PLANT-ENVIRONMENT INTERACTIONS

Targeted metabolomics unveil alteration in accumulation and root exudation of flavonoids as a response to interspecific competition

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ABSTRACT

This study aimed to elucidate the chemical response of hairy vetch (Vicia villosa Roth) to interspecific competition by rye (Secale cereale L.). Hairy vetch plants were grown as target plants in a mixture with different densities of rye plants (0, 1, 2, and 4). Twelve flavonoids in the shoot, root, and root exudate of hairy vetch plants were quantified by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Kaempferol 3-O-[(β-D-rhamnosyl-(1->6)→[(β-D-xylosyl)-(1→2)]→β-D-galactoside) (Kaempferol-Rha-Xyl-Gal) was the predominant flavonoid found in shoots, roots, and root exudate of hairy vetch plant. Co-cultivation of hairy vetch with one rye plant increased the concentration of kaempferol-Rha-Xyl-Gal in hairy vetch root exudates. However, the concentration was reduced when co-cultivated with 2 or 4 rye plants. This study revealed that low levels of competition increased root exudation of kaempferol-Rha-Xyl-Gal, while higher levels of competition negatively affected production and root exudation of kaempferol-Rha-Xyl-Gal by hairy vetch.

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1. Introduction

Hairy vetch (Vicia villosa Roth) is a legume native to Europe and western Asia, which has been used as a cover crop grown alone or in mixtures with cereals (Ebelhar et al. 1984; Lawson et al. 2015). The ability of legumes to fix and convert atmospheric nitrogen into a plant-available form of nitrogen makes them an excellent option for cover cropping (Clark 2008; Tosti et al. 2012). The Winter hardiness of hairy vetch and its ability to provide large amounts of N to the following crop through nitrogen fixation has increased its popularity as a cover crop among the legumes (Brainard et al. 2012). Growing hairy vetch with rye (Secale cereale L.) as a cover crop mixture is common due to high biomass production and ecosystem benefits (Hayden et al. 2014). It has been reported that the total biomass of ryegrass-hairy vetch mixtures tended to be dominated by rye (Hayden et al. 2012), which could be a result of high competitiveness or allelopathic effects of rye (Barnes and Putnam 1986; Hazrati et al. 2019). There are studies investigating facilitation effects and ecosystem benefits of rye-hairy vetch mixture (Hayden et al. 2012; Baraibar et al. 2018; Bashyal et al. 2019), but there is a lack of research on the response of hairy vetch to competition by rye.

Interactions between plant species depend mainly on resource competition, but there is increasing evidence showing allelochemicals’ involvement (Fiorentino et al. 2009; Hazrati et al. 2017; Hazrati et al. 2018; Hu et al. 2018). Plants sense and respond to neighboring plants’ roots by changing the chemical profile of their root and root exudate (Dayan 2006; Fernandez et al. 2016; Kong et al. 2018; Hazrati et al. 2020). Many studies report an increase of secondary metabolite production by allelopathic plants such as rye and wheat in response to co-cultivation with other plants (Macías et al. 2014; Kong et al. 2018). However, studies on how a target
plant, such as hairy vetch, responds to an allelopathic neighbor (e.g. rye) are scarce.

Flavonoids are a group of secondary metabolites synthesized by plants with diverse chemical structures caused by the attached substituents (Mierziak et al. 2014). They have essential functions in plant interactions with other organisms such as microbes, insects, and neighboring plants (Star 1980; Koes et al. 1994; Dai et al. 1996). Some of the flavonoids, such as catechin and flavones, can negatively affect other plant species’ germination and growth (Kong et al. 2004; Kong et al. 2007). Moreover, kaempferol and several kaempferol glycosides extracted from Lobularia maritima (L.) Desv. can cause both growth stimulatory and inhibitory effects on the co-existing plant species (Fiorentino et al. 2009). Phytotoxicity of hairy vetch against weed species in both field and laboratory studies has been proved (Ercoli et al. 2005; Ercoli et al. 2007). Growth inhibitory activity of the hairy vetch crude extract is mainly explained by cyanamide, which is suggested to be the possible allelochemical in the hairy vetch (Kamo et al. 2003). The lack of studies on hairy vetch’s chemical composition has created a need to identify and quantify flavonoids in hairy vetch.

Active root exudation of flavonoids in response to abiotic and biotic signals has been reported (Armero et al. 2001; Cesco et al. 2010; Khashi U Rahman et al. 2019; Sugiyama 2019b). However, there is a lack of studies on the chemical profiling of flavonoids in hairy vetch root exudate and their role in plant-plant interactions. In the present study, two questions for a better understanding of the underlying mechanism of hairy vetch-rye interaction are addressed: (i) How does hairy vetch biomass respond to competition with rye? and (ii) How does the concentration of flavonoids in hairy vetch plant tissue and root exudate change as a result of competition with rye? These questions were addressed by measuring biomass accumulation and determining the identity and quantity of flavonoids in root and shoot tissue and root exudates of hairy vetch plants.

2. Material and methods

2.1. Chemicals

Kaempferol (96%) and formononetin (99%) were purchased from Fluka (Brøndby, Denmark). Biochanin A (97%), genistin (95%), catechin (97%), epicatechin (98%), orientin/homoorientin (97%), naringin (95%), nicotiflorin (98%) and astragalin (99%) were purchased from Sigma-Aldrich (Brøndby, Denmark). Rutin (99%), apigenin (99%), saponarin (99%), luteolin (99%), luteolin 4-O-Glc (95%), naringenin (99%), and daidzin (90%) were purchased from Extrasynthèse (Genay, France). Kaempferol-Rha-Xyl-Gal and quercetin-Rha-Xyl-Gal were isolated from white clover, purified, and identified by their UV, mass, and NMR spectra as part of a previous study (Carlson et al. 2008). Medicarpin was obtained from Dr. Paul M. Dewick at the University of Nottingham, England. Stock flavonoid solutions of 1 g/L were prepared by dissolution in methanol. Working standard solutions of the 23 compounds were obtained by serial dilution of the stock standard solutions in 35% MeOH and 65% MilliQ water (v/v) containing 0.2% formic acid. Mixed standard curves for the negative and positive modes were generated from 10 concentrations of each standard and used for quantification.

2.2. Pot experiment (soil growth media)

Plants were grown in three-liter pots filled with sandy loam field soil (2.8% organic matter, 11.5% clay, 28.4% silt, and 57.2% sand) under controlled conditions in a climate chamber at a 16/8 h photoperiod with a day/night temperature of 20/16°C. The soil was collected from a field where hairy vetch had never been cultivated. The pots were watered with tap water. One hairy vetch plant as target species was placed in the center of the pot and rye plants, as neighboring species at four densities (0, 1, 2, and 4 plants per pot), were placed in a ring around the hairy vetch plant (a so-called target-neighborhood design). Seven replicates were used for each treatment. Five-week-old plants were harvested; the roots were washed with distilled water and then carefully separated from shoots and immediately immersed in liquid nitrogen to prevent any enzymatic reaction before being transferred to a freezer at −80°C. Finally, samples were freeze-dried and kept at room temperature until analysis.

2.3. Plant growing system for root exudate extraction (glass bead growth media)

Root exudate extraction was performed using the method developed by Hazrati et al. (2020). Fifty ml plastic tubes were used as containers for growing plants. A 7-mm drainage hole was made in the bottom of the tubes and covered with a 7-cm² piece of mesh (pore size 30 µm) to avoid any loss of growth media, to prevent roots from growing outside the tubes, and to prevent contamination of the extracted root exudate by small pieces of root tissue. Tubes were filled with 40 g of glass beads with a size of 250–425 micron. Black colored packing tape was wrapped around the tubes to prevent algal growth and stop light exposure of the roots. Seeds of rye and hairy vetch were pre-germinated on water-saturated Whatman filter papers in Petri dishes (one seed in each Petri dish) and placed in a growth chamber at 20°C with 16 h photoperiod for 72 h. Germinated seedlings were transplanted into the tubes that were placed in a growth chamber (8 h light/16 h dark, day and night temperatures of 20/16°C, and an average light intensity of 120 µmol m⁻² s⁻¹ photons at the plant level and relative humidity of 60%). Plants were watered every second day with 5-ml of half-strength Hoagland solution until root exudate extraction. Three different set-ups were investigated; hairy vetch growing alone, hairy vetch growing together with two rye plants, hairy vetch growing together with four rye plants with ten replications per treatment. 17-day-old hairy vetch plants were harvested, their roots were carefully washed with distilled water to make sure all the glass beads were removed, and roots were subsequently rinsed with Milli-Q water. All plants’ shoots and roots were placed in separate boxes and immediately immersed into liquid nitrogen to stop enzymatic reactions before being transferred to −80°C for storage. Roots and shoots were freeze-dried and kept at room temperature until analysis.

2.4. Root exudate extraction

A solvent containing 70% methanol (v/v) and 0.2% formic acid (v/v) was used for root exudate extraction. Fifteen ml
extraction solution was applied through a syringe to the top of the tube, and vacuum pressure was used to accelerate the flushing of the solution through the glass beads. Flushing of the growth media for collecting root exudates was performed in 30 s for all the samples to reduce root cell damage by methanol. Each tube contained 10-ml water, which, together with the extraction solution, added up to a final volume of root exudate of ~25-ml for each tube. The fresh root exudates were filtered through a 0.22 µm cellulose acetate syringe filter and transferred into glass vials before HPLC-MS analysis.

2.5. Metabolite extraction for identification and quantification of flavonoids

Ground root and shoot materials (~20 mg) were transferred into an Eppendorf tube (with safe-lock), and 1-ml of 80% methanol containing 0.2% formic acid was added instantly. Samples were sonicated with ultrasonic for 45 min and centrifuged at 4500 g (Sigma 1–14 K micro-centrifuge, Buch and Holm, Herlev, Denmark) for 10 min. The addition of solvent, sonication, and centrifuge was repeated. The samples and the insoluble plant materials were discarded and the supernatant was transferred to fresh dark 4-ml glass vials. 500 µl of the insoluble plant materials were discarded and the supernatant was taken and diluted 2 and 100 times in 100% methanol containing 0.2% formic acid was added instantly. The samples were sonicated with ultrasonic for 45 min and centrifuged at 14,000 g for 5 min. The supernatant was transferred into glass vials before HPLC-MS analysis.

2.6. Compounds and MS source optimization for flavonoids

Liquid chromatography-tandem mass spectrometry method development for identification and quantification of flavonoids was conducted. An Agilent 1200 series HPLC (California, USA) connected to an AB Sciex 3200 triple-quadrupole trap mass spectrometer (QTRAP/MS) (AB Sciex, Framingham, USA) with electrospray ionization (ESI) in both positive and negative polarity was used for method development. Compound optimization was performed as follows: First, the individual reference standards were introduced to an electrospray source by direct infusion through the continuous flow of the analyte via a syringe pump (µl min⁻¹ at a concentration of 1 mg l⁻¹) (in 5% methanol with 20 mmol l⁻¹ acetic acids). Next, Q1MS scan mode was employed to check if the protonated or deprotonated configuration of each molecule was present and achieve the analyte’s maximum sensitivity by optimizing the compound dependent MS parameters such as de-clustering potential (DP) and entrance potential (EP). Subsequently, the fragments associated with the targeted precursor ions (quantifier (the most abundant ion) and qualifier ions) were determined by optimizing collision energy (CE) and collision cell exit potential (CXP) (Tables 1 and 2). Analyst Software (version 1.6.2) was used for instrument control, data acquisition, and subsequent quantifications. Quantifications were done based on standard curves prepared in the range of 0.39–400 ng ml⁻¹. Data points of the standard curves were weighted according to x⁻¹.

2.7. LC-MS/MS method development for flavonoids

Some of the analytes of interest were only charged either in positive or negative mode. Therefore, two LC-MS/MS methods were developed, in which 18 analytes were quantified in negative mode and 5 in positive mode. A reversed-phase Synergi Fusion-C18, 80A column (250×2 mm id, 4 µm particle size) was used for both modes to separate analytes. The mobile phase for both modes was a binary solvent mixture compost of solvent A (100% Milli Q water with 0.2% formic acid) and solvent B (100 acetonitrile with 0.2% formic acid) with the flow rate of 0.35 ml min⁻¹ and the injection volume of 30µl.

2.8. Negative mode

LC conditions for (ESI -) were as follows: column oven was set at 40°C, curtain gas, 30 psi; ion spray voltage, - 4200 V; and spray voltage, - 3 000 V.
temperature, 560°C; ion source gas 1, 60 psi; ion source gas 2, 50 psi.

Binary gradient for ESI was as follows: 0-3 min, column equilibration (80% A), 3-24 min, ramping to (55% A), 24-26 min, reduced to (0% A), 26-29 min, isocratic hold (0% A), 29-29.5 min, increased to (80% A), 29.5-40 min, hold (80% A). Nitrogen gas was used as a collision gas to generate MS/MS fragmentation. The separation of flavonoids by LC-QTRAP-MS in negative mode is shown in Figure 1.

2.9. Positive mode

LC conditions for (ESI +) optimized as follows: column oven was set at 40°C, curtain gas, 30 psi; ion spray voltage, - 4500 V; temperature, 500°C; ion source gas 1, 60 psi; ion source gas 2, 50 psi. Binary gradient for ESI - was as following: 0-3 min, column equilibration (80% A), 3-4 min, ramping to (45% A), 4-11 min, reduced to (25% A), 11-12 min, reduced to (0% A) 12-15 min, isocratic hold (0% A), 15-15.5 min, increased to (80% A), 15.5-25 min, hold (80% A). The separation of flavonoids by LC-QTRAP-MS in positive mode is shown in Figure 2.

2.10. Statistical analysis

A generalized linear model (GLM) was fitted to the data. Differences between means were separated...
using the Tukey honestly significant difference (HSD) test at the significance level of 95%. Assumption of normality and homogeneous variance were visually inspected. Data analysis was conducted using the lme4 (1.1-23) package in open-source software R (4.0.2).

3. Results

3.1. Response of hairy vetch grown in soil to interspecific competition

Dry biomass of five-week-old hairy vetch plants grown in soil media with different number of rye plants is shown in
Figure 3. Root and shoot dry biomass of hairy vetch plants decreased as the number of neighboring rye plants increased. The decrease in dry shoot biomass was more pronounced than roots. The hairy vetch’s dry shoot biomass was significantly reduced even when hairy vetch was co-cultivated with just one rye plant. In contrast, a significant reduction in root dry biomass only occurred when hairy vetch was grown with two and four rye plants (Figure 3). Among the 23 flavonoid compounds that were quantified by LC-MS/MS analysis, 8 (kaempferol-Rha-Xyl-Gal, astragalin, daidzein, nicotiflorin, rutin, apigenin, luteolin, and quercetin-Rha-Xyl-Gal) were identified and quantified in root and shoot of five-week-old hairy vetch plants grown in soil (Figures 4 and 5). The dominant flavonoid in root and shoot of hairy vetch plants grown in soil was kaempferol-Rha-Xyl-Gal (Figure 5). The chemical structure of kaempferol-Rha-Xyl-Gal is shown in Figure 6. The concentration of kaempferol-Rha-Xyl-Gal in roots of hairy vetch plants was significantly reduced in response to an increasing number of neighboring rye plants, but there was no significant difference in kaempferol-Rha-Xyl-Gal concentration in shoots (Figure 4). Daidzein and luteolin concentrations in hairy vetch’s roots growing with two and
four rye plants increased significantly. In contrast, the concentrations of nicotinidine and quercetin-Rha-Xyl-Gal in hairy vetch’s roots and shoots were negatively affected by the increased number of rye plants (Figure 5). There was no significant difference in concentrations of other flavonoids in hairy vetch in response to co-cultivation with rye (Figure 5).

3.2. Response of hairy vetch grown in glass beads to interspecific competition

Twelve flavonoids: kaempferol-Rha-Xyl-Gal, astragalin, daidzein, nicotinidine, rutin, kaempferol, apigenin, luteolin, quercetin-Rha-Xyl-Gal, genistein, luteolin-4-O-Glc, and orientin/ homoorientin, were identified and quantified in roots and shoots of 17-day-old hairy vetch plants grown in glass beads (Figures 7 and 8). Kaempferol-Rha-Xyl-Gal was the dominant flavonoid in roots and shoots of 17-day-old hairy vetch plants (Figure 7). The concentration of kaempferol-Rha-Xyl-Gal in 17-day-old hairy vetch plants grown in glass beads was higher than in five-week-old plants grown in soil (Figures 4 and 7). Growing hairy vetch with rye plants significantly reduced the kaempferol-Rha-Xyl-Gal concentration in hairy vetch root compared with control (Figure 7). The concentration of kaempferol-Rha-Xyl-Gal in hairy vetch shoot did not change significantly in response to co-cultivation with different densities of rye (Figure 7). Quercetin-Rha-Xyl-Gal concentration in roots decreased significantly in response to co-cultivation with rye (Figure 8). Apigenin concentration in roots significantly reduced when growing hairy vetch with four rye plants. Changes in the concentration of other flavonoids in response to co-cultivation with rye were not significant.

Kaempferol-Rha-Xyl-Gal was the only flavonoid identified in hairy vetch’s root exudate. The concentration of kaempferol-Rha-Xyl-Gal (per plant and mg root dry weight) in root exudate of hairy vetch significantly increased when hairy vetch plants were co-cultivated with one rye plant, but it decreased by co-cultivation of hairy vetch with two and four rye plants (Figure 9, A and B). Root exudation ratios of kaempferol-Rha-Xyl-Gal were calculated as the amount of kaempferol-Rha-Xyl-Gal in root exudates relative to the amount in the root tissue. According to the results (Figure 9, C), the root exudation ratio of kaempferol-Rha-Xyl-Gal from hairy vetch increased about twofold in response to co-cultivation with one rye plant. However, co-cultivation with two and four rye plants did not have any significant effects on the root exudation ratio. As a part of this experiment, the root and root exudate of rye plants growing alone were tested, and it was confirmed that kaempferol-Rha-Xyl-Gal was not produced by rye.

4. Discussion

In this study, we quantified, for the first time, 12 flavonoids present in hairy vetch’s root and shoot and one flavonoid in the root exudate. The presence of myricetin, quercetin, and kaempferol in hairy vetch flower has been reported previously (Burghardt et al. 1997). Until now, no other studies have quantified flavonoids in plant tissue or root exudate of hairy vetch plants. Composition and quantity of flavonoids

Figure 9. Concentration of kaempferol-Rha-Xyl-Gal in 17-day-old hairy vetch plants grown in glass beads alone (Vetch) and mixture with one (Vetch + Rye), two (Vetch+2Rye), and four (Vetch+4Rye) plants: concentration/plant (A), concentration/mg dry weight of root (B), exudation ratio (C). Exudation ratio was calculated as the concentration of kaempferol-Rha-Xyl-Gal in root exudate relative to the concentration in the root tissue. Values plotted are means (n = 10) ± standard errors (SE). Columns with different letters indicate significant difference among treatments at P < 0.05.
in 17-day-old hairy vetch plants grown in glass beads were different from five-week-old hairy vetch plants grown in soil. Concentration of kaempferol-Rha-Xyl-Gal was higher in plants grown in glass beads than in soil-grown plants. We hypothesize that difference in the content of flavonoids between soil and glass bead-growing plants is due to their growth stage and/or microbial community of their growing media. As an example of the effect of the growing stage on the presence of secondary metabolites, it was reported that the content of isoflavones and soyasaponin in root and root exudate of soybean plants reach a maximum in one-week-old seedlings (Sugiyama et al. 2016; Tsuno et al. 2017). An effect of growth stages on the content of phenolic compounds and flavonoids were also reported in other plant species, including *Trifolium pretense* L. (Vlaisavljević et al. 2017), *Achillea millefolium* L. (Farhadi et al. 2020) and *Tithonia diversifolia* Hems. (Pretti et al. 2018). The isoflavone daidzein was only found in the hairy vetch plants grown in soil, while orientin/homoorientin, kaempferol, and luteolin 4-O-GLc were only found in plants grown in glass beads. The presence of different microbial communities in the soil and glass bead media could explain the differences in flavonoids’ content in hairy vetch plants. Bais et al. (2004) stated that plants could detect their adjacent microbial community in the rhizosphere and respond accordingly. We assume that hairy vetch plants can detect the microbial community in the rhizosphere and respond by altering flavonoids production and that daidzein production was triggered by the presence of specific microorganisms that were absent in the semi-sterile glass bead media. In accordance with our results, it was reported that isoflavones, including daidzein, act as signaling metabolites in plant-microbe interactions (Kossak et al. 1987; Sugiyama et al. 2017). Another study unveiled that roots of soybean plant exude signaling isoflavones such as daidzein and genistein into the rhizosphere to establish symbiotic interaction with rhizobia for nodulation (Sugiyama 2019a).

Among the 12 identified flavonoids, kaempferol-Rha-Xyl-Gal was the most abundant flavonoid compound in shoot, root and root exudates of hairy vetch. We hypothesized that the accumulation and exudation of a large amount of kaempferol-Rha-Xyl-Gal was a result of its biological importance to hairy vetch. It has been reported that many bioactive compounds are glycosides (Kren 2008), and that the presence of glycoside conjugate is essential for the bioactivity of many of these compounds (Kren and Martiníková 2001). We suggest that high water solubility of kaempferol-Rha-Xyl-Gal ease its root exudation and thus increase its distribution in the rhizosphere, where it could play various roles in plant interactions. In support of our argument, it was shown that glycosylation of flavonoids strongly increases their water solubility, which improves their bioavailability and metabolism (Pandey et al. 2014; Slámová et al. 2018).

An increasing number of rye plants negatively affected hairy vetch plants’ dry biomass in the present study. It was reported that the high competitiveness of rye could result in growth suppression of other plants (Barnes and Putnam 1986). For instance, it was shown that cover cropping of rye suppressed winter annual weeds biomass by more than 90% (Werle et al. 2017). In our study, we assumed that an increasing number of rye plants increased competition for resources and limited hairy vetch growth.

Many studies reported an increase in the concentration of flavonoids in response to biotic and abiotic stresses (Yang et al. 2018). In contrast, our study showed that concentrations of kaempferol-Rha-Xyl-Gal in roots of hairy vetch plants were negatively affected by co-cultivation with rye. Alteration in the kaempferol-Rha-Xyl-Gal concentration in five-week-old hairy vetch plants grown in soil (no extra nutrient applied) was more pronounced than with 17-day-old hairy vetch plants grown in glass beads (regularly irrigated with Hoagland solution). We speculate that competition for resources, in particular nutrients, is responsible for decreasing kaempferol-Rha-Xyl-Gal concentration in hairy vetch’s root. A previous study by Coronado et al. (1995) indicated that nitrogen deficiency increases root flavonoid and isoflavonoid production. We hypothesize that severe competition from rye may result in a deficit of several nutrients and not only nitrogen in hairy vetch plants and that multi-nutrient deficiency may have reduced the production of flavonoids in hairy vetch plants. In consistency with our result, it has been reported that intra- and interspecific competition in *Rosmarinus officinalis* L. plants reduced the concentration and emission of terpenes (Ormeño et al. 2007). Furthermore, it has been shown that increasing intraspecific competition reduced concentrations of the allelochemicals chlorogenic acid, rutin and tomatine in tomato plants (Stamp et al. 2004). In several of the above-mentioned studies, the authors concluded that a decrease in the availability of nutrients under competitive stress could explain the reduction in the secondary metabolites’ concentration (Cipollini and Bergelson 2001; Stamp et al. 2004).

Our study is the first study reporting exudation of kaempferol-Rha-Xyl-Gal from roots. Kaempferol-Rha-Xyl-Gal was the only identified flavonoid compound in the root exudate of hairy vetch. We assume that the other flavonoids were also present in the root exudate of hairy vetch but their concentration was below the detection level. The presence of flavonoid compounds such as daidzein, naringenin, genistein, methoxychalcone, and 4',7-dihydroxylavone in the root exudate of legume plant species has been reported previously (Bolaños-Vásquez and Werner 1997; Li et al. 2016; Liu and Murray 2016). Among the 12 identified flavonoids in hairy vetch root and shoot, we observed a negative correlation between increases in rye density and kaempferol-Rha-Xyl-Gal concentrations in hairy vetch’s root and shoot. Shading by neighboring rye plants of hairy vetch could be one of the reasons for this observation. It has been shown that changes in light quality (wavelength and photoperiod of radiations) and intensity alter flavonoids’ biosynthesis (Zoratti et al. 2014). For example, shading significantly reduced biosynthesis of O-glycosylated flavonols and proanthocyanins in leaves of tea *Camellia sinensis* (L.) O. Kuntze (Wang et al. 2012). Among the various environmental and physical variables, UV irradiation was considered the main cause of enhanced flavonoid content (Chaves et al. 1997). Similarly, exposure of several *Passiflora* species’ calluses to UV irradiation increased glycosyl flavonoids’ production (Antognoni et al. 2007).

The current study showed that root exudation of kaempferol-Rha-Xyl-Gal from hairy vetch plants significantly increased at a low level of competition (hairy vetch growing with one rye plants), but it decreased under more competitive conditions (hairy vetch growing with two and four rye plants). This observation reinforces results from a previous
study stating that medium plant competition had positive effects on allelochemicals content in *Pinus halepensis* Mill., whereas a negative effect was observed at higher levels of competition (Rivoal et al. 2011). Besides, root exudation of kaempferol-Rha-Xyl-Gal could have been affected by the changes occurring in the nutrient status and environmental conditions due to increasing competition from ry e. It was previously reported that a wide range of environmental variables such as light intensity, drought, nutrient deficiency or metal toxicity can alter root exudation patterns (Rovira 1959; Ma et al. 2001; Carvalhais et al. 2011; Canarini et al. 2016). Furthermore, exposure of *Alnus glutinosa* L. roots to white light during the 16 h photoperiod increased quercetin, kaempferol and kaempferol conjugate concentrations in root exudate 46, 4 and 70 fold, respectively (Hughes et al. 1999). Similarly, the root exudation of flavonoids such as catechin by *Centaurea stoebe* Lam. altered response to the light intensity with the highest concentration six hours after exposure to sunlight (Tharayil and Triebwasser 2010). Nonetheless, our results on the effect of competition on secondary metabolites production and biomass are, for the most part, in agreement with the growth-differentiation balance hypothesis (GDBH), which describes how low resource availability limits both growth and production of secondary metabolites (Daniel and William 1992).

5. Conclusion

This study was undertaken to better understand physio-chemical changes occurring in hairy vetch in response to interspecific competition by rye. It revealed that interspecific competition affected both biomass production and content of kaempferol-Rha-Xyl-Gal in hairy vetch negatively. In addition, the study showed that the concentration of kaempferol-Rha-Xyl-Gal in root exudates and the root exudation ratio increased at the low level of competition and decreased at the high level of competition. Future studies would be of interest to examine whether the response of hairy vetch to the intraspecific and interspecific competition is similar to its response to rye. Finally, further studies should be undertaken to elucidate the bioactivity of kaempferol-Rha-Xyl-Gal and its role in plant-plant interactions.

Disclosure statement

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References

Antognoni F, Zheng S, Pagnucco C, Baraldi R, Poli F, Biondi S. 2007. Induction of flavonoid production by UV-B radiation in *Passiflora quadrangularis* callus cultures. Fitoterapia. 78:345–352.
Armiero J, Requejo J, Jorrin J, López-Valbuena R, Tena M. 2001. Release of phytoalexins and related isoflavonoids from intact chick-pea seedlings elicited with reduced glutathione at root level. Plant Physiol Biochem. 39:785–795.
Bais HP, Park S-W, Weir TL, Callaway RM, Vivanco JM. 2004. How plants communicate using the underground information superhighway. Trends Plant Sci. 9:26–32.
Baralbar B, Hunter MC, Chipanski ME, Hamilton A, Mortensen DA. 2018. Weed suppression in cover crop monocultures and mixtures. Weed Sci. 66:121–133.
Barnes JP, Putnam AR. 1986. Evidence for Allelopathy by Residues and Aqueous extracts of Rye (Secale cereale). Weed Sci. 34:384–390.
Bashyal M, Ferguson JC, Perez-Hernandez O, Hoillet N. 2019. Effect of cereal Rye and hairy vetch on pest suppression and corn yield. Commun Soil Sci Plant Anal. 50:1093–1105.
Bolaños-Vásquez MC, Werner D. 1997. Effects of *Rhizobium* tropici, *R. etli*, and *R. leguminosarum* bv. phaseoli on nod gene-inducing flavonoids in root exudates of *Phaseolus vulgaris*. Molecular Plant-Microbe Interactions®. 10:339–346.
Brainard D, Henshaw B, Snapp S. 2012. Hairy vetch varieties and bi-cultural influences cover crop services in strip-tillaged sweet corn. Agron J. 104:629–638.
Burghardt F, Fiedlert K, Proksch P. 1997. Uptake of flavonoids from Vicia villosa (Fabaceae) by the lycaenid butterfly, *Polygonumiscus icarus* (Lepidoptera: Lycaenidae). Biochem Syst Ecol. 25:527–536.
Canarini A, Merchant A, Dijkstra FA. 2016. Drought effects on *Helianthus annuus* and Glycine max metabolites: from phloem to root exudates. Rhizosphere. 2:85–97.
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Carlsen SCK, Mortensen AG, Olesen W, Piacente S, Stochmal A, Fomsgaard IS. 2008. Variation in flavonoids in leaves, stems and flowers of white clover cultivars. Nat Prod Commun. 3:1299–1306.

Carvalhais LC, Dennis PG, Fedoseyenko D, Hajirezaei M-R, Borriss R, Von Wirén N. 2011. Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. J Plant Nutr Soil Sci. 174:3–11.

Cesco S, Neumann G, Tomasi N, Pinton R, Weiskopf L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil. 329:1–25.

Chaves N, Escudero JC, Gutiérrez-Merino C. 1997. Role of ecological variables in the seasonal variation of flavonoid content of Cistus ladanifer exudate. J Chem Ecol. 23:579–603.

Cipollini DF, Bergelson J. 2001. Plant density and nutrient availability constrain constitutive and wound-induced expression of Trpysin inhibitors in Brassica napus. J Ecol. 27:593–610.

Clark A. 2008. Managing cover crops profitably. Darby, PA: Diane Publishing.

Coronado C, Zuanazzi J, Sallaud C, Quirion JC, Esnault R, Husson HP, Cipollini DF, Bergelson J. 2007. Allelopathic effects of the soybean rhizosphere: metabolites, microbes, and VOCs in plant–plant interaction. J Plant Interact. 14:630–636.

Coronado C, Zang S-Z, Li Y-H, Xia Z-C, Yang X-F, Meiners SJ, Wang P. 2018. Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. Nat Commun. 9:3867.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kamo T, Hiradate S, Fujii Y. 2003. First Isolation of Natural cyanamide as a possible allelochemical from hairy vetch Vicia villosa. J Chem Ecol. 29:275–283.

Kong C-H, Zhang S-Z, Li Y-H, Xia Z-C, Yang X-F, Meiners SJ, Wang P. 2018. Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. Nat Commun. 9:3867.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong C-H, Zhang S-Z, Li Y-H, Xia Z-C, Yang X-F, Meiners SJ, Wang P. 2018. Plant neighbor detection and allelochemical response are driven by root-secreted signaling chemicals. Nat Commun. 9:3867.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.

Kong CH, Zhao H, Xu XH, Wang P, Gu Y. 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J Agric Food Chem. 55:6007–6012.
Tosti G, Benincasa P, Farneselli M, Pace R, Tei F, Guiducci M, Thorup-Kristensen K. 2012. Green manuring effect of pure and mixed barley – hairy vetch winter cover crops on maize and processing tomato N nutrition. Eur J Agron. 43:136–146.

Tsuno Y, Fujimatsu T, Endo K, Sugiyama A, Yazaki K. 2017. Soyasaponins: A New class of root exudates in soybean (Glycine max). Plant Cell Physiol. 59:366–375.

Vlaisavljević S, Kaurinović B, Popović M, Vasiljević S. 2017. Profile of phenolic compounds in Trifolium pratense L. extracts at different growth stages and their biological activities. Int J Food Properties. 20:3090–3101.

Wang Y, Gao L, Shan Y, Liu Y, Tian Y, Xia T. 2012. Influence of shade on flavonoid biosynthesis in tea (Camellia sinensis (L.) O. Kuntze). Sci Hortic. 141:7–16.

Werle R, Burr C, Blanco-Canqui H. 2017. Cereal rye cover crop suppresses winter annual weeds. Can J Plant Sci. 98:498–500.

Yang L, Wen K-S, Ruan X, Zhao Y-X, Wei F, Wang Q. 2018. Response of plant secondary metabolites to environmental Factors. Molecules (Basel, Switzerland). 23:762.

Zoratti L, Karppinen K, Luengo Escobar A, Häggman H, Jaakola L. 2014. Light-controlled flavonoid biosynthesis in fruits. Front Plant Sci. 5:534.