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Aboveground phytochemical responses to belowground herbivory in poplar trees and the consequence for leaf herbivore preference

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Abstract

Belowground (BG) herbivory can influence aboveground (AG) herbivore performance and food preference *via* changes in plant chemistry. Most evidence for this phenomenon derives from studies in herbaceous plants but studies in woody plants are scarce. Here we investigated whether and how BG herbivory on black poplar (*Populus nigra*) trees by *Melolontha melolontha* larvae influences the feeding preference of *Lymantria dispar* (gypsy moth) caterpillars. In a food choice assay, caterpillars preferred to feed on leaves from trees that had experienced attack by BG herbivores. Therefore, we investigated the effect of BG herbivory on the phytochemical composition of *P. nigra* trees alone and in combination with AG feeding by *L. dispar* caterpillars. BG herbivory did not increase systemic AG tree defences like volatile organic compounds, protease inhibitors and salicinoids. Jasmonates and salicylic acid were also not induced by BG herbivory in leaves but abscisic acid concentrations drastically increased together with proline and few other amino acids. Leaf coating experiments with amino acids suggest that proline might be responsible for the caterpillar feeding preference *via* presumptive phagostimulatory properties. This study shows that BG herbivory in poplar can modify the feeding preference of AG herbivores *via* phytochemical changes as a consequence of root-to-shoot signaling.

KEYWORDS

abscisic acid (ABA), belowground–aboveground interaction, induced resistance, *Lymantria dispar*, *Melolontha melolontha*, proline, Salicaceae, water stress

1 | INTRODUCTION

The role of belowground (BG) herbivory for aboveground (AG) plant defence chemistry and associated effects on insect herbivores and higher trophic levels has gained increasing attention (reviewed by Bezemer & van Dam, 2005; van Dam, 2009; van Dam & Heil, 2011). BG herbivory can shape AG arthropod community structure in individual plants (Johnson, Mitchell, McNicol, Thompson, & Karley, 2013) and influence plant species composition in more complex communities (Stein et al., 2010). Furthermore, BG herbivore damage affects growth

(Tsunoda, Kachi, & Suzuki, 2014) and the nutritional content of the AG plant tissues (Blossey & Hunt-Joshi, 2003). There is a rather general pattern in herbaceous species that BG herbivory positively affects AG piercing and sucking insects such as aphids and spider mites (Johnson et al., 2012 and references therein; Johnson et al., 2013; Hoysted et al., 2017; Kammerhofer et al., 2015), but negatively affects AG chewing insects (Bakhtiari, Glauser, & Rasmann, 2018; Bezemer, Wagenaar, Van Dam, & Wackers, 2003; Erb et al., 2011; van Dam, Raaijmakers, & van der Putten, 2005).

Plant chemical defence responses to insect attack are mediated by the hormones salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA). Although JA and ABA are often induced systemically in AG tissues following BG herbivory (Erb et al., 2011; Erb, Ton, Degenhardt, & Turlings, 2008; Soler, Erb, & Kaplan, 2013), SA plays a minor role in BG–AG interactions (Erb et al., 2009; Erb et al., 2011; Pieterse, Van der Does, Zamioudis, Leon-Reyes, & Van Wees, 2012; Soler et al., 2013). An increase in plant defence hormones following root herbivory can lead to the production of secondary metabolites such as glucosinolates, benzoxazinoids, phenolics and alkaloids in systemic AG tissues (Papadopoulou & van Dam, 2016 and references therein). This defence induction can ultimately affect the preference and performance of AG herbivores (Brown & Gange, 1990; Poveda, Steffan-Dewenter, Scheu, & Tscharntke, 2003; Rasmann & Turlings, 2007).

Primarily, herbaceous plants, and here, mostly crop species, have been studied in the context of BG–AG interactions and herbivore induced defences (reviewed by Papadopoulou & van Dam, 2016). In long-lived woody plants like trees, the consequence of BG herbivory on AG phytochemistry and insect herbivore preference and performance has so far attained only little attention (but see Huang, Siemann, Carrillo, & Ding, 2015; Li et al., 2016; Huang, Siemann, Xiao, Yang, & Ding, 2014). Differences in defence response to BG herbivory between herbaceous plants and trees are very likely, considering the life histories of these plants. Trees are long-lived and often large-sized, which makes them generally more apparent to insects than annual herbaceous plants with shorter life times (Lämke & Unsicker, 2018). Herbivorous insects with very short life cycles in comparison with trees can adapt to the tree defences within only a few generations. Hence, Haukioja and Koricheva (2000) argue that trees should exhibit more tolerance to insect herbivory than herbaceous plants due to different recovery potentials. Tree tolerance and resistance traits were so far mainly studied in response to AG herbivory, and our knowledge on the role of BG herbivory is scarce (Zvereva & Kozlov, 2012).

Here, we investigated BG–AG interactions in black poplar (*Populus nigra*). Trees within the genus *Populus* possess a large diversity of anti-herbivore defence compounds that are either constitutively present or induced upon insect herbivore attack (Philippe & Bohlmann, 2007). Salicinoids, a group of two-component phenolics unique to the Salicaceae, are repellent and/or toxic for specifically generalist insect herbivores (Boeckler, Gershenzon, & Unsicker, 2011; Boeckler, Paetz, Feibicke, Gershenzon, & Unsicker, 2016 and references therein). The results from studies investigating whether or not these constitutively present phenolics are also inducible by insect herbivores are inconsistent (e.g. Boeckler, Gershenzon, & Unsicker, 2013; Rubert-Nason, Couture, Major, Constabel, & Lindroth, 2015). Systemic induction of salicinoids upon insect herbivore feeding was reported in a few previous studies (Rubert-Nason et al., 2015; Stevens & Lindroth, 2005), and one study also investigated the effect of AG defoliation on root salicinoids (Stevens, Gusse, & Lindroth, 2014). However, to our knowledge, the role of BG herbivory on AG salicinoid patterns in poplar trees is unknown.

Poplar trees also induce a number of defence-related proteins such as polyphenol oxidases, endochitinases and Kunitz-type protease inhibitors (PIs) (Philippe & Bohlmann, 2007) upon insect feeding. Kunitz-type PIs are a well-studied group of defence-related proteins in poplars (Haruta, Major, Christopher, Patton, & Constabel, 2001; Major & Constabel, 2006; Talyzina & Ingvarsson, 2006), and genes of the Kunitz-type PI gene family are among the most highly upregulated genes after insect herbivory or methyl jasmonate treatment (Christopher, Miranda, Major, & Constabel, 2004; Major & Constabel, 2007). Simulated AG herbivory with methyl jasmonate resulted in root induction of the trypsin inhibitor gene *PtdTI3* suggesting systemic shoot-to-root signalling (Major & Constabel, 2007).

Additionally, poplar trees also produce a large number of volatile organic compounds (VOCs) specifically when they are under attack by herbivores (Arimura, Huber, & Bohlmann, 2004; Brilli et al., 2009; Clavijo Mccormick, Irmisch, et al., 2014). The constitutive and herbivore-induced emission of poplar VOCs, as well as their biosynthesis and their role in direct and indirect defence, have been intensively studied in the past (Clavijo McCormick, Boeckler, Köllner, Gershenzon, & Unsicker, 2014; Clavijo Mccormick, Irmisch, et al., 2014; Danner et al., 2011; Eberl, Hammerbacher, Gershenzon, & Unsicker, 2017; Irmisch, Jiang, Chen, Gershenzon, & Köllner, 2014; Lackus, Lackner, Gershenzon, Unsicker, & Köllner, 2018; Unsicker, Gershenzon, & Köllner, 2015). However, so far, no study investigated the consequences of BG herbivory on AG VOC emission in poplar.

Here, we studied the single and combined effects of AG and BG herbivory on the phytochemistry of young *P. nigra* trees and tested whether BG herbivory by larvae of the beetle *Melolontha melolontha* (cockchafer) has an effect on the feeding preference of generalist gypsy moth (*Lymantria dispar*) caterpillars. Under controlled laboratory conditions, we first investigated whether caterpillars discriminate between black poplar leaves from root-infested (BG herbivory) *versus* non-infested trees. Due to a significant preference of *L. dispar* for leaves from BG infested trees, we then investigated the phytochemical profiles of leaves from trees that experienced (a) BG herbivory by *M. melolontha*, (b) AG herbivory by *L. dispar* caterpillars, (c) a combination of BG and AG herbivory and (d) no herbivory (controls). We measured volatile organic compounds, salicinoids, protease inhibitor activity, defence hormones, free sugars and free amino acids to elucidate which of these primary and secondary metabolites could be responsible for caterpillar food preference.

Our results show that BG herbivory alone did not induce major defences such as VOCs, salicinoids or protease inhibitor activity in AG tissues of *P. nigra*. Upon AG caterpillar damage, leaves responded, as previously described, with an induction of major volatile groups and protease inhibitor activity. Although there was no induction of

defence compounds by BG herbivory alone, beetle larvae feeding substantially increased the amount of ABA and proline in *P. nigra* leaves. A food choice assay in which we offered leaf discs of undamaged trees, that were either coated or uncoated with different amino acids, to *L. dispar* caterpillars suggests, that the originally observed preference for leaves of BG infested trees is most likely due to higher proline concentrations.

2 | MATERIAL AND METHODS

2.1 | Plants and insects

Populus nigra (black poplar) trees were cultivated from cuttings of one genotype growing in a common garden near Jena, Germany. Sixty trees were rooted in 2 L pots filled with pure sand and maintained in the greenhouse under summer conditions (24 °C, 60% relative humidity, 16 h/8 h light cycle) for 4 months until the start of the experiment. By then, the trees were around 1 m tall. Twenty-four hours before the experiment started, the trees were acclimatized in a climate chamber (humidity: 60%, day/night temperature: 20 °C/16 °C; 16 h light). Due to limited space, the experiment was split in three blocks (three time points) with 20 trees in each, consisting of an equal number of replicates for each treatment. Time between the start of the experiments in the first block and the third block was 4 weeks.

Cockchafer (*Melolontha melolontha*) larvae (grubs), collected from meadows in Germany and Switzerland (Huber et al., 2016), were reared individually in 200 mL plastic cups filled with a mix of potting soil and grated carrots and kept in a wine cooler at 13 °C and 70% humidity. Gypsy moth (*Lymantria dispar*) caterpillars were hatched from egg batches and reared on artificial wheat germ diet (MP Biomedical, Eschwege, Germany) in a climate chamber (25 °C, 60% humidity, 14:10 L:D period) until they entered the experiment in 4th instar.

2.2 | Experimental root and shoot herbivory

The 60 trees used for this experiment were split in three blocks with 20 individuals each. Half of the 20 trees in each block were induced with one *M. melolontha* larva (grub). The grub was allowed to feed on the roots for 6 d. After 4 d, six 4th instar *L. dispar* caterpillars were released AG on 10 of 20 trees and allowed to feed for 40 h. Each experimental block thus consisted of five replicates of four different treatments: non-damaged trees (control), trees with BG herbivory by *M. melolontha* (grub), trees with AG *L. dispar* caterpillar infestation (caterpillar) and trees with combined AG–BG infestation by both herbivores (grub + caterpillar; Figure S1 in the Supporting Information).

2.3 | Food choice experiments with *Lymantria dispar* caterpillars

To investigate whether *L. dispar* caterpillars discriminate between leaves of trees previously infested by *M. melolontha* BG and non-infested control trees, food choice experiments were performed in modified 90 mm Petri dishes (Figure 1b). Four leaf discs (16 mm in diameter) of each of the two treatments (grub and control) were alternately stuck on pins glued to the Petri dish equidistantly 3 cm around the middle of the dish. One 2nd instar *L. dispar* caterpillar (starved overnight) was then released in the centre of the Petri dish and allowed to feed for 24 h. Altogether, 20 caterpillars were tested in each treatment. Thereafter, leaf discs were photographed and leaf area loss was determined by reconstructing the leaf blades with Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

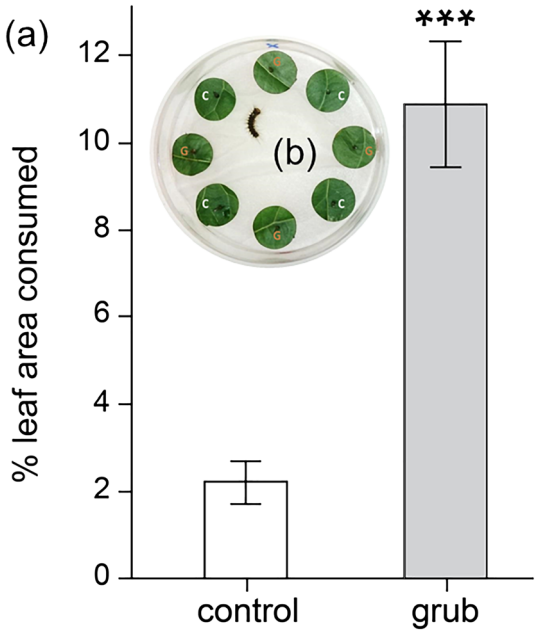

FIGURE 1 (a) Damage caused by *Lymantria dispar* caterpillars to black poplar leaf discs after belowground damage by one *Melolontha melolontha* larva (grub) compared with non-damaged control plants (control). Asterisks indicate significant differences between treatments based on a related-samples Wilcoxon signed rank test (W-value = 3.921; $p \leq 0.001$). Bars represent means ± SEM; $n = 20$. (b) Arena used for caterpillar food choice assays. A 90 mm petri dish was modified with office pins and padded with moist filter paper. The cut leaf discs (16 mm in diameter) were pushed on the pins in an alternating fashion. Afterwards, one caterpillar per petri dish was released in arena for 1 d

2.4 | Volatile collection and analysis

Six days after the onset of the experiment (6 d of grub feeding and 40 hours of caterpillar feeding), PET bags were installed on the poplar foliage and volatiles in the headspace were collected for 4 h with Poropak traps (Alltech, Florida, USA), as described in (Clavijo McCormick, Irmisch, et al., 2014). After the volatile collections, traps were eluted twice with 100 µL dichlormethane, containing an internal standard (nonyl acetate, concentration, 10 ng × μL^{-1}; Sigma Aldrich, Seelze, Germany). For identification of compounds, 2 µL of the eluate

was injected splitless into a gas chromatograph (GC; 6890 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 250 µm × 0.25 µm DB5-MS column (Wicom GmbH, Heppenheim, Germany) coupled to a quadrupole mass spectrometer (MS; 5973 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA; in short, GC/MS). The injector was held at 230 °C with helium used as carrier gas at 1 mL/min. The oven temperature of the GC/MS was held at 50 °C for 3 min after injection and then heated up to 95 °C at a rate of 4 °C/min. Afterwards, the oven temperature was increased to 145 °C with a 15 °C/min gradient and then to 180 °C with a 10 °C/min gradient. Finally, the oven temperature was kept stable for 3 min at 300 °C. Mass spectra were recorded (transfer line temperature: 230 °C, source temperature: 230 °C, quadrupole temperature: 150 °C, ionization energy: 70 eV, mass range: 40–500 m/z). Compounds were identified by comparing their mass spectra with authentic standards and three libraries (Wiley275, NIST, ADAMS). For quantification, the samples were separated with the same GC method as described above with hydrogen as the carrier gas. Afterwards, the samples were analysed with a flame ionization detector (9200 Hydrogen detector, Packard, Agilent Technologies, Santa Clara, CA, USA) operating at 300 °C. Absolute amounts of all compounds were calculated based on the relation of their flame ionization detector peak area and the area of the internal standard according to the 'effective carbon number (ECN) concept' (Scanlon & Willis, 1985).

2.5 | Leaf harvest

Right after volatile collection, leaves of each tree were cut and photographed to determine leaf area and experimental leaf area loss by caterpillar herbivory. Then leaf midribs were cut and discarded. Pooled leaf halves were transferred to 5 mL vials and then immediately flash-frozen in liquid nitrogen. After lyophilization, the samples were stored at −20 °C until further analysis.

2.6 | Protease inhibitor analysis via radial diffusion assay

10 mg ground leaf material was extracted with 400 µL HEPES-buffer (25 mM, pH 7.2, containing 3% PVPP, 2% PVP, 0.8 Triton X100 and 1 mM EDTA). A 3 mm metal ball was added to the extracts. Then extracts were shaken for 8 min in a paint shaker before centrifugation for 10 min at maximum speed, at 4 °C. The supernatant was used for analysis.

To identify PI activity, a radial diffusion assay was performed (modified from (Jongsma, Bakker, & Stiekema, 1993; Van Dam, Horn, Mareš, & Baldwin, 2001). A 1.8 % plant agar gel was prepared with HEPES-KOH (25 mM, pH 7.2) buffer containing 2 µL trypsin (0.2 mg/mL) per mL gel. With a 4 mm diameter cork borer, wells were punched in the gel 2 cm apart. The wells were filled with leaf extracts or a standard trypsin inhibitor from soybean (Sigma Aldrich, Seelze, Germany). The loaded gel was incubated for 22 h at 4 °C. After incubation, the gel was washed once with HEPES-KOH buffer (25 mM, pH 7.2, containing 10 mM $CaCl_2$) and stained with a freshly prepared staining solution [72 mg Fast Blue B Salt in 90 mL HEPES-KOH, 25 mM (pH 7.2) combined with 60 mg N-acetyl-DL-phenylalanine β-naphthyl ester (Sigma Aldrich, Seelze, Germany) in 10 mL DMF]. Gel and staining solution were incubated for 30 to 90 min at 37 °C. When a sufficient colouring was achieved, the staining solution was poured off; the gel was rinsed with water and then photographed for later analysis with Adobe Photoshop (San José, CA, USA). Levels of trypsin inhibition were calculated based on comparisons with standard inhibitor activity. A standard Bradford assay (Biorad, Hercules, CA, USA) was performed to calculate protein content in each sample.

2.7 | Salicinoid analysis

Phenolic compounds were extracted in parallel to phytohormones (see below). As internal standard, 0.8 mg/mL phenyl-β-glucopyranoside was added additionally to the internal phytohormone standards. The raw extracts of 2 × 200 µL separated during phytohormone extraction were combined and 400 µL of Milli-Q H_2O was added before measuring the analytes using high-performance liquid chromatography–ultraviolet detection (HPLC–UV). 20 µL analyte was injected onto a chromatographic column (EC 250 × 4.6 mm NUCLEODUR Sphinx RP, 5 µm, Macherey Nagel, Germany) connected to a pre-column (C18, 5 µm, 4 × 3 mm, Phenomenex, USA). The mobile phases consisting of two solvents, solvent A (Milli-Q H_2O) and solvent B (acetonitrile), were run with solvent B in gradient mode. The time/concentration (min/%) of the gradient was set to 0/14; 22.00/58; 22.10/100; 25.00/100; 25.10/14; 30.00/14 with a constant flow rate of 1 mL/min. The column oven temperature was set to 25 °C. The signal was detected with Photo Diode Array (PDA) and Evaporative Light Scatter (ELSD) detectors (Varian, USA). Using these settings, the retention times (RT) of the compounds of interest were: 5.10 min (salicin), 10.20 min (salicortin) and 15.20 min (homaloside D). One more unidentified salicinoid with a retention time at 18.50 min was isolated for subsequent structure elucidation by nuclear magnetic resonance spectroscopy (NMR). NMR spectral data were acquired using an Avance III HD 500 MHz spectrometer equipped with a 5 mm TCI cryoprobe (Bruker Biospin, Rheinstetten, Germany). Data aquisition and processing was accomplished using Bruker TopSpin software suite, ver. 3.5. Standard pulse programmes as implemented in TopSpin were used. The NMR-sample was measured in MeOH-d_4.

2.8 | Phytohormone and free sugar analysis

Phytohormones were extracted from 10 mg of freeze-dried *P. nigra* leaf material. 1 mL of methanol (MeOH) containing internal standards of labelled phytohormones [40 ng/mL JA (D2-JA), ABA (D6-ABA), SA (D4-SA), and 8 ng/mL JA-13C6-Ile] was added to each sample in a 96-

well plate (Mironic, Lelystad, the Netherlands). Afterwards, the suspension was homogenized by shaking in a paint shaker together with one steel ball for 1 min before centrifuging it at 2.057 g for 1 min. 400 µL of the supernatant was taken out and transferred to a new 96-well plate. The remaining pellet was suspended with another 1 mL of MeOH without internal standards, and the procedure (shaking and centrifuging) was repeated. Again, 400 µL were taken out and combined with the first 400 µL extract to retrieve in sum 800 µL of extract per sample. Phytohormones were analysed via HPLC (Agilent 1100 Varian ELSD, Varian, USA) coupled to a MS/MS system (API 5000 LC/MS/MS System, AB Sciex, USA). The analytes were injected onto a chromatographic column (XDB-C18, 1.8 µm, 4.6 × 50 mm, Agilent, USA) connected to a pre-column (C18, 5 µm, 4 × 3 mm, Phenomenex). The injection volume was set to 2 µL. Two solvents, solvent A (0.05% formic acid in H_2O) and solvent B (acetonitrile) were used. Solvent B was injected in a gradient mode driving the following gradient (time in min/concentration of solvent B in %): 0.00/5, 0.50/5, 9.50/58, 9.52/100, 11.00/100, 11.10/5 and 14.00/5. The constant flow rate was set to 1100 µL/min. The temperature of the column oven was set to 25 °C.

Phytohormones were ionized in negative electrospray ionization mode, and chromatograms were analysed using the software Analyst 1.6 (AB Sciex, MA, USA). The peak integration was performed automatically by the software after adjusting the peak areas manually. The quantification was realized by comparing the peak areas of the samples with the peak area of the internal standards. Soluble sugars were measured from the same raw extracts, but diluted 1:10, that were used for phytohormone analysis as described in (Madsen et al., 2015).

2.9 | Amino acid analysis

Free amino acids were analysed from the same raw extracts as used for phytohormone analysis. The raw extracts were diluted 1:10 with water containing an isotopically labelled amino acid mix (^{13}C, ^{15}N-labelled amino acid mix at a concentration of 10 µg of the mix per mL; from Isotec, Miamisburg, OH, USA). The extracts were measured with HPLC (Agilent 1200, Agilent Technologies, Waldbronn, Germany) coupled to a triple-quadrupole mass spectrometer (API 5000 LC/MS/MS System, AB Sciex, USA). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 mm × 4.6 mm, 1.8 µm, Agilent Technologies, Germany). Two solvents, solvent A (H_2O) and solvent B (acetonitrile), were used. Solvent B was injected in a gradient mode driving the following gradient (time in min/concentration of solvent B in %): 0.00/3, 1.00/3, 2.70/100, 3.00/100, 3.10/3, 6.00/3. The constant flow rate was set to 1100 µL/min. The temperature of the column oven was set to 25 °C. Analytes were ionized in negative electrospray ionization mode. Multiple reactions monitoring (MRM) was used to monitor analyte parent ion → product ion: MRMs were chosen as in (Jander et al., 2004) except for Arg (*m/z* 175 → 70) and Lys (*m/z* 147 → 84). Analyst 1.5 software (AB Sciex, Darmstadt, Germany) was used for data acquisition and processing. Individual amino acids in the sample were quantified by the respective ^{13}C, ^{15}N-labelled amino acid internal standard, except for tryptophan and asparagine: tryptophan was quantified using ^{13}C, ^{15}N-Phe applying a response factor of 0.42 and asparagine was quantified using ^{13}C, ^{15}N-Asp applying a response factor of 1.0.

2.10 | Caterpillar food choice with enhanced amino acid concentrations

To experimentally enhance the concentration of selected amino acids in poplar leaves, 20 µL of an amino acid-ethanol solution was pipetted onto 16 mm leaf discs and allowed to dry before the discs were offered to the caterpillars (method adapted from Ximénez-Embún, Ortego, & Castañera, 2016).

In a first preference assay where only proline was tested, the concentration applied to the leaf surfaces was based on mean *in planta* leaf proline concentration measured after 6 d of root herbivory by one *M. melolontha* grub (~500 nmol/g or 14.39 µg/g fresh weight). Control leaf discs were treated with ethanol only. Proline-coated leaf discs and control leaf discs were placed in Petri dish arenas and a food choice experiment with 3rd instar *L. dispar* caterpillars feeding for 48 h was performed as described above (Figure 1 b) with the exception that the caterpillars were not starved prior to the experiment.

In a second assay, proline and three additional amino acids, leucine, alanine, and tryptophan, were tested in the same way. Here, amino acid concentrations applied to the leaf surfaces were based on the medians of *in planta* amino acid concentrations measured after 6 d of root herbivory by one *M. melolontha* grub (Table S3). Alanine was pre-dissolved in a 0.05 formic acid solution as it was not soluble in ethanol. To evaluate whether the amino acid concentrations in the artificially coated leaf discs yielded the desired increase, bulk samples of five leaf discs were freeze-dried, extracted and amino acid concentrations were analysed as described above.

2.11 | Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS, Chicago, IL, USA). All data were checked for statistical assumptions such as normal distribution and heterogeneity of variances. Whenever necessary, log transformation (analyte concentration data) or *arcus sinus* transformation (percent leaf area loss) was applied. In case of simple two group comparisons, *t* tests, Mann–Whitney U tests or related samples Wilcoxon rank tests were performed. ANOVA followed by Tuckey *post hoc* comparison or Welch-ANOVA followed by Games–Howell *post hoc* testing was performed in case of normally distributed data with homogeneous variances. In case of non-parametric data, Kruskal–Wallis tests followed by Dunn's *post hoc* tests were carried out.

3 | RESULTS AND DISCUSSION

3.1 | *Lymantria dispar* caterpillars prefer leaves from trees that experienced belowground herbivory

In a food choice assay, *L. dispar* caterpillars preferred to feed on leaf discs from *P. nigra* trees that were previously infested BG with a *M. melolontha* larva over discs from non-damaged control trees (Mann–Whitney U test: U = 4.021 $p \leq 0.001$, Figure 1a). A positive effect of BG herbivory on AG insect herbivore preference has been observed in recent studies in herbaceous plant species (Huberty & Denno, 2004; Soler, Bezemer, Van Der Putten, Vet, & Harvey, 2005), mostly for sucking insects (Hol et al., 2013; Hoysted et al., 2017; Kutyniok & Muller, 2012). However, in the majority of recent studies on interactions between chewing insects and crops, BG feeding negatively affected AG herbivores *via* systemically induced defence induction (Anderson, Sadek, & Wackers, 2011; Bakhtiari et al., 2018; Johnson et al., 2012).

To understand the molecular mechanisms underlying the preference of *L. dispar* caterpillars for black poplar leaves from BG infested trees, we characterized and compared the leaf phytochemistry including volatile emission, protease inhibitor activity and salicinoid accumulation in uninfested trees, in trees infested either BG with one *M. melolontha* larva or AG with *L. dispar* caterpillars, and in trees infested with both herbivores.

3.2 | BG herbivory did not induce AG VOC emission in *Populus nigra* trees

We identified 44 different VOCs in the headspace of *P. nigra* trees in the four experimental treatments (Table S1). These VOCs fall into six major groups, namely, green leaf volatiles, monoterpenes, sesquiterpenes, aromatic compounds, nitrogenous compounds and the homoterpene (*E*)-4,8-Dimethyl-1,3,7-nonatriene (*E*-DMNT; Figure 2 and Table 1). The emission of green leaf volatiles, monoterpenes, aromatic and nitrogenous compounds and *E*-DMNT significantly increased after AG caterpillar feeding and after combined BG–AG

TABLE 1 Statistical results of the Kruskal Wallis test for VOCs, phytohormones and salicinoids in black poplar leaves after belowground damage by a cockchafer grub, aboveground damage by gypsy moth caterpillars and a combination of both herbivores compared with non-damaged control plants

Analyte	H	*p*
GLV	**32.281**	**<0.001**
MT	**13.077**	**0.004**
ST	7.311	0.63
E-DMNT	**39.009**	**<0.001**
Aromatics	**21.707**	**<0.001**
Nitrogenous	**40.076**	**<0.001**
SA[a]	1.352	0.717
ABA	**22.04**	**≤0.001**
Jasmonates	**33.802**	**≤0.001**
Salicin	4.722	0.193
Salicortin	5.313	0.15
Homaloside D	**10.825**	**0.013**
6′-*O*-benzoylsalicortin	5.753	0.124

Note. Bold values indicate statistically significant differences; *n* = 12–15.

Abbreviations: ABA, abscisic acid; GLV, green leaf volatiles; MT, monoterpenes; ST, sesquiterpenes; SA, salicylic acid.

[a]One outlier in the grub treatment was removed from the dataset.

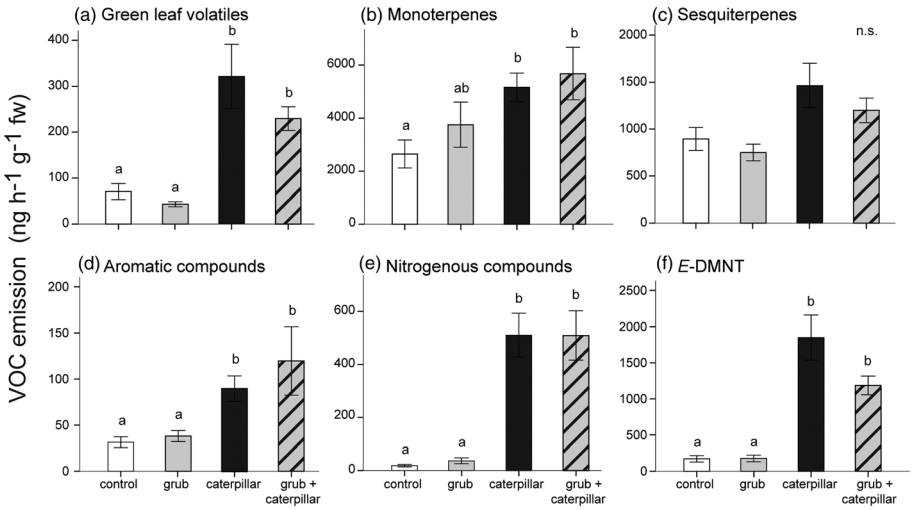

FIGURE 2 (a) Green leaf volatile, (b) monoterpene, (c) sesquiterpene, (d) aromatic compound, (e) nitrogenous compound and (f) *E*-DMNT emissions from black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar), and a combination of both herbivores (grub + caterpillar) compared with non-damaged control plants (control). Different letters indicate significant differences between treatments based on a Kruskal–Wallis test (a: $p < 0.001$, b: $p = 0.004$, c: $p = 0.63$, d: $p < 0.001$, e: $p < 0.001$ and f: $p < 0.001$) with Dunn's post hoc test. Details of the statistical results are shown in Table 1. Bars represent means ± SEM; *n* = 13–15

attack (Figure 2 and Table 1). There was also a trend for an increase in sesquiterpene emission upon caterpillar feeding alone and in combination with BG herbivory; however, this was non-significant (Figure 2c).

BG herbivory by *M. melolontha* alone did not induce systemic AG VOC emission (Figure 2). In a few recent studies in maize, apple and *Brassica* species, BG herbivory led to an induction of AG volatiles (Abraham, Giacomuzzi, & Angeli, 2015; Neveu, Grandgirard, Nenon, & Cortesero, 2002; Rasmann et al., 2005; Soler et al., 2007), but there are also examples where BG herbivory did not induce AG VOCs (Rasmann & Turlings, 2007; review by Papadopoulou & van Dam, 2016 and references therein). When *P. nigra* was attacked by BG and AG herbivores, the emission of green leaf volatiles, sesquiterpenes and DMNT was slightly reduced (non-significantly; Figure 2). Numerous studies in the past have shown that green leaf volatiles, DMNT and sesquiterpenes function as attractants for higher trophic-level predators and parasitoids (Brodmann et al., 2008; Kappers et al., 2005; Schnee et al., 2006). A reduction in VOC emission by BG herbivory could negatively affect the indirect tree defence properties, as this was shown for a number of herbaceous plants (e.g. Bezemer & van Dam, 2005; Holopainen & Gershenzon, 2010; Tariq, Wright, Bruce, & Staley, 2013). We argue, however, that BG feeding by *M. melolontha* would not alter the efficacy of *L. dispar* parasitoids in finding their hosts because the most important volatiles for parasitoid attraction in poplar, namely, nitrogenous compounds (Clavijo McCormick, Boeckler, et al., 2014), are not affected by BG feeding (Figure 2e).

The VOC emission patterns from *P. nigra* did not explain the observed *L. dispar* caterpillar preference for leaves from BG-infested trees. We therefore measured protease inhibitor activity, as these are considered efficient anti-herbivore poplar defences.

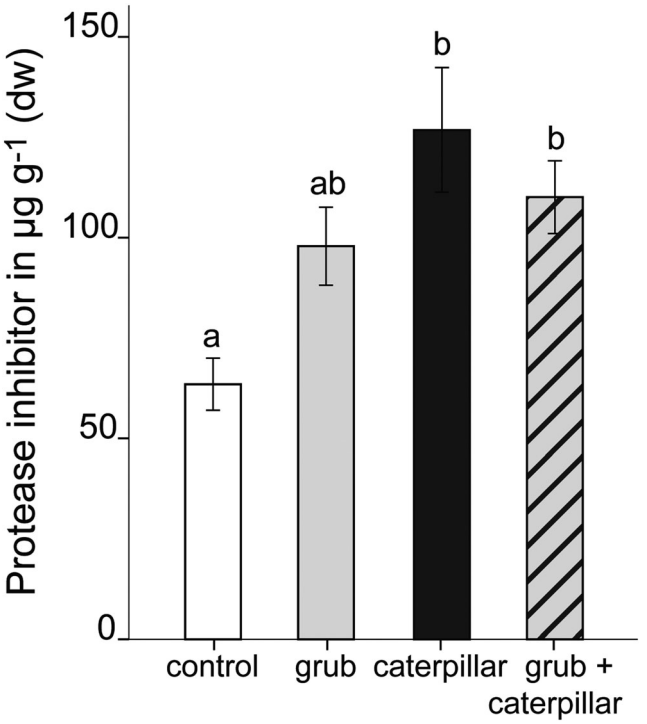

FIGURE 3 Protease inhibitor concentration in black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar) and a combination of both herbivores (grub + caterpillar) compared with non-damaged control plants (control). Different letters indicate significant differences between treatments based on an ANOVA ($F_{3, 53} = 9.187$, $p < 0.001$) with Tuckey's post hoc test. The data was log transformed before all analyses. Bars represent means ± SEM; $n = 13–15$

3.3 | AG but not BG herbivory increases leaf protease inhibitor activity

L. dispar caterpillar feeding significantly increased the trypsin inhibitor activity in leaves of *P. nigra* (Figure 3). The activity increase in caterpillar-infested leaves was more than two-fold in comparison with that in non-infested control trees. PIs are efficient defence proteins against insect herbivores (Howe & Jander, 2008; Zhu-Salzman & Zeng, 2015). In the artificial diet of *Eurygaster integriceps*, PIs had a negative impact on nymph developmental time, adult weight and survival (Saadati & Bandani, 2011). The consumption of artificial diet containing only 1% soybean trypsin inhibitor (of total dietary protein) resulted in a 40% reduction of trypsin activity in the guts of this hemipteran pest (Saadati & Bandani, 2011). Major and Constabel (2006) showed that the PI genes in hybrid poplar (*Populus trichocarpa x P. deltoids*) belong to the most strongly induced genes after mechanical wounding and insect herbivory. Kunitz-type PIs in *Populus trichocarpa x P. deltoides* strongly inhibited proteases in mid-gut extracts of the lepidopteran generalist *Malacosoma disstria* (Major & Constabel, 2008). Recent studies in tomato and maize report a systemic induction of PI activity upon BG herbivory (Arce et al., 2017; Erb et al., 2011). Although non-significant, we observed a trend for slightly higher PI activity in leaves of *P nigra* trees that were infested BG with *M. melolontha* larvae (Figure 3). Future studies will reveal whether such slight induction in PI activity by BG herbivory is detrimental for leaf chewing insects in poplar trees.

Because the PI activity was not significantly higher after BG herbivory, it cannot serve as an explanation for the previously observed *L. dispar* caterpillar preference for leaves from BG infested trees. It is also still unclear whether *L. dispar* caterpillars are able to taste the presence of PIs.

3.4 | Leaf salicinoid concentrations in *P. nigra* were slightly affected by BG and AG herbivory

Concentrations of four different salicinoids in *P. nigra* leaves namely salicin, salicortin, homaloside D and 6'-*O*-benzoylsalicortin were measured. The latter compound is first described here as a salicinoid in *P. nigra*. Details on the NMR identification of 6'-*O*-benzoylsalicortin are given in the Supporting Information (Figures S4–S8). Among all salicinoids, salicortin was the most abundant compound (Figure 4b).

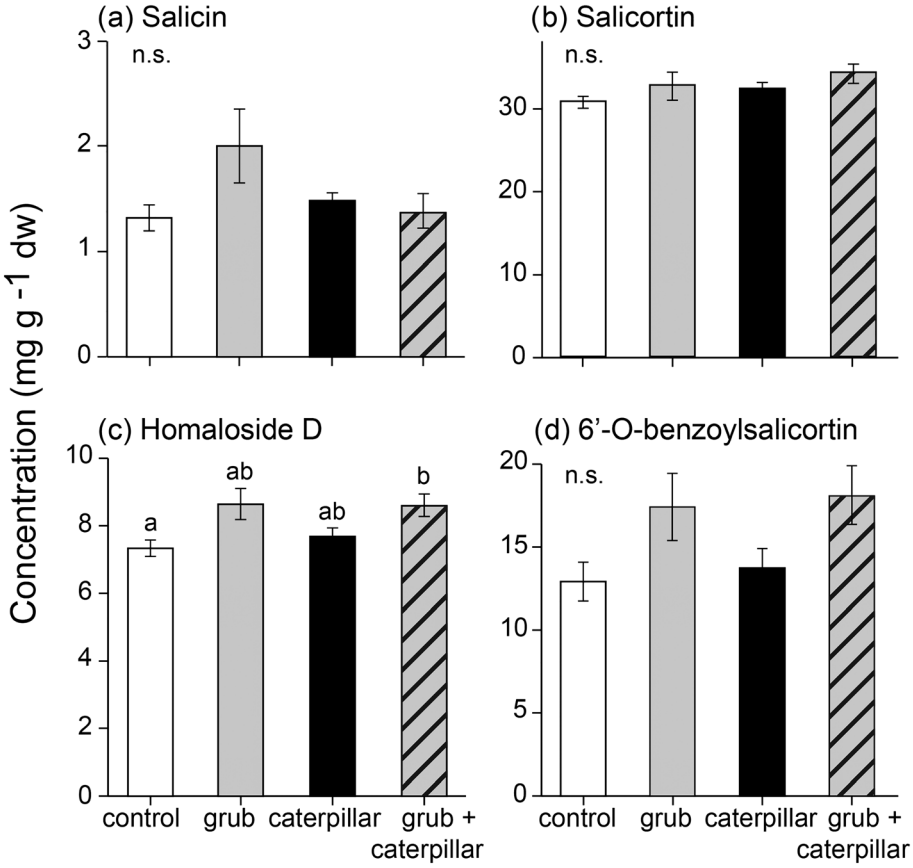

FIGURE 4 (a) Salicin, (b) salicortin, (c) homaloside D and (d) 6'-*O*-benzoylsalicortin concentrations in black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar) and a combination of both herbivores (grub + caterpillar) compared with non-damaged control plants (control). Different letters indicate significant differences between treatments based on a Kruskal–Wallis test (a: $p = 0.193$, b: $p = 0.15$, c: $p = 0.013$, d: $p = 0.124$) with Dunn's post hoc test. Details of the statistical results are shown in Table 1. Bars represent means ± SEM; $n = 13–15$

Salicin, salicortin and 6'-*O*-benzoylsalicortin concentrations were not affected by any treatment. However, there was a trend for an increase in foliar salicin concentrations in the grub treatment (Figure 4a). 6'-*O*-benzoylsalicortin shows a similar trend and additionally an increase after combined BG–AG attack. Details of the statistical results are shown in Table 1. Homaloside D concentrations significantly differed between the four treatments and *post hoc* comparisons revealed that the combined AG and BG herbivore treatment (grub + caterpillar) significantly differed from the control trees but not from the grub and caterpillar treatments (Figure 4c). AG herbivory by *L. dispar* did not lead to an increase in salicinoid levels in damaged leaves. This is coherent to the findings of Boeckler et al. (2013). However, Rubert-Nason et al. (2015) reported an induction of salicinoids in *P. tremuloides* leaves attacked by gypsy moth and an even higher induction in systemic leaves of the herbivore treatment.

The salicinoid patterns from *P. nigra* in our experiment did not explain the observed *L. dispar* caterpillar preference for leaves from BG infested trees. On the contrary, the slight but non-significant increase after BG feeding would hint at the opposite outcome of the food choice experiment. We therefore searched for changes in nutritional values in leaves like available carbon and nitrogen sources.

3.5 | Phytohormone, sugar and amino acid measurements in *P. nigra* leaves suggest water stress symptoms inflicted by BG herbivory

We measured three major defence hormones in leaves of black poplar, namely, SA, ABA and JA and its derivatives (JA-Ile-1, JA-Ile-2, cis-12-oxo-phytodienoic acid, OH-JA, OH-JA-Ile and COOH-JA-Ile presented here together as jasmonates). SA levels were not influenced by any of the treatments (Figure 5a), in contrast to ABA and jasmonates. There was a significant increase in ABA concentrations in *P. nigra* leaves following BG herbivory (twofold) but only moderately after AG caterpillar feeding (Figure 5b). Combined BG–AG herbivory resulted in an even more pronounced ABA induction in the leaves (threefold; Figure 5b). The jasmonates were significantly induced fourfold after caterpillar feeding compared with the control (Figure 5c, details of the statistical results are shown in Table 1) and there was a non-significant trend for higher systemic JA levels in leaves of *P. nigra* that received BG herbivory (Figure 5c and Table 1).

Coherent to results from studies in herbaceous plants, BG herbivory in *P. nigra* did not have an impact on SA levels in leaves (Erb et al., 2009; Erb et al., 2011). It was argued before that SA does not act as a signal in systemic defence induction following BG herbivory (Erb et al.,

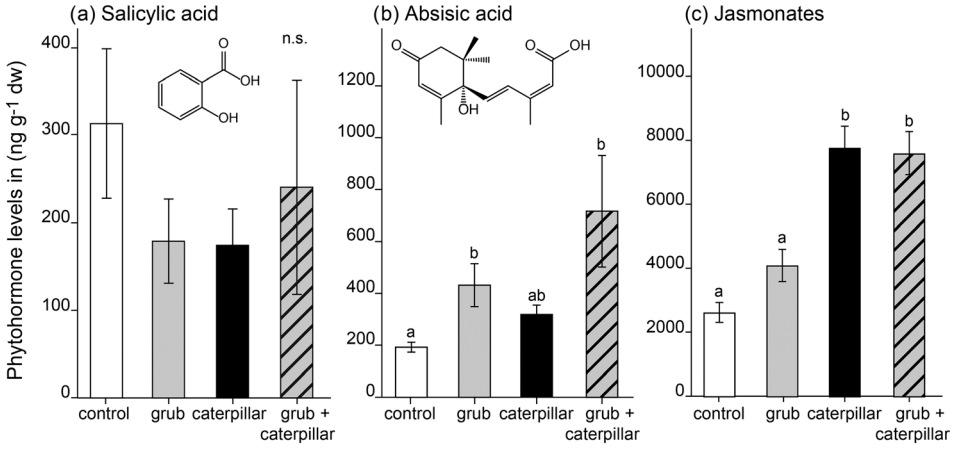

FIGURE 5 (a) Salicylic acid, (b) abscisic acid and (c) jasmonates concentrations in black poplar leaves after belowground damage by a *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar) and a combination of both herbivores (grub + caterpillar) compared with non-damaged control plants (control). The group jasmonates consist of JA and its derivatives (JA-Ile-1, JA-Ile-2, cis-12-oxo-phytodienoic acid, OH-JA, OH-JA-Ile and COOH-JA-Ile). Different letters indicate significant differences between treatments based on a Kruskal Wallis test (a: $p = 0.717$, b: $p \leq 0.001$, c: $p \leq 0.001$) with Dunn's post hoc test. Details of the statistical results are shown in Table 1. Bars represent means ± SEM; $n = 12–15$

2009; Pierre et al., 2012; Pieterse et al., 2012), although SA could affect BG and/or BG–AG defence mechanisms by interactions with other phytohormones (Pieterse et al., 2012). It has long been known that ABA can be transported from roots to shoots *via* the vascular system (Jackson, 1997 and references therein). However, ABA is also *de novo* synthesized in AG foliage following BG herbivory, as a study by (Erb et al., 2011) in maize plants showed. Whether the systemic increase in ABA in *P. nigra* following BG *M. melolontha* herbivory is due to vascular transport or *de novo* biosynthesis awaits further elucidation. JA is a major signal in plant inducible defences against chewing insects (Bruce & Pickett, 2007; De Vos et al., 2005; Erb, Meldau, & Howe, 2012). We observed jasmonate induction following AG caterpillar herbivory, and this is consistent to numerous recent studies in herbaceous and woody plant species (Boeckler et al., 2013; Clavijo Mccormick, Irmisch, et al., 2014; Eberl et al., 2017; Nabity, Zavala, & DeLucia, 2013). The overall phytohormone patterns in our study are comparable with results from studies by (Erb et al., 2009; Erb et al., 2011) in maize plants that were infested BG with larvae of the beetle *Diabrotica virgifera virgifera*. In these studies, BG feeding also substantially increased ABA levels, whereas SA, JA, JA-Ile and OPDA levels were not systemically induced in leaves.

Root herbivory often goes hand in hand with water stress due to the loss of the fine root system and thus a decrease in the overall water uptake (Blossey & Hunt-Joshi, 2003; Erb et al., 2011 and references therein). In our experiment, there was also substantial loss of fine roots in *M. melolontha* larva-infested black poplar trees. When grubs are allowed to feed longer on the poplar trees, this will result in leaf desiccation and leaf fall (S. B. Unsicker, personal observation). A drastic increase of ABA in AG plant tissues is a consequence of water stress in plants (Seki, Umezawa, Urano, & Shinozaki, 2007) often accompanied by an increase of free amino acids and free sugars in AG tissues (Masters, Brown, & Gange, 1993; reviewed by Blossey & Hunt-Joshi, 2003). To cope with water stress, many plants perform an osmotic adjustment, where carbohydrates are mobilized into free sugars, and proteins into amino acids (Bowne et al., 2012; Brown & Gange, 1990; Hummel et al., 2010; Poveda et al., 2003; Rosa et al., 2009). The formed low-molecular-weight compounds act as osmolytes and can protect fragile proteins from degradation by forming a hydration shell around them (Hajlaoui, Ayeb, Garrec, & Denden, 2010).

To test whether BG herbivory also influences the pool of free amino acids and sugars in *P. nigra*, we measured these compounds in leaf material collected from the differently treated trees. The experimental treatments had no effect on fructose, trisaccharide and tetrasaccharide concentrations in black poplar leaves. However, glucose and sucrose concentrations were significantly lower in the caterpillar treatment as compared with the controls. Sucrose concentrations in the combined treatment (caterpillar + grub) were also significantly lower than in non-infested control trees (Table 2). In contrast to the other free sugars, the pentasaccharides significantly increased after combined BG and AG herbivory. Additionally, we observed a non-significant trend of increased tri-, tetra- and pentasaccharide concentrations after BG feeding alone (Table 2).

Five out of the 18 analysed amino acids (Table S2), glutamine, proline, serine, threonine and tryptophan, showed a significant response to the experimental treatments (Figure 6). Caterpillar feeding alone significantly decreased concentrations of proline, serine and threonine, whereas tryptophan was significantly increased in the caterpillar treatment (Figure 6). The combined caterpillar + grub treatment showed the highest concentrations of glutamine, proline and tryptophan as compared with the non-damaged controls. In contrast, serine and threonine concentrations significantly decreased in the combined caterpillar + grub treatment. Proline was the only amino acid that showed a significant increase in the grub treatment as compared with the control treatment (Figure 6). Total leaf protein concentrations did not differ between the four treatments (Figure S3).

TABLE 2 Soluble sugar concentrations after belowground damage by a cockchafer grub, aboveground damage by gypsy moth caterpillars and a combination of both herbivores compared with non-damaged control plants in μg mg^{-1} dw

Analyte	Control	Grub	Caterpillar	Grub + caterpillar	*p*-value
Glucose	7.06 ± 0.74[a]	6.33 ± 1.49[ab]	3.94 ± 0.50[b]	7.12 ± 1.40[ab]	**0.04**
Fructose	2.78 ± 0.32	3.16 ± 0.93	2.17 ± 0.17	3.14 ± 0.44	0.198
Sucrose	20.09 ± 0.34[a]	19.51 ± 0.59[ab]	17.98 ± 0.31[b]	17.39 ± 0.72[b]	**0.001**
Trisaccharide	0.88 ± 0.18	1.23 ± 0.28	0.99 ± 0.19	1.70 ± 0.31	0.186
Tetrasaccharide	0.60 ± 0.19	1.25 ± 0.40	0.71 ± 0.21	1.43 ± 0.35	0.193
Pentasaccharide	0.004 ± 0.002[a]	0.016 ± 0.007[ab]	0.006 ± 0.002[ab]	0.024 ± 0.007[b]	**0.022**

Bold *p*-values indicate significant differences based on Kruskal–Wallis tests. Different letters indicate significant differences between treatments based on Dunn´s post hoc test. Shown is the mean ± SEM; *n* = 13–15.

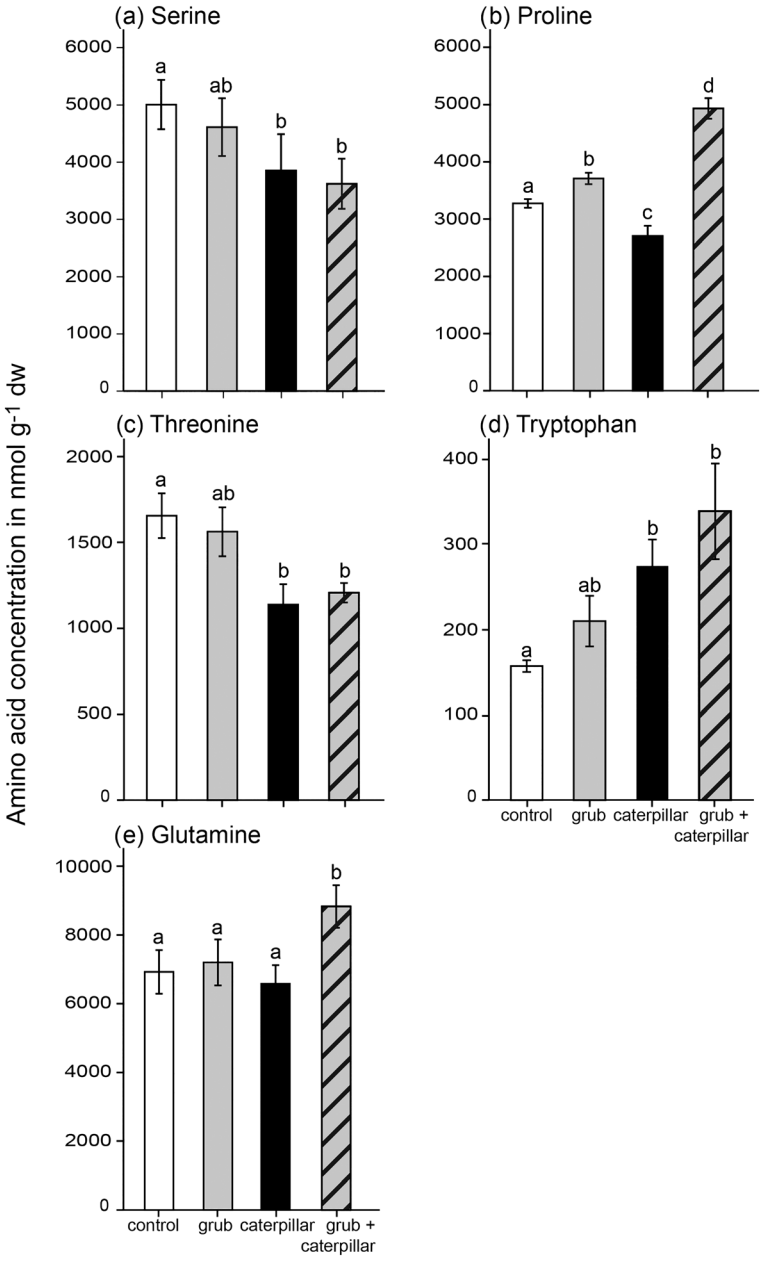

FIGURE 6 (a) Serine, (b) proline, (c) threonine, (d) tryptophan and (e) glutamine concentrations in black poplar leaves after belowground damage by one *Melolontha melolontha* larva (grub), aboveground damage by *Lymantria dispar* caterpillars (caterpillar) and a combination of both herbivores (grub + caterpillar) compared with non-damaged control plants (control). Different letters indicate significant differences between treatments based on a Welch ANOVA (a: $p = 0.032$, b: $p \leq 0.001$, c: $p = 0.002$, d: $p \leq 0.001$, e: $p = 0.007$) with Games–Howell post hoc test. Details of the statistical results are shown in Table S2. Bars represent means ± SEM; n = 14–15

3.6 | Higher proline levels in leaves of BG damaged trees likely explain the caterpillar preference

When we analyzed the experimental leaf area loss in both caterpillar treatments (caterpillar and caterpillar + grub), we found significantly lower damage in the combined treatment with AG and BG herbivory than in the treatment with only caterpillar feeding (Figure S2, Mann Whitney U-test: U = 42.00; $p < 0.01$). This observation together with the initial observation that *L. dispar* caterpillars prefer feeding on leaves from trees that were infested BG with a *M. melolontha* larva lead us to speculate that *L. dispar* consume less biomass from these leaves as their nutritional requirements are more easily met there. The BG treated trees showed signs of water stress with significantly increased ABA levels in the foliage and increased levels of for example proline, an amino acid considered to be a reliable marker for drought stress in herbaceous plants (Ximénez-Embún et al., 2016; Yamada et al., 2005) that most likely exists in woody plants as well. However, in drought-stressed herbaceous plants, the increase in proline concentrations in relation to the control plants (three- to ninefold, Delauney & Verma, 1993) was much stronger compared to our observations in *P. nigra* trees. Proline is reported to have an impact on several developmental processes in plants like flowering, pollen and seed productions and root growth (reviewed by Kavi Kishor & Sreenivasulu, 2014). Proline can be transported to the roots through the phloem *via* specific transporters (Lee et al., 2009). It is conceivable that the rather minor increase of proline we observed in leaves of *P. nigra* upon BG herbivory is due to an increased transport of proline to the damaged roots to aid compensatory growth.

We argue that our data support the 'plant stress hypothesis' (Joern & Mole, 2005), which predicts a better performance and higher abundance of herbivorous insects on plants suffering from abiotic stresses such as drought. Insect preference, however, does not necessarily reflect the performance as was shown in a recent study by Gutbrodt, Mody, and Dorn (2011). There, *Pieris brassicae* caterpillars chose well-watered over drought-stressed *Alliaria petioloata* plants but they performed better on drought stressed plants. In our example, the data on caterpillar performance is missing and further studies have to reveal whether BG herbivory by *M. melolontha* larvae also positively affects *L. dispar* performance and fitness.

The concentrations of all putative anti-herbivore defence metabolites we measured in *P. nigra* leaves cannot explain the initially observed preference of *L. dispar* caterpillars for leaves from *P. nigra* trees infested BG with a *M. melolontha* larva. The only metabolite that could explain caterpillar feeding preference for leaves of the 'grub' treatment was the amino acid proline. This compound is also known to act as a phagostimulant for leaf-chewing insects (e.g. Behmer & Joern, 1994; Meyer, Roces, & Wirth, 2006; Ximénez-Embún et al., 2016). Proline concentrations in *P. nigra* leaves influenced by *M. melolontha* BG herbivory were significantly higher in comparison with those in non-damaged control trees (Figure 6). We therefore performed a choice assay with proline-supplemented leaf discs and solvent-control discs to investigate whether proline could be responsible for the preference of *L. dispar* caterpillars exhibited for trees suffering BG herbivory. Indeed, *L. dispar* caterpillars inflicted twice as much damage on proline-supplemented leaf discs than on the controls (Mann Whitney U-test $p = 0.013$, Figure 7a).

To test whether other amino acids also have phagostimulatory effects, the choice assay was repeated. Proline was again included into the experiment as well as alanine, leucine and tryptophan because these showed the most prominent though non-significant changes between control and grub treatment (Figure 7b). The caterpillars fed significantly more leaf material only when the discs were supplemented with proline. None of the other tested amino acids had a significant effect on caterpillar preference. Both proline and alanine were reported to trigger a response in a phagostimulatory neuron of arctiid moth caterpillars (Bernays, Chapman, & Singer, 2000). We did not observe a preference for alanine-coated leaf discs in *L. dispar*

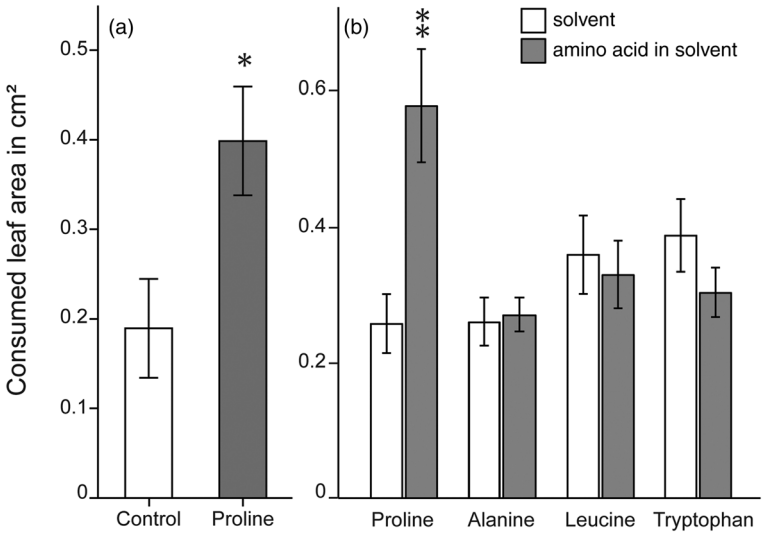

FIGURE 7 (a) Feeding preference of *Lymantria dispar* caterpillars to leaf discs with enhanced proline levels compared to control leaf discs (b) and to leaf discs supplemented with other amino acids. 20 µL of an amino acid dissolved in ethanol was pipetted onto leaf discs to increase amino acid concentration. Control leaf discs were treated with ethanol only. The leaf discs were allowed to dry before they were offered to the caterpillars. Asterisks indicate significant differences between treatments based on a related-samples Wilcoxon signed rank test for a (W-value = 3.053; $p = 0.002$) and paired t-test for b (proline: $t = 3.618$, $p = 0.002$; alanine: $t = 0.27$, $p = 0.79$; leucine: $t = -0.449$, $p = 0.658$; tryptophan: $t = -0.177$, $p = 0.255$). Bars represent means ± SEM; $n = 17$ (a), $n = 18–20$ (b)

caterpillars, which is most likely due to the fact that phagostimulatory effects of single amino acids are herbivore species-specific (Agnihotri, Roy, & Joshi, 2016). Our results suggest that the change in concentration of a single amino acid might be responsible for the initially observed caterpillar preference for leaves from trees with BG herbivory by one *M. melolontha* larva. Furthermore, this could also explain the significantly higher leaf area loss in the caterpillar treatment as compared with the combined herbivore treatment (grub + caterpillar). The leaves that were affected by BG feeding had a higher nutritional value compared to control leaves. Thus, caterpillars feeding on control leaves might have to ingest more tissue to reach their nutrient intake target. Future studies will have to show whether this will also result in a fitness benefit for the caterpillars.

4 | CONCLUSION

Besides putative signs of water stress as a consequence of BG *M. melolontha* larva feeding (induction of ABA and certain amino acids), there was no pronounced systemic induction of typical anti-herbivore poplar defence metabolites (VOCs, PIs and Salicinoids) or defence signaling hormones (JA and SA) in leaves of young *P. nigra* trees. Only in the combined treatment with simultaneous BG herbivory and AG feeding by *L. dispar* caterpillars, the defence related metabolites homaloside D was increased. The non-essential amino acid proline, however, significantly accumulated in leaves of trees suffering from BG herbivory. When *L. dispar* caterpillars had the choice between black poplar leaf discs coated with proline and leaf discs without this amino acid, they preferred the first, supporting previous observations where this compound has been reported to be a phagostimulant. Our results provide a first insight into the defence chemistry of AG–BG interactions in poplar trees and comprise a basis for future chemical ecological studies in trees under more complex, natural conditions.

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The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

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

Table S1: Volatile organic compounds released from black poplar leaves

Figure S1: Schematic view of the experimental treatments

Figure S2: Leaf area loss caused by *Lymantria dispar* caterpillars

Figure S3: Total protein content of black poplar leaves

Table S2: Amino acid concentrations

Table S3: Verification of the amino acid supplementation

NMR structure elucidation of 6'-O-benzoylsalicortin

Figure S4: 1H-NMR spectrum of 6'-O-benzoylsalicortin

Figure S5: 1H-1H COSY NMR spectrum of 6'-O-benzoylsalicortin

Figure S6: 1H-13C HSQC NMR spectrum of 6'-O-benzoylsalicortin

Figure S7: 1H-13C HMBC NMR spectrum of 6'-O-benzoylsalicortin

Figure S8: Structure of 6'-O-benzoylsalicortin with chemical shifts

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