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Temporal and spatial variation in chemical composition of susceptible and resistant alternate hosts of *Cronartium pini*, *Melampyrum sylvaticum* and *M. pratense*

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Abstract *Cronartium pini* infects ca. 60 alternate host species among which *Melampyrum sylvaticum* is a susceptible and *M. pratense* a resistant species. Variation in rust resistance among these species may be connected to natural or induced leaf chemicals. In this study, we investigated the chemical variation of 11 compounds between two *C. pini*-resistant and -susceptible *Melampyrum* species in leaves of different age, temporally within growing season and spatially among different geographic locations. LC–MS (liquid chromatography mass spectrometry) was used to quantify the compounds. Concentrations of chlorogenic acid, syringic acid, hyperoside and quercitrin were significantly higher in *M. pratense*, while those of p-coumaric acid, rutin, ferulic acid and luteolin were significantly higher in *M. sylvaticum*. Temporal variation occurred in concentrations of the compounds. Old leaves of both *Melampyrum* species contained mostly higher concentrations than young leaves. Spatial variation occurred for individual compounds in both *Melampyrum* species. In conclusion, age of leaves, time of collection during growing season and geographic location affect concentrations of chemical compounds in leaves of both *Melampyrum* species, which should be taken into consideration when exploring the potential of plant chemicals in rust resistance.

Keywords Alternate hosts · Leaf compounds · Mass spectrometry · Rust resistance · Scots pine blister rust

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Introduction

Cronartium pini (Willd.) Jørst. is a pathogenic rust species that causes damage on pines (*Pinus* spp.) in Europe and Asia and is a quarantine species in North America (CABI, 2020; Kim et al., 2022). The rust has caused severe epidemics especially on Scots pine (*Pinus sylvestris* L.) in northern Fennoscandia in the 2000s (Kaitera, 2000; Wulff et al., 2012). The rust spreads via alternate host plants of which over 50 susceptible species are recorded from 14 plant families (Kaitera et al., 2015). Among the plant genera commonly found in northern forests, *Melampyrum* contains several susceptible species (Kaitera, 1999; Kaitera et al., 1999, 2012, 2015, 2017, 2018), such

as *M. sylvaticum* L., *M. nemorosum* L., *M. arvense* L. and *M. cristatum* L., whereas *M. pratense* is a highly resistant species (Kaitera, 1999; Kaitera & Nuorteva, 2003a, 2003b; Kaitera et al., 1999, 2012).

Variation in rust resistance among the closely related *Melampyrum* species may be due to several factors, such as leaf chemical traits or their internal microbial communities. However, recent research suggests that the culturable leaf endophytic fungi do not explain or co-vary with rust-resistance (Piispanen et al., 2024). On the other hand, *C. pini* infection has been found to induce a 1.3–108 fold increase in concentrations of monoterpenes, resin acids and several sesquiterpenes in *P. sylvestris* wood (Kaitera et al., 2021) indicating that terpenoid chemicals are strongly associated with *C. pini* infection. Less is known about the potential role of terpenoids or other common secondary chemicals in rust interactions with alternate hosts. Recent studies addressing the secondary chemical variation in *Melampyrum* species have shown that especially the concentration of chlorogenic acid (a phenolic compound) is high in leaves of *M. pratense* and low in *M. sylvaticum* (Kaitera & Witzell, 2016; Piispanen et al., 2023). The apigenin derivate acacetin was also richer in *M. pratense* than in *M. sylvaticum*, while luteolin derivate chrysoeriol was vice versa (Kaitera & Witzell, 2016). Recently, *C. pini*-inoculation increased significantly only p-coumaric acid concentration in a rust-susceptible alternate host, *Impatiens balsamina* L., indicating that early phenolic accumulation mostly lacked during early infection stages of the rust (Piispanen et al., 2025).

Plant chemicals, such as phenolics, could provide material for development of environmentally friendly, low-risk products for control of rusts. Yet, the extent of variation in phenolic chemicals of *Melampyrum* species in relation to the observed resistance patterns are poorly studied. For instance, rust occurrence is known to differ between leaves of different age, with young leaves being more susceptible than old ones. Rust occurrence also shows temporal variation (increase as the growth period proceeds), and there can be spatial variation between different geographic locations. These variations should be taken into consideration when evaluating the potential of leaf chemistry as a factor determining the susceptibility patterns of alternate hosts of *Cronartium* rusts. Therefore, the aim of this study was to investigate variation in leaf phenolic compounds leaves in susceptible and resistant alternate hosts of *Cronartium pini*, *M. sylvaticum* and *M. pratense*, between 1) leaves of different age i.e. old versus young leaves, 2) time of collection during growing season, and 3) spatially between various geographic locations.

Material and methods

Collection of leaves

To study the temporal variation, ten plants of *M. sylvaticum* and *M. pratense* each were collected from the same five locations in the Oulu city area in late June 2021 (early season collection) and mid-July 2021 (mid-season). The coordinates of the geographic locations were: 1 — 65°2,69 N, 25°28,04E, 2 — 65°1,29 N, 25°25,38E, 3 — 65°1,70 N, 25°25,01E, 4 — 64°59,69 N, 25°32,16E, and 5 — 65°1,23 N, 25°25,86E. The plants were collected in paper bags and transported to the Lynet laboratory in Oulu (Luke). Young, healthy and green leaves from the two youngest whorls along both the main stem and side branches of the plants represented young leaves, while leaves of the older leaf whorls represented old leaves. The collected leaves were dried in open paper bags and stored at − 20 °C prior to analysis.

Extraction and analysis of the leaves

The dry leaf samples were crushed manually inside a paper bag to obtain a homogenous powder. A subsample (ca. 15 mg of powder) was weighed into an Eppendorf vial and kept in 1 ml of cold (+ 4 °C) methanol containing 5 mg/l of ampicillin as an internal standard for one hour, shaken for 5 min in Eppendorf MixMate (1400 rpm) and centrifuged at +4 °C (5 min, 12,000 rpm, Hettich Mikro 200). The supernatant was transferred to a new Eppendorf vial and the pellet was re-extracted with 0.5 ml of pure methanol. After combining the supernatants, the extracts were filtered (syringe filter pore size 0.2 µm, Pall Corporation) and stored at − 20 °C. A total of 400 samples (10 plants per each of the two species, 5 locations, 2 time points and young and old leaves) were prepared for LC–MS analysis.

The analyses were conducted using a Waters Aquity UPLC-system (pump 186,016,007, autosampler 186,015,001 and column oven model

186,015,010) that was coupled with Q-Exactive plus orbitrap mass spectrometer (Thermos Fisher Scientific), operated in negative polarity at resolution set to 70,000 A in m/z range from 115 to 1200. Injection volume was 5 µl and the separation was achieved in Waters Aquity Premier HSS T3 column (2.1 ×100 mm, 1.8 µm, Part No. 186009468) using a gradient (0–14 min: 0.1% formic acid in water and acetonitrile from 3 to 70%) at column temperature 40 °C. Data processing was done with the X-calibur and its Quanbrowser option (Thermo).

A Millipore Synergy UV instrument (Cat.no. SYNSV0000), equipped with a LC-PAK Polisher (Cat.no. LCPAK 0001) cartridge was used to purify water for the chromatography. HPLC grade methanol (Merck) was used in extractions and OPTIMA LCMS grade solvents, acetonitrile (A955 -212) and formic acid (A117 -50) were used for the gradient (Fisher Chemical). The standard compounds used in LC–MS analyses were supplied by TCI, Tokyo Chemical Industry (caffeic acid, syringic acid, p-coumaric acid, luteolin, apigenin, kaempherol, and rutin), Acros Organics (chlorogenic acid), Sigma (ferulic acid, ampicillin sodium crystalline), PanReac Appli Chem (hyperoside) and Cayman Chemical Company (quercitrin). For a more thorough description of the protocols used, see Piispanen et al. (2023).

Statistical analysis

Concentrations of the selected compounds in the samples were compared between age of the leaves (old vs. young leaves), sampling times (early and mid-season), locations (5) and between resistant and susceptible species (*M. pratense* vs. *M. sylvaticum*) using ANOVA with F-test. Statistical analyses were conducted using SPSS (IBM Corp. Released 2023. IBM SPSS Statistics for Windows, Version 29.0.0.0 Armank, NY: IBM Corp) software.

Results

Phenolic acids

Concentration of chlorogenic acid in leaves of *M. pratense* was 300–500 times higher than in *M. sylvaticum* (F-test, F = 756.22, df = 1, $p < 0.001$; Figs. 1 and 2, Table 1). In *M. sylvaticum* leaves, chlorogenic acid concentration was very low, regardless of the age of the leaves or time of collection. Concentration of chlorogenic acid was significantly higher (F = 8.67, df = 1, $p < 0.01$) in old leaves than in young leaves of *M. pratense* in early and mid-summer. Generally, chlorogenic acid concentrations increased significantly from early to mid-season (F = 19.14, df = 1, $p < 0.001$). These changes were similar both in young and old leaves. In *M. pratense,* the chlorogenic acid concentrations did not differ between collection locations (F = 1.50, df = 4, $p = 0.205$).

Concentration of caffeic acid was very low in all samples of both *M. sylvaticum* and *M. pratense*. Detectable concentrations were found only in early season. No statistical comparisons using ANOVA could be calculated between leaves of different age, geographic areas or times of collection due to high numbers of zero values.

Concentration of syringic acid was generally very low, but in leaves of *M. pratense* it was higher (F = 77.23, df = 1, $p < 0.001$, Table 1) than in *M. sylvaticum* leaves (Figs. 1 and 2). In old leaves and in early season, the concentration of syringic acid was higher than in young leaves (F = 48.87, df = 1, $p < 0.001$) and in mid-season (F = 207.81, df = 1, $p < 0.001$). Significant differences occurred also between locations (F = 31.51, df = 4, $p < 0.001$): in one of the locations (no. 3) the concentration was higher than in the other areas.

Concentration of p-coumaric acid was generally high and in leaves of *M. sylvaticum* it was higher than in leaves of *M. pratense* (F = 29.24, df = 1, $p < 0.001$; Figs. 1 and 2, Table 1). In early season, the concentration was higher than in mid-season (F = 137.23, df = 1, $p < 0.001$) and also in young leaves it was higher than in old leaves (F = 98.20, df = 1, $p < 0.001$). Age of the leaf had a significant interaction with the location and the sampling time, and *Melampyrum* species. Significant differences were found also between locations (F = 4.11, df = 4, $p < 0.01$).

Concentration of ferulic acid was generally high and significantly higher in leaves of *M. sylvaticum* than of *M. pratense* (F = 117.32, df = 1, $p < 0.001$; Figs. 1 and 2, Table 1). They were significantly higher in mid-season compared to early season (F = 9.37, df = 1, $p < 0.001$). Significant differences occurred also between areas (F = 9.40, df = 4, $p < 0.001$). Concentrations were equal (F = 6.13, df = 1, $p = 0.014$) in old and young leaves in early and mid-season.

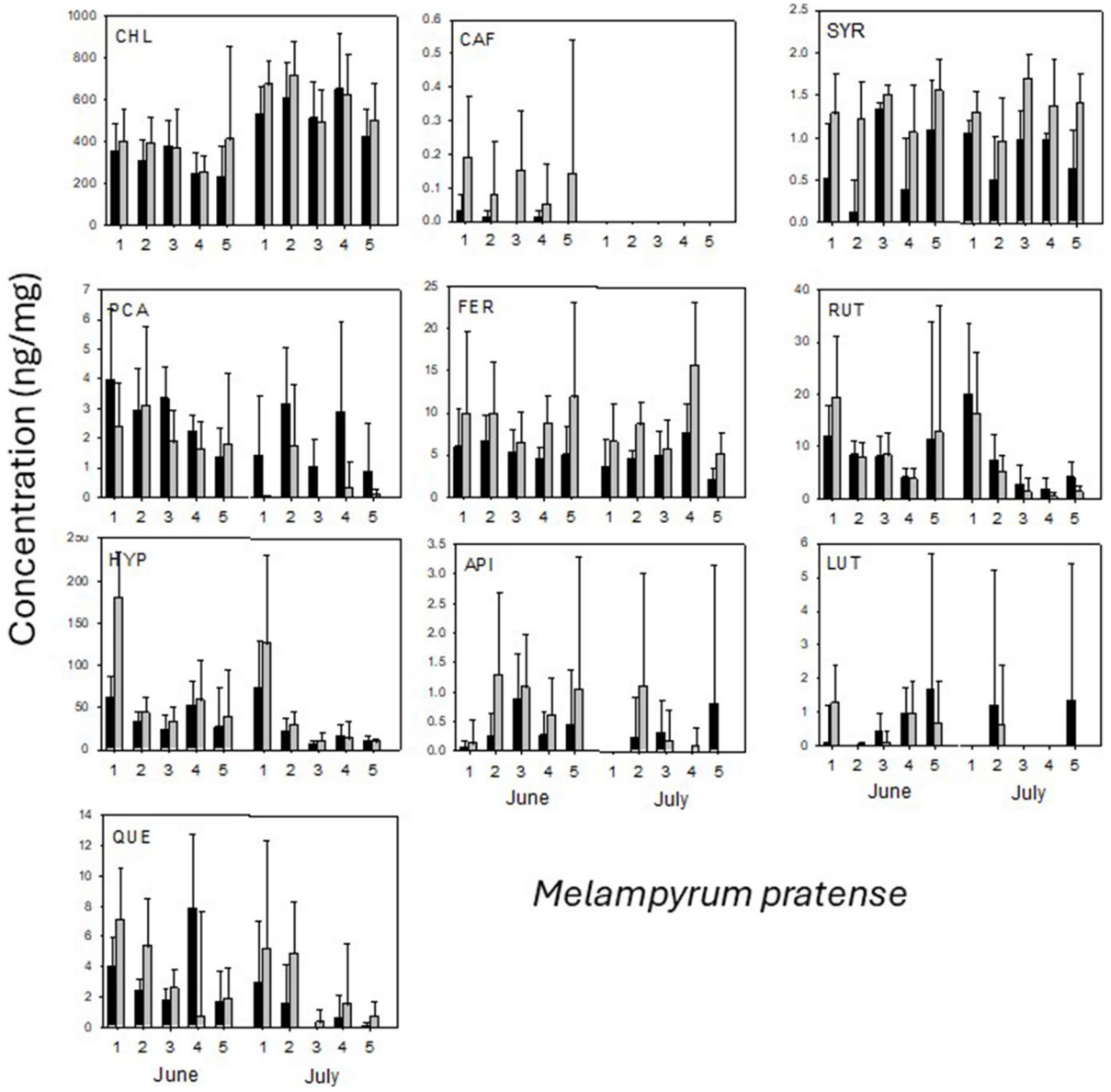

Fig. 1 Mean concentrations and standard deviations (ng/mg, n = 10) of 10 phenolic compounds, CHL = Chlorogenic acid, CAF = Caffeic acid, SYR = Syringic acid, PCA = p-Coumaric acid, FER = Ferulic acid, RUT = Rutin, HYP = Hyperoside, QUE = Quercitrin, LUT = Luteolin, and API = Apigenin, in young (black bars) and old (grey bars) leaves of *M. pratense* in late June (bars 1–5 on the left in x-axis) and late July (bars 1–5 on the right in x-axis). The numbers (1–5) on the x-axis denote collection areas (1 = 65º2,69 N, 25º28,04E, 2 = 65º1,29 N, 25º25,38E, 3 = 65º1,70 N, 25º25,01E, 4 = 64º59,69 N, 25º32,16E, and 5 = 65º1,23 N, 25º25,86E)

Flavonoids

Concentration of rutin was higher in leaves of *M. sylvaticum* than in leaves of *M. pratense* ($F = 60.18$, $df = 1$, $p < 0.001$; Figs. 1 and 2, Table 1). It was also higher in early season as compared to mid-season ($F = 79.82$, $df = 1$, $p < 0.001$). No differences were found in rutin concentration in young and old leaves ($F = 0.03$, $df = 1$, $p = 0.861$), nor between locations ($F = 2.39$, $df = 4$, $p = 0.053$).

Concentration of hyperoside in leaves of *M. pratense* was higher than in leaves of *M. sylvaticum*, but

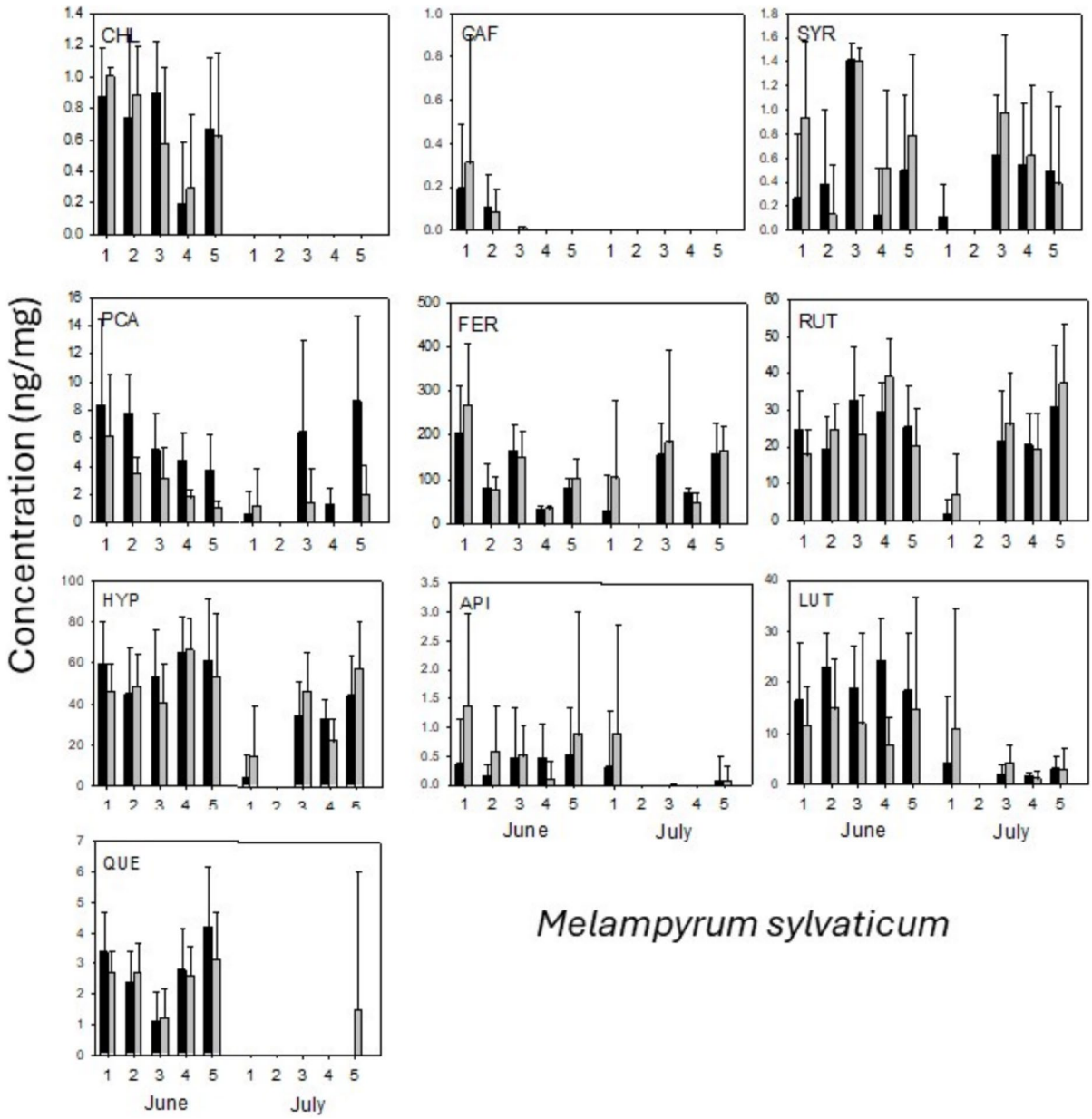

Fig. 2 Mean concentrations and standard deviations (ng/mg, n = 10) of 10 phenolic compounds, CHL = Chlorogenic acid, CAF = Caffeic acid, SYR = Syringic acid, PCA = p-Coumaric acid, FER = Ferulic acid, RUT = Rutin, HYP = Hyperoside, QUE = Quercitrin, LUT = Luteolin, and API = Apigenin, in young (black bars) and old (grey bars) leaves of *M. sylvaticum* in late June (bars 1–5 on the left in x-axis) and late July (bars 1–5 on the right in x-axis). The numbers (1–5) on the x-axis denote collection areas (1 = 65º2,69 N, 25º28,04E, 2 = 65º1,29 N, 25º25,38E, 3 = 65º1,70 N, 25º25,01E, 4 = 64º59,69 N, 25º32,16E, and 5 = 65º1,23 N, 25º25,86E)

several zero values in *M. sylvaticum* restricted reliability of statistical analysis (Figs. 1 and 2). Hyperoside concentrations in old leaves were higher than in young leaves across early and mid-season (F = 34.53, df = 1, $p < 0.001$, Table 1). Significant interaction was found between leaf age, *Melampyrum* species and location ($p < 0.001$). Hyperoside concentration was equal between early and mid-season (F = 0.29,

Table 1 Arrows and colors show significant (α = 0.01) differences (upwards arrow in blue = higher; downwards arrow in red = lower), "o" indicates non-significant differences between plant species (MP = *M. pratense* and MS = *M. sylvaticum*), age of leaves (young and old leaves), seasonal collection (late June and mid-July) and spatial areas according to F-test of ANOVA. "– "= not calculated. CHL = chlorogenic acid, CAF = caffeic acid, SYR = syringic acid, PCA = p-coumaric acid, FER = ferulic acid, RUT = rutin, HYP = hyperoside, QUE = quercitrin, LUT = luteolin, API = apigenin

Compound	Plant species		Age of leaves		Seasonal		Spatial
	MP	MS	Young	Old	Late June	Mid-July	
CHL	↑	↓	↓	↑	↓	↑	o
CAF	-	-	-	-	-	-	-
SYR	↑	↓	↓	↑	↑	↓	↑
PCA	↓	↑	↑	↓	↑	↓	↑
FER	↓	↑	o	o	↓	↑	↑
RUT	↓	↑	o	o	↑	↓	o
HYP	↑	↓	↓	↑	o	o	↑
QUE	↑	↓	↓	↑	↑	↓	↑
LUT	↓	↑	o	o	o	o	o
API	o	o	↓	↑	o	o	o

df = 1, p = 0.593) and differed between locations (F = 14.59, df = 4, p < 0.001).

Quercitrin was present at low concentration, and showed seasonal and species-specific fluctuation, being higher in leaves of *M. pratense* than in *M. sylvaticum* (F = 27.08, df = 1, p < 0.001, Figs. 1 and 2, Table 1). Some *M. sylvaticum* samples had, however, zero values, which restricts reliability of the statistical analysis. Compared to young leaves, old leaves contained higher concentrations of quercitrin (F = 29.89, df = 1, p < 0.001) and the concentrations were higher in early season compared to mid-season (F = 60.25, df = 1, p < 0.001). Significant differences were also found between locations (F = 7.10, df = 1, p < 0.001).

Concentration of luteolin was higher in leaves of *M. sylvaticum* than in leaves of *M. pratense* (F = 39.30, df = 1, p < 0.001; Figs. 1 and 2, Table 1), but there was no difference between young and old leaves (F = 1.14, df = 1, p = 0.2888). In early season, the concentrations did not differ significantly from those in mid-season (F = 4.04, df = 1, p = 0.046). There were no differences between locations (F = 0.71, df = 4, p = 0.588). A few samples had zero values, which restricts reliability of statistical analysis.

Apigenin concentration was similar in leaves of both species (F = 0.63, df = 1, p = 0.428; Figs. 1 and 2, Table 1), but its concentration in old leaves was higher than in young leaves (F = 10.52, df = 1, p < 0.001). Apigenin concentration was also stable during the season (F = 5.45, df = 1, p = 0.021) and across locations (F = 1.09, df = 4, p = 0.365). However, the zero values restricted the reliability of statistical analysis.

Concentrations of kaempferol were very low in leaves of both *M. sylvaticum* and *M. pratense* (data not shown). Therefore, no statistical comparisons could be calculated between leaves of different age, spatially or seasonally.

Discussion

The results of our study underscore the significant variation in phenolic metabolites across different plant species, within individual plants, and over temporal and spatial scales. Moreover, the results reinforce the potential role of chlorogenic acid in host plant-rust interactions, aligning with previous research that has linked this compound to rust resistance mechanisms in different species. Earlier, chlorogenic acid concentration in leaves of rust-resistant *M. pratense* has been found to be higher than in the leaves of rust-susceptible *M.*

sylvaticum (Kaitera & Witzell, 2016; Piispanen et al., 2023), and the same pattern was found in the current study. Increasing chlorogenic acid concentration during the growing season, and the higher concentrations in older, more resistant leaves (see also Ragazzi et al., 1989) further suggest that this compound could be linked to resistance against *C. pini* in *Melampyrum* species. We also found that the chlorogenic acid concentrations were stable across the different locations suggesting that its synthesis and accumulation were not readily affected by environmental variations. The bioactive role of chlorogenic acid in various stress conditions has been shown earlier (Petkovsek et al., 2009; Soviguidi et al., 2022). In a recent study, chlorogenic acid had no inhibitive effect on *Cronartium* spore germination at low concentrations (50 ppm and 100 ppm) but it inhibited germination at high concentration (500 ppm) (Piispanen, unpublished). Chlorogenic acid is reported to occur mainly in vacuoles and in apoplastic space (Baker et al., 2017) and thus it or its metabolites may influence the haustorial phase inside the leaves more than the spore germination on the leaf surface.

Of the other phenolic acids, only syringic acid with its higher concentration in *M. pratense* and in old leaves could be associated with rust resistance patterns. As similar result was reported previously by Piispanen et al. (2023), who found that concentration of syringic acid was higher in *M. pratense* than in *M. sylvaticum* (Piispanen et al., 2023). Thus, despite of its low concentration in *Melampyrum*, syringic acid may be involved in rust resistance in *Melampyrum*. The other tested phenolic acids (i.e., caffeic acid, syringic acid, p-coumaric acid and ferulic acid) did not show clear and consistent associations with rust resistance patterns. In some cases, this may have reflected their low concentration and zero values.

Most of the flavonoid compounds studied showed a lack of or an inconsistent relationship to rust resistance patterns, and for example rutin and luteolin tended to be present in higher concentrations in the more susceptible species, in agreement with earlier reports (Kaitera & Witzell, 2016; Piispanen et al., 2023). However, the concentrations of quercitrin and hyperoside followed the pattern of rust resistance, being higher in *M. pratense* and in old leaves. In a previous study, concentrations of quercitrin were also found to be higher in *M. pratense* than in *M. sylvaticum* in early season (Piispanen et al., 2023). Moreover, quercitrin had no inhibitive effect on *Cronartium* spore germination at low concentrations but it highly inhibited germination at high concentration (Piispanen, unpublished). Thus, our results support the view that quercitrin may play some role in rust resistance, although the generally low concentrations, zero values in some samples of *M. sylvaticum* and the high spatial variation restricted our ability to draw strong conclusions. In other studies, quercitrin concentrations were high in leaves of *Frangula alnus* Mill. and *Peganum harmola* L. playing a significant role in bioactivity of these plants against cancer cells (Elansary et al., 2020). The evidence regarding hyperoside is less clear. In an earlier study, its concentration was higher in *M. sylvaticum* as compared to *M. pratense* (Piispanen et al., 2023). Spatial variation observed in this study may explain difference between results of these two studies. The low concentrations of some *M. sylvaticum* samples and high spatial variation does not allow strong conclusions to be drawn from the role of hyperoside in rust-resistance based on the present results.

In conclusion, the study provides additional confirmation to the earlier results regarding the role of chlorogenic acid and quercitrin in rust-resistant *M. pratense,* with syringic acid and hyperoside being other potentially interesting compounds. As the major new conclusions from this study, both the spatial and seasonal variation seemed to be important regulators of individual phenolic chemicals in *Melampyrum* species. The high temporal and spatial variation of leaf compounds should be taken into consideration when exploring the potential of secondary chemicals as environmentally safe plant protection agents. In the future, the effect of individual chemicals on *Cronartium* infection on susceptible and resistant alternate host plants should be further studied. Future studies should also explore the molecular mechanisms activated by rusts in the alternate hosts.

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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 ab-

sence 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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