis833.09714.477.8.262.166.06LSArguathiotastis ASecondrictis57.13017.017.01.56.167.00LSArguathiotastis ASecondrictis57.13012.207.4937.396.07LSCoosed matryl ester-related C23+B6014Excendrictis57.13017.017.0.156.167.00LSCoosed matryl ester-related C23+B6014Excendrictis57.13017.207.345.00LSCoosed matryl ester-related C34-B6014Excendrictis57.13017.407.1356.130Coosed matryl ester-related C34-B6014Excendrictis57.13017.4018.242.786.05LSCoosed matryl ester-related C34-B6014Excendrictis57.13017.401	Unknown C26H34O13 hexoside	-	553.187	25.31	88.84	5.53E-11	LS
Uhrown-387, 16443.9165.943.3615.UhrownSecondocids13.2412.6485.653.315.1015.Calcaclaroscie BPheryleinancias477, 13617.758.61.36.185.1015.Laptosocid-validoSecondocids909.29427.1858.59.96.98.94.98.1015.Laptosocid-validoJuncova77.2071.1413.054.006.1015.Quinte acid-valideJuncova12.04413.1212.449.469.46615.Quinte acid-valideSecondocids88.10711.2212.429.469.46615.Anguatiloloxida ASecondocids88.10711.2212.429.469.46615.Anguatiloloxida BSecondocids88.10711.207.43.27.196-0715.Singanena bene-splucasiteLiprone679.10812.057.43.27.196-0715.Singanena bene-splucasiteLiprone851.28117.1610.176.015.15.16Singanena bene-splucasiteLiprone851.28114.4467.3311.276.015.Indexiste BSinganena bene-splucasiteLiprone851.28114.4467.3311.276.015.Indexiste BSinganena bene-splucasiteLiprone851.28114.4467.3311.276.015.Indexiste BSinganena bene-splucasiteLiprone851.28114.4467.3311.276.015.Indexiste BS	Esculetin	Coumarins	177.018	14.47	88.14	1.07E-10	LS
Uhenxom         Securitorish         1133.244         18.64         68.64         3.31F-10         I.S.           Calcolationalda B         Provybanouda         477.136         17.75         86.13         6.13E-10         I.S.           Uhenxom C21+1000         —         405.030         4.27         86.13         6.015E-10         I.S.           Synapsenio bata - diglucoside rolatod         Uprane         77.220         15.14         83.65         4.09E-06         I.S.           Synapsenio bata - diglucoside rolatod         Uprane         77.220         16.47         83.24         9.46E-06         I.S.           Synapsenio bata - diglucoside rolatod         Secoireccids         701.222         18.02         82.45         9.46E-06         I.S.           Manasotide         Secoireccids         701.222         18.07         0.85E-06         I.S.           Finado in housade         Counarina         383.007         14.47         78.25         1.05E-07         I.S.           Finado in housade         Lignans         597.32         19.09         7.15E-07         I.S.           Onceade manthy ester-relator discoundo         Uprans         697.302         19.09         7.35E-06         I.S.           Stondamanthy ester-relator discoundo </td <td>Unknown</td> <td>-</td> <td>387.154</td> <td>43.91</td> <td>86.94</td> <td>3.09E-10</td> <td>LS</td>	Unknown	-	387.154	43.91	86.94	3.09E-10	LS
Calcadardosina B         Phonyathanolas         477, 18         17, 75         85,41         4,845-10         LS           Lightonade-valuad         Secoliciadis         003,024         27,83         85,98         6,076         LS           Syringaresinol betro-reglucoside-related         Lightonade         787,260         15,14         83,65         4,045,108         LS           Syringaresinol betro-reglucoside-related         Sibilinates         463,146         8,19         82,44         9,285-08         LS           Colicie acd-related         Columins         221,144         13,122         124,42         2,111-06         LS           Facial Intercation         Columins         83,077         13,47         78,26         1,205-07         LS           Anguithfoliciasis B         Columins         83,037         11,47         78,26         1,207-07         LS           Syringaresinol bate-reglucositis         Lignaris         57,122         10,09         7,126         1,207-07         LS           Syringaresinol bace-reglucositis         Lignaris         57,222         10,09         7,265         2,105-06         LS           Syringaresinol bace-reglucositis         Lignaris         57,222         10,09         2,00         0,005	Unknown	Secoiridoids	1133.324	18.64	86.86	3.31E-10	LS
Unknown (21111009)         -         405.029         4.27         86.13         6.13E-10         LS           Syringaresinol bota - diglucoside related         Lignanse         707.220         15.14         83.65         4.00E-03         LS           Syringaresinol bota - diglucoside related         Sinikimates         403.146         9.19         82.48         9.28E-09         LS           Serindroll         Councrins         22.104         15.82         22.45         9.46E-09         LS           Angustrificiaride A         Serindroll         701.222         18.07         9.32E-08         LS           Angustrificiaride A         Serindrolls         652.23         18.16         7.639         2.5E-07         LS           Angustrificiaride B         Serindrolds         507.19         12.20         7.43.3         7.19E-07         LS           Syringaresinol back of glucoside         Lignans         579.202         10.90         7.25         2.10E-08         LS           Serindrolls         Serindrolds         S89.109         9.46         68.95         9.98E-06         LS           Serindrolds         S89.109         9.46         66.81         2.28E-06         LS           Unknown C17P-28011         -         <	Calceolarioside B	Phenylethanoids	477.136	17.75	86.41	4.84E-10	LS
LightenderSecuritdods90.2.8427.8385.980.007-101.5Ounine acid-relatedDifferenter473.1401.5182.480.4287-081.5SecuritdodsCommarine221.0441.5182.480.4287-081.5Anguatifolicaida ASecuritdods701.22218.0281.242.11E.081.5Anguatifolicaida ASecuritdods701.22218.0281.242.11E.081.5Anguatifolicaida ASecuritdods657.1891.2.077.4361.2.07-071.5Cleacide methy ester-related C23+080016Securitdods657.1891.2.077.4952.1.0E-071.5Syntegramine hata-regucasitaLiparas579.30219.397.2.652.1.0E-061.5EtacksamplerideGamaria579.30219.399.3259.4646.3021.5EtacksamplerideLiparas69.1381.4.4467.391.822.051.5Inderson di diglucosideLiparas69.3099.3029.622.788-061.5Instancind AccassidaSecuritdods95.3881.4.4467.391.822.051.5Instancind AccassidaSecuritdods95.399.3642.2.788-061.5Instancind AccassidaSecuritdods95.391.3.240.00761.5Instancind AccassidaSecuritdods95.3971.7.334.6.350.00761.5Instancind AccassidaSecuritdods93.1711.7.334.6.350.0076<	Unknown C21H10O9	-	405.029	4.27	86.13	6.13E-10	LS
Syringssein bata         Lignans         772,00         15.14         82.85         4.00E.00         LS           lenhacid-wested         Cournearins         473.146         13.82         82.45         9.48E-09         LS           lenhacid-wested         Secondoids         701.22         18.02         81.24         2.11E-08         LS           Argustfiloissie B         Secondoids         662.28         1.447         73.26         1.26E-07         LS           Argustfiloissie B         Cournarins         630.097         1.4.47         73.28         7.19E-07         LS           Argustfiloissie B         Cournarins         673.202         7.4.93         7.19E-07         LS           Syringession beta-e-glucositie         Lignans         675.188         17.01         70.15         6.17E-06         LS           Elockampferio         Elgnans         665.188         17.41         7.015         6.17E-06         LS           Elockampferio         Elgnans         661.285         1.444         67.39         1.82E-05         LS           Elockampferio         Secoindoids         89.128         1.8.40         66.81         9.98E-06         LS           Elockampferio         Clucoside         Secoindoids </td <td>Ligstroside-related</td> <td>Secoiridoids</td> <td>909.294</td> <td>27.83</td> <td>85.98</td> <td>6.90E-10</td> <td>LS</td>	Ligstroside-related	Secoiridoids	909.294	27.83	85.98	6.90E-10	LS
Qainic addix valued         Shiemates         483.146         9.18         92.463         9.286.09         1.5           Angusithiciscile A         Secoindoids         701.222         18.02         81.24         2.116.48         1.5           Angusithiciscile A         Coumarins         383.097         14.47         78.26         1.206-07         1.5           Angusithiciscile C         Coumarins         383.097         14.47         78.26         1.206-07         1.5           Closadia mutuy leater-roluted C29.19200         Sociridoids         657.180         1.20         77.39         7.112-07         1.5           Syringareshol bataglucositie         Lignans         679.02         1.9.09         72.85         2.106.08         1.5           Sociedondry for diguositie         Lignans         658.188         17.011         7.015         61.70-06         1.5           Closadia         Bayonods         290.05         8.3.2         69.70         9.996.06         1.5           Interacted 21.294011         -         407.15         10.44         86.30         9.906.06         1.5           Sociridoids         59.108         9.92         65.22         9.706.5         1.5           Forulacaid         Maronids<	Syringaresinol beta-D-diglucoside-related	Lignans	787.260	15.14	83.65	4.09E-09	LS
Isofraxian         Coursains         221 044         18.62         82.45         9.48E-05         LS           Angustilolisatio A         Secolididids         701 222         18.102         81.24         2.11E 6.09         LS           Fraxid Interoscile         Coursarins         38.077         1.4.47         75.26         1.20E-07         LS           Angustilolisatile B         Secolididids         657.189         12.20         7.4.83         7.19E-07         LS           Syngaresino Detacolide         Secolididids         57.198         12.20         7.4.83         7.19E-07         LS           Syngaresino Detacolide         Lignans         57.302         10.90         7.265         L210E-06         LS           Isokaempferide         Cocolidio         Sasocilidioids         9.33.22         68.05         9.99E-06         LS           Insultaroscide S (6 <sup>-1</sup> -dijuccoside         Lignans         681.25         14.44         67.39         1.82E-05         LS           Insultaroscide S (6 <sup>-1</sup> -dijuccoside         Secolididids         913.24         62.52         9.87E-05         LS           Insultaroscide S (6 <sup>-1</sup> -dijuccoside         Secolididids         53.177         12.78         43.03         0.0016         LS	Quinic acid-related	Shikimates	463.146	9.19	82.48	9.28E-09	LS
Angustificiasión A         Secciridoids         70.222         10.47         81.24         2.11E 0.68         LS           Unknown hexoside         -         489.159         14.47         78.26         1.28E-07         LS           Angustificiaside B         Secciridoids         682.28         18.16         70.99         2.51E-07         LS           Syningersinol beta-r-glucoside         Ugnans         573.022         19.09         72.65         2.10E-06         LS           Traixaisnin flowsoide         Ugnans         573.022         19.09         72.65         2.10E-06         LS           Traixaisnin flowsoide         Ugnans         563.188         17.01         7.015         6.17E-06         LS           Casadia         Sacciridoids         389.100         9.08         69.05         LS           Promesind diglucoside         Lignans         691.25         14.44         67.39         182E-05         LS           Unknown C17/128011         -         -         407.155         10.44         63.20         0.0016         LS           Secciridoids         Sacciridoids         59.128         18.40         64.21         0.0016         LS           Unknown C17/128001         Sacciridoids	Isofraxidin	Coumarins	221.044	13.82	82.45	9.46E-09	LS
ukunown haonaida         -         48.150         14.47         80.27         3.85.08         LS           Fraxdin hexoside         Goumarins         383.097         14.47         78.26         1.28E-07         LS           Angustifoliacide B         Secorididids         665.283         18.16         76.99         2.51E-07         LS           Orecaske methyl ester-neisele C23480.016         Secorididids         665.188         17.01         74.83         7.19E-07         LS           Syngaresinol bexoside         Liprans         57.202         19.09         72.65         2.10E-06         LS           Stokempferide         Heynorodds         299.053         33.22         69.72         7.34E-06         LS           Onceaside         Liprans         681.255         14.44         67.39         1.82E-05         LS           Insultaroadida (31/1-128011         -         407.155         10.44         66.31         2.24E-05         LS           Insultaroadida (31/1-128011         -         -         407.155         10.44         67.39         0.3016         LS           Insultaroadida (31/1-128011         -         -         407.158         10.44         63.70         0.60016         LS	Angustifolioside A	Secoiridoids	701.222	18.02	81.24	2.11E-08	LS
Fraxilin henoxidie         Cournames         33.007         1.4.7         7.8.2         1.2.6.7         L.S           Angustiolisside B         Seconidoids         685.28         18.16         76.99         2.51E-07         L.S           Syningaresinol bataglucoside         Lipans         570.202         19.00         72.453         2.10E-06         L.S           Exvicesinol hoscolide         Lipans         565.188         17.01         70.15         6.17E-06         L.S           Exvicesinol hoscolide         Lipans         681.235         14.44         68.35         9.99E-06         L.S           Decaide         Seconidoids         39.010         9.06         68.35         9.99E-06         L.S           Innularosids 37.47         diptoresinol         681.235         14.44         66.34         2.20E-05         L.S           Reamplerol-3-Oglucoside         Cimemic acid         193.049         23.05         63.70         6.70E-05         L.S           Naringerin         Seconidoids         23.017         19.73         43.40         0.0016         L.S           Calcolarioside         Accolidids         23.176         25.44         45.20         0.0076         L.S           Calcolarioside <t< td=""><td>Unknown hexoside</td><td>_</td><td>489.159</td><td>14.97</td><td>80.27</td><td>3.85E-08</td><td>LS</td></t<>	Unknown hexoside	_	489.159	14.97	80.27	3.85E-08	LS
Angustfoliciside B         Secontribulics         685 228         18.16         76.90         2.51.07         LS           Discide methyl ester-nelated C23H3BO16         Secontribulics         577.120         19.09         72.65         2.10E-06         LS           Frakteenol hexocide         Lignans         579.202         19.09         72.65         2.10E-06         LS           Exokampfairloi         Lignans         567.188         17.01         70.15         6.17.7e-06         LS           Disordin digucoside         Lignans         681.235         14.44         67.39         1.82E-05         LS           Innutarosida 3.6 <sup>+,*</sup> -diglucoside         Secontribulics         91.09         9.06         68.24         2.27E-05         LS           Inautarosida 3.6 <sup>+,*</sup> -diglucoside         Crimemic acid         193.049         23.05         63.70         6.70E-05         LS           Inautarosida 3.6 <sup>+,*</sup> -diglucoside         Crimemic acid         193.049         19.32         64.20         0.0016         LS           Secontridoids         Se33.171         19.79         64.30         0.0016         LS           Inautaroside A         Provenolds         52.3176         25.64         45.20         0.00076         LS	Fraxidin hexoside	Coumarins	383.097	14.47	78.26	1.26E-07	LS
Decisional methyl ester-related C28H86O16         Secoindolds         SF7 H20         74.03         7.10E-07         LS           Syninganesnio blataglucoside         Lignans         S79.202         7.463         2.10E-06         LS           Eaxlesnion brataglucoside         Lignans         856.188         17.01         70.15         6.17E-06         LS           Elsokampfarida         Resconidolds         389.010         9.06         68.95         9.09E-06         LS           Obeside         Lignans         681.235         14.44         67.39         1.82E-05         LS           Unknown C17H2SD11         -         407.155         10.64         66.81         2.26E-05         LS           Keempferol-3-Cglucoside         Elgranoids         951.288         18.40         66.24         2.76E-05         LS           Neindreprin         Elevanoids         477.089         19.32         62.52         9.87E-05         LS           Oleuropein         Secoindoids         271.058         30.64         52.49         0.0016         LS           Ligstroside         Secoindoids         523.176         25.64         45.20         0.0076         LS           Ligstroside         Secoindoids         55.166	Anaustifolioside B	Secoiridoids	685.228	18.16	76.99	2.51E-07	LS
Syringaresinon beta-glucoside         Lignans         579.202         19.09         72.65         2.10E-06         LS           Eraximesino hexceside         Lignans         565.188         17.01         70.15         6.17E-00         LS           Elosteamplante         Filevonoids         299.053         33.32         09.72         7.34E-00         LS           Dinoscind diplucoside         Lignans         681.255         14.44         67.39         1.98E-055         LS           Invaluencidis 3, 67, 4iglucoside         Secoindiolds         99.1288         18.40         66.44         2.78E-05         LS           Invaluencidis 3, 67, 4iglucoside         Secoindiolds         599.171         19.78         64.30         0.0016         LS           Vernopein         Cinumitica acid         193.049         23.05         6.37         0.0016         LS           Vernopein         Feavancids         579.176         2.564         45.20         0.0016         LS           Singlastroaide         A Secoindiodis         523.177         2.476         44.17         0.00265         LS           Upstroside         Secoindiodis         553.177         2.564         4.52.9         0.0076         LS           Ligstroside </td <td>Oleoside methyl ester-related C23H36O16</td> <td>Secoiridoids</td> <td>567.189</td> <td>12.20</td> <td>74.93</td> <td>7.19E-07</td> <td>LS</td>	Oleoside methyl ester-related C23H36O16	Secoiridoids	567.189	12.20	74.93	7.19E-07	LS
Fraktesinch hexoside         Lignans         665.188         17.01         70.15         6.17E-06         LS           lsokeempleride         Flavonidis         299.053         33.32         09.72         7.34E-06         LS           Clocaide         Secolridoids         380.109         9.96         68.95         9.99E-06         LS           Pinoresind diglucoside         Lignans         681.235         14.44         67.39         1.82E-05         LS           Inknown C17H28D11         -         407.1528         18.40         66.24         2.78E-05         LS           Stampferol-2-O-glucoside         Flavanoids         937.128         18.40         66.24         2.78E-05         LS           Naringenin         Secoiridoids         539.171         19.79         54.30         0.0010         LS           Calceolarioside A         Pherylethanoids         271.058         30.64         63.50         0.0016         LS           Lasingistroside         Secoiridoids         523.176         25.64         45.20         0.0076         LS           Lasingistroside         Secoiridoids         523.177         24.76         44.17         0.0038         LS           Lositomethylester-related         Secoirid	Svringaresinol beta-p-glucoside	Lignans	579.202	19.09	72.65	2.10E-06	LS
Isokaempferide         Favonoids         299.053         33.32         69.72         7.34E-08         LS           Okoside         Secoritodids         389.109         9.96         68.95         9.99E-06         LS           Dinorestinol diglucoside         Lignans         681.235         14.44         67.39         1.82E-05         LS           Unknown C17H28O11         -         407.155         10.64         66.84         2.78E-05         LS           Evaluacide 37,6"-diglucoside         Sociridoids         931.28         18.40         66.24         2.78E-05         LS           Gluaropain         Sociridoids         531.71         19.79         54.30         0.0010         LS           Naringenin         Flavanoids         271.058         30.64         52.49         0.0016         LS           Calcaclarioside A         Secoiridoids         523.176         25.64         45.20         0.0076         LS           Ligstroside         Secoiridoids         523.176         27.54         41.36         0.0155         LS           Unknown         -         943.335         30.43         38.17         0.0265         LS           Unknown         -         943.035         30.43	Fraxiresinol hexoside	Lignans	565.188	17.01	70.15	6.17E-06	LS
Diseaside         Saccinidadids         389.109         9.96         88.95         9.98-06         LS           Pinnensind idglucoside         Lignans         681.235         14.44         67.39         1.22E-05         LS           Invitavion C17H2E011         -         407.155         10.64         66.81         2.28E-05         LS           Invitavioside 3', 6''-diglucoside         Saccinidadis         951.288         18.40         66.24         2.78E-05         LS           Keenpiter-3-C-glucoside         Fievanoids         447.089         19.32         62.52         9.87E-05         LS           Oleuropein         Saccinidadis         231.76         26.44         52.49         0.0016         LS           Calceolarioside A         Phenylethanoids         477.136         17.33         46.35         0.00016         LS           Ligstroside         Saccinidadis         523.176         25.64         45.20         0.0076         LS           Unknown         -         -         443.35         0.444         13.81         0.4040         LS           Oleoside methylester-related         Sacciridadis         852.27         12.53         24.68         0.1072         LS           Unknown <t< td=""><td>Isokaemoferide</td><td>Flavonoids</td><td>299.053</td><td>33.32</td><td>69.72</td><td>7.34E-06</td><td>15</td></t<>	Isokaemoferide	Flavonoids	299.053	33.32	69.72	7.34E-06	15
Pinoresinol diglucoside         Lignams         681.235         14.44         67.39         1.82E-05         LS           Unknown C17/128O11         -         407.155         10.64         66.81         2.26E-05         LS           Ferulic acid         Ginnamic acid         193.049         23.05         63.70         6.70E-05         LS           Ferulic acid         Cinnamic acid         193.049         23.05         63.70         6.70E-05         LS           Kaempferol-3-O-glucoside         Flavanoids         447.089         19.32         62.52         9.87E-05         LS           Naringenin         Ravanoids         271.058         30.64         52.49         0.0016         LS           Calcacolarioside A         Secoriticolids         523.176         24.76         44.17         0.0003         LS           Ligstroside         Secoriticolids         523.176         24.76         44.17         0.00061         LS           Ligstroside         Secoriticolids         952.817         12.63         38.17         0.2265         LS           Unknown         -         -         943.385         10.43         38.17         0.2265         LS           Oleoside methylester-related         Secor	Oleoside	Secoiridoids	389.109	9.96	68.95	9.99E-06	15
Uhrnown C17+28011         -         407.155         10.84         6.81         2.26E-05         LS           Insularoside 3', 6'-diglucoside         Secoirdoids         951.288         18.40         66.24         2.78E-05         LS           Forulia caid         193.049         23.05         63.70         6.70E-05         LS           Kaempferol-3-O-glucoside         Flavanoids         247.089         19.32         62.52         9.87E-05         LS           Naringenin         Flavanoids         271.058         30.64         52.49         0.0016         LS           Lagtoside         Secoirdoids         523.177         24.76         44.17         0.0093         LS           Io-HyhHildroxybeuropein         Secoirdoids         523.177         24.76         44.17         0.0285         LS           Uhrknown         -         943.335         30.43         38.17         0.0285         LS           Oleoside methylester-related         Secoirdoids         685.227         12.53         28.68         0.1017         LS           Uhrknown         -         943.35         9.071         13.75         0.4376         LS           Uhrknown         -         94.090         13.71         13.75<	Pinoresinol dialucoside	Lignans	681,235	14.44	67.39	1.82E-05	15
Insularoside 3',6''-díglucoside         Secolidoids         951.288         18.40         66.24         2.78E-05         LS           Ferulic acid         Cinnamic acid         193.049         23.05         63.70         6.70E-05         LS           Staemplerol-3-O-glucoside         Flavanoids         47.089         19.32         62.52         9.87E-05         LS           Oleuropein         Secolridoids         539.171         19.79         54.30         0.0010         LS           Naringenin         Flavanoids         277.1058         30.64         52.49         0.0076         LS           calceolar/osoide A         Phenylethanoids         477.138         17.33         46.35         0.00076         LS           calceolar/osoide         Secoiridoids         553.176         25.64         45.20         0.0076         LS           Ligstroside         Secoiridoids         55.186         17.74         41.43         0.0255         LS           Unknown         –         943.335         30.43         38.17         0.0265         LS           Unknown         –         943.335         30.43         38.17         0.285         LS           Oleoside-metated         Secoiridoids         65.227 <td>Unknown C17H28O11</td> <td>-</td> <td>407.155</td> <td>10.64</td> <td>66.81</td> <td>2.26E-05</td> <td>15</td>	Unknown C17H28O11	-	407.155	10.64	66.81	2.26E-05	15
Farulia acid         Cinnamica         Cinnamica <thcinnamica< th=""> <thcinnamica< th="">         &lt;</thcinnamica<></thcinnamica<>	Insularoside 3' 6''-dialucoside	Secoiridoids	951,288	18.40	66.24	2.78E-05	LS
Name         Environ         E	Ferulic acid	Cinnamic acid	193 049	23.05	63 70	6 70E-05	1.5
Nature of generation         Securit/dolds         Sign 71         19.79         54.30         0.0010         LS           Naringenin         Flavanolds         271.058         30.64         52.49         0.0016         LS           Calceolarioside A         Phenylethanoids         477.136         17.33         46.35         0.00016         LS           Ligstroside         Secoiridolds         523.176         25.64         45.20         0.00076         LS           Ligstroside         Secoiridolds         555.166         17.54         41.13         0.0265         LS           Unknown         -         943.335         30.43         38.17         0.0265         LS           Unknown         -         943.355         12.53         24.09         0.1772         LS           Inknown         Securit/olds         665.227         18.55         24.09         0.161         4	Kaempferol-3-0-alucoside	Flavanoids	447 089	19.32	62.52	9.87E-05	LS
Naringenin         Flavancids         271.058         30.84         52.49         0.0016         LS           Calcedarioside A         Phenylethanoids         477.136         17.33         46.35         0.0061         LS           Isoligstroside         Secoridoids         523.176         25.64         45.20         0.0076         LS           Ligstroside         Secoridoids         523.177         24.76         44.17         0.0033         LS           10-HyhHHdroxyoleuropein         Secoridoids         555.166         17.54         41.36         0.0155         LS           Oleoside related         Secoridoids         787.226         14.13         34.85         0.0440         LS           Oleoside related         Secoridoids         655.287         12.53         28.58         0.1017         LS           Nuzhenide         Secoridoids         655.287         13.75         0.4376         LS           Vedroxytycsol 1-0-glucoside         Phenylethanoids         315.108         9.77         12.81         0.4696         LS           Quercetin 3-0-rutinoside         Flavonoids         609.141         16.57         90.16         1.48E-111         HS           Couraryishilimin caid         Monolignols	Oleuropein	Secoiridoids	539,171	19.79	54.30	0.0010	1.5
Catagoal         Description         Description <thdescription< th=""> <thdescription< th=""> <t< td=""><td>Naringenin</td><td>Flavanoids</td><td>271.058</td><td>30.64</td><td>52.49</td><td>0.0016</td><td>15</td></t<></thdescription<></thdescription<>	Naringenin	Flavanoids	271.058	30.64	52.49	0.0016	15
Calculation         Interfactor	Calceolarioside A	Phenylethanoids	477 136	17.33	46.35	0.0061	LS
Longmodule         Second divids         Second divi	Isoliastroside	Secoiridoids	523 176	25.64	45.20	0.0076	LS
Light Statut         Decknade         Second data	Liastroside	Secoiridoids	523 177	24.76	44 17	0.0093	LS
Number of the second	10-HyhHHdroxyoleuropein	Secoiridoids	555 166	17.54	41.36	0.0155	LS
Onescient         Output         Outp	Linknown	_	943 335	30.43	38.17	0.0265	LS
Biologian         Biologian <t< td=""><td>Oleoside methylester-related</td><td>Secoiridoids</td><td>787 226</td><td>14 13</td><td>34 85</td><td>0.0440</td><td>LS</td></t<>	Oleoside methylester-related	Secoiridoids	787 226	14 13	34 85	0.0440	LS
Outcome (all of all o		Secoiridoids	955 287	12.53	28 58	0.1017	LS
National Columbria         Columation         Columation <th< td=""><td>Nuzbenide</td><td>Secoiridoids</td><td>685 227</td><td>18.95</td><td>24.09</td><td>0.1702</td><td>LS</td></th<>	Nuzbenide	Secoiridoids	685 227	18.95	24.09	0.1702	LS
Hydroxytyrosol 1-O-glucoside         Phenylethanoids         315.108         9.77         12.81         0.4896         LS           Quercetin 3-O-rutinoside         Flavonoids         609.141         16.57         90.16         1.46E-11         HS           5-O-Caffeoylshikimate         Coumarins         335.075         15.05         88.08         1.12E-10         HS           4-Coumaroylshikimic acid         Monolignols         319.080         17.43         85.21         1.27E-09         HS           Formate adduct of tyrosol glucoside         Phenylethanoids         345.118         11.39         81.73         1.53E-08         HS           Citric acid         Small organic acid         191.020         3.14         79.63         5.69E-06         HS           Soquercetin         Monolignols         353.087         10.88         71.43         3.59E-06         HS           Isoquercetin         Flavonoids         463.084         17.52         65.13         4.13E-05         HS           Verbascoside         Cinnamic acid esters         623.193         16.79         63.81         6.46E-05         HS           Isoacteoside         Cournarins         339.071         11.99         60.72         0.0002         HS	Frazin-related	Coumarins	911 240	13 71	13 75	0.4376	LS
Number of glasses       Flavnoids       609.141       16.57       90.16       1.46E-11       HS         Quercetin 3-O-rutinoside       Flavnoids       609.141       16.57       90.16       1.46E-11       HS         4-Coumarins       335.075       15.05       88.08       1.12E-10       HS         4-Coumarolyshikimite acid       Monolignols       319.080       17.43       85.21       1.27E-09       HS         Formate adduct of tyrosol glucoside       Phenylethanoids       345.118       11.39       81.73       1.53E-08       HS         Citric acid       Small organic acid       191.020       3.14       79.63       5.69E-08       HS         Isoquercetin       Flavonoids       463.084       17.52       65.13       4.13E-05       HS         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Isoquercetin       Coumarins       339.071       11.99       60.72       0.0002       HS         Piorossinol 4-O-beta-p-glucopyranosyle       Lignans       519.181       18.78       59.96       0.0002       HS         Isoacteoside       Secoiridoids       623.193       17.20       55.35       0.0008       HS	Hydroxytyrosol 1-0-alucoside	Phenylethanoids	315 108	9.77	12.81	0.4696	LS
Cadronal of a function of the second of the secon		Flavonoide	609 1/1	16.57	90.16	1.46E-11	LO HS
4-Coumaroylshikimic acid       Monolignols       319.080       17.43       85.21       1.227E-09       HS         Formate adduct of tyrosol glucoside       Phenylethanoids       345.118       11.39       81.73       1.53E-08       HS         Citric acid       Small organic acid       191.020       3.14       79.63       5.69E-08       HS         Chlorogenic acid       Monolignols       353.087       10.88       71.43       3.59E-06       HS         Isoquercetin       Flavonoids       463.084       17.52       65.13       4.13E-05       HS         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-p-glucopyranosyle       Lignans       519.181       18.78       59.96       0.0002       HS         Isoacteoside       Secoiridoids       662.193       17.20       55.35       0.0008       HS         Frameroside       Secoiridoids       61.164       15.71       54.27       0.0010       HS         So-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83	5-0-Caffeovlsbikimate	Coumarins	335.075	15.05	88.08	1.40E-11 1.12E-10	HS
Formate adduct of tyrosol glucoside       Phenylethanoids       345.118       11.39       81.73       1.512 00       HS         Citric acid       Small organic acid       191.020       3.14       79.63       5.69E-08       HS         Citric acid       Monolignols       353.087       10.88       71.43       3.59E-06       HS         Isoquercetin       Flavonoids       463.084       17.52       65.13       4.13E-05       HS         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-p-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         Isoacteoside       Secoiridoids       663.193       17.20       55.35       0.0008       HS         Isoacteoside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         Frameroside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       <	4-Coumarov/shikimic acid	Monolianols	319.080	17.43	85.21	1.72E-10	HS
Citric acid       Small organic acid       191.020       3.14       79.63       5.69E-08       HS         Chlorogenic acid       Monolignols       353.087       10.88       71.43       3.59E-06       HS         Isoquercetin       Flavonoids       463.084       17.52       65.13       4.13E-05       HS         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-D-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         Isoacteoside       Secoiridoids       662.193       17.20       55.35       0.0002       HS         Isoacteoside       Secoiridoids       61.104       15.71       54.97       0.0002       HS         Isoacteoside       Secoiridoids       61.108       24.11       54.97       0.0009       HS         Farmeroside       Secoiridoids       61.104       15.71       54.27       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS      <	Formate adduct of tyrosol alucoside	Phenylethanoids	345 118	11.39	81.73	1.53E-08	HS
Chlorogenic acid       Monolignols       353.087       10.88       71.43       3.59E-06       HS         Isoquercetin       Flavonoids       463.084       17.52       65.13       4.13E-05       HS         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-D-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         Isoacteoside       Secoiridoids       665.174       13.32       59.50       0.0002       HS         Isoacteoside       Secoiridoids       663.193       17.20       55.35       0.0002       HS         Isoacteoside       Secoiridoids       663.193       17.20       55.35       0.0002       HS         Frameroside       Secoiridoids       623.193       17.20       55.35       0.0009       HS         Formate adduct of Phenylethyl primeveroside       Phenylethanoids       461.164       15.71       54.97       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029	Citric acid	Small organic acid	191 020	3.14	79.63	5.69E-08	нs
Inclugence and       Inclugences       500.007       10.00       11.40       0.0012       110         Isoquercetin       Flavonoids       463.084       17.52       65.13       4.13E-05       HS         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-D-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         7-beta-D-Glucopyranosyl-11-methyloleoside       Secoiridoids       565.174       13.32       59.50       0.0002       HS         Isoacteoside       Secoiridoids       623.193       17.20       55.35       0.0008       HS         Frameroside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         Formate adduct of Phenylethyl primeveroside       Phenylethanoids       461.164       15.71       54.27       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS         8-Hydroxypinoresinol 8-glucoside       Lignans       535.178       16.30	Chlorogenic acid	Monolignols	353 087	10.88	73.00	3.59E-06	нs
Notified       Fractioneds       400.004       11.32       60.10       4.102.00       110         Verbascoside       Cinnamic acid esters       623.193       16.79       63.81       6.46E-05       HS         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-D-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         7-beta-D-Glucopyranosyl-11-methyloleoside       Secoiridoids       565.174       13.32       59.50       0.0002       HS         Isoacteoside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         Frameroside       Secoiridoids       601.208       24.11       54.97       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS         8-Hydroxypinoresinol 8-glucoside       Lignans       535.178       16.30       49.07       0.0034       HS         Formate adduct of unknown C18H26O11       -       463.141       17.93       48.22       0.0041       HS         Uhdenown       Secoiridoids       525.158       16.42       44.71       0.0		Flavonoida	463 084	17.52	65 13	0.00E-00	HS
Verbasedside       Coumarine acid esters       622.130       10.13       60.01       61.402       60       110         Esculin       Coumarins       339.071       11.99       60.72       0.0002       HS         Pinoresinol 4-O-beta-p-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         7-beta-p-Glucopyranosyl-11-methyloleoside       Secoiridoids       565.174       13.32       59.50       0.0002       HS         Isoacteoside       Secoiridoids       623.193       17.20       55.35       0.0008       HS         Frameroside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         Formate adduct of Phenylethyl primeveroside       Phenylethanoids       461.164       15.71       54.27       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS         8-Hydroxypinoresinol 8-glucoside       Lignans       535.178       16.30       49.07       0.0034       HS         Uhdenoside       Secoiridoids       525.158       16.42       44.71       0.0084       HS	Verbascoside	Cinnamic acid esters	623 193	16.79	63.81	4.10E-05	HS
Pinoresinol 4-O-beta-D-glucopyranoside       Lignans       519.181       18.78       59.96       0.0002       HS         7-beta-D-Glucopyranosyl-11-methyloleoside       Secoiridoids       565.174       13.32       59.50       0.0002       HS         Isoacteoside       Secoiridoids       623.193       17.20       55.35       0.0008       HS         Frameroside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         Formate adduct of Phenylethyl primeveroside       Phenylethanoids       461.164       15.71       54.27       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS         8-Hydroxypinoresinol 8-glucoside       Lignans       535.178       16.30       49.07       0.0034       HS         Formate adduct of unknown C18H26O11       -       463.141       17.93       48.22       0.0041       HS         Uhdenoside       Secoiridoids       525.158       16.42       44.71       0.0084       HS	Esculin	Coumarine	339.071	11 99	60.72	0.402-00	HS
Thickes ind 4-0-beta biglical states       Eignah states       515.161       10.16       50.56       0.0002       HS         7-beta-b-Glucopyranosyl-11-methyloleoside       Secoiridoids       565.174       13.32       59.50       0.0002       HS         Isoacteoside       Secoiridoids       623.193       17.20       55.35       0.0008       HS         Frameroside       Secoiridoids       601.208       24.11       54.97       0.0009       HS         Formate adduct of Phenylethyl primeveroside       Phenylethanoids       461.164       15.71       54.27       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS         8-Hydroxypinoresinol 8-glucoside       Lignans       535.178       16.30       49.07       0.0034       HS         Formate adduct of unknown C18H26O11       -       463.141       17.93       48.22       0.0041       HS         Uhdenoside       Secoiridoids       525.158       16.42       44.71       0.0084       HS		Lignans	519 181	18.78	59.96	0.0002	нs
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Isolate side       Secondods       622.135       11.20       53.55       0.0005       115         Frameroside       Secondods       601.208       24.11       54.97       0.0009       HS         Formate adduct of Phenylethyl primeveroside       Phenylethanoids       461.164       15.71       54.27       0.0010       HS         3-O-Feruloylquinic acid       Cinnamic acid esters       367.101       15.63       49.83       0.0029       HS         8-Hydroxypinoresinol 8-glucoside       Lignans       535.178       16.30       49.07       0.0034       HS         Vhdenoside       Secoiridoids       525.158       16.42       44.71       0.0084       HS		Secoiridoido	602 102	17.02	55.25	0.0002	
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Universide         Securitudius         323,130         10.42         44.71         U.U084         HS           Linknown	I Unitate auduct of unknown CT8H20UT	- Socoiridaida	400.141 525 159	16.40	40.22	0.0041	но ЦС
	Unknown	_	569 181	22.84	42.99	0.0116	HS

 Table 1. Continued

Compound identity	Biochemical class	[M-H] <sup>-</sup> m/z	RT (min)	Coefficient mean score	<i>P</i> -value from <i>t</i> -test	More abundant in group
Unknown	-	445.121	3.01	37.19	0.0309	HS
N2	Secoiridoids	565.174	12.60	26.55	0.1295	HS
Quinic acid-related	Cinnamic acid esters	353.086	12.94	24.33	0.1659	HS
Ligstroside-related	_	909.292	28.45	20.12	0.2538	HS
Oleoside-11-methyl ester	Secoiridoids	403.122	14.16	15.20	0.3904	HS
Fraxin	Coumarins	369.081	13.69	6.47	0.7155	HS

For each of the candidates, compound identification, biochemical class, molecular mass ([M-H]<sup>-</sup> m/z), retention time in minutes (RT), and in which group (HS or LS) the metabolite is more abundant. The importance measure (coefficient mean score) represents on a scale from 0 to 100 the relative importance of each compound at separating the LS and HS groups and is based on a weighted sum of the PLS regression coefficients. The weights are a function of the reduction of the sums of squares across the number of principal least square components.



**Fig. 2.** Effects of methanolic extracts of bark from five HS and five LS *F. excelsior* genotypes on growth of *H. fraxineus*. Colony area after 15 d of growth of the *H. fraxineus* isolate Erk2D on control (ADA medium), ADA supplemented with 4% MeOH, ADA supplemented with 4% methanolic extract of five high-susceptibility (HS) ash genotypes, and ADA supplemented with 4% methanolic extract of five low-susceptibility (LS) ash genotypes (*n*=3).

#### Discussion

Plant metabolites are key components in the regulation of biotic interactions at cell level between pathogens and hosts. Our results indicate that LS and HS F. excelsior genotypes represent two different chemotypes in F. excelsior, with large differences in their metabolomics profiles, some of which were associated with the susceptibility to H. fraxineus. Biotic interactions are major factors driving natural selection (Wheat et al., 2007). These interactions have been associated with the presence of different chemotypes in several plant species in response to the action of pests and pathogens (Christensen et al., 2014; Calf et al., 2019). Previous studies showed that susceptibility to ADB in F. excelsior is a quantitative trait (Lobo et al., 2014; Muñoz et al., 2016; Stocks et al., 2019) that depends on multiple individual factors, each with a small effect. This study identified a total of 64 metabolites associated with increased and reduced susceptibility to H. fraxineus (Table 1). As can be appreciated in Fig. 1c and Supplementary Fig. S2, individual genotypes showing HS or LS might contain or lack only a portion of these factors, making signals difficult to identify and associate with a given phenotype. This difficulty can be present in experiments where population admixture occurs in the experimental set-up (Deng, 2001), leading to incorrect associations between signals and phenotype. Similar to Villari et al. (2018), a

broad selection of ash genotypes from multiple provenances in several countries was used in this study to avoid confounding effects. Furthermore, HS and LS plants were grown as grafted plants from healthy scion wood in the same conditions, and samples for extractions were made in triplicate per genotype in order to minimize environmental error sources. Villari et al. (2018) could not observe an environmental footprint in bark chemistry in samples taken from six different European locations. Similarly, a study in Quercus agrifolia showed no differences in phenolics between healthy bark tissue and bark tissue sampled 60 cm away from canker caused by Phytophtora ramorum (Ockels et al. 2007). While there is enough evidence that the samples in this study could be considered free from footprints left by their previous contact with H. fraxineus or their original environment, this hypothesis cannot be completely discarded. As genome-wide genotyping tools for ash become available in the future (Sollars et al., 2017; Stocks et al., 2019), it will be possible to associate increased or reduced levels of specific metabolites with DNA variations, with the aim of understanding the genetic basis behind the biosynthesis of specific metabolites responsible for ash defense.

Genotype selection was further validated by the results of the bioassay, which show that the addition of the methanolic bark extract of HS to ADA medium resulted in a similar colony growth to the control, while the addition of the methanolic extract of LS genotypes to ADA resulted in a complete growth inhibition of *H. fraxineus* (Fig. 2). The results of this experiment showed that the factors causing fungal inhibition were found in the methanolic extract of F. excelsior bark. We observed that addition of bark extracts or of coumarins to the medium resulted in a brownish coloration in the medium with the fungus. Future studies may investigate how H. fraxineus detoxifies ash metabolites and increase the understanding of this plant-pathogen interaction. It is known that H. fraxineus in Europe has a very low genetic diversity (Burokiene et al., 2015; McMullan et al., 2018). In the present study, the results of the bioassay to test the individual effect of fraxetin and esculetin on fungal growth showed a variation in the tolerance to the different treatments in the different isolates (Table 2). This indicates that the pathogen has a certain capacity to adapt to some of the natural defense barriers in F. excelsior. As a consequence, new strains of *H. fraxineus* should be prevented from arriving in Europe in the future as a larger genetic diversity might

Table 2.	Effect of p	ohysiolog	ical con	centrations	of esculetin	and
fraxetin o	n growth (	(mm <sup>2</sup> ) of	three isc	lates of H.	fraxineus	

а	Area d15±SD	b	Control	EsHS	EsLS	FrHS	FrLS
21c		21c					
Control	1680±60	Control	1	0.009	0.007	0.053	0.009
EsHS	1532±53	EsHS		1	0.182	0.875	0.326
EsLS	1277±269	EsLS			1	0.191	0.321
FrHS	1545±122	FrHS				1	0.930
FrLS	1529±277	FrLS					1
Erk2D		Erk2D					
Control	1711±68	Control	1	0.000	0.000	0.010	0.000
EsHS	1452±34	EsHS		1	0.048	0.417	0.005
EsLS	1279±101	EsLS			1	0.052	0.127
FrHS	1510±106	FrHS				1	0.009
FrLS	1126±94	FrLS					1
Ö3		Ö3					_
Control	1651±105	Control	1	0.007	0.000	0.062	0.006
EsHS	1224±249	EsHS		1	0.018	0.156	0.374
EsLS	625±91	EsLS			1	0.000	0.000
FrHS	1492±93	FrHS				1	0.133
FrLS	1292±159	FrLS					1

(a) Colony areas after 15 ds of growth for fungal isolates: 21c, Erk2D, and O3 on ADA medium (control) and ADA supplemented with physiological concentrations of esculetin (Es) and fraxetin (Fr), estimated in high susceptibility (HS) and low susceptibility (LS) groups: esculetin (EsHS; 53.3 µg ml<sup>-1</sup> and EsLS, 152.2 µg ml<sup>-1</sup>) and fraxetin (FrHS, 58 µg ml<sup>-1</sup> and FrLS, 165.6 µg ml<sup>-1</sup>). (b) *P*-value of the comparisons between different treatments. Significant differences are highlighted in grey.

allow *H. fraxineus* to adapt more efficiently to the natural defense barriers in *F. excelsior*.

A total of eight coumarins were found among our candidate metabolites, with six of them showing high coefficient scores. Among them, fraxetin and esculetin were tested against the fungus. Both coumarins showed a fungistatic effect on H. fraxineus at physiological concentrations, validating their role as biochemical markers for tolerance to H. fraxineus in F. excelsior. Coumarins are naturally abundant in Fraxinus species (Kostova and Iossifova, 2007), and at the same time their levels are highly variable (Whitehill et al., 2012), making them ideal candidates for intra- and interspecific variation in pest resistance in ash. Interestingly, levels of the glycosylated forms of fraxetin and esculetin (fraxin and esculin, respectively) were less abundant in LS genotypes. Similarly, a study on emerald ash borer (EAB) resistance in ash found reduced levels of the glycoside esculin in EAB-resistant F. mandshurica compared with EAB-susceptible F. nigra (Villari et al., 2016). A previous study showed that the glycosylated coumarins have a much lower antifungal activity compared with their respective aglycones (Mercer et al., 2013). Our results may suggest that LS genotypes can retain a higher amount of coumarins in their nonglycosylated form, thus increasing the antimicrobial capacity of the coumarinic portion in LS genotypes. The importance of coumarins in ash defense might involve more organisms than just ADB. EAB is an invasive insect that has brought several ash species in North America to the brink of extinction (Herms and McCullough, 2014). Studies have found that those ash species susceptible to EAB show a lack of coumarins, with the

exception of scopoletin, compared with EAB-resistant ash species (Whitehill et al., 2012; Villari et al., 2016; Qazi et al., 2018). A recent study showed proof of convergent evolution in substrate recognition sites for an enzyme targeting *p*-coumarate, a coumarin precursor, in EAB-resistant ash species (Kelly et al. 2020). Phenylcoumaran benzylic ether reductase, an enzyme that targets coumarin precursors (Niculaes et al., 2014), was identified in a proteomics study as a candidate enzyme linked to the resistance of *F. mandshurica* to EAB (Whitehill *et al.*, 2011). Studies in other plant species cover the biosynthesis of coumarins (Karamat et al., 2014; Shimizu, 2014; Vanholme et al., 2019) and its regulation (Chezem et al., 2017), but the topic remains poorly understood. Because of the natural abundance and diversity of coumarins and their role in plant defense, Fraxinus species are ideal model species to study the mechanisms behind the biosynthesis and regulation of these compounds. As more genomic resources are made available (Sollars et al., 2017), this objective should be achievable.

Candidate metabolites from other chemical families including secoiridoids, flavonoids, lignans, and phenylethanoids were identified in this study. This broad range of metabolites reflects the diversity in mechanisms on which F. excelsior's defense against H. fraxineus relies. Among the candidate metabolites, 21 were secoiridoids. This large diversity of secoiridoids reflects the importance of this compound family in defense against H. fraxineus in F. excelsior. Two secoiridoids previously reported as N2 (Excelside A-like compound) and N4 (Nuzhenide) (Sambles et al., 2017; Sollars et al., 2017) were also identified in our study, but neither of them showed significant differences between the HS and LS groups. Two other secoiridoids associated with LS genotypes, oleuropein and 10-hydroxyoleuropein, were previously reported to associate with LS for *F. excelsior* seedlings after treatment with a H. fraxineus fungal elicitor (Cleary et al., 2014). Collectively, secoiridoids have been associated with increased susceptibility to ADB in ash (Sambles et al., 2017; Sollars et al., 2017) and with reduced susceptibility to ADB (Cleary et al., 2014) and to EAB (Villari et al., 2016). In this study, 15 secoiridoid candidates were associated with LS genotypes and six were associated with HS genotypes. Secoiridoids vary in their antimicrobial and antioxidant properties, which depend on small changes in their structure (Božunović et al., 2018). It is possible that a different transcriptional regulation in secoiridoid biosynthesis leads HS and LS genotypes to accumulate secoiridoids with different activity against H. fraxineus.

The present study showed that five flavonoids were identified and associated with different susceptibility to ADB. Two quercetin glycosides, rutin and isoquercetin, were associated with HS genotypes, while naringenin and kaempferide were associated with LS genotypes. Interestingly, rutin, which is associated with the HS group in our study, has been previously associated with increased fungal susceptibility in apple (Petkovsek *et al.*, 2007). Flavonoids play an important role in plant–microbe interactions (Hassan and Mathesius, 2012) including plant–microbe crosstalk and antimicrobial functions. This study indicates that flavonoids play a role in susceptibility to ADB. Six lignans were annotated in this study. Lignans have been reported previously in resistance to EAB in ash (Whitehill *et al.*, 2011, 2012; Villari *et al.*, 2016; Qazi *et al.*, 2018). Apart from their role in insect defense, lignans have antifungal properties (Cho *et al.*, 2007), which could play a role in the interaction between ash and *H. fraxineus*. Lastly, six phenylethanoids were annotated in this study as candidate metabolites, two of which were present to a greater extent in HS genotypes while four were in HS genotypes, including calceolarioside A and B, which were previously associated with resistance against EAB in *F. mandshurica* (Eyles *et al.*, 2007; Whitehill *et al.*, 2012).

In conclusion, this study presents the most complete list of ash metabolites associated with reduced and increased susceptibility to ADB including metabolites identified in previous studies. Within each of the chemical families into which the candidate metabolites were classified, a portion of the candidates associated with either the HS or the LS genotypes. Our findings suggest that differences between the chemotypes associated with the HS and LS groups are unlikely to be caused by one or a few enzymes within biosynthetic pathways but rather by the action of transcriptional regulatory elements. The differences in the levels of metabolites between HS and LS ash groups were present in bark samples which had not been previously inoculated or treated with fungal elicitors, suggesting that at least a part of the defense mechanisms against H. fraxineus in ash are constitutive chemical barriers. Additionally, an important portion of the candidate metabolites in this study have been reportedly associated with resistance to EAB in ash, suggesting the possibility of parallel mechanisms conferring resistance against EAB and ADB simultaneously. The study presents new biochemical markers in ash and, for the first time, validates the fungistatic properties of fraxetin and esculetin on H. fraxineus and their role as markers for tolerance in ash.

#### Supplementary data

Supplementary data are available at JXB online.

Table S1. List of the genotypes used in this study.

Fig. S1. MS/MS fragmentation of the ions of the 64 relevant metabolites in negative ionization mode

Fig. S2. A boxplot representation for each of the 64 candidate metabolites of the relative levels in HS and LS genotypes.

Fig. S3. Heatmap representation of the levels of 64 candidate metabolites identified in this study.

Fig. S4. Fungistatic activity of the methanolic extracts from HS and LS genotypes of *F. excelsior* on the isolate Erk2D of *H. fraxineus*.

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#### **Data availablity**

The dataset for this study has been deposited in the Dryad Data Repository at doi:10.5061/dryad.7m0cfxpqv (Nemesio-Gorriz *et al.*, 2020).

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