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Research

A glimmer of hope – ash genotypes with increased resistance to ash dieback pathogen show cross-resistance to emerald ash borer

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Summary

- Plants rely on cross-resistance traits to defend against multiple, phylogenetically distinct enemies. These traits are often the result of long co-evolutionary histories. Biological invasions can force naïve plants to cope with novel, coincident pests, and pathogens. For example, European ash (*Fraxinus excelsior*) is substantially threatened by the emerald ash borer (EAB), *Agrilus planipennis*, a wood-boring beetle, and the ash dieback (ADB) pathogen, *Hymenoscyphus fraxineus*. Yet, plant cross-resistance traits against novel enemies are poorly explored and it is unknown whether naïve ash trees can defend against novel enemy complexes via cross-resistance mechanisms.
- To gain mechanistic insights, we quantified EAB performance on grafted replicates of ash genotypes varying in ADB resistance and characterized ash phloem chemistry with targeted and untargeted metabolomics.
- Emerald ash borer performed better on ADB-susceptible than on ADB-resistant genotypes. Moreover, changes in EAB performance aligned with differences in phloem chemical profiles between ADB-susceptible and ADB-resistant genotypes.
- We show that intraspecific variation in phloem chemistry in European ash can confer increased cross-resistance to invasive antagonists from different taxonomic kingdoms. Our study suggests that promotion of ADB-resistant ash genotypes may simultaneously help to control the ADB disease and reduce EAB-caused ash losses, which may be critical for the long-term stability of this keystone tree species.

Introduction

With the intensification and expansion of global trade, thousands of species have been moved outside their native ranges and into naïve ecosystems (Seebens *et al.*, 2017, 2018; Pyšek *et al.*, 2020). Non-native invasive pests and pathogens have destroyed or damaged substantial parts of the world's forests, causing considerable ecological and economic costs (Roy *et al.*, 2014; Brockerhoff & Liebhold, 2017; Panzavolta *et al.*, 2021). Simultaneous invasions by taxonomically divergent organisms may further exacerbate the impact on native tree species (Harrington *et al.*, 2008; Tisserat *et al.*, 2009; Meyer *et al.*, 2015; Paap *et al.*, 2018; Davydenko *et al.*, 2022). For example, chewing insect herbivores and biotrophic pathogens can trigger hormonal pathways associated with different plant defensive responses (Stout *et al.*, 2006; Erb *et al.*, 2012). Sequential or simultaneous attacks inducing different plant hormonal pathways may result in antagonistic crosstalks between pathways (Pieterse *et al.*, 2006; Erb *et al.*, 2012; Thaler *et al.*, 2012). Consequently, several studies have reported that an initial attacker increased the performance or damage of a subsequential, taxonomically distinct attacker associated with a different defensive pathway (Preston *et al.*, 1999; Bacher *et al.*, 2002; Musser *et al.*, 2003). However, the finding that taxonomically distinct attackers sharing the same plant host benefit each other is not universal (Moreira *et al.*, 2018). For example, research on the interaction between plants and native plant consumers has shown that plants can defend themselves against multiple enemies simultaneously, even if these enemies are from different taxonomic

kingdoms and possess different modes of attack (Biere *et al.*, 2004; Andrew *et al.*, 2007; Luu *et al.*, 2017). Traits conferring cross-resistance against phylogenetically distinct enemies are often the product of a long co-evolutionary history between plants and their enemies (Krischik *et al.*, 1991; Strauss *et al.*, 2005). A critical knowledge gap in our understanding of the resilience of native plant hosts to biological invasions is whether native trees can rely on cross-resistance to defend against multiple novel enemies.

European ash (*Fraxinus excelsior* Linnaeus) is a widespread, keystone species that has critical importance for biodiversity and the functioning of temperate broadleaved ecosystems in Europe (Pautasso *et al.*, 2013; Littlewood *et al.*, 2015; Beck *et al.*, 2016; Hultberg *et al.*, 2020). Besides its ecological relevance, ash is also of high economic value due to its fast growth and elastic wood (Dobrowolska *et al.*, 2011; Beck *et al.*, 2016). Currently, European ash is heavily impacted by ash dieback (ADB), which is caused by the East Asia-native, invasive fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Gross *et al.*, 2014; McMullan *et al.*, 2018). The first symptoms of ADB were observed in the early 1990s in Poland (Gil *et al.*, 2017). Since then, the disease has rapidly spread and is now threatening ash populations throughout Europe (Gross *et al.*, 2014; Enderle *et al.*, 2019).

More recently, a new threat is encroaching on European ash. The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is a wood-boring beetle that was first introduced from China to the USA sometime in the 1990s (Siegert *et al.*, 2014) and was first detected in the early 2000s (Haack *et al.*, 2002). In North America, EAB has killed hundreds of millions of ash trees (Klooster *et al.*, 2018), resulting in severe population reductions of many ash species and effective extirpation from parts of the invaded range (Herms & McCullough, 2014). Since its discovery in Western Russia in 2003, EAB has expanded its range toward Central Europe, posing a similar threat of large-scale losses to European ash in the near future as was realized in North America (Orlova-Bienkowskaja & Bieńkowski, 2018; Orlova-Bienkowskaja *et al.*, 2020).

Despite the widespread presence of ADB in Europe, a small proportion of the population of *F. excelsior* in countries such as Denmark, Sweden, and Switzerland (McKinney *et al.*, 2014) shows remarkable field resistance to ADB, even when growing in heavily infested stands. This resistance seems to be under strong genetic control (Stener, 2018). Ash dieback-resistant trees display comparatively less shoot necrosis and dieback in the crown (Enderle *et al.*, 2019). The intraspecific differences in ADB resistance may relate to genotypic variations in shoot-specialized metabolites (Villari *et al.*, 2018; Nemesio-Gorriz *et al.*, 2020). Studies on Green ash (*Fraxinus pennsylvanica* Marshall) showed that ash genotypes can also strongly differ in EAB resistance (Knight *et al.*, 2012; Koch *et al.*, 2015). These findings suggest that there may also be substantial intraspecific variation in EAB resistance in other ash species including European ash. However, whether increased ADB resistance among European ash genotypes also results in an increased resistance against the EAB is unclear.

Emerald ash borer resistance has been linked to differences in phloem phenolic profiles (Whitehill *et al.*, 2012, 2014; Qazi *et al.*, 2018), similar to the results observed for ADB resistance (Villari *et al.*, 2018; Nemesio-Gorriz *et al.*, 2020). Taken together, these findings suggest that both EAB and ADB resistance in ash is partly determined by differences in phenol chemistry, giving reason to speculate that ash genotypes with increased resistance against ADB may also possess increased resistance to EAB. By contrast, Showalter *et al.* (2020) argued that EAB and ADB resistance are probably not related as they found no difference in EAB resistance among European ash populations varying in ADB prevalence. Despite these contradictory conclusions, a possible link between ADB and EAB resistance remains to be experimentally tested. ADB-EAB cross-resistance would be of immense value for limiting damages by these invasive species and for conservation and restoration of ash (Sniezko & Koch, 2017).

In this study, we tested the hypotheses that (i) European ash genotypes differ significantly in their resistance against EAB, similar to results observed for resistance to *H. fraxineus*; (ii) ash genotypes with an increased ADB resistance show cross-resistance against EAB (i.e. resistant to both ADB and EAB); and (iii) ADB-resistant and ADB-susceptible ash genotypes differ in phloem phytochemistry, and these differences correlate with differences in EAB performance. To test these hypotheses, we grafted genotypically distinct scions from Swiss and Scandinavian *F. excelsior* displaying either resistance or susceptibility to ADB. The grafted scions were then inoculated with EAB eggs, and we measured EAB performance as a proxy for resistance. Phytochemical traits from ADB-resistant and ADB-susceptible genotypes were measured and linked to EAB performance.

Materials and Methods

Ash genotype selection, beetles, and fungal pathogen

We selected 28 European ash genotypes that either showed relatively high ADB resistance or susceptibility in the field. There were 19 genotypes from Switzerland (10 ADB-resistant, nine ADB-susceptible), six from Sweden (three ADB-resistant and three ADB-susceptible), and three from Denmark (all ADB-resistant; Table 1). Resistant and susceptible genotypes from Switzerland were selected from forest stands based on crown health assessments during the summer of 2018. Study plots were chosen throughout Switzerland in regions where ADB was found to be fully established by forest surveys (Queloz & Gossner, 2019). In 2019, we selected 10 ash genotypes from 10 different locations in Switzerland that showed a maximum of 25% crown defoliation and no stem necroses, even though the selected trees were growing adjacent to ADB-infested trees. These genotypes were assigned to the ADB-resistant category. One genotype each (totally nine) of highly symptomatic trees (50–75% crown defoliation) was selected from the same locations and assigned to the ADB-susceptible category (Table 1). Ash dieback resistance of the Swedish genotypes was determined based on the periodic evaluation of two clonal seed orchards of *Fraxinus excelsior* Linnaeus in southern Sweden since 2006, which showed considerable

Table 1 Overview of the studied ash genotypes (*Fraxinus excelsior)* from Switzerland (19 genotypes), Denmark (three genotypes), and Sweden (six genotypes).

Genotype	ADB-resistance category	Location	Location latitude/ longitude	Country	Phytochemically analyzed
G1-R	Resistant	Kemmental	47.63N 9.12E	Switzerland	No
G1-S	Susceptible	Kemmental	47.63N 9.12E	Switzerland	No
G2-R	Resistant	Kesswil	47.58N 9.31E	Switzerland	No
G2-S	Susceptible	Kesswil	47.58N 9.31E	Switzerland	No
G3-R	Resistant	Ilanz	46.76N 9.22E	Switzerland	No
G3-S	Susceptible	Ilanz	46.76N 9.22E	Switzerland	No
G4-R	Resistant	La Chaux-de-Fonds	47.15N 6.85E	Switzerland	Yes
G4-S	Susceptible	La Chaux-de-Fonds	47.15N 6.85E	Switzerland	Yes
G5-R	Resistant	Frauenfeld	47.58N 8.88E	Switzerland	No
G5-S	Susceptible	Frauenfeld	47.58N 8.88E	Switzerland	No
G6-R	Resistant	Tuggen	47.21N 8.96E	Switzerland	Yes
G6-S	Susceptible	Tuggen	47.21N 8.96E	Switzerland	Yes
G7-R	Resistant	Bassersdorf	47.46N 8.63E	Switzerland	No
G7-S	Susceptible	Bassersdorf	47.46N 8.63E	Switzerland	No
G8-R	Resistant	Quarten	47.11N 9.22E	Switzerland	Yes
G8-S	Susceptible	Quarten	47.11N 9.22E	Switzerland	Yes
G9-R	Resistant	Ermatingen	47.65N 9.08E	Switzerland	No
G9-S	Susceptible	Ermatingen	47.65N 9.08E	Switzerland	No
G10-R	Resistant	Murten	46.94N 7.17E	Switzerland	No
G11-R	Resistant	Randers	56.50N 10.04E	Denmark	No
G12-R	Resistant	Randers	56.50N 10.04E	Denmark	No
G13-R	Resistant	Randers	56.50N 10.04E	Denmark	No
G14-R	Resistant	Trolleholm	55.94N 13.02E	Sweden	No
G15-R	Resistant	Trolleholm	55.94N 13.02E	Sweden	No
G16-R	Resistant	Trolleholm	55.94N 13.02E	Sweden	No
G17-S	Susceptible	Trolleholm	55.94N 13.02E	Sweden	No
G18-S	Susceptible	Trolleholm	55.94N 13.02E	Sweden	No
G19-S	Susceptible	Trolleholm	55.94N 13.02E	Sweden	No

ADB, ash dieback.

genotypic variation in susceptibility to ADB, and where clonal resistance over time of select resistant genotypes has shown remarkable stability (Stener, 2013, 2018). The ADB resistance of the three Danish genotypes was determined based on yearly assessments since 2008 of the progeny trial 'Randers' where the three genotypes have shown consistently minimal disease severity (< 10% of the crown) over the years (Kjær *et al.*, 2012).

To generate replicates of each genotype, 1-yr-old, asymptomatic, similar-sized scions were collected from the mother trees. Scions from the Swiss genotypes were collected in March 2019 and 2020. Danish and Swedish genotypes (henceforth: Scandinavian genotypes) were sampled in February and March 2020. Scions were kept on ice in the field and were subsequently stored in a cool room (4°C) until further processing. All scions were grafted onto common ash rootstocks 1–2 wk after collection. Scions from the Swiss and Danish genotypes were grafted in equal numbers (50 replicates) onto 2-yr-old rootstocks (obtained from Josef Kressibucher AG, Berg, Switzerland) from two Swiss *F. excelsior* provenances. Scions from Swedish genotypes were grafted onto *F. excelsior* rootstocks obtained from a nursery in Hungary. All grafted trees were planted in 4 l pots filled with humus-rich soil (Potting soil, Ökohum, Germany) containing 2 g long-term fertilizer (18% N, 12% K_2O, 6% P_2O_5 Tardit-Top, Hauert, Switzerland). Trees were kept in outdoor foil tunnels until the beginning of the bioassays.

We obtained EAB eggs from two insect colonies maintained at the Great Lakes Forestry Centre, Sault Ste. Marie (Glfc:IPQL:AplaPPP01 and Glfc:IPQL:AplaPPP02; Roe *et al.*, 2018). These two families of EAB were initiated from adult insects flushed from green ash log bolts (*Fraxinus pennsylvanica* Marshall) collected in Presque'lle Provincial Park, Brighton, ON, Canada, and reared according to Roe *et al.* (2018). The import permit was issued by Federal Office for Agriculture FOAG, Switzerland in 2019 (Letter of authority No. 01/19), and renewed in 2020 (Letter of authority No. 24/20) and 2021 (Letter of authority No. 36/21). Upon arrival in Switzerland, all eggs were transferred to a level 3 laboratory in the Plant Protection Lab at WSL (Ecogen nr: A182420) and kept at 25°C, 55% RH, 16 h : 8 h, light : dark. The eggs were stored for 4–6 d until inoculation for EAB resistance screening.

The ADB-pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya, was freshly isolated in 2020 from an infected tree in Switzerland (47°21′42.3″N, 8°27′25.3″E). The isolate was cultivated on ash leaf malt agar (50 g fresh, *F. excelsior* leaves, removed after autoclavation (121°C), 15 g l^{-1} agar (Plant Propagation Agar, Condalab, Spain), 20 g l^{-1} malt extract (DiaMalt 'trocken'; Hefe Schweiz AG, Stettfurt, Schweiz), 1000 ml deionized H_2O). After 10–14 d of incubation at room temperature, autoclaved (in a solution of 20 g l^{-1} malt extract (DiaMalt 'trocken'; Hefe Schweiz AG) and deionized water) fresh ash wood plugs (size *c.* 1.5 × 5 × 5 mm^3) were placed onto the mycelium under sterile conditions. The plates were then incubated at room temperature for 20–22 d to allow *H. fraxineus* to colonize the wood plugs, which were then used for tree inoculations (see: ADB bioassay).

EAB resistance screening

Resistance against EAB of various ash genotypes was assessed using EAB performance in bioassays as a proxy for natural infestation where high EAB performance on an ash genotype indicates a low genotypic EAB resistance and vice versa. The grafted scions (hereafter referred to as trees) were moved from the foil tunnels to climate chambers (24°C, 70% RH, 16 h : 8 h, light : dark) 10–14 d before the bioassays were implemented. Swiss genotypes were assayed in four runs (7–25 August 2020, 18 August–5 September 2020, 3 October–21 October 2020, and 17 October–4 November 2020). Therefore, all tests ran for 18 d. Bioassays with Scandinavian genotypes were also conducted in four runs 1 yr later (23 August–9 September 2021, 23 August–10 September 2021, 27 August–13 September 2021, and 27 August–14 September 2021). In this case, two tests ran for 17 d and two for 18 d. For each test, one tree of each genotype was inoculated with EAB eggs. To keep EAB infestation densities similar among trees, we inoculated trees that were 90–125 cm tall with an average stem diameter of 0.93 cm at 3–4 sites along the stems and trees that were smaller than 90 cm with an average stem diameter of 0.89 cm at two to three sites. Inoculation sites were at least 10 cm apart and 10 cm above the graft union. At each inoculation site, we placed four EAB eggs attached to coffee filter strips directly onto the bark. Coffee filter strips were affixed and held in place with parafilm.

For each inoculation site, the stem age (current year or older than current year) was noted and the stem cross-section area was approximated using the stem diameter. Stem age and cross-section area were quantified since they might affect EAB performance.

To determine the most likely date of egg eclosion, 20–30 EAB eggs were placed in a ventilated Petri dish located next to the experimental trees. We observed the dishes twice a day between 09:00–11:00 h and 16:00–18:00 h and counted the number of freshly hatched EAB larvae. The starting date of a test was defined as the first date on which 50% of all EAB eggs in the dish had hatched.

At the end of each bioassay, we counted the number of hatched eggs; all trees were debarked and the EAB larvae were recovered. We quantified three EAB larval performance parameters: larval survival per tree; the number of larvae per larval instar; and the final dry weight of each larva. Larval survival per tree was calculated by subtracting the number of recovered and still-living larvae from the number of hatched eggs. The few larvae that were cannibalized or got stuck on the parafilm were excluded from the survival calculations. Larval instars were determined based on peristomal width (Cappaert *et al.*, 2005). Finally, larvae were oven-dried for 5 d at 70°C and weighed individually.

ADB-resistance screening

In addition to the field-estimated ADB-resistance categories, we also quantified resistance as the resultant proximal lesion length following stem inoculation with *H. fraxineus*, with longer ADB lesions indicating low genotypic resistance to ADB and the reverse for high resistance ash genotypes (McKinney *et al.*, 2012).

Ash trees were moved to a glasshouse 10–14 d before the bioassays. The assays with the Swiss genotypes were conducted in one test lasting 14 wk (21 August 2020–27 November 2020). Due to graft failures, two Swiss genotypes (G1-S and G4-R, Table 1) could not be assayed. Bioassays with Scandinavian genotypes were conducted on 23 August 2021–7 December 2021 and were successful only for very few trees due to unknown reasons. Hence, only bioassay data generated from Swiss genotypes were analyzed. At the beginning of a bioassay, five trees from each genotype were inoculated with *H. fraxineus* by inserting ADB colonized wood plugs into small incisions in the tree bark of the stem according to McKinney *et al.* (2012). To keep ADB inoculation densities consistent, we inoculated trees that were shorter than 30 cm with one plug, trees between 30 cm and 60 cm with two plugs, and taller trees with three plugs. Where individual trees were inoculated more than once on the stem, incisions were made at least 20 cm apart and on opposite sides of the stem to minimize interactions between inoculation sites. Two trees per genotype were treated with uncolonized wood plugs as negative controls. At the end of each run, inoculated stem sections were cut in half lengthwise and the ADB lesion lengths along the vascular cambium were measured. To confirm that the observed lesions were caused by *H. fraxineus*, re-isolations were attempted from all samples with lesion length ≥ 0.5 cm and species identity was confirmed using morphological traits as well as sequencing the ITS region as in Queloz *et al.* (2011).

Phloem chemistry

To explore whether differences in ADB and EAB resistance were associated with phloem chemistry, we collected phloem samples from ADB-resistant and ADB-susceptible ash genotypes that were either infested with EAB (induced phytochemical responses) or remained uninfested (constitutive phytochemistry). To reliably link phytochemistry and EAB performance, we depended on ADB-resistant and ADB-susceptible genotypes that were available in sufficiently high replicate numbers. Due to tree losses during the ash grafting, the only genotype pairs (resistant and susceptible ash genotypes from the same location) that were available in suitable numbers were the three Swiss genotypes G4-R, G6-R, and G8-R that showed increased ADB resistance and the three co-occurring ADB-susceptible genotypes G4-S, G6-S, and G8-S (Table 1). We infested five to seven trees of each genotype with EAB larvae as described above and retained a set of uninfested trees as a control. Four bioassays were conducted in a glasshouse (24°C, 70% RH) on 12 June–27 July, 14 June–29 July, 19 June–3 August, and 29 June–13 August 2022. In each test, we sampled one to two trees for each treatment combination. EAB eggs were only placed on stem sections that were at least 1 yr old. Trees were harvested 45 d after the estimated egg-hatching date. Phloem tissue was collected next to beetle galleries in EAB-infested trees. Phloem tissue from comparable stem sections was collected from uninfested trees. The phloem samples were collected using ice-cold razor blades. Collected samples were immediately flash-frozen in liquid nitrogen, pulverized in a mortar using liquid nitrogen, and stored at −80°C until chemically analyzed. Beetles were sampled as described above (EAB bioassay).

Phloem chemistry was analyzed using both targeted and untargeted approaches. In the targeted approach, we focused on the phenolics fraxin, verbascoside, and calceolarioside B as well as the terpenes oleuropein and ligustroside. These compounds have been previously identified as being involved in ash resistance against EAB (Whitehill *et al.*, 2012, 2014; Chakraborty *et al.*, 2014; Qazi *et al.*, 2018) or ADB (Nemesio-Gorriz *et al.*, 2020). To quantify these compounds, 100 mg (±5) of frozen phloem powder was extracted in 500 µl of methanol (MeOH) containing 5 mM butylated hydroxyanisole (BHA; Sigma-Aldrich) as an internal standard. MeOH containing BHA was added to the ground tissue and vortexed for 10 s, sonicated for 30 min in an ice bath, and centrifuged for 5 min at 4°C at 12 000 *g*. Supernatants were transferred to new tubes. From each vial, 300 µl of extract was lyophilized for shipment to Ohio State University, where the samples were resuspended and analyzed via UPLC-MS using an ACQUITY H-Class UPLC™ system coupled to a Waters ACQUITY H-Class Triple-quadrupole™ mass spectrometer with single reaction monitoring of mass traces. Targeted quantification of compounds was conducted in Waters Targetlynx™ feature, using commercially available standards from Extrasynthese (Lyon, France; fraxin, verbascoside, oleuropein, all > 98% purity) and Sigma-Aldrich (calceolarioside, ligustroside, > 95% purity). Further analytical details are described in Supporting Information Methods S1.

In our untargeted approach, we compared the specialized metabolomes of ADB-resistant and ADB-susceptible as well as EAB-infested and uninfested ash trees. Untargeted analyses of the phytochemical profiles were performed as described in Defossez *et al.* (2023) with some modifications. In brief, 500 µl of extraction solvent (methanol : water : formic acid, 80 : 19.5 : 0.5, v/v; Sigma-Aldrich) and five to eight glass beads were added to 65 (±5) mg of frozen phloem powder in individual Eppendorf tubes. The mixture was shaken in a Qiagen TissueLyser for 3 min at 30 Hz, and centrifuged at 12 000 *g* for 3.0 min, and the supernatant was collected and analyzed via ultra-high-performance liquid chromatography – quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS) using an ACQUITY UPLC™ I-Class coupled to a Synapt XS mass spectrometer (Waters, Milford, CT, USA). Further analytical details are described in Methods S1. The high-resolution tandem MS data were processed with MZmine2 (v.2.53; Pluskal *et al.*, 2010). For feature identification, we used Sirius5 (v.5.6.4; Dührkop *et al.*, 2019) utilizing CSI:fingerID fingerprint prediction (Dührkop *et al.*, 2015) and CANOPUS (Djoumbou Feunang *et al.*, 2016; Dührkop *et al.*, 2021; Kim *et al.*, 2021). In addition, for the statistically discriminant features, we also used the MASST search interface provided in the Global Natural Products Social Molecular Networking (GNPS) environment.

Statistical analysis

All analyses were conducted in R 4.1.2 (R Core Team, 2021). Linear models (LMs) were fitted with R-base, and linear mixed

effects models (LMMs) were fitted with the LME4 package (v.1.1–27.1, Bates *et al.*, 2014). Fitted LMs and LMMs were subjected to type II (or type III in the presence of interactions) analyses of variance (ANOVAs) with Kenward–Roger's method to produce a summary of the *F*- and *P* statistics (CAR package, v.3.0–12, Fox & Weisenber, 2018). Generalized linear mixed effects models (GLMMs) were fitted with the LME4 package and then subjected to type II chi-squared tests using the CAR package. Response variables were transformed if necessary to meet LMs and LMMs assumptions for normality and homoscedasticity. All models were checked for multicollinearity with the performance package (Lüdecke *et al.*, 2021). GLMMs were checked for zero inflation and overdispersion. Conditional R^2 values were computed using the MuMIn package (v.1.47.1, Bartoń, 2022). The mixOmics package (v.6.22.0; Rohart *et al.*, 2017) was used to conduct (sparse) partial least squares discriminant analysis (s)PLS-DA and sparse partial least squares regression (sPLSR) models. Models were tuned via a 10-fold, 100 repeat cross-validated balanced error rates, and mean average error values, respectively. Since PLS-DA is prone to overfitting, it is important to validate the models before the score plots can be reliably interpreted (Hervé *et al.*, 2018). We validated all tuned PLS-DA models via permutation tests (999 permutations) using the RVAideMoire package (v.0.9-81-2, Hervé, 2022).

First, we tested for the effect of host genotype on EAB performance (hypothesis i) separately for the Swiss and Scandinavian provenances. This was necessary because Scandinavian genotype effects could not be statistically disentangled from rootstock effects. We used three different performance parameters as response variables: EAB survival, EAB larval instar, and EAB dry weight. To test for effects on EAB survival and EAB larval instar, we used GLMMs for binomial distribution with ash genotype, rootstock provenance (only for Swiss genotypes), stem age, and stem cross-section area as fixed effects. Larval instar was determined as number of larvae that developed within 18 d into the second instar (only first- and second-instar larvae were found, binomial distribution). To test for effects on EAB dry weight, we used LMMs with the same fixed effects. Individual trees nested in experimental runs were included as random intercepts in all models.

Second, we explored the relationship between ADB resistance and EAB performance (hypothesis ii). The genotype variable was not included in any model due to high multicollinearity between ADB resistance and genotype (variance inflation factor > 10; Ziegler & Myers, 1990). GLMMs were used to test for the effects of ADB resistance, rootstock provenance, stem age, and stem cross-section area on EAB survival (binomial distribution) and larval instar (binomial distribution). LMMs were used to test for the same fixed effects on EAB dry weight. ADB resistance was modeled either as a categorical (qualitative) variable using ADB-resistance categories, based on field observations (ADB-resistant vs ADB-susceptible), or as a quantitative variable, that is, mean ADB lesion length, based on the results of the ADB-resistance screening. The effect of ADB-resistance categories on EAB performance was explored for: EAB after 18 d on Swiss genotypes; EAB after 18 d on Scandinavian genotypes; or EAB after 45 d on Swiss genotypes. Models that included EAB performance after 45 d on Swiss genotypes did not include stem age as a model parameter since all trees were infested at stem sections of the same age. Moreover, the response variable 'larval instar' was determined as number of larvae that developed into the last (fourth) instar. Individual trees nested in experimental runs were included as random intercepts in all models.

The relationship between ADB lesion lengths and EAB performance was explored using only the data from the 18-d EAB bioassay on Swiss genotypes. Individual trees nested in EAB bioassay experimental runs were included as random intercepts in all models. To test the validity of our ADB-resistance field categories, we used a LM comparing lesion lengths between trees categorized as ADB-resistant and ADB-susceptible.

Finally, we analyzed whether differences in phloem chemistry between ADB-resistant and ADB-susceptible genotypes aligned with differences in genotype resistance against EAB (hypothesis iii; only the data from the 45-d EAB bioassay on Swiss genotypes). We first tested whether constitutive or EAB-induced levels of phenolics and terpenes that were quantified via the targeted approach differed between ADB-resistant and ADB-susceptible genotypes. We used LMMs with individual compounds as response variables and ADB-resistance categories (ADB-resistant vs ADB-susceptible) interacting with EAB damage treatments (EAB-infested vs uninfested) as well as genotype and rootstock provenance as fixed effects. Experimental runs were modeled as random intercepts. For compounds that were significantly affected by ADB resistance, we then explored the relationship between EAB performance parameters and plant compounds in LMMs and GLMMs with compound concentration, rootstock provenance, and stem cross-section area as fixed effects and trees nested in experimental runs as random intercepts.

We then compared the (untargeted) metabolomic phytochemical profiles of ADB-resistant and ADB-susceptible genotypes that were either infested or uninfested using PLS-DA. Zero or near-zero variance phytochemical features were removed. The remaining features were Pareto scaled for the analysis. The scores from the first two components of the PLS-DA confidence ellipse plots that separated ADB-resistant and ADB-susceptible trees infested with EAB were extracted. The scores were then used in LMMs and GLMMs with EAB performance parameters as response variables, scores, rootstock provenance, and stem cross-section area as fixed effects and trees nested in experimental runs as random intercepts. We applied sPLS-DA (Lê Cao *et al.*, 2011) to identify the metabolomic features that were most influential in explaining the phytochemical differences between ADB-resistant and ADB-susceptible trees that were infested with EAB. The most influential features were only identified for the first PLS-DA component, which discriminated resistant and susceptible trees much better than the second component. Feature importance was determined based on weight coefficients (plot loadings; Lê Cao & Welham, 2021). Sparse partial least squares regression (Lê Cao *et al.*, 2008), which can deal with highly collinear explanatory variables, was used to identify metabolomic features that were most strongly associated with variation in beetle dry weight. The relationship between beetle dry weight and feature peak intensities was explored in LMMs as described above.

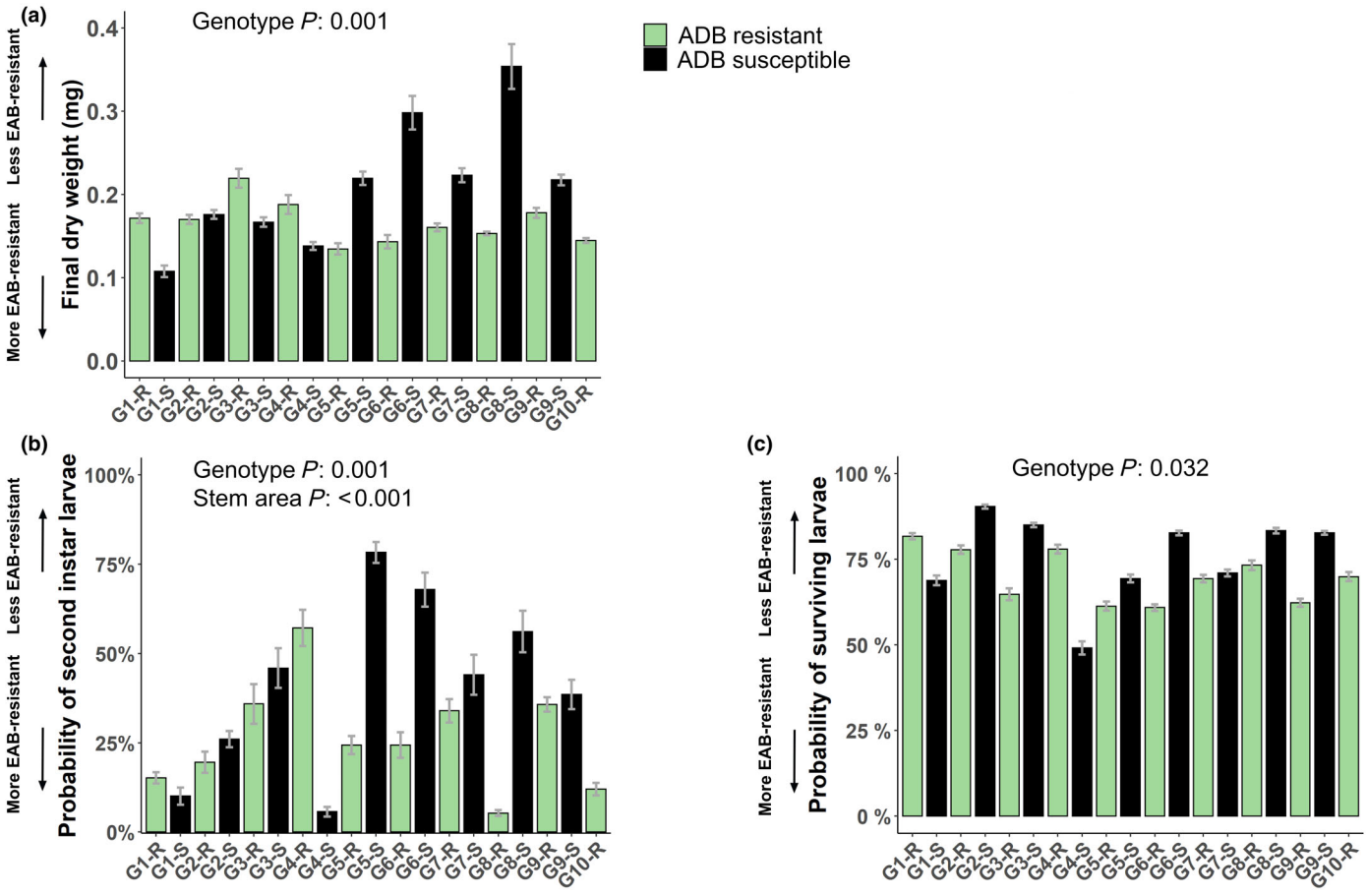

Fig. 1 Emerald ash borer (EAB) larval performance (predicted mean ± SE) on individual European ash genotypes from Switzerland with variable resistance to ash dieback (ADB). Relative ADB resistance was based on crown assessments in the field. Larval performance was either quantified as (a) final dry weight, (b) the probability of larvae reaching the second instar, or (c) the probability of larvae survival after feeding 18 d on ash trees. Only fixed effects with $P < 0.050$ are shown. *P*-values were calculated via a linear mixed effects model (dry weight) or generalized linear mixed effects models (larval instar, mortality) subjected to a type II analysis of variance or chi-squared tests. Statistics for all fixed effects are provided in Supporting Information Table S1.

Results

Ash intraspecific variation in EAB resistance

Emerald ash borer performance differed significantly among ash genotypes (Fig. 1; Table S1), supporting our hypothesis i. At the end of the 17/18-d bioassays, we observed up to a 250% difference in EAB larval dry weight among Swiss ash genotypes (Fig. 1a). Genotypic variability also affected larval developmental speed. The percentage of larvae that reached the second instar differed more than five-fold among ash genotypes (Fig. 1b). Finally, we observed up to a 38% difference in EAB survival among ash genotypes (Fig. 1c). Similar patterns were observed among ash genotypes from Scandinavia, with significant intraspecific variation in EAB resistance (Fig. S1). However, EAB larvae that fed on Scandinavian genotypes were on average 20% heavier than larvae on Swiss genotypes.

Impact of ash ADB resistance on EAB performance

Ash genotypes that were categorized as ADB-resistant possessed a higher resistance against EAB than genotypes of the category 'ADB-susceptible', supporting hypothesis ii. For Swiss genotypes, EAB showed better overall performance on ADB-susceptible genotypes than on genotypes classified as ADB-resistant. Emerald ash borer on ADB-susceptible genotypes were 39% heavier than those reared on resistant genotypes after 18 d on the trees (Fig. 2a; Table S2). Moreover, 19% more second-instar larvae were found on ADB-susceptible than on ADB-resistant genotypes at the end of the bioassays (Table S2; Fig. S2a). However, the survival of EAB was not significantly affected by ADB resistance (Table S2; Fig. S2d). Similarly, for Scandinavian genotypes EAB larval weights were up to 50% higher on ADB-susceptible than on ADB-resistant genotypes after 18 d (Fig. 2b). Neither the percentage of second-instar larvae nor EAB survival was affected by ash ADB resistance (Table S2; Fig. S2b,e). Our 45-d EAB bioassay confirmed the results of the 18-d bioassay and thus hypothesis ii. The average EAB larval dry weight was 42% higher on ADB-susceptible than on ADB-resistant ash genotypes after 45 d (Fig. 2c; Table S2). Although not significant, the number of fourth-instar larvae and the larval survival tended to be higher on ADB-susceptible trees (Table S2; Fig. S2c,f).

Swiss genotypes that were categorized as ADB-susceptible developed pathogen-induced stem lesions that were > 300%

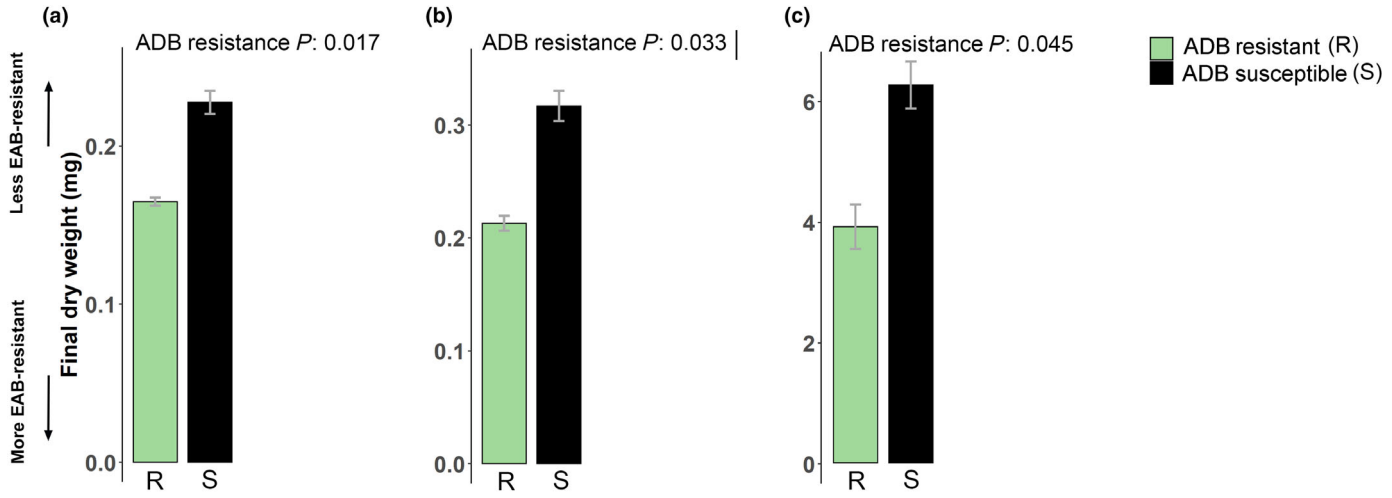

Fig. 2 Emerald ash borer (EAB) larval performance (predicted mean ± SE) on ash genotypes that either possess increased resistance to ash dieback (ADB) or are susceptible to ADB. Relative ADB resistance was based on crown assessments in the field. The average larval performance on ADB-susceptible or ADB-resistant genotypes was quantified as final dry weight averaged over (a) all ADB-susceptible and all ADB-resistant Swiss genotypes (18-d bioassay), over (b) all ADB-susceptible and all ADB-resistant Scandinavian genotypes (18-d bioassay) or (c) over three ADB-susceptible (G4-S, G6-S, and G8-S) and three ADB-resistant (G4-R, G6-R, and G8-R) Swiss genotypes (45-d bioassay). Only *P*-values of fixed effects with $P < 0.050$ are shown. *P*-values were calculated via linear mixed effects models subjected to type II analyses of variance. Statistics for all fixed effects are provided in Supporting Information Table S2.

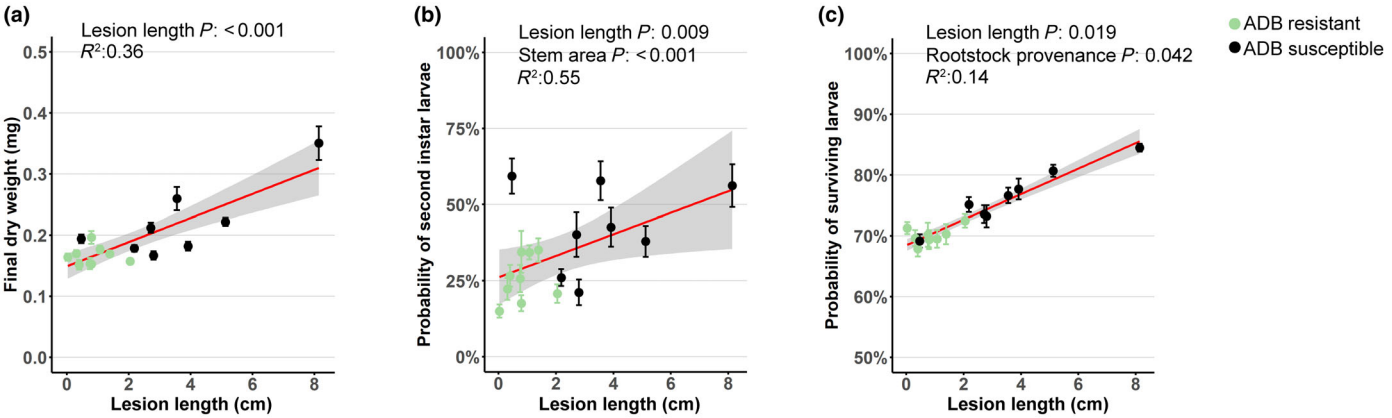

Fig. 3 Linear regression models, with 95% confidence band (gray area), of relationships between resistance to ash dieback (ADB) based on lesion lengths and emerald ash borer (EAB) larval performance quantified as (a) final dry weight; (b) the probability of larvae reaching the second instar; or (c) the probability of larval survival after feeding for 18 d on ash trees. Each point represents the predicted mean (±SE) of EAB and ADB performance of an ash genotype. Conditional R^2-values and *P*-values of fixed effects with $P < 0.05$ are shown. *P*-values were calculated via a linear mixed effects model (dry weight) or generalized linear mixed effects models (larval instar, mortality) subjected to a type II analysis of variance or chi-squared tests. Statistics for all fixed effects are provided in Supporting Information Table S3.

longer than on ADB-resistant genotypes, thereby confirming our ADB-resistance phenotypes (Fig. S3a,b). Importantly, genotypes that developed shorter stem lesions also hosted lighter EAB larvae, fewer larvae that reached the second instar and fewer larvae that survived (Fig. 3a–c; Table S3).

Phloem chemistry explains ADB-EAB cross-resistance

In our phytochemical analyses of six ash genotypes from Switzerland, we found that ash resistance to ADB and EAB was associated with specific constitutive and induced phytochemical phloem profiles. Our targeted approach examined compounds previously identified as involved in EAB or ADB resistance. We found that ADB resistance affected overall verbascoside levels with ADB-resistant ashes showing 16% higher verbascoside levels than ADB-susceptible ashes (Table 2; Fig. 4a). Verbascoside levels were also induced by EAB damage (Table 2; Fig. 4a). In addition, we observed that verbascoside was marginally (*F* value; $P = 0.086$), but negatively, associated with EAB dry weight (Fig. 4b). However, the negative relationship between verbascoside and EAB dry weight was largely driven by four ADB-susceptible trees with low verbascoside and high EBA-dry weight levels (Fig. 4b).

Calceolarioside and ligustroside levels did not differ between ADB-resistant and ADB-susceptible ashes (Table 2; Fig. S4a,c). Fraxin levels were slightly increased by EAB infestation in ADB-resistant but not in ADB-susceptible ashes (interaction between ash resistance category and damage treatment, Table 2; Fig. S4b). Oleuropein levels, however, were 28% lower in ADB-resistant

Table 2 Summary statistics (linear mixed effects models subjected to type III analyses of variance) on the effects of ash resistance (R) to ash dieback (ADB), emerald ash borer (EAB) larval damage (D), the interactions of both variables, ash genotype, and rootstock provenance on specialized metabolites in the ash phloem.

	ADB resistance (R) (crown assessment)		EAB damage (D)		R × D		Genotype		Rootstock provenance	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Verbascoside	4.40	0.041	79.86	< 0.001	1.54	0.221	1.82	0.140	1.95	0.173
Fraxin	4.62	0.036	3.48	0.068	4.79	0.033	6.70	< 0.001	6.65	0.014
Calceolarioside	0.685	0.170	48.58	< 0.001	0.47	0.496	2.31	0.071	2.99	0.093
Ligustroside	2.04	0.160	3.09	0.085	0.17	0.684	4.70	0.003	9.13	0.004
Oleuropein	6.96	0.011	1.59	0.213	2.05	0.159	4.30	0.005	0.92	0.342

The plotted verbascoside data are shown in Fig. 4(a). The data of the remaining compounds are shown in Supporting Information Fig. S4. ADB resistance was approximated based on crown assessments.

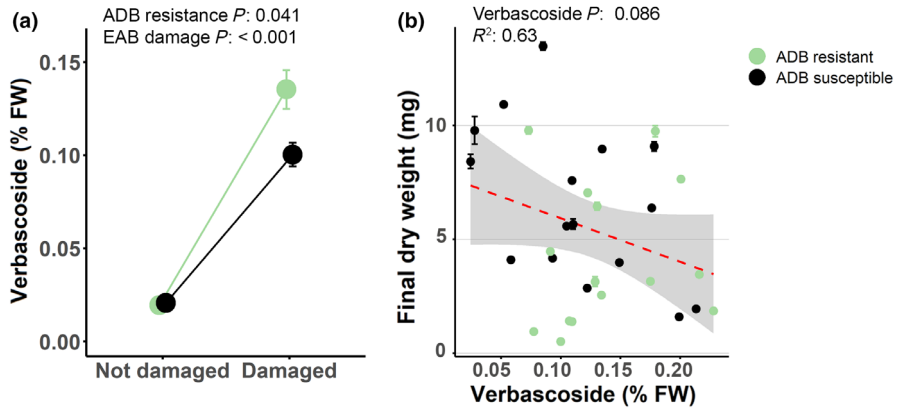

Fig. 4 Relationships between resistance to ash dieback (ADB), verbascoside levels, and emerald ash borer (EAB) performance in ADB-susceptible and ADB-resistant genotypes. (a) Predicted mean (±SE) effects of ADB resistance on EAB-induced verbascoside levels. *P*-values are only shown for effects with $P < 0.050$. *P*-values were calculated via a linear mixed effects model (LMM) subjected to a type III analysis of variance (ANOVA). *F*- and *P* statistics are provided in Table 2; (b) Linear regression model, with 95% confidence band (gray area), visualizing the relationship between verbascoside levels and EAB dry weight after feeding for 45 d. Each point represents the predicted mean (±SE) EAB dry weight for each replicate ADB-resistant and ADB-susceptible genotype. Conditional R^2-values and *P*-values of fixed effects with $P < 0.050$ are shown. *P*-values were calculated via a LMM subjected to type II ANOVA.

than in ADB-susceptible ashes (Table 2; Fig. S4d). Yet, neither fraxin nor oleuropein affected EAB performance (data not shown).

PLS-DA ellipsoid plots (95% CI) revealed that ADB-resistant and ADB-susceptible genotypes as well as EAB-infested and non-infested trees differed in their composite specialized phytochemical profiles, which supports hypothesis iii (Figs 5a, S5a,b). Phytochemical profiles of ADB-resistant and ADB-susceptible genotypes that were infested with EAB were discriminated along the first PLS-DA component axis, which explained 8% of the observed variance (Fig. 5a). Differences in EAB dry weight varied significantly with the first PLS-DA components scores that separated ADB-resistant and ADB-susceptible genotypes (Fig. 5b). Among > 5000 phytochemical features, *c.* 900 features were identified as being important for explaining the phytochemical differences between resistant and susceptible genotypes. Using sPLSR, followed by LMM, we identified 10 compounds that were significantly associated with variation in beetle dry weight (Fig. 5c; Table S4). The levels of six compounds were higher in ADB-susceptible genotypes and positively associated with EAB weight, whereas the levels of four compounds were higher in ADB-resistant ash and negatively associated with beetle weight (Fig. 5c).

Discussion

As the number of biological invasions continues to increase world-wide (Seebens *et al.*, 2017, 2018), native tree species in many regions have to cope with multiple non-native invasive pests and pathogens simultaneously. Yet, research documenting tree cross-resistance against multiple invasive organisms is rare and the potential causes and consequences of tree cross-resistance remain elusive. This study documents native tree cross-resistance against two highly invasive, non-native species and explores the relevance of intraspecific variation in ash phloem chemistry for mediating resistance against two invasive organisms.

Intraspecific variation is a well-documented key driver of plant stress adaptation (Petit & Hampe, 2006; Westerband *et al.*, 2021). For example, different genotypes from the same species of *Populus tremuloides* Michaux (Hemming & Lindroth, 1995; Eisenring *et al.*, 2023), *Brassica juncea* (Linnaeus) Czernohorsky (Qadir *et al.*, 2004), or *Zea mays* Linnaeus (Chen *et al.*, 2016;

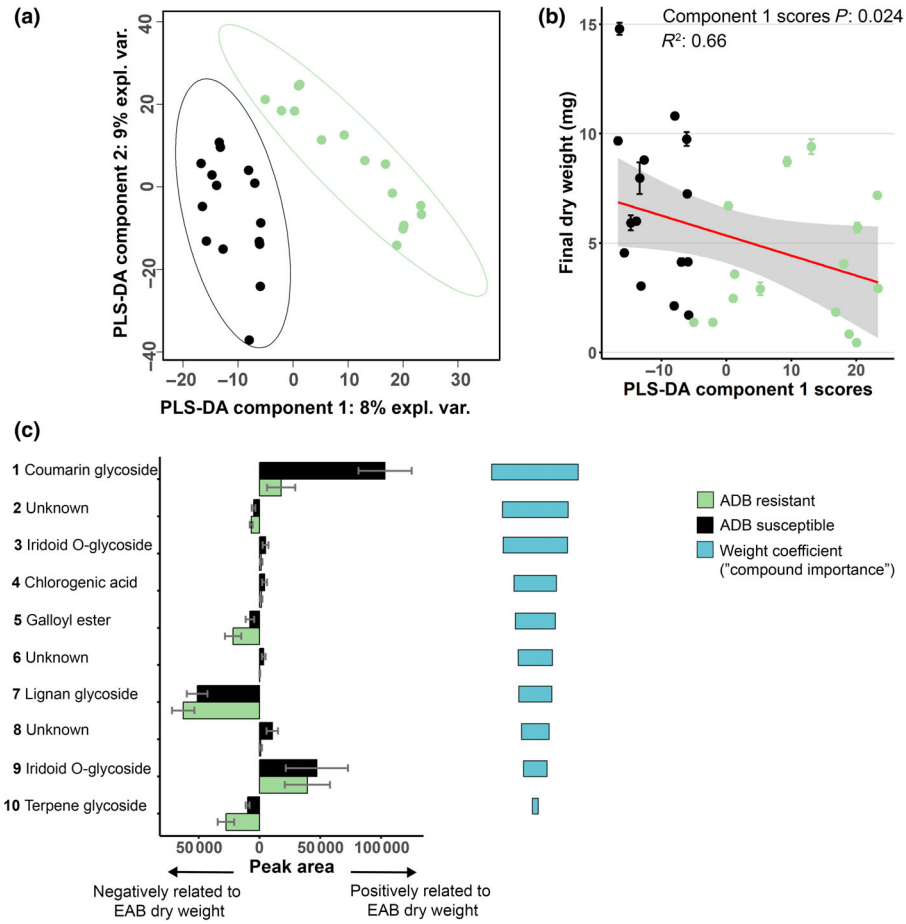

Fig. 5 Specialized phytochemical profiles of ash dieback (ADB)-resistant and ADB-susceptible ash genotypes and the effect of tree metabolomic differences on emerald ash borer (EAB) performance. (a) 95% confidence ellipse plots of a partial least squares discriminant analysis (PLS-DA) discriminating composite phytochemical profiles of ADB-resistant and ADB-susceptible trees induced by EAB attack. Each point represents the predicted mean (±SE) EAB dry weight for each replicate ADB-resistant and ADB-susceptible genotype; (b) Linear regression model, with 95% confidence band (gray area), visualizing the relationship between PLS-DA component 1 scores and EAB dry weight. Each point represents the predicted mean (±SE) of EAB dry weight for each replicate of an ADB-resistant or an ADB-susceptible genotype. The conditional R^2-value is shown. (c) Compounds quantified via untargeted metabolite analysis that are most strongly associated with variation in beetle dry weight (sparse partial least squares regression (sPLSR)) and that significantly ($P < 0.05$) affected beetle dry weight when tested in linear mixed effects models. Compounds are ordered by sPLSR weight coefficients ('compound importance'), which are visualized in blue. Compounds were annotated using CSI: FingerID and CANOPUS. Mass and retention times of each compound are provided in Supporting Information Table S4.

Yang *et al.*, 2019) show marked differences either in constitutive trait expression or in induced trait expression under environmental stress. In agreement with this precept, we found that different European ash genotypes varied considerably in EAB resistance (hypothesis i). Our results align with Koch *et al.* (2015), who showed that there were significant intraspecific differences in EAB resistance among genotypes of North American green ash.

A key finding of our study was that ash genotypes with a higher ADB resistance also possess an increased resistance against EAB (hypothesis ii). Importantly, the results were consistent across Swiss and Scandinavian genotypes and for early and late instar EAB larvae (Fig. 2). In our 18-d bioassays, EAB larvae were much heavier on Scandinavian than on Swiss genotypes (Fig. 2), suggesting that Scandinavian genotypes are more susceptible to EAB than Swiss provenances. However, since the experiments on Swiss and Scandinavian genotypes were conducted in two different years, the experimental eggs were produced by different female generations, so we cannot rule out maternal effects on EAB performance (Mousseau & Dingle, 1991). Intraspecific genotypic variation also explained the positive, albeit weak, relationship between ADB resistance quantified as lesion length and EAB performance (Fig. 3). However, EAB performed poorly on some ADB-susceptible genotypes (Fig. 3), which suggests that, as expected, there are exceptions to the general positive relationship between EAB and ADB resistance (Fig. 2). This finding indicates that multiple traits may contribute to EAB resistance in European ash. Some traits that confer increased ADB resistance also provide increased EAB resistance (Fig. 2), while other ADB-resistance traits might not be involved in EAB resistance. Genotypic variation in these latter traits may obscure the relationship between ADB resistance and EAB performance as shown in Fig. 3.

In alignment with hypothesis iii, we found that ADB-resistant and ADB-susceptible ash genotypes differed in the expression of constitutively and EAB-induced phytochemical traits (Figs 4a, 5a, S4, S5). In our targeted approach, verbascoside showed the most interesting pattern as it was stronger induced by EAB attack in ADB-resistant trees than in ADB-susceptible trees and tended to be negatively associated with EAB performance (Fig. 4). Our results align with Whitehill *et al.* (2014), who demonstrated that verbascoside is likely involved in the defense of several North American ash species against EAB. Moreover, larval survival and growth were reduced, in a dose-dependent manner, when feeding on artificial diet amended with verbascoside (Whitehill *et al.*, 2014). Together with the results of Whitehill *et al.* (2014), our findings suggest that verbascoside contributes, albeit weakly, to defense against EAB and to ADB-EAB cross-resistance in European ash.

In our untargeted approach, we found that ADB-resistant and ADB-susceptible trees can be discriminated based on their

constitutive specialized metabolome (Fig. 5a). However, the explained amount of variance along the most discriminant first axis component of the PLS-DA plot was < 10%. This finding indicates that differences in ADB-resistance classes are probably not the major source of variation among the different tree genotypes. Our untargeted approach allowed us to identify additional compounds that were associated with EAB performance (Fig. 5c) and that varied among resistant and susceptible genotypes. Many of these compounds were specialized metabolites that were positively related to EAB weight (Fig. 5c). Specialized metabolites are traditionally associated with protective functions against both biotic and environmental stress (Hartmann, 2007). However, such a classification falls short, as specialized metabolites are often multifunctional (Erb & Kliebenstein, 2020), and in several cases, have been shown to promote herbivore performance (Richards *et al.*, 2012; Marti *et al.*, 2013; Hu *et al.*, 2018). Overall, our targeted and untargeted phytochemical results indicate that rather than being determined by a few highly potent defense compounds, the ADB-EAB cross-resistance seems to be driven by multiple phytochemical traits from different chemical classes. Some traits provide enhanced resistance against EAB and occur at higher levels in ADB-resistant trees. Other traits stimulate EAB performance and occur at lower levels in ADB-resistant trees (Fig. 5c). The idea that EAB performance is determined by a suite of phytochemicals is further supported by the finding that EAB weight varied significantly with the scores of the first PLS-DA component separating composite phytochemical profiles between ADB-resistant and ADB-susceptible trees (Fig. 5b). Hence, composite phytochemistry rather than individual flagship defense compounds seem to drive ADB-EAB cross-resistance patterns, as shown in other systems (e.g. Wallis *et al.*, 2008; Sherwood & Bonello, 2013).

The alignment found between ADB and EAB resistance traits suggests that a large-scale promotion of ash genotypes with increased ADB resistance (Cleary *et al.*, 2020; Pike *et al.*, 2021), can help to future-proof ash populations for the imminent arrival of EAB in central Europe (Orlova-Bienkowskaja *et al.*, 2020). In addition, promotion of ADB-resistant genotypes may also support EAB management strategies in already infested regions. For example, reduced EAB larval weight as observed on our ADB-resistant ash genotypes may result in increased overwintering mortality (Leather *et al.*, 1995), and lighter adult females with reduced fecundity (Honěk, 1993), although this hypothesis remains to be tested. Furthermore, reduced EAB developmental rates may widen the window of opportunity for EAB biocontrol via parasitoids or for identifying and destroying EAB-infested trees.

To implement EAB-ADB cross-resistance-based management, it is crucial to identify and promote suitable ash genotypes in the field. Consequently, our study highlights the importance of efficient resistance screening approaches to rapidly detect ADB-resistant trees. These can include spectroscopy-based phenotyping (Villari *et al.*, 2018) or phenotyping via molecular markers (Harper *et al.*, 2016; Stocks *et al.*, 2019), particularly when combined with visual field monitoring (Menkis *et al.*, 2020). Our findings on ADB-EAB cross-resistance are important not only for ensuring the continued ecological functionality of European ash but also to promote the conservation of other ash species. For example, North American populations of *Fraxinus americana* Linnaeus and *F. pennsylvanica* have been severely depleted by the EAB and may face additional threats, such as ADB, or cottony ash psyillid (Wamonje *et al.*, 2022), if they were to be introduced in the future.

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Competing interests

None declared.

Author contributions

ME and MMG conceived the study. ME, MMG, AP-G, EB, and VQ designed the experiments. ME, AP-G, and EB performed the experiments. ME, GG, SKG, and PB performed chemical analyses. LRN, MC, and ML contributed ash genotypes. TL and ADR provided a continuous supply of emerald ash borer eggs. ME, MMG, SKG, and PB interpreted the data. ME wrote the first draft of the manuscript. All authors contributed to revisions.

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Data availability

Data on phytochemistry, emerald ash borer, and ash dieback performance are deposited at *EnviDat*: 10.16904/envidat.405.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Emerald ash borer larval performance on Scandinavian ash genotypes after 18 d on ash.

Fig. S2 Emerald ash borer larval performance (developmental speed and mortality) on Swiss and Scandinavian ash genotypes after 18 or 45 d on ash.

Fig. S3 Ash dieback lesion length measured on Swiss ash genotypes.

Fig. S4 Effects of ash dieback resistance and emerald ash borer damage on individual specialized metabolites.

Fig. S5 Confidence ellipse plots discriminating composite phytochemical profiles of ash-dieback resistant and susceptible trees and emerald ash borer-damaged and undamaged trees.

Methods S1 Analytical details phloem chemistry.

Table S1 Summary statistics on the effects of Swiss genotype, stem area, stem age, and rootstock provenance on emerald ash borer performance parameters.

Table S2 Summary statistics on the effects of ash dieback resistance approximated based on crown assessments, stem area, stem age, and rootstock provenance on emerald ash borer performance.

Table S3 Summary statistics on the effects of ash dieback resistance approximated based on lesion length, stem area, stem age, and rootstock provenance on emerald ash borer performance.

Table S4 Compounds quantified via untargeted specialized metabolite analysis that are most strongly associated with variation in beetle dry weight.

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