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UV-B radiation affects flavonoids and fungal colonisation in *Fagopyrum esculentum* and *F. tataricum*

Research Article

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Abstract: In the present study, we have evaluated the effects of increased UV-B radiation that simulates 17% ozone depletion, on fungal colonisation and concentrations of rutin, catechin and quercetin in common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tataricum*). Induced root growth and reduced shoot:root ratios were seen in both of these buckwheat species after enhanced UV-B radiation. There was specific induction of shoot quercetin concentrations in UV-B-treated common buckwheat, whereas there were no specific responses for flavonoid metabolism in tartary buckwheat. Root colonisation with arbuscular mycorrhizal fungi significantly reduced catechin concentrations in common buckwheat roots, and induced rutin concentrations in tartary buckwheat, but did not affect shoot concentrations of the measured phenolics. Specific UV-B-related reductions in the density of microsclerotia were observed in tartary buckwheat, indicating a mechanism that potentially affects fungus–plant interactions. The data support the hypothesis that responses to enhanced UV-B radiation can be influenced by the plant pre-adaptation properties and related changes in flavonoid metabolism.

Keywords: *Buckwheat • Fungal endophytes • Quercetin • Rutin • UV-B*

1. Introduction

Research into the effects of ultraviolet (UV) radiation on terrestrial organisms can be split to two main themes: the effects of increased UV-B radiation arising from depletion of the stratospheric ozone concentration, and the effects of solar UV radiation as a source of information about the environment for microbes, plants and animals [1]. The UV-C region of the UV spectrum includes wavelengths under 280 nm, which are effectively absorbed by ozone in the stratosphere. In contrast, UV-A (320-400 nm) and UV-B (280-320 nm) radiation do reach ground level [2]. As the stratospheric ozone concentration decreases, the UV-B portion of the sunlight will increase [3].

Common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tataricum*) are frequently used for human consumption and they receive additional attention through medicinal research. They are particularly rich in flavonoids, which have numerous beneficial effects on human health. Among the flavonoids found in both of these buckwheat species, rutin and quercetin have a broad range of physiological activities in humans and other animals, such as anti-inflammatory [4], anti-tumour [5,6], and anti-bacteria [7,8] effects and thus they might also be of interest to the pharmaceutical industry.

Tartary buckwheat is frequently cultivated at higher altitudes, and thus under intense UV radiation, where the environment is not suitable for rice or other major crops. Plants exposed to elevated UV-B radiation frequently show reduced growth as a consequence of induced morphological changes [9,10]. Although the efficiency of damage caused by UV-A radiation is much

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lower, it appears to lead to similar inactivation of the light reactions of photosynthesis as seen for UV-B radiation [11,12]. At the cellular level, UV-B radiation initiates the formation of superoxide radicals, resulting in oxidative damage that has to be counteracted by antioxidants and protective pigments before adequate shielding by the flavonoids is achieved [13,14].

In a genetic approach to evaluate the relative importance of the proposed UV-B protective mechanisms in flowering plants, it was demonstrated that two distinct classes of phenylalanine-derived, UV-absorptive secondary products provide UV-B protection to *Arabidopsis*: flavonols and sinapic acid esters [15]. Among the secondary products in buckwheat plants, the catechins are known to increase differentially in plants exposed to enhanced UV-B conditions, although they might not provide sufficient protection against excessive UV-B radiation. Higher quercetin concentrations are also seen in plant populations with higher UV-B exposure. These were thus shown to increase under enhanced UV-B radiation in correlation with plant-growth reduction, conferring protection to the plants against UV-B-induced damage [16]. In contrast, rutin showed dependence on the irradiation levels used, as its concentrations can be either lower or higher than in plants grown under ambient conditions [17,18].

There is increasing evidence that UV radiation can affect trophic interactions and, in turn, influence a variety of ecosystem functions through both direct and indirect effects. A reduction in plant diseases has been seen under enhanced UV-B conditions, as this can directly kill spores of casual fungi [1]. In addition, with the changes that can occur in the plant chemistry, the host plant is frequently more resistant to pathogens and may also change their environment *via* root exudation [1,19]. Through these effects variations in UV radiation might impact on the interactions of plants with beneficial microorganisms.

The majority of plants form associations with arbuscular mycorrhizal (AM) fungi and/or dark septate endophytes (DSEs) [20]. These provide plants with mineral nutrients [21,22] in exchange for carbon compounds [23,24]. Although there are several reports that indicate the absence of AM colonisation in buckwheat [25-27], its colonisation by AM fungi and DSE was recently reported [28]. The susceptibility of AM fungi to UV-B stress might be partially attributed to the changes in plant hormone levels, and partially to host changes in the phenylpropanoid pathway [29,30].

The main objectives of the present study were: (i) to evaluate the effects of enhanced UV radiation on the selected flavonoid concentrations; (ii) to establish root colonisation levels with fungal endophytes under enhanced UV-B radiation conditions; and (iii) to shed more light on flavonoid metabolism and the interactions of both of these buckwheat species with their endophytes under enhanced UV-B radiation.

2.Experimental Procedures

2.1 Plant growth conditions and fungal inoculations

Seeds of common buckwheat (*Fagopyrum esculentum* Moench, cv. Siva) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.; domestic population from Luxembourg) were surface sterilised for 5 min in Na-hypochlorite solution (3% active chlorine, in water), and rinsed with sterile water. These surface-sterilised seeds were sown in plastic trays (30 seeds per tray) containing a sterilised soil and vermiculite mixture (1:3, v/v), and germinated in a growing chamber (22°C, 80% humidity, 16-h day period, under 325 µmol/m^2/s illumination). One half of the trays (for each treatment) was layered with 1 cm fungal inoculum prepared from an indigenous fungal mixture from a buckwheat field with maize (*Zea mays* L.) as the inoculum host plant. AM fungi in the inoculum were identified by spore morphology as *Glomus mosseae, G. fasciculatum* (Thaxt.) *Gerd.&Trappe, G. etunicatum* W. N. Becker &Gerd., *G. intraradices* N.C. Schenck& G.S. Smith and *Scutellospora sp.* Inoculation of the maize growing on the inoculum was F% 100±0%, M% 69.5±3.6% and A% 69.5±4.2% (Mean ± SE, n=10). The experiments were conducted over two consecutive years (2002 and 2003), with the same trends observed; therefore, only the results from 2002 are shown here.

2.2 UV-B simulation

Trays with 2-week-old buckwheat plants were transferred to the research plot in the Ljubljana Botanical Garden for the duration of the experiment. At the stage of two to three leaves per plant, the plants were thinned to 15 plants per tray, with the trays filled with 10 litres of substrate and subjected to three different light treatments, as described previously [17,31]. A UV-B supplementary system for outdoor experiments was designed as described in [32]. Three different treatments were applied: (1) Enhanced UV-B treatment [UV-B] that simulated 17% ozone depletion, using Q-Panel UV-B 313 lamps (Cleveland, OH, USA) filtered with cellulose diacetate filters (to block the UV-C range, as wavelengths <280 nm) that allowed transmission of both UV-A and UV-B radiation. (2) Reduced UV-B treatment achieved by Q-Panel UV-B 313 lamps filtered with Mylar foil, which cuts out wavelengths approx. <320 nm and allows transmission of only UV-A radiation

[UV-A], therefore allowing for responses to be specifically attributed to UV-B radiation [33,34]. (3) Exposure to ambient radiation, with no interference [ambient].

The lamps were placed 140 cm above ground level, to provide sufficient aeration and to exclude possible phytotoxic effects of cellulose diacetate [35]. The system was timer controlled. The UV-B doses were calculated and adjusted weekly using the programme of Björn and Murphy [32] and based on the generalised plant action spectrum of Caldwell [36]. The filters were replaced every two weeks to ensure uniformity of UV transmission. The plants of common and tartary buckwheat were harvested at seed maturity (84 and 91 days after germination, respectively).

2.3 Fungal colonisation

At harvest, the roots of the buckwheat plants were washed thoroughly with water, and stained with Trypan Blue [37]. The level of colonisation by AM fungi and DSEs was estimated under light microscopy on 1-cm-long root fragments (15 fragments per plant, 15 plants per treatment). The fungal colonisation parameters of colonisation frequency (F%), global intensity of colonisation of the root system (M%), and density of the arbuscules (A%), were assessed according to Trouvelot *et al.* [38]. The density of microsclerotia (MS%), as typical structures of DSEs, was calculated in the same way, as the density of arbuscules. The fungi that had colonised the roots of both of these buckwheat species were identified using molecular tools [28].

2.4 Flavonoid analysis

At harvest, four plants per treatment were sampled randomly (10 to 20 mg/plant for both shoots [stems with leaves] or plant roots). They were immersed in liquid nitrogen, lyophilised, and extracted with methanol: water (60:40, v/v) at room temperature for 45 min. After centrifugation for 10 min at 10000×*g*, the supernatants were filtered (Millipore, Durapore membrane filters; 0.22 µm GVPP).

For the analysis of shoot and root flavonoids, a Waters HPLC system (Separation Module 2960 with PDA 996 detector) was used in combination with Millenium 32 (Waters) software. The flavonoids in 50 µl supernatant were separated on 5 mm Waters Spherisorb, ODC-II, C18 column (250x4.6 mm) by high-performance liquid chromatography, with elution with a two-step linear gradient using acetonitrile (solvent A) and water – methanol – 1.5% H_3PO_4 in water (1:1:1, v/v/v, solvent B). The mobile phase started at 100% solvent A, and was increased linearly to 40% solvent B in 20 min, followed by a further linear increase to 100% solvent B in 20 min. The flow rate was 1 ml/min, and the detection wavelengths for quantification were 350 nm for rutin and quercetin, and 280 nm for catechin (Figure 1).

2.5 Statistical analyses

The effects of UV radiation on plant biomass, flavonoid concentrations, and fungal colonisation levels were examined by analysis of variance according to a general linear-model procedure. The differences among the various treatment means were separated by Holm-Sidak *post-hoc* tests at the 0.05 level of probability. Differences in flavonoid concentrations between both of the buckwheat species were evaluated using the t-test. All analyses were performed in SigmaPlot (Systat Software Inc.).

3. Results

3.1 Fresh weight of common and tartary buckwheat

Exposure of the plants to enhanced UV-B radiation specifically increased the shoot (stems with leaves) biomass in common buckwheat and the root biomass of both buckwheat species, when compared to the UV-A plants; in contrast, the shoot biomass in tartary

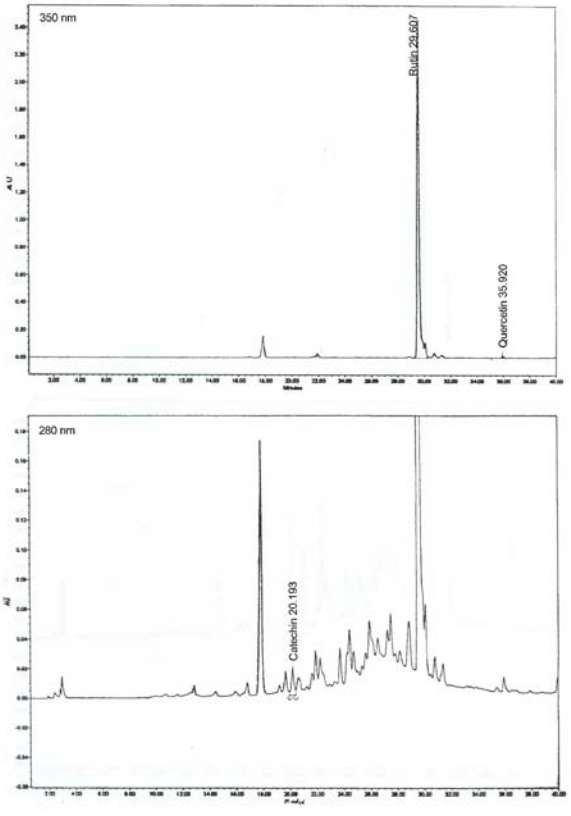

Figure 1. HPLC chromatograms of common buckwheat leaf extracts separated by C18 reverse-phase chromatography. Chromatograms were analyses at 350 nm for rutin and quercetin quantification (upper panel), and at 280 nm for catechin quantification (lower panel). The retention times are shown next to the relevant peaks.

buckwheat was significantly reduced (Table 1). The shoot:root ratios of the UV-B plants of both buckwheat species were therefore significantly decreased (Table 1).

3.2 Colonisation with AM and DSE fungi

Root fungal colonisation of both buckwheat species was characterised by hyphae and distinct microsclerotia of DSEs. In addition, occasional arbuscules, as typical AM structures, were seen, although these appeared in numbers that were too low for any reliable statistical analysis. Colonisation frequencies (F%) and densities of arbuscules (A%) were not affected by the exposure to enhanced UV radiation (Table 2). In contrast, the density of microsclerotia (MS%) in the root system of tartary buckwheat was specifically reduced after enhanced UV-B radiation, when compared to the UV-A plants. In addition, the global intensity of colonisation of the root system (M%) and the density of microsclerotia (MS%) were reduced in both buckwheat species under the enhanced UV-B radiation, when compared to the non-treated (ambient) plants.

3.3 Flavonoid concentrations

Tartary buckwheat contained higher root rutin and shoot catechin and quercetin concentrations as compared to common buckwheat (Table 3). The root quercetin

Treatments	Biomass (g)					
	Shoots		Roots		Shoot/root ratio	
	Common buckwheat	Tartary buckwheat	Common buckwheat	Tartary buckwheat	Common buckwheat	Tartary buckwheat
Ambient	3.73 ± 0.14bc	3.97 ± 0.21b	0.58 ± 0.06yz	0.8 ± 0.16xy	7.3 ± 0.6y	6.7 ± 0.8y
UV-A	3.26 ± 0.29c	4.82 ± 0.28a	0.44 ± 0.04z	0.4 ± 0.04z	7.7 ± 0.6y	13.2 ± 1.4x
UV-B	3.81 ± 0.25b	2.92 ± 0.30c	0.90 ± 0.08x	0.8 ± 0.13xy	4.4 ± 0.3z	4.8 ± 0.4z

Table 1. The effects of the UV treatments on the biomass of shoots (stems with leaves) and roots in common and tartary buckwheat plants. Data are means ±SE (n=15). Different letters in each pair of columns represent statistically significant differences at $P \leq 0.05$.

Buckwheat species	Treatment	F%	M%	A%	MS%
Common	Ambient	96.19 ± 1.35	35.42 ± 4.06 b	0.00 ± 0.00	9.38 ± 2.54 bc
	UV-A	95.24 ± 2.15	20.29 ± 3.72 c	0.00 ± 0.00	3.16 ± 0.51 d
	UV-B	97.62 ± 1.13	24.07 ± 3.05 c	0.00 ± 0.00	3.92 ± 0.61 d
Tartary	Ambient	96.41 ± 1.22	47.05 ± 4.65 a	0.26 ± 0.22	18.76 ± 3.08 a
	UV-A	98.79 ± 1.21	36.28 ± 2.67 b	0.00 ± 0.00	13.20 ± 1.91 b
	UV-B	98.79 ± 0.81	30.06 ± 4.30 bc	0.06 ± 0.06	5.47 ± 1.57 cd

Table 2. The effects of the UV treatments on colonisation of common buckwheat and tartary buckwheat with fungal endophytes. Data are means ±SE (n=8). Different letters in each pair of columns represent statistically significant differences at $P \leq 0.05$.

Flavonoid	Flavonoid concentration (% dw)			
	Shoots		Roots	
	Common buckwheat	Tartary buckwheat	Common buckwheat	Tartary buckwheat
Rutin	2.808 ±0.127	2.998 ±0.078	0.065 ±0.004 a	0.207 ±0.024 b
Catechin	0.080 ±0.007 a	0.106 ±0.006 b	0.025 ±0.002 a	0.018 ±0.002 b
Quercetin	0.016 ±0.004 a	0.047 ±0.007 b	nd[1]	nd[1]

Table 3. The mean flavonoid concentrations in the shoots (stems with leaves) and roots of the common and tartary buckwheat plants. [1]nd = not detected. Data are means ±SE (n=8). Different letters in each pair of columns represent statistically significant differences of t-test at $P < 0.05$.

concentrations in both of these buckwheat species were under the detection limits.

UV radiation did not affect the shoot rutin, catechin and quercetin concentrations in tartary buckwheat plants (Figure 2a-c). In common buckwheat, the only UV-B-specific effect was an increase in shoot quercetin concentrations of the UV-B-treated plants, when compared to the UV-A-treated plants (Figure 2c).

Inoculation with the indigenous fungal mixture from the buckwheat field resulted in a significant reduction in root catechin concentrations in the common buckwheat roots (Figure 3a), whereas there was a significant increase in root rutin concentrations in the tartary buckwheat roots when compared to the non-inoculated plants (Figure 3b). No changes in shoot flavonoid concentrations were seen as a result of the fungal inoculations in either of the species.

4. Discussion

Both reductions as well as enhancements in plant biomass have been reported as responses to enhanced UV-B radiation under field conditions [9]. The UV-B-specific induction of root biomass and reduction in shoot:root ratios seen for both of these buckwheat species were attributed to changes in water relations and carbon partitioning, as previously reported for common buckwheat plants [31]. The reduction in shoot biomass of the tartary buckwheat is also in line with previous observations [18], and might have been accompanied by a reduced number of nodes, reduced branching, and reduced length of the petiole [10].

The flavonoid content in buckwheat species depends on the plant genotype, the plant organ, the phenological state, and the time of sowing [17,39,40]. Therefore, both common and tartary buckwheat plants were deliberately collected at the stage of seed maturity (e.g. 84 and 91

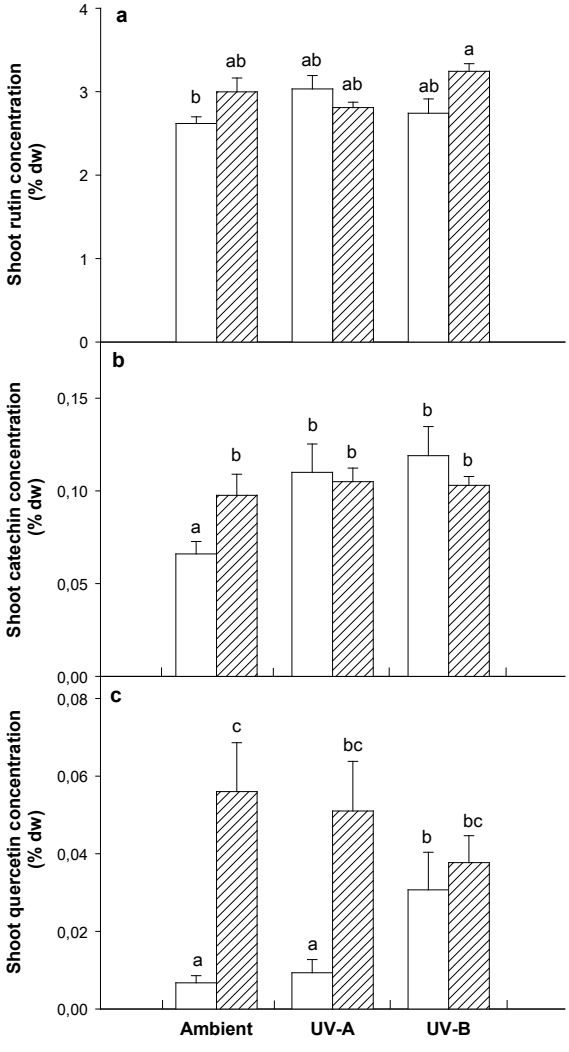

Figure 2. Shoot (stems with leaves) flavonoid concentrations. (a) rutin, (b) catechin and (c) quercetin concentrations in common buckwheat ☐ and tartary buckwheat ▨ under ambient radiation, UV-A and UV-B treatments. Data are means ±SE (n=8). Different letters represent statistically significant differences at $P<0.05$

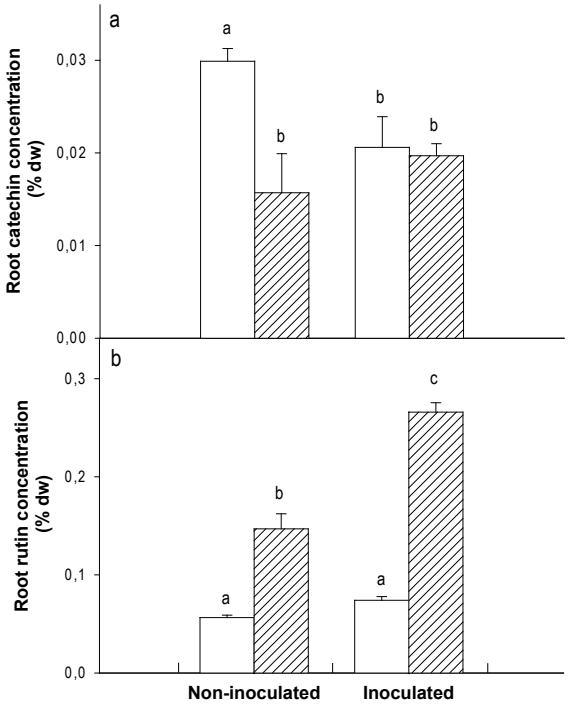

Figure 3. Root flavonoid concentrations. (a) catechin and (b) rutin concentrations in common buckwheat ☐ and tartary buckwheat ▨ non-inoculated and inoculated with indigenous fungi. Data are means ±SE (n=8). Different letters represent statistically significant differences at $P<0.05$

days after sowing). The tartary buckwheat showed higher shoot catechin and quercetin concentrations and root rutin concentration. Increased concentrations of these measured secondary metabolites can be viewed as the result of genetic pre-adaptation of tartary buckwheat to higher altitudes, where they would serve for protection against UV radiation [41]. As a consequence, no further UV-B-specific changes in flavonoid concentrations were seen. In contrast, the common buckwheat showed a specific increase in shoot quercetin concentrations when exposed to enhanced UV-B radiation. The lower overall flavonoid concentrations in this common buckwheat and the more intensive responses in flavonoid metabolism to enhanced UV-B radiation suggest that the common buckwheat is less pre-adapted than the tartary buckwheat.

Various flavonoid compounds are known to specifically affect the growth of mycorrhizal fungi [42,43]. Rutin has been reported not to impact on the growth of AM fungal hyphae, but to have an impact on fungal colonisation of tomato plants when exogenously applied [42,44]; however, rutin has also been demonstrated to enhance hyphal growth of some ectomycorrhizal fungi [45]. Knowledge of the changes in plant-root flavonoid concentrations induced after fungal inoculation is even more limited. The few reports point to complex pathways of metabolism of the flavonoids and their glycoside forms after fungal colonisation. Reduced catechin contents were found in roots of *Fagus sylvatica* L. and *Larix decidua* Mill. after colonisation by ectomycorrhizal fungi [46-48]. The reduced catechin concentrations in common buckwheat roots and increased rutin concentrations in tartary buckwheat roots seen after fungal colonisation in the present study indicate specific changes in the metabolic pathways related to fungal infections. The roots of both buckwheat species used in our experiments were colonised by Ascomycota, Basidiomycota and Chytridiomycota, with tartary buckwheat roots also colonised by AM fungi (Glomeromycota) [28]. It is not yet possible to relate observed root flavonoid concentration changes to root colonisation by specific representatives of the fungal groups. A more extensive study would be needed to relate these root flavonoid concentrations to the root fungal colonisation levels. Due to the importance of flavonoid metabolism for plants, fungi and fungus–plant interactions, this area of study clearly deserves further attention.

Reports on the impact of elevated UV-B radiation on soil and endophytic microbial communities are rare, despite the vital role of these communities in ecosystem functioning [29,49-51]. In tartary buckwheat roots, a UV-B-specific reduction in density of microsclerotia (M%) was observed, when compared to the UV-A-treated plants. Reductions in both the global colonisation intensity (M%) and the density of microsclerotia (MS%) were seen for both of these buckwheat species exposed to enhanced UV-B and UV-A radiation, when compared to the ambient-grown plants. This emphasises the subtle receptiveness of fungus–plant interactions to changes in UV radiation in general. Reductions in root colonisation by AM fungi in gramineous species exposed to enhanced UV-B radiation, accompanied by unexpected shifts in the competitive balance between pigmented and non-pigmented saprobic fungi, have already been reported [19,30]. The responses seen in the present study might have resulted from changes in plant rhizodeposition, changes in host hormonal balance, and/or changes in the phenylpropanoid pathway [29]. The effects of the flavonoid levels on the root fungal colonisation are far from being one-sided, as endophytic fungi can also affect the metabolism of the host. Clearly, more investigations are needed before we can decipher the complex interactions between flavonoid metabolism and root fungal colonisation under changed UV radiation of environmental conditions.

5. Conclusions

Increases in UV-B radiation due to reductions in the ozone layer are expected to affect plants and fungi and their interactions. In the present study, repartition of carbohydrates toward more intensive root growth and changed shoot:root ratios were seen for both the common and tartary buckwheat species. The variability of the responses in flavonoid metabolism, which provide protection against harmful UV-B radiation, might arise from pre-adaptation of the plant to environmental UV-B conditions. Reduction in the root fungal colonisation levels due to the enhanced UV-B radiation might, as a consequence, reducte the effectiveness of plant interactions with their beneficial fungal colonisers. More detailed studies on flavonoid metabolism in plants inoculated with specific fungal colonisers are needed if we are to find the fungus–plant combinations that have the greatest beneficial effects under such changed UV environmental conditions.

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