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Variation of compounds in leaves of susceptible and resistant alternate hosts of *Cronartium pini* and *C. ribicola*

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Abstract Leaf compounds may contribute to plant defense against *Cronartium* rusts. Secondary compounds are either natural or induced in leaves. We studied the variation of compounds in leaves of six alternate hosts of *Cronartium pini* and two of *C. ribicola* that represented either susceptible or resistant species to these rusts. Extracts from the plant leaves were analyzed using LC-MSMS (liquid chromatography tandem mass spectrometry) and compounds were compared between susceptible and resistant species of the same plant genera to identify significant differences between resistant and susceptible species. Also, LC–MS (liquid chromatography mass spectrometry) with external calibration was used to quantify 12 candidate compounds known from the literature. Among these compounds, the most abundant significant ones in *C. pini* -resistant *Melampyrum pratense* were chlorogenic acid and quercitrin, in *Veronica chamaedrys* ferulic acid, quercitrin and luteolin and in *Impatiens glandulifera* quercitrin, ferulic acid, kaempferol, rutin and hyperoside. In *C. ribicola* -resistant *Ribes rubrum* the most abundant significant compounds were caffeic acid, p-coumaric acid and quercitrin. Among all extracted leaf compounds, concentrations of three compounds were over 1000 times greater in rust-resistant *M. pratense*, three compounds in *V. chamaedrys*, eight compounds in *I. glandulifera*, and one compound in *R. rubrum* than in rust-susceptible species. Among the compounds, the most promising possibly linked to rust resistance were chlorogenic acid and quercitrin.

Keywords Alternate hosts · Leaf compounds · Rust resistance · Scots pine blister rust · White-pine blister rust

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Introduction

Tree rusts of *Cronartium* are important pathogens of *Pinus* spp. in the northern hemisphere (Gäumann, 1959; Ziller, 1974). *Cronartium pini* (Willd.) Jørst. is a significant rust disease that kills *Pinus* spp. in Europe and Asia (CABI, 2020), while the rust is a quarantine species in North America (Kim et al., 2022). *Cronartium pini* causes severe damage especially on *Pinus sylvestris* L. in northern Fennoscandia (Kaitera, 2000; Samils et al., 2021; Wulff et al., 2012). In the 2000s, *C. pini* has caused severe losses especially on young Scots pine plantations in nutrient-rich soils (Wulff et al., 2012). The chemical compounds enriched in the wood after *Cronartium*

infection have been investigated (Bullington et al., 2018; Kaitera et al., 2021). The results suggested that terpenes and resin acids are produced by the host to protect it from *Cronartium* rust. The rust spreads via alternate hosts, and over 50 susceptible species are known from 13 plant families (Kaitera et al., 2015; Kim et al., 2022). Important species belong especially to Orobanchaceae, Paeoniaceae and Balsaminaceae (Kaitera et al., 2015).

Another *Cronartium*, *C. ribicola* Fisch., is a serious pathogen of five-needle pines in North America (Zambino, 2010). It spreads via *Ribes* (Grossulariaceae), of which *R. nigrum* L. is a highly susceptible species, while *R. rubrum* L. is a resistant species. In Finland, nearly all *R. nigrum* cultivars are susceptible and *R. rubrum* cultivars are resistant to *C. ribicola* (Kaitera & Nuorteva, 2006).

Among plant genera, *Melampyrum* is one of the most susceptible ones to *C. pini* (Kaitera, 1999; Kaitera et al., 1999, 2012, 2015, 2017, 2018). *M. sylvaticum* L., *M. nemorosum* L., *M. arvense* L. and *M. cristatum* L. are highly susceptible species, while *M. pratense* L. is a resistant species (Kaitera, 1999; Kaitera & Nuorteva, 2003a, b; Kaitera et al., 1999, 2012). Other important alternate host genera are *Pedicularis*, *Euphrasia*, *Impatiens* and *Veronica*.

Reasons for the differences in susceptibility and resistance are mostly unknown, but certain intrinsic compounds are likely to play a role. Terpenes are among such compounds (Bullington et al., 2018). They may be normally present or induced by pathogens and other factors. Variation in rust resistance among closely related species may be due to variation in leaf chemistry as was proposed for *M. sylvaticum* and *M. pratense* (Kaitera & Witzell, 2016). Especially chlorogenic acid was abundant in the resistant *M. pratense*, while the compound was lacking in *M. sylvaticum*. Chemical variation on *M. pratense* and *M. sylvaticum* in leaves of different ages, time of collection and among locations have not been studied.

Secondary compounds such as phenolics may be involved in plant resistance. In *Melampyrum scardicum* Wettst, luteolin and apigenin flavonoids were found to be rich (Naumov et al., 1998). These compounds have an antimicrobial impact on bacterial, viral and fungal pathogens (Cushnie & Lamb, 2005). Phenolics are also potential defensive compounds on *Pinus* against *Cronartium* rusts (Boyer, 1964; Hanover & Hoff, 1966; Hudgins et al., 2005; Sniezko et al., 2014).

Based on literature concerning plant resistance 12 compounds were selected to study concentration differences in the plant species: chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, syringic acid, luteolin, kaempferol, myricetin, quercitrin, rutin, apigenin and hyperoside. Chlorogenic acid is a polyphenol and the ester of caffeic acid and quinic acid. In humans, it has been reported to have antioxidant, antibacterial, chemopreventive, antiviral and neuroprotective characteristics (Clifford et al., 2017; Magana et al., 2021). In the human diet coffee, fruits and vegetables are its major sources (Upadhyay & Rao, 2013). Chlorogenic acid has been shown to have bioactivity against various plant pathogens playing a defensive role against biotic and abiotic stresses (Kundu & Vadassery, 2019; Soviguidi et al., 2022). Caffeic acid is a hydroxycinnamic acid derivative and polyphenol. Like chlorogenic acid it also has many beneficial effects on human health. Caffeic acid is found at high levels in some herbs and fruits, and its bioactivity has been shown (Kiokias et al., 2020). Ferulic acid is a phenolic acid that can be found in the seeds of coffee, apple, artichoke, peanut and orange (Kiokias et al., 2020). p-Coumaric acid is found in many natural plants and organisms like fungi, peanuts, beans, tomatoes, carrots, basil and garlic (Kiokias et al., 2020). In addition, most fruits contain p-coumaric acid. Evidence for its high bioactivity has been reported. Syringic acid is a derivative of gallic acid. Its bioactivity for suppression of chronic diseases like human leukemia (HL)-60 and DV-145 human prostate carcinoma cells has been shown (Shahidi & Yeo, 2018). Luteolin, kaempferol, myricetin, quercitrin, rutin, apigenin and hyperoside are hydroxyflavones having potential bioactivity (Adamczak et al., 2020; Cirak et al., 2007; Dall'Agnol et al., 2003; Elansary et al., 2020; Gharibi et al., 2019).

In addition, an unbiased liquid chromatography-tandem mass spectrometry (LC-MSMS) approach was chosen to characterize differences in the compound spectrum of the different plant species. Here all detectable compounds were characterized by their accurate mass and chromatographic retention time and differences in signal intensity (integrated ion counts of chromatographic peaks of the SICs) were compared between susceptible and resistant species. Data were collected in data dependent acquisition

mode, in which the instrument control software recognizes signals and switches automatically to MSMS mode to select and fragmentate the corresponding ions. Accurate mass and MSMS spectra were then used to search different compound databases to identify compounds of interest.

The aim of this study was 1) to investigate the variation of compounds in leaves of alternate hosts species susceptible and resistant to *C. pini* and *C. ribicola*, 2) to compare compounds of resistant and susceptible species groups to one another, and 3) to characterize compounds that may be linked to rust resistance. These compounds may be important in developing control of rust diseases and might be utilized against other pathogens in the future.

Material and methods

Plant material

Circa 10–20 young leaves of eight species were harvested from 20 randomly selected wild or cultivated plants per species from the city area of Oulu. Resistant wild species of *C. pini* were *M. pratense*, *Impatiens glandulifera* Royle and *Veronica chamaedrys* L., and a resistant cultivated species of *C. ribicola* was *Ribes rubrum*. Susceptible wild species of *C. pini* were *M. sylvaticum* and *V. longifolia* L., while a susceptible cultivated species was *I. balsamina* L. Susceptible cultivated species of *C. ribicola* was *R. nigrum*. The cultivated plants were located in the Botanical Gardens of the University of Oulu (65°3,86 N, 25°27,79E). All plants of *Melampyrum*, *Impatiens* and *Veronica* were collected first into paper bags and transported to the laboratory prior to leaf collection. Ten leaves of *Ribes* spp. per species were collected directly into paper bags and transported similarly to the laboratory. The collection locations of the plants in Oulu were: *M. sylvaticum* and *M. pratense* (65°2,69 N, 25°28,04E), *V. longifolia* (65°2,27 N, 25°29,16E), *V. chamaedrys* (65°1,30 N, 25°25,96E), *I. glandulifera* (65°3,03 N, 25°25,20E), *I. balsamina* (garden plants grown from seed in the botanical Garden), *R. nigrum* and *R. rubrum* (cultivated plants in the botanical garden). The plants were collected in late June 2021. The habitats of the wild species were determined by the personnel of the Botanical Gardens. The leaves were collected mainly during flowering of the plants to ensure correct identification of the plants described in Hämet-Ahti et al. (1998).

Standards and reagents

The standard compounds used in the quantitative LC–MS analysis were as follows: caffeic acid (TCI, Tokyo Chemical Industry, C002), p-coumaric acid (TCI, C0393), syringic acid (TCI, G0014), luteolin (TCI, T2682), myricetin (TCI, M2131), rutin (TCI, R0035), kaempferol (TCI, K0018), apigenin (TCI, A1514), quercitrin (Cayman Chemical Company, CAYM19866), hyperoside (PanReac Appli Chem, A1791,0100), chlorogenic acid (Acros Organics, 109,240,010), ferulic acid (Sigma, PHR1791) and ampicillin sodium crystalline (Sigma, A9518). Methanol was HPLC grade (Merck, 1.06007.2500).

Water for the chromatography was produced in house with a Synergy UV instrument (Millipore, cat.no SYNSV0000), equipped with a LC-PAK Polisher (Cat.No. LCPAK 0001) cartridge for the final purification step. Acetonitrile and formic acid were OPTIMA LCMS grade (Fisher Chemical, code A955-212 and A117-50, respectively).

Pretreatment and extraction of leaves

In the laboratory, healthy green leaves of the plants without any sign of fungal or insect damage, were separated from the rest of the plant material in a laminar cabin with sterile tweezers. Then the leaves were air-dried for ca. a week in a laminar cabin in open paper bags and stored at -20 °C prior to analysis. The leaf samples were crushed manually inside a paper bag until a powdery consistency was achieved. About 15 mg of each plant material was weighed into Eppendorf vials. Methanol, containing 5 mg/l of internal standard (ampicillin), was used as an extraction solvent. The solvent was cooled to + 4 °C before usage. 1 ml of the solvent was added to vials which were kept at + 4 °C for one hour. The samples were shaken using Eppendorf MixMate (5 min, 1400 rpm) after which they were centrifuged at + 4 °C (5 min, 12,000 rpm, Hettich Mikro 200). The supernatant was transferred to another vial and the residue was extracted with 0.5 ml of pure methanol (without internal standard). The supernatants were combined and

filtered through a disposable syringe filter (pore size 0.2 µm, Pall Corporation). The extracts were stored at -20 °C.

Chemical analysis

20 biological repeats i.e. all collected leaves from individual plants per species, 160 samples in total, were analyzed. In the LC–MS approach compounds were characterized by retention time, accurate mass and peak area. This dataset was used to obtain maximal data points for quantitation and quantify the 12 candidate compounds with external calibration. Additionally, the same samples were analyzed with an LC-MSMS approach to obtain data for the identification of unknown compounds. 5 µl sample aliquots were eluted from a Waters Aquity Premier HSS T3 column (2.1 × 100 mm, 1.8 µm, Part No. 186009468)) with a gradient made with 0.1% formic acid in water and acetonitrile from 3 to 70% over 14 min, column temperature 40 °C (Waters Aquity UPLC-system comprising column oven (186015010), binary, high pressure mixing pump module (186016007), and autosampler (186015001)). The detector was a Q-Exactive plus orbitrap mass spectrometer in biopharma configuration (Thermos Fisher Scientific) operated in negative polarity at resolution set to 70,000 A in m/z range from 115 to 1200. For the MSMS acquisition, the same conditions were used with the addition of DDA- controlled fragmentation with stepped collision energy (nce) of 25 and 35 and fragment acquisition with m/z range 200 to 2000. The DDA data were processed with Compound Discoverer (Thermo) using standard settings for natural compound analysis. Mz vault, mz cloud, ChemSpider and mass lists were applied in compound identification.

For the quantitation of the candidate compounds a calibration curve with 8 levels from 1 µg/ml to 1 ng/ml was established, data processing was done with the X-calibur and its Quanbrowser option (Thermo). As quality controls two pools comprising 30 µl aliquots of 50 different samples were applied. The compounds, known to occur in leaves of *M. pratense* and *M. sylvaticum* (Kaitera and Witzell 2017), were: chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, rutin, hyperoside, ferulic acid, quercitrin, myricetin, luteolin, apigenin and kaempferol.

Statistical analysis

The data set for negative ionization, filtered for features (compounds) with intensity counts over 10^6 in any of the individual samples, was used in the statistical analyses. About 40 features (compounds) with the highest ratios at minimum 25 (e.g. *M. pratense*:*M. sylvaticum* > 25) distinguishing the susceptible and resistant plant groups at $p < 0.001$ (Compound Discoverer) were listed with the identification suggested by the data base search. Identification leading to compounds non-existing in plant leaves (e.g. fluorine compounds) were left unnamed like compounds without identification. The concentrations of the 12 compounds selected prior to the chemical analysis were compared between resistant and susceptible species with Welch two-sample t-test with unequal variances using the R program (R Core Team 2019, Version 3.6).

Results

Variation of compounds in plants

In the *C. pini* -susceptible-resistant species pair *M. sylvaticum-M. pratense* among the 40 compounds with the highest loadings differentiating the groups, 26 compounds were listed as significant ones (Table 1). The compounds that significantly distinguished *M. pratense* and *M. sylvaticum* were: afzelechin 3–0-alpha-L-rhamno-pyranoside, apigenin 7,4'-diglucuronide, acacetin 7-glucuronosyl-(1 = > 2)-glucaronide, 6-hydroxyapigenin 7-glucuronosyl (1 = > 2)-glucuronide and chrysoeriol 7,4'-diglucuronide. Thirteen compounds remained unidentified (Table 1).

In another *C. pini* -susceptible-resistant species pair *V. longifolia – V. chamaedrys*, 30 compounds were listed as significant ones (Table 2). The significant compounds that distinguished *V. chamaedrys* and *V. longifolia* were: methyl 3-O-({1-[(1S)-1-carboxy-2-(1H-indol-2-yl)ethyl]-1H-1,2,3-triazol-4-yl} methyl)-alpha-D-galactopyranoside, phloretin 2'-O-(6"-O-acetylglucoside), okanin 3,4,3'-trimethyl ether 4'-glucoside and luteolin 4'-methyl ether 7-(4G-rhamnosylneohesperidoside). Fourteen compounds remained unidentified (Table 2).

Table 1 Significant compounds found in the *C. pini* -susceptible-resistant species pair *M. sylvaticum – M. pratense*

Molecular Weight (g/mol)	RT (min)	Name	Formula	Group CV (%) *M.pratense*	Group CV (%) *M.sylvaticum*	Group CV (%) Qual A	Ratio *M.pratense*/ *M.sylvaticum*
140.082	4.751	5-Ethylcyclohexane-1,3-dione	$C_8H_{12}O_2$	85.02	51.45	5.23	0.002
184.073	4.751	Unidentified		92.65	54.35	0.62	0.002
238.047	5.150	3,5-Diacetoxybenzoic acid	$C_{11}H_{10}O_6$	75.83	102.29	8.65	0.011
362.913	5.168	Unidentified	$C_{10}H_5Cl_2N_3O_4P_2$	82.17	39.58	16.39	0.018
394.184	4.380	Diethyl 4,4'-(3,4-dioxo-1-cyclobutene-1,2-diyl) di(1-piperazinecarboxylate)	$C_{18}H_{26}N_4O_6$	54.24	53.18	6.76	0.023
398.889	5.121	Unidentified	$C_{10}H_6Cl_3N_3O_4P_2$	90.16	78.61	18.05	0.024
420.142	5.318	Afzelechin 3-O-alpha-L-rhamnopyranoside	$C_{21}H_{24}O_9$	82.53	103.12	62.92	1558.06
440.189	4.381	Unidentified	$C_{17}H_{27}F_3N_4O_6$	70.51	52.75	1.06	0.015
450.153	5.464	4-Methoxyphlorizin	$C_{22}H_{26}O_{10}$	126.85	23.48	10.70	0.001
457.158	4.209	Unidentified	$C_{17}H_{28}FNO_{12}$	103.49	19.15	4.96	225.68
472.355	13.812	Unidentified	$C_{30}H_{48}O_4$	24.25	45.79	6.63	26.27
476.096	5.658	Unidentified	$C_{21}H_{18}O_{10}$	131.19	46.49	1.74	0.009
486.130	5.485	Unidentified	$C_{24}H_{24}ClFN_4O_2S$	141.16	19.31	5.20	0.001
496.158	5.466	2-[(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinyl) (2-methoxyethyl)amino]-2-oxoethyl 1,3-benzodioxole-5-carboxylate	$C_{24}H_{24}N_4O_8$	130.61	26.03	3.29	0.001
497.162	5.599	Unidentified	$C_{17}H_{30}FN_5O_7P_2$	132.72	154.03	150.27	0.005
497.162	5.471	Unidentified	$C_{19}H_{30}N_7O_3P_3$	132.47	24.64	7.01	0.001
498.174	5.206	1,2-Ethanediylbis(imino-2-oxo-2,1-ethanediyl) bis[(benzoylamino) acetate]	$C_{24}H_{26}N_4O_8$	135.56	36.83	4.01	0
513.148	5.465	6,8-dimethyl-N-[4-[(5-methyl-3-isoxazolyl) sulfamoyl]phenyl]-2-(2-pyridinyl)-4-quinolinecarboxamide	$C_{27}H_{23}N_5O_4S$	142.98	24.42	19.18	0.001
575.018	5.535	Unidentified	$C_{21}H_{20}Cl_2F_2N_5O_2P_3$	137.41	25.13	7.43	0.004
622.118	4.469	Apigenin 7,4'-diglucuronide	$C_{27}H_{26}O_{17}$	61.89	77.59	3.59	357.15
623.121	4.470	Unidentified	$C_{22}H_{24}FN_9O_8P_2$	56.15	79.61	6.18	522.38
636.133	5.843	Acacetin 7-glucuronosyl-(1->2) -glucuronide	$C_{28}H_{28}O_{17}$	35.32	336.89	76.58	39,665.46
638.113	4.554	Luteolin 7,3'-diglucuronide	$C_{27}H_{26}O_{18}$	79.42	76.01	4.53	0.001
638.113	3.991	6-Hydroxyapigenin 7-glucuronosyl- (1->2) -glucuronide	$C_{27}H_{26}O_{18}$	90.23	98.48	10.30	31.08
652.128	4.758	Chrysoeriol 7,4'-diglucuronide	$C_{28}H_{28}O_{18}$	55.13	250.59	14.37	1265.20
900.305	5.452	Unidentified	$C_{39}H_{49}N_8O_{15}P$	30.34	58.62	10.08	0

In the third *C. pini* -susceptible-resistant species pair *I. balsamina – I. glandulifera*, 27 compounds were listed as significant ones (Table 3). The significant compounds that distinguished *I. glandulifera* and *I. balsamina* were: peniisocoumarin I, eriodictyol, (2S,3S)-2-{[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl] oxy}-3,4-dihydroxy-2-methylbutanoic acid, sinensin, 6-O-[(2E)-3-(4-Hydroxyphenyl)-2-propenoyl]-D-glucopyranose, 1-O-feruloyl-beta-D-glucose, astragalin, quercetin 3- (3"-acetylrhamnoside) and

Table 2 Significant compounds found in the *C. pini* -susceptible-resistant species pair *V. longifolia – V. chamaedrys*

Molecular Weight (g/mol)	RT (min)	Name	Formula	Group CV (%) *V.chamaedrys*	Group CV (%) *V.longifolia*	Group CV (%) Qual A	Ratio *V.chamaedrys*/ *V.longifolia*
164.046	3.187	4-Coumaric acid	$C_9H_8O_3$	27.83	30.36	9.09	0.045
170.057	1.287	Unidentified	$C_9H_{11}FS$	98.91	26.17	12.22	0.016
182.057	3.124	3,4-Dihydroxyphenyl-propionic acid	$C_9H_{10}O_4$	29.02	18.39	8.47	0.030
182.057	1.287	Unidentified	$C_{10}H_{11}FS$	83.36	30.44	14.24	0.017
200.067	3.123	Unidentified	$C_{10}H_{13}FOS$	36.50	18.79	7.58	0.001
258.089	6.426	Kappaxan (VAN)	$C_{15}H_{14}O_4$	14.61	20.52	10.25	0.001
270.053	5.493	Apigenin	$C_{15}H_{10}O_5$	98.57	21.76	7.81	0.002
304.095	6.425	3,4,4',alpha-Tetrahydroxy-2'-methoxy-dihydrochalcone	$C_{16}H_{16}O_6$	14.13	18.78	9.08	0.001
394.184	4.365	Unidentified	$C_{16}H_{25}F_3N_4O_4$	33.31	16.65	9.72	0.016
398.121	5.884	Unidentified	$C_{17}H_{17}F_3N_4O_4$	37.00	19.65	10.77	0.028
416.168	3.773	Unidentified	$C_{18}H_{23}F_3N_4O_4$	15.92	50.97	10.94	588.49
446.085	5.498	Norwogonin 8-glucuronide	$C_{21}H_{18}O_{11}$	21.37	25.20	8.83	0.010
462.174	3.855	Methyl 3-O-({1-[(1S)-1-carboxy-2-(1H-indol-2-yl) ethyl]-1H-1,2,3-triazol-4-yl}methyl)-alpha-D-galactopyranoside	$C_{21}H_{26}N_4O_8$	34.77	38.18	10.65	204.34
463.083	4.871	4-[(4E)-3-Methyl-5-oxo-4-{[5-(1-oxo-1,3-dihydro-2-benzofuran-5-yl)-2-furyl] methylene}-4,5-dihydro-1H-pyrazol-1-yl] benzenesulfonamide	$C_{23}H_{17}N_3O_6S$	17.36	27.98	8.16	0.005
478.147	4.260	Phloretin 2'-O-(6"-O-acetylglucoside)	$C_{23}H_{26}O_{11}$	12.63	20.03	9.62	6020.77
480.151	4.274	Unidentified	$C_{13}H_{26}N_{10}O_6P_2$	26.38	20.55	7.94	407.25
492.163	7.506	Okanin 3,4,3'-trimethyl ether 4'-glucoside	$C_{24}H_{28}O_{11}$	77.52	24.62	10.55	0.001
492.163	5.072	Okanin 3,4,3'-trimethyl ether 4'-glucoside	$C_{24}H_{28}O_{11}$	21.41	30.35	12.25	245.45
496.158	6.585	2-[(6-Amino-1-benzyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinyl)(2-methoxyethyl) amino]-2-oxoethyl 1,3-benzodioxole-5-carboxylate	$C_{24}H_{24}N_4O_8$	86.75	21.22	17.40	0.002
500.143	4.030	Unidentified	$C_{14}H_{21}F_4N_{10}O_4P$	78.32	31.68	7.49	0
508.158	5.441	Scutellarioside II	$C_{24}H_{28}O_{12}$	20.90	16.24	10.46	0

Table 2 (continued)

Molecular Weight (g/mol)	RT (min)	Name	Formula	Group CV (%) *V.chamaedrys*	Group CV (%) *V.longifolia*	Group CV (%) Qual A	Ratio *V.chamaedrys*/ *V.longifolia*
534.174	6.511	3,4-Dihydroxychalcone 4-beta-L-arabino-pyranosyl- (1- > 4) -galactoside	$C_{26}H_{30}O_{12}$	30.86	27.14	15.18	0.009
542.164	6.586	(2S)-({2-Amino-3-[4-(4-hydroxy-phenoxy)phenyl] propanoyl}amino) [(2R,3S,4R,5R)-5-(2,4-dioxo-3,4-dihydro-1(2H)-pyrimidinyl)-3,4-dihydroxytetrahy-dro-2-furanyl]acetic acid	$C_{25}H_{26}N_4O_{10}$	55.61	21.74	17.95	0
561.133	3.980	Unidentified	$C_{22}H_{27}NO_{16}$	146.66	11.35	16.50	0
562.136	4.015	Unidentified	$C_{16}H_{22}N_{10}O_{13}$	115.16	22.66	13.23	0
570.185	7.525	Unidentified	$C_{15}H_{28}F_5N_{10}O_6P$	10.93	20.42	8.13	0
587.149	4.738	Unidentified	$C_{24}H_{28}F_2N_3O_{10}P$	35.83	15.01	10.85	0
592.140	4.266	Unidentified	$C_{23}H_{23}F_2N_8O_7P$	13.96	20.55	9.77	857.16
754.232	5.002	Luteolin 4'-methyl ether 7- (4G-rhamnosylneo-hesperidoside)	$C_{34}H_{42}O_{19}$	21.34	16.16	11.57	2071.88
859.284	4.264	Unidentified	$C_{37}H_{49}F_3N_5O_{11}PS$	13.97	25.78	23.90	1034.77

luteolin 7-O- (6"-malonylglucoside). Sixteen compounds remained unidentified (Table 3).

In the fourth *C. ribicola* -susceptible-resistant species pair *R. nigrum* – *R. rubrum*, 37 compounds were listed as significant ones (Table 4). The significant compounds that distinguished *R. rubrum* and *R. nigrum* were: (+) -maackiain 3-O-glucoside, 1-({[3,5-bis(hydroxymethyl)-2,4,6-trioxo-1,3,5-triazinan-1-yl]methoxy}methyl)-3-(hydroxymethyl)urea, gliricidol, quercetin 3- (2"-p-coumarylglucoside) and quercetin 3- (2Gal-apiosylrobinobioside). Five compounds remained unidentified (Table 4).

Concentrations of pre-selected compounds in the samples

Concentrations of chlorogenic acid were significantly 570 times higher in samples of *M. pratense* compared to those in *M. sylvaticum* ($p < 0.0001$, Table 5). They were also insignificantly two times higher in samples of *V. chamaedrys* compared to samples of *V. longifolia* (Table 6), 17 times higher in samples of *I. glandulifera* compared to samples of *I. balsamina* (Table 7), and 5 times higher in samples of *R. rubrum* compared to samples of *R. nigrum* (Table 8).

The mean concentration of caffeic acid in samples of *M. sylvaticum* was 0.04 ng/mg, whereas samples of *M. pratense* did not contain measurable amounts (NF, not found; Table 5). Concentrations of caffeic acid were insignificantly 1.1 times higher in samples of *V. longifolia* compared to those in *V. chamaedrys* and 1.8 times higher in samples of *I. balsamina* compared to those in *I. glandulifera* (Tables 6 and 7). However, concentrations of caffeic acid were significantly 21 times higher in samples of *R. rubrum* than in samples of *R. nigrum* ($p < 0.0001$, Table 8).

Concentrations of syringic acid were insignificantly 3.5 times higher in samples of *M. pratense* compared to those in *M. sylvaticum* (Table 5). The mean concentrations in samples of *V. chamaedrys* and *I. balsamina* were 0.28 ng/mg and 0.12 ng/mg (Tables 6 and 7). Samples of *V. longifolia*, *I.*

Table 3 Significant compounds found in the *C. pini* -susceptible-resistant species pair *I. balsamina – I. glandulifera*

Molecular Weight (g/mol)	RT (min)	Name	Formula	Group CV (%) *I.glandulifera*	Group CV (%) *I.balsamina*	Group CV (%) Qual A	Ratio *I.glandulifera*/ *I.balsamina*
230.151	5.549	Dodecanedioic acid	$C_{12}H_{22}O_4$	71.50	41.69	9.47	0.019
282.074	3.471	Peniisocoumarin I	$C_{13}H_{14}O_7$	46.50	78.72	35.07	1348.81
285.040	5.658	Unidentified	$C_{10}H_8FN_3O_6$	22.55	55.34	21.84	85.08
288.063	5.360	Eriodictyol	$C_{15}H_{12}O_6$	46.18	52.54	22.62	907.17
288.064	6.695	Eriodictyol	$C_{15}H_{12}O_6$	45.22	155.44	8.61	3750.04
301.034	4.784	Unidentified	$C_4H_9FN_7O_6P$	35.24	82.27	22.21	68.69
312.084	3.780	(2S,3S)-2-{[(2E)-3-(3,4-Dihydroxyphenyl)-2-propenoyl] oxy}-3,4-dihydroxy-2-methylbutanoic acid	$C_{14}H_{16}O_8$	28.22	26.10	15.01	5658.38
326.100	3.211	6-O-[(2E)-3-(4-Hydroxyphenyl)-2-propenoyl]-D-glucopyranose	$C_{15}H_{18}O_8$	32.51	55.55	27.42	45.11
329.228	7.804	Unidentified	$C_{12}H_{27}N_9O_2$	199.98	96.24	92.64	0.040
356.111	3.519	1-O-feruloyl-beta-D-glucose	$C_{16}H_{20}O_9$	37.27	77.28	8.60	30.00
400.973	3.785	Unidentified	$C_{14}H_{11}ClFN_3O_2P_2S$	25.98	28.68	8.45	483.38
448.101	5.143	Astragalin	$C_{21}H_{20}O_{11}$	56.31	69.71	18.70	201.30
450.116	5.359	Sinensin	$C_{21}H_{22}O_{11}$	42.25	53.52	24.79	4681.54
486.093	5.385	Unidentified	$C_{22}H_{19}ClN_4O_7$	35.82	57.58	27.18	181.12
490.111	5.660	Quercetin 3-(3"-acetylrhamnoside)	$C_{23}H_{22}O_{12}$	20.12	54.16	23.81	73.31
491.114	5.663	Unidentified	$C_{16}H_{20}F_2N_7O_7P$	18.15	53.88	34.61	73.33
492.116	5.661	Unidentified	$C_{16}H_{21}F_3N_8O_3P_2$	26.12	60.81	11.30	72.73
494.200	4.891	Unidentified	$C_{22}H_{30}N_4O_9$	31.99	62.65	10.88	27.91
506.265	13.102	Unidentified	$C_{23}H_{39}FN_2O_9$	292.18	38.12	60.74	0.003
513.112	5.365	Unidentified	$C_{21}H_{22}F_2N_3O_8P$	23.97	54.59	13.70	1739.48
514.115	5.372	Unidentified	$C_{19}H_{20}N_{10}O_4P_2$	24.60	51.80	23.16	285.49
534.101	5.659	Luteolin 7-O-(6"-malonylglucoside)	$C_{24}H_{22}O_{14}$	23.88	56.12	31.51	98.63
568.052	5.371	Unidentified	$C_{29}H_{17}F_4O_4PS$	25.48	55.36	51.32	208.01
634.026	5.653	Unidentified	$C_{39}H_{10}N_2O_6S$	14.74	67.40	29.82	128.39
898.217	5.342	Unidentified	$C_{29}H_{45}N_{10}O_{17}P_3$	44.49	51.80	7.45	2007.92
928.192	4.759	Unidentified	$C_{50}H_{36}F_5N_4O_3P_3$	75.08	17.86	6.51	13,900.09
1068.202	5.655	Unidentified	$C_{53}H_{42}N_4O_{17}P_2$	33.82	118.06	8.99	4323.93

glandulifera, *R. rubrum* and *R. nigrum* did not contain any syringic acid (Tables 6, 7 and 8).

Concentrations of p-coumaric acid were insignificantly 1.1 times higher in samples of *M. pratense* compared to those in *M. sylvaticum*, and 1.5

Table 4 Significant compounds found in the *C. ribicola* -susceptible-resistant species pair *R. nigrum – R. rubrum*

Molecular Weight (g/mol)	RT (min)	Name	Formula	Group CV (%) *R.rubrum*	Group CV (%) *R.nigrum*	Group CV (%) Qual A	Ratio *R.rubrum*/ *R.nigrum*
270.053	7.755	Apigenin	$C_{15}H_{10}O_5$	56.35	27.97	10.01	0.026
272.068	7.683	Naringenin	$C_{15}H_{12}O_5$	31.41	51.75	9.60	0.004
284.068	10.050	Glycitein	$C_{16}H_{12}O_5$	56.33	23.52	6.56	0.033
286.084	10.065	Brazilin	$C_{16}H_{14}O_5$	63.11	43.65	16.00	0.004
287.087	10.063	Clitocine	$C_9H_{13}N_5O_6$	77.74	44.33	15.42	0.004
300.100	11.659	Methylnissolin	$C_{17}H_{16}O_5$	298.96	22.14	12.37	0
308.093	3.847	12-Sulfooxy-9,10-dihydrojasmonic acid	$C_{12}H_{20}O_7S$	81.30	32.10	9.60	0.026
316.204	14.369	13,14-Dihydro-15-keto Prostaglandin J2	$C_{20}H_{30}O_4$	88.56	25.77	22.49	0
316.204	14.939	13,14-Dihydro-15-keto Prostaglandin J2	$C_{20}H_{30}O_4$	101.10	22.85	25.97	0
320.089	3.261	Gliricidol	$C_{16}H_{16}O_7$	50.04	23.21	13.70	295.53
321.093	3.261	1-({[3,5-Bis(hydroxymethyl)-2,4,6-trioxo-1,3,5-triazinan-1-yl]methoxy}methyl)-3-(hydroxymethyl)urea	$C_9H_{15}N_5O_8$	50.23	27.13	13.70	367.16
332.199	10.071	4,4'-{[(4-Methyl-1-piperazinyl)methyl]phosphoryl}dimorpholine	$C_{14}H_{29}N_4O_3P$	20.78	53.03	5.90	0
346.069	9.096	Taxifolin 3-acetate	$C_{17}H_{14}O_8$	184.87	19.42	5.48	0
346.178	10.392	Gibberellin A24	$C_{20}H_{26}O_5$	9.22	21.34	10.34	0
348.194	10.116	14,16-Dimethoxy-3-methyl-3,4,5,6,9,10,11,12-octahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione	$C_{20}H_{28}O_5$	107.77	18.68	7.54	0
362.210	12.307	Humulone	$C_{21}H_{30}O_5$	192.79	16.11	12.60	0.001
362.210	11.456	Humulone	$C_{21}H_{30}O_5$	17.29	20.90	15.86	0
368.256	9.935	Carboprost	$C_{21}H_{36}O_5$	7.28	24.08	6.42	0
378.189	4.690	1,3-Dihydroxy-2-propanyl 6-O-(cyclohexylacetyl)-beta-D-galactopyranoside	$C_{17}H_{30}O_9$	138.82	25.38	9.75	0.017
381.223	11.157	2,2',2''-(1,3,5-Triazine-2,4,6-triyltri-1,3-diazetidine-3,1-diyl)triethanol	$C_{15}H_{27}N_9O_3$	4.42	22.43	5.89	0.001
412.246	8.700	Rhodotoxin	$C_{22}H_{36}O_7$	7.88	18.57	14.14	0
412.246	9.982	3-[5-(3-Methylbenzyl)-1,3,4-oxadiazol-2-yl]-1-[3-(4-morpholinylmethyl)-1-piperidinyl]-1-propanone	$C_{23}H_{32}N_4O_3$	22.25	19.99	23.71	0
446.122	5.758	(+) -Maackiain 3-O-glucoside	$C_{22}H_{22}O_{10}$	98.96	38.78	18.95	275.41
448.231	6.928	12-Oxo-12-{[(2R,5S)-5-(6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydro-2-furanyl]methoxy}dodecanoic acid	$C_{22}H_{32}N_4O_6$	173.14	25.83	15.55	0.003
458.252	10.007	Ethyl 4-[({1-[(2-methyl-2,3-dihydro-4H-1,4-benzoxazin-4-yl)carbonyl]-4-piperidinyl}carbonyl)amino]-1-piperidinecarboxylate	$C_{24}H_{34}N_4O_5$	37.09	30.05	10.44	0
458.252	9.934	Ethyl 4-[({1-[(2-methyl-2,3-dihydro-4H-1,4-benzoxazin-4-yl)carbonyl]-4-piperidinyl}carbonyl)amino]-1-piperidinecarboxylate	$C_{24}H_{34}N_4O_5$	38.91	86.36	6.17	0
459.255	9.934	N-{(R)-4-Biphenylyl[(1R,2R)-2-butylcyclopropyl]methyl}-4-biphenylcarboxamide	$C_{33}H_{33}NO$	7.35	92.08	4.59	0
475.242	10.023	2-[8-(3-Aminopropyl)-6-(1-benzothiophen-3-yl)imidazo[1,2-a]pyrazin-2-yl]-N-cyclohexyl-2-methylpropanamide	$C_{27}H_{33}N_5OS$	10.07	20.90	8.70	0
494.237	6.860	2-[3,8-Dihydroxy-8-(hydroxymethyl)-3-methyl-2-oxodecahydro-5-azulenyl]-2-propanyl hexopyranoside	$C_{21}H_{36}O_{10}$	81.52	81.69	14.17	0.002

Table 4 (continued)

Molecular Weight (g/mol)	RT (min)	Name	Formula	Group CV (%) *R.rubrum*	Group CV (%) *R.nigrum*	Group CV (%) Qual A	Ratio *R.rubrum*/ *R.nigrum*
610.133	6.285	Quercetin 3- (2"-p-coumarylglucoside)	$C_{30}H_{26}O_{14}$	69.25	27.48	100.63	64.45
616.119	4.770	Unidentified	$C_{26}H_{28}O_{15}$	95.44	16.34	6.51	583.66
659.134	4.321	Unidentified	$C_{31}H_{27}N_5O_8P_2$	58.81	12.44	8.65	1009.55
696.388	10.119	4,4'-[(2,4,6-Trihydroxy-5-isobutyryl-1,3-phenylene)bis(3-methyl-1,1-butanediyl)]bis(5-hydroxy-2,2,6,6-tetramethyl-4-cyclohexene-1,3-dione)	$C_{40}H_{56}O_{10}$	8.92	28.61	10.25	0
698.079	4.767	Unidentified	$C_{41}H_{18}N_2O_8S$	96.12	15.74	6.51	704.89
712.095	4.731	Unidentified	$C_{44}H_{27}O_2P_3S$	106.90	13.24	6.51	389.65
714.074	4.317	Unidentified	$C_{33}H_{24}N_4O_9P_2S$	60.40	12.44	8.65	343.94
742.197	3.070	Quercetin 3- (2Gal-apiosylrobinobioside)	$C_{32}H_{38}O_{20}$	95.76	18.46	48.68	470.52

Table 5 Mean concentrations of selected 12 compounds in species pair *M. pratense—M. sylvaticum* (NF, not found). Significantly different mean concentrations compared between resistant and susceptible species using t-test are marked with different levels of significance, $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*)

	M. pratense Mean (ng/mg)	*M. sylvaticum* Mean (ng/mg)	t	df	p-value
Chlorogenic acid	182.48	0.32	7.33	19	< 0.0001***
Caffeic acid	NF	0.04	-1.39	19	0.18
Syringic acid	0.73	0.21	2.06	28.43	0.049
p-Coumaric acid	2.00	1.90	0.10	26.24	0.92
Rutin	2.30	29.66	-8.65	19.81	< 0.0001***
Hyperoside	12.89	23.72	-3.94	24.54	< 0.001**
Ferulic acid	19.14	381.17	-13.94	19.53	< 0.0001***
Quercitrin	2.38	0.13	4.84	20.74	< 0.0001***
Myricetin	0.11	0.21	-2.71	20.44	0.013
Luteolin	2.29	33.38	-4.55	19.28	< 0.001**
Apigenin	2.64	3.75	-1.09	22.58	0.29
Kaempferol	0.60	0.56	0.28	36.34	0.78

Table 6 Mean concentrations of selected 12 compounds in species pair *V. chamaedrys – V. longifolia* (NF, not found). Significantly different mean concentrations compared between resistant and susceptible species using t-test are marked with different levels of significance, $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*)

	V. chamaedrys Mean (ng/mg)	*V. longifolia* Mean (ng/mg)	t	df	p-value
Chlorogenic acid	0.40	0.20	2.10	33.96	0.043
Caffeic acid	34.83	37.62	-0.88	36.37	0.38
Syringic acid	0.28	NF	2.81	19.00	0.011
p-Coumaric acid	44.46	159.06	-9.00	23.69	< 0.0001***
Rutin	NF	NF	-	-	-
Hyperoside	1.17	NF	1.48	19.00	0.16
Ferulic acid	24.22	NF	21.71	19.00	< 0.0001***
Quercitrin	0.50	NF	9.81	19.00	< 0.0001***
Myricetin	0.06	0.10	-2.19	37.80	0.035
Luteolin	2.96	0.76	4.31	38.00	< 0.001**
Apigenin	21.21	1.55	1.54	19.02	0.14
Kaempferol	22.60	0.02	1.55	19.00	0.14

Table 7 Mean concentrations of selected 12 compounds in species pair *I. glandulifera* – *I. balsamina* (NF, not found). Significantly different mean concentrations compared between resistant and susceptible species using t-test are marked with different levels of significance, $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*)

	I. glandulifera Mean (ng/mg)	*I. balsamina* Mean (ng/mg)	t	df	p-value
Chlorogenic acid	0.52	0.03	1.44	19.20	0.17
Caffeic acid	14.67	26.70	-2.19	29.27	0.04
Syringic acid	NF	0.12	-1.00	19.00	0.33
p-Coumaric acid	88.54	132.16	-1.90	23.09	0.07
Rutin	179.39	32.11	6.58	22.30	<0.0001***
Hyperoside	5717.31	164.87	15.51	19.27	<0.0001***
Ferulic acid	NF	4726.37	-7.94	19.00	<0.0001***
Quercitrin	5493.58	1567.80	11.67	36.81	<0.0001***
Myricetin	NF	NF	-	-	-
Luteolin	0.35	NF	1.29	19.00	0.21
Apigenin	NF	NF	-	-	-
Kaempferol	217.35	71.32	5.93	27.34	<0.0001***

Table 8 Mean concentrations of selected 12 compounds in species pair *R. rubrum* – *R. nigrum* (NF, not found). Significantly different mean concentrations compared between resistant and susceptible species using t-test are marked with different levels of significance, $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*)

	R. rubrum Mean (ng/mg)	*R. nigrum* Mean (ng/mg)	t	df	p-value
Chlorogenic acid	58.78	11.98	1.35	19.74	0.19
Caffeic acid	15.36	0.72	9.25	19.81	<0.0001***
Syringic acid	NF	NF	-	-	-
p-Coumaric acid	39.47	0.90	7.90	19.14	<0.0001***
Rutin	63.12	459.97	-7.72	28.41	<0.0001***
Hyperoside	288.84	207.93	2.17	22.64	0.04
Ferulic acid	2.79	4.62	-2.86	31.72	<0.01*
Quercitrin	451.34	96.02	3.11	19.06	<0.01*
Myricetin	NF	NF	-	-	-
Luteolin	NF	NF	-	-	-
Apigenin	NF	20.25	-13.50	19.00	<0.0001***
Kaempferol	NF	0.53	-3.23	19.00	<0.01*

times higher in samples of *I. balsamina* than those in *I. longifolia* (Tables 5 and 7). However, concentrations were significantly 3.6 times higher in samples of *V. longifolia* than in samples of *V. chamaedrys* ($p < 0.0001$, Table 6), and 44 times higher in samples of *R. rubrum* than those in *R. nigrum* ($p < 0.0001$, Table 8).

Concentrations of rutin were significantly 13 times higher in samples of *M. sylvaticum* compared to those in *M. pratense* ($p < 0.0001$), 6 times higher in samples of *I. glandulifera* than those in *I. balsamina* ($p < 0.0001$), and 7 times higher in samples of *R. nigrum* than those in *R. rubrum* ($p < 0.0001$; Tables 5,7 and 8). Samples of *V. chamaedrys* and *V. longifolia* did not contain any rutin (Table 6).

Concentrations of hyperoside were significantly 1.8 times higher in samples of *M. sylvaticum* compared to those in *M. pratense* ($p < 0.001$), and 35 times higher in samples of *I. glandulifera* compared to those in *I. balsamina* ($p < 0.0001$; Tables 5 and 7). Concentrations were insignificantly 1.4 times higher in samples of *R. rubrum* than those in *R. nigrum* (Table 8). The mean concentration of hyperoside in samples of *V. chamaedrys* was 1.17 ng/mg, whereas samples of *V. longifolia* did not contain measurable amount (Table 6).

Concentrations of ferulic acid were significantly 20 times higher in samples of *M. sylvaticum* compared to those in *M. pratense* ($p < 0.0001$; Table 5). Concentrations in samples of *V. chamaedrys* and

I. balsamina were also significantly higher compared to those in *V. longifolia* and *I. glandulifera* ($p < 0.0001$, Tables 6 and 7). Concentrations were insignificantly 1.7 times higher in samples of *R. nigrum* than those in *R. rubrum* (Table 8).

Concentrations of quercitrin were significantly 18 times higher in samples of *M. pratense* compared to those in *M. sylvaticum* ($p < 0.0001$; Table 5), 3.5 times higher in samples of *I. glandulifera* compared to those in *I. balsamina* ($p < 0.0001$; Table 7), and 4.7 times higher in samples of *R. rubrum* compared to those in *R. nigrum* ($p < 0.01$; Table 8). The mean concentration of quercitrin in samples of *V. chamaedrys* was 0.50 ng/mg compared to NF for *V. longifolia* ($p < 0.0001$; Table 6).

Concentrations of myricetin were insignificantly 1.9 times higher in samples of *M. sylvaticum* compared to those in *M. pratense*, and 1.7 times higher in samples of *V. longifolia* than those in *V. chamaedrys* (Tables 5 and 6). Samples of *I. glandulifera, I. balsamina, R. rubrum* and *R. nigrum* did not contain any myricetin (Tables 7 and 8).

Concentrations of luteolin were significantly 15 times higher in samples of *M. sylvaticum* compared to those in *M. pratense* ($p < 0.001$; Table 5), and 4 times higher in samples of *V. chamaedrys* compared to those in *V. longifolia* ($p < 0.001$; Table 6). The mean concentration of luteolin in samples of *I. glandulifera* was 0.35 ng/mg (Table 7). Samples of *I. balsamina*, *R. rubrum* and *R. nigrum* did not contain any luteolin (Table 7 and 8).

Concentrations of apigenin were insignificantly 1.4 times higher in samples of *M. sylvaticum* compared to those in *M. pratense*, and 14 times higher in samples of *V. chamaedrys* compared to those in *V. longifolia* (Tables 5 and 6). The mean concentration in samples of *R. nigrum* was 20.25 ng/mg being significantly higher compared to *R. rubrum* ($p < 0.0001$, Table 8). Samples of *I. glandulifera*, *I. balsamina* and *R. rubrum* did not contain any apigenin (Tables 7 and 8).

Concentrations of kaempferol were significantly three times higher in samples of *I. glandulifera* than those in *I. balsamina* ($p < 0.0001$; Table 7). The mean concentration of kaempferol in samples of *R. nigrum* was 0.53 ng/mg being significantly higher compared to *R. rubrum* ($p < 0.01$; Table 8). Concentrations were insignificantly 1130 times higher in samples of *V. chamaedrys* than those in *V. longifolia*, and 1.1 times higher in samples of *M. pratense* compared to those in *M. sylvaticum* (Tables 5 and 6).

Discussion

The highest concentration of chlorogenic acid (5-O-caffeoylquinic acid) was found in *M. pratense* (182.48 ng/mg, Table 5). *R. rubrum* (58.78 ng/mg) and *R. nigrum* (11.98 ng/mg) contained also significant amounts of chlorogenic acid (Table 8). In the other studied plant species the concentrations were low (Tables 6 and 7). It is well known that chlorogenic acid plays a defensive role against biotic and abiotic stresses in plants. Petkovsek et al. (2009) noticed seasonal changes in phenolic compound concentrations in the leaves of scab-resistant and susceptible apple cultivars. The mean chlorogenic acid concentrations found from the resistant and susceptible species were 365–3262 ng/mg and 184–500 ng/mg, respectively. They discovered that concentrations of total phenolics as well as single phenolic compounds, like chlorogenic acid, were statistically significantly higher in resistant than in susceptible apple varieties during the growing season. The concentration of chlorogenic acid was also higher in leaves of rust-resistant *M. pratense* compared to those of rust-susceptible *M. sylvaticum* in a recent study (Kaitera & Witzell, 2016). The role of chlorogenic acid in plant response to abiotic stresses (heavy metal, UV light, heat, cold, salinity and drought) has also been extensively studied (Soviguidi et al., 2022). Our results clearly support the significant role of chlorogenic acid in plant defense mechanism against biotic stresses.

In this study, the highest concentrations of quercitrin were measured from *I. glandulifera* (5493.58 ng/mg) and *I. balsamina* (1567.80 ng/mg) (Table 7). The concentration in the resistant species *I. glandulifera* was significantly higher than in the susceptible species *I. balsamina*. Also, high concentrations were found in *R. rubrum* (451.34 ng/mg) and *R. nigrum* (96.02 ng/mg; Table 8). The concentrations of quercitrin in other tested plant species were low, but statistically significantly higher in the resistant species, *M. pratense* and *V. chamaedrys*, compared to the susceptible species *M. sylvaticum* and *V. longifolia* (Tables 5 and 6). Elansary et al. (2020) studied polyphenols of *Frangula alnus* Mill. and *Peganum harmala* L. leaves and their bioactivity. They studied the concentrations

of several polyphenolic compounds and reported that quercitrin was the main flavonoid in *F. alnus* (11,323 mg/kg). Leaf extracts of both species showed cytotoxic effects against Jurkat, MCF-7, HeLa and HT-29 cancer cells. They concluded that the polyphenolic composition of leaves including quercitrin, trifolin and cymaroside play a significant role in the bioactivity of these plants.

Also, other statistically significant compounds that distinguish the selected resistant vs. susceptible plant species pairs were searched from the LC-MSMS data in this study. In the *M. pratense* and *M. sylvaticum* species pair, the most significant compounds were afzelechin 3–0-alpha-L-rhamno-pyranoside, apigenin 7,4'-diglucuronide, acacetin 7-glucuronosyl-(1 = > 2)-glucaronide and chrysoeriol 7,4'-diglucuronide (Table 1). Afzelechin 3–0-alpha-L-rhamno-pyranoside is a flavonoid glycoside. Based on earlier studies, this compound has been isolated from e.g. *Artocarpus sepicanus* Diels leaves (Radwan et al., 2009), *Cassipourea malosana* (Baker) Alston bark (Drewes et al., 1992) and *Averrhoa bilimbi* L. leaves (Ahmed et al., 2018). *Averrhoa bilimbi* is widely used in traditional medicine. Ahmed et al. (2018) found that the n-butanol fraction of *A. bilimbi* crude methanol leaf extract showed significant antioxidant properties. They concluded that afzelechin 3–0-alpha-L-rhamno-pyranoside and cucumerin A likely cause this bioactivity in the methanol leaf extract of *A. bilimbi*. Radwan et al. (2009) studied the compounds antimicrobial activity against the fungi *Candida albicans* (C.-P. Robin) Berkhout, *Aspergillus fumigatus* Fresen, *Cryptococcus neoformans* (San Felice) Vuill., and the bacteria *Escherichia coli* (Migula) Castellani & Chalmers, *Pseudomonas aeruginosa* (Schroeter) Migula, *Mycobacterium intracellulare* Runyon and MRSA (methicillin-resistant *Staphylococcus aureus* Rosenbach). Afzelechin 3–0-alpha-L-rhamno-pyranoside was inactive against all microbes tested. Apigenin 7,4'-diglucuronide and chrysoeriol 7,4'-diglucuronide are members of flavonoids and a glucosiduronic acid. Ichimura et al. (2021) investigated the effects of temperature and light intensity on anthosyanin biosynthesis in snapdragons (*Antirrhinum majus* L.). In this study they also measured the apigenin 7,4'-diglucuronide content of the flowers of the plants. They concluded that the high temperature affects anthocyanin synthesis more than flavone biosynthesis in snapdragon petals. Acacetin, chrysoeriol and their respective glycosides are common flavones in *Citrus* fruits and juices with good pharmacological effects (Barreca et al., 2020), but for acacetin 7-glucuronosyl-(1 = > 2)-glucaronide and chrysoeriol 7,4'-diglucuronide, we couldn't find any literature. The apigenin derivate acacetin was reported to be richer in leaves of rust-resistant *M. pratense* compared to those of rust-susceptible *M. sylvaticum* and luteolin derivate chrysoeriol vice versa in a previous study (Kaitera & Witzell, 2016).

For the *V. chamaedrys* and *V. longifolia* species pair the most significant compounds were phloretin 2'-O- (6"-O-acetylglucoside), okanin 3,4,3'-trimethyl ether 4'-glucoside and luteolin 4'-methyl ether 7- (4G-rhamnosylneohesperidoside) (Table 2). Methylated okanin derivatives can be found from *Bidens torta* Sherff (McCormick et al., 1984). McCormick et al. (1984) determined the structures of four methylated chalcones including okanin 3,4,3'-trimethyl ether 4'-glucoside. Rao et al. (2020) studied the response of phenolic compounds in rice to different growing conditions. Luteolin 4'-methyl ether 7- (4G-rhamnosylneohesperidoside) was one of the compounds determined from different rice varieties and growing locations. They discovered that the effect of cultivation environment on the concentration and antioxidant activity of this compound varied between rice varieties indicating the influence of both genetics and environment on the compound. Earlier, luteolin was reported to be richer in leaves of rust-susceptible *M. sylvaticum* compared to those of rust-resistant *M. pratense* (Kaitera & Witzell, 2016). Phloretin can be found in apple tree leaves. Antifungal activity of phloretin against several plant pathogenic fungi has been reported (Shim et al., 2010). Phlorizin, a glucoside of phloretin, is also present in the apple tree (root bark, shoots, leaves) and experimental evidence suggests that it plays a significant role in apple tree physiology (Ehrenkranz et al., 2005). For the bioactivity of phloretin 2'-O- (6"-O-acetylglucoside) we couldn't find any literature.

For the *I. glandulifera* and *I. balsamina* species pair the most significant compounds were peniisocoumarin I, eriodictyol, (2S,3S)-2-{[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]oxy}-3,4-dihydroxy-2-methylbutanoic acid, sinensin, 1-O-feruloyl-beta-D-glucose, astragalin and luteolin 7-O- (6"-malonylglucoside (Table 3). Peniisocoumarin 1 is a natural product in *Penicillium commune* Charles Thom. Eriodictyol is a tetrahydroxyflavanone

that can be found from wide range of medicinal plants, citrus fruits and vegetables. The medicinal properties of eriodictyol have been extensively studied (Deng et al., 2020; Islam et al., 2020; Khan et al., 2014). (2S,3S)-2-{[(2E)-3-(3,4-dihydroxyphenyl)-2-propenoyl]oxy}-3,4-dihydroxy-2-methylbutanoic acid is a hydroxycinnamic acid. Sinensin is a flavonoid and a glycoside. Baek et al. (2015) identified kaempferol, astragalin, quercetin, isoquercitrin, sexangularetin and sinensin from the calyx of *Fragaria ananassa* Duchesne ex Rozier. Quercetin showed the highest radical-scavenging activity whereas sinensin showed the lowest activity. Astragalin (kaempferol 3-glucoside) is a bioactive compound found in several medicinal plants such as *Cuscuta chinensis* Lam. (Riaz et al. 2018). Astragalin is well known for its pharmacological properties. Kaempferol was reported to be richer in leaves of rust-susceptible *M. sylvaticum* compared to those of rust-resistant *M. pratense* recently (Kaitera & Witzell, 2016). 1-O-feruloyl-beta-D-glucose is a natural product having a role as an antioxidant and a plant metabolite (Arnaldos et al., 2001; Delazar et al., 2017; Du et al., 2006; Jia et al., 2017). Luteolin 7-O-(6"-malonylglucoside) is a trihydroxyflavone. Luteolin was reported to be richer in leaves of rust-susceptible *M. sylvaticum* compared to those of rust-resistant *M. pratense* recently (Kaitera & Witzell, 2016).

For the *R. rubrum* and *R. nigrum* species pair the most interesting compounds were (+) -maackiain 3-O-glucoside, gliricidol, quercetin 3- (2"-p-coumarylglucoside) and quercetin 3- (2Gal-apiosylrobinobioside) (Table 4). (+) -Maackiain 3-O-glucoside, also called sophojaponicin, belongs to the pterocarpans group of compounds. It has been isolated from the roots of *Cicer judaicum* Baksier, which is an annual herb from the Middle East (Stevenson & Veitch, 1996). Gliricidol is a flavonoid found from the methanolic extract of *Gliricidia sepium* (Jacq.) Steud. bark (Rastrelli et al., 1999). It has shown bioactivity against *Artemia salina* L. larvae. For the two quercetin derivatives we couldn't find any literature about their bioactivity in plants, but generally, the quercetin compounds are known to have many possible health effects on humans. Quercetin compounds were also rich in leaves of *Melampyrum* spp. in a recent study (Kaitera & Witzell, 2016). In conclusion, our quantitative results of the pre-selected compounds revealed two compounds, chlorogenic acid and quercitrin, whose concentrations differ significantly between rust-resistant and susceptible plant species. The literature also supported the probable bioactivity of these compounds against rust diseases. From the discovery approach, we could find additional compounds with a putative role in the plant defense against rust disease. It is also known that in infected wood of mature *P. sylvestris*, *C. pini* induced a 1.3–108 fold increase in concentrations of monoterpenes, resin acids and several sesquiterpenes compared to control wood (Kaitera et al., 2021). In *P. albicaulis* Engelm. seedlings, terpene concentrations were higher in *C. ribicola* -resistant trees compared to susceptible ones (Bullington et al., 2018). Also *C. quercuum* f.sp. *fusiforme* Burds. & G.A.Snow -susceptible *P. elliotii* Engelm. trees contained lower amounts of some monoterpenes than resistant ones (Michelozzi et al., 1991). Therefore, monoterpenes are important compounds in *Cronartium* resistance to *Pinus* spp. Further research is needed to describe the temporal and spatial variation of the compounds in alternate host plants of *Cronartium*. Inoculation tests in controlled environment should be done to study the induced chemical changes in alternate hosts due to rust infections. Also the effect of leaf extracts and individual compounds of extracts of rust-resistant species should be tested against *Cronartium* rusts in controlled experiments.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

The authors bear all the ethical responsibilities of this manuscript. They declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest and that it does not include any animal and/or human trials.

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