

CHANGES IN ISOFLAVONOID AND FLAVONOID CONTENT IN SOYBEAN LEAVES AFFECTED BY UV-B OR COPPER

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The production of antioxidant flavonoids is an important aspect of plant defence mechanisms under stress conditions. The hypothesis of increased antioxidant flavonol quercetin and isoflavone content in soybean leaves subjected to UV-B irradiation and copper toxicity was proposed, and their accumulation was investigated using HPLC analysis. However, the accumulation of antioxidant quercetin was not induced by either UV-B irradiation or copper treatment. UV-B irradiation strongly induced isoflavone aglycones, mainly genistein, daidzein, formononetin, and to a lesser extent, biochanin A. Isoflavone glycosides, genistin and daidzin were also induced. Copper treatment, whether applied to leaves or root media, resulted in the accumulation of genistin and its malonylated form, whereas aglycones were only slightly affected. The higher accumulation of genistein over daidzein after copper treatment could be related to the higher chelating ability of genistein and its glycosides. Soybean isoflavones, which possess antioxidant and chelating properties, rather than flavonols, play a crucial role in the plant's defence mechanisms against both UV-B irradiation and copper toxicity.

Key words: soybean, abiotic stress, ultraviolet irradiation, copper stress, flavonoids, isoflavonoids

Plants produce a diverse range of specialized metabolites, among which flavonoid compounds represent one of the largest and most studied classes. Structurally, they can be divided into two main groups: 2-phenylchromans, flavonoids (euflavonoids) that are widely distributed in the plant kingdom and 3-phenylchromans, isoflavonoids that are typically produced in legumes (Du *et al.* 2010).

Plant flavonoids are involved in environmental adaptation as defence compounds, allelopathic compounds, signalling molecules, antioxidants, chelating agents, ultraviolet (UV) radiation absorbents, and others (Zhuang *et al.* 2023). Isoflavonoids often

contribute to protection against biotic stresses, in plant-microbe interaction, particularly in plant symbioses with rhizobia and mycorrhizal fungi, as well as in the interactions between pathogenic organisms and plants (Mierziak *et al.* 2014). Moreover, growing evidence suggests their potential role in responding to various abiotic factors such as UV-irradiation, drought, salinity, or heavy metals toxicity (Trush & Paľove-Balang 2023).

Isoflavones are the main class of isoflavonoids found predominantly in soy. The main and most studied compounds are daidzein, genistein, glycitein, and their glycosides, malonylglycosides and

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acetylglycosides. Biochanin A and formononetin have also been described there (Karki *et al.* 2020). Flavonols are an important and widespread class of flavonoids. They are highly abundant in soy mainly as diverse glycosides of quercetin, kaempferol, or, to lesser extent isorhamnetin. Their presence can vary widely in both quality and quantity among different cultivars (Lee *et al.* 2022).

Isoflavone content is controlled by multiple genes and affected by environmental factors (Chu *et al.* 2017), including drought (Lozovaya *et al.* 2005), light conditions (Kirakosyan *et al.* 2006), fertilisation (Vyn *et al.* 2002; Kim *et al.* 2005) temperature and CO₂ levels (Kitamura *et al.* 1991; Kim *et al.* 2005; Lozovaya *et al.* 2005). Cold and osmotic stress increased the levels of isoflavones (daidzin, genistin, daidzein, and genistein) in the roots of soybean seedlings (Swigonska *et al.* 2014). High-temperature stress led to the accumulation of different isoflavones during seed development and to the expression of key genes encoding enzymes involved in isoflavone synthesis (Chennupati *et al.* 2012). The increase in isoflavone content in germinating soybean is dose-dependent and accompanied by the increased activities of the key enzymes in isoflavone biosynthesis (Ma *et al.* 2018; Ma *et al.* 2019). On the other hand, a reduction in seed isoflavone concentration was determined during a period of water stress (Gutierrez-Gonzalez *et al.* 2010). Seed isoflavone content dropped dramatically due to water stress, high temperature and solar radiation (Carrera & Dardanelli 2015).

In *Lotus japonicus*, certain types of abiotic stress conditions, such as UV, salinity, or nitrogen deficiency increased isoflavonoid content, but not the flavonol content (Kaducová *et al.* 2019). However, other types of abiotic stress mainly drought, but also salinity also induced the accumulation of quercetin glycosides (García-Calderón *et al.* 2015; Mrázová *et al.* 2017). In those non-legume species that do not accumulate isoflavonoids, UV light-induced the synthesis of flavonol compounds. For instance, in *Ligustrum vulgare* (Agati *et al.* 2011), *Vitis vinifera* (Berli *et al.* 2010), *Petunia* sp. (Ryan *et al.* 2002), and *Arabidopsis* (Stracke *et al.* 2010; Kusano *et al.* 2011).

The aim of the present work was to determine the changes in the accumulation of major flavonoids

and isoflavonoids under abiotic stress in soybean leaves. The accumulation of flavonols and isoflavones was compared under UV stress and Cu toxicity. The results obtained were incorporated into the overall context concerning the specific response of legumes to abiotic stress.

MATERIAL AND METHODS

Plant materials, growth and stress treatments

The soybean *Glycine max* (L.) Merrill. cv. ‘Williams 82’ was used as experimental material. Plants were grown from seed in pots filled with an inert medium, Vermiculite. Plants were grown for 30 days at proton flux density at the plant level of about 150 µmol/m²/s (4000 K) under 16/8 h day/light cycle and 22°C with a constant relative humidity of 70%. Plants were watered with Hornum medium (Handberg & Stouggard 1992), which was optimized for the model plant *Lotus japonicus* but proved to be suitable for soybean cultivation under our conditions. Three days before the treatment, all the plants were transferred to hydroponics (1/4 strength Hornum medium) to 1-liter columns (one plant per column). Three randomly selected plants were used for each treatment as three separate biological replicates.

UV-B type of radiation (280–315 nm) was applied as the irradiation from above using a UV-B G8T5E fluorescent lamp (Sayko Denki, Tokyo, Japan, 310nm) together with the continuous visible light (treatment 1). The distance between the leaves and the UV-B source was approximately 23 cm (±3). A spectral radiometer (Sola-Scope 2000 from Solatell, resolution 0.5 nm) was used to determine the spectral output of the UV tubes at the top of the plant canopy. Values were weighted using the biological spectral weighting function (Flint & Caldwell 2003). The bioactive UV-B dose was 0.68 mW/cm².

A spray of CuCl₂ was applied to the plants, which was prepared at a volume of 20 mL with a concentration of 20 mM. The spray was applied once and from a distance of approximately 20 cm (treatment 2). The addition of CuCl₂ to a 1 liter hydroponic solution to the final concentration of 50 µM was used to apply copper to the medium. The duration of copper exposure was 48 hours (treatment 3). The

control plants were exposed to the same conditions, without the application of the copper.

HPLC

For phenolic compound extraction, the harvested *G.max* samples were dried at 60°C for 24 h. For the biological replicate, all leaves from one individual plant were ground and pooled. Three biological replicates were analysed for each treatment, with two technical samples prepared for each biological replicate. 50 mg of dry tissue was extracted with 1.5 mL of 80% (v/v) methanol. The extracts were centrifuged at 12,000 rpm for 5 min and filtered using a 0.2 µm nylon membrane. Flavonoid and isoflavonoid contents were analysed by gradient reversed-phase HPLC using an Agilent 1260 Infinity Quaternary LC system with 1260 Infinity DAD detector and Kromasil C₁₈ 250 × 4.6 mm 5 µm column at a 0.7 mL/min flow rate. Two-mobile-phase gradient elution system was used: 5% (v/v) acetonitrile with 3% (v/v) trifluoroacetic acid (A) and 80% (v/v) acetonitrile (B). The programme used for the determination of flavonoids was as follows: 0 min A/B (90: 10); 5 min A/B (86: 14); 30 min A/B (76: 24); 35 min A/B (60: 40); 50 min A/B (0: 100); 55 min A/B 41 (0: 100); 60 min A/B (90: 10). And the programme used for determination of isoflavonoids was as follows: 0 min A/B (90: 10); 5 min A/B (60: 40); 24 min A/B (0: 100); 30 min A/B (0: 100); 35 min A/B (90: 10). Flavonols were evaluated at λ=350 nm and isoflavonoids at λ=220 nm. Values were expressed as µmol/g dry mass (DM).

The identification of the compounds was based on peaks retention time measurements and UV-VIS spectra carried out during the analysis with the DAD detector against commercially available standards of quercetin-3-O-rutinoside, kaempferol-3-O-glucoside, (Sigma Aldrich), formononetin, malonyl-genistin (Chem Faces), medicarpin (Plantech, UK), dadzein, daidzin, genistein, genistin, glycitein, glycitin (Extrasynthese), or standards that were previously isolated from *L. japonicus* and identified by the methods of NMR (García-Calderón *et al.* 2015).

Statistical analysis

MINITAB 18 software was used for statistical analysis. One-way ANOVA analysis of variance was chosen to determine significant differences in all measured parameters with Tukey's post hoc tests

to compare estimates of variance between and within each of the sample groups.

RESULTS AND DISCUSSION

The idea that flavonols may counteract oxidative damage under abiotic stress conditions and attenuate the high-energy UV-B photons has been supported by several lines of evidence. Flavonol biosynthesis and accumulation are often induced by strong irradiation in different plant species (Kreft *et al.* 2002; Ebisawa *et al.* 2008; Agati *et al.* 2011; Kusano *et al.* 2011; Eichholz *et al.* 2012; Bidel *et al.* 2015). Exogenously applied quercetin had a protective effect likely related to its antioxidant properties (Yao *et al.* 2006) or interactions with thylakoid membranes (Dobrikova & Apostolova 2015).

The response of plants to UV-B irradiation and copper toxicity may share an increase of reactive oxygen species as a common signal, resulting in some similarities in response to different stress factors (Babu *et al.* 2003). Under the conditions of abiotic stress, the biosynthesis of ortho-dihydroxy B-ring-substituted flavonoids (especially quercetin!) is often favoured over that of their monohydroxy B-ring-substituted equivalents (Tattini *et al.* 2005; Agati *et al.* 2009, 2013). Quercetin is an efficient scavenger of reactive oxygen species (ROS) that can mitigate stress as plants are often strongly harmed by oxidative cell damage. External application of potassium quercetin can have a stimulating effect on the physiological processes, flavonoid content and antioxidative properties of plants (Jańczak-Pieniążek *et al.* 2021). In tobacco seedlings, the stimulatory effect of quercetin on the expression of antioxidant enzymes was reported (Mahajan & Yadav 2013). Quercetin also interacted with the glutathione *S*-transferase (GST) gene (*GmGSTL1*) in transgenic *A. thaliana* alleviating salt-stress symptoms (Chan & Lam 2014). In addition, flavonoid-metal complexes can act as superoxide dismutase mimetics (Kostyuk *et al.* 2004) and also correlate with the overall antioxidant properties of plant tissues (Jacobo-Velázquez *et al.* 2009). All this evidence points to an important role for flavonoids, especially quercetin in plant response to different types of abiotic stress.

In legumes, however, the typical isoflavonoid pathway may act as an alternative to flavonol production. In *Lotus* sp., UV-B irradiation induced the isoflavonoid pathway, resulting in the accumulation of several isoflavonoids, especially vestitol. The accumulation of flavonols was not affected (Kaducová *et al.* 2019; Kaducová *et al.* 2022). Similarly, other stress factors such as metal toxicity, or salinity-induced isoflavonoid accumulation in this genus (Trush *et al.* 2020; Trush & Paľove-Balang 2023). Soybean leaves of cultivar Williams 82 contained several quercetin and kaempferol glycosides. Predominant were quercetin-3-O-rutinoside and kaempferol-3-O-rutinoside (Table 1). Six other quercetin and two kaempferol derivatives were also detected according to their UV absorption spectra (Figure 1). A strong decrease in quercetin glycosides was detected after UV-B irradiation (treatment 1) as well as after both Cu treatments (treatments 2 and 3), which was most apparent in the case of the predominant quercetin-3-O-rutinoside (Table 1). Both copper treatments

negatively affected the levels of kaempferol-3-O-rutinoside and another kaempferol-glycoside. However, the total kaempferol content was much less affected, than the quercetin content after both UV-B irradiation and the copper treatments (Table 1).

In *Pisum sativum*, blue light was found to induce the accumulation of quercetin and kaempferol glycosides, whereas the UV-B irradiation had no significant effect except for a 28% reduction in the concentration of quercetin-3,7-di-O-glucoside in apical leaves (Siipola *et al.* 2015). In alfalfa, UV-B irradiation induced the accumulation of kaempferol, apigenin, and daidzein (Gao *et al.* 2019). In *Trifolium repens*, quercetin glycoside levels increased strongly in response to UV-B irradiation and correlated with tolerance to UV-B-induced growth reduction (Hofmann *et al.* 2000). All these findings suggest that species-specific preferences in the production of flavonols or isoflavonoids in legumes may exist in response to abiotic stress.

Soybean is generally rich in isoflavonoids,

T a b l e 1

Effect of UV-B and Cu treatments on the quercetin and kaempferol glycosides in soybean leaves

Compound	Treatment			
	Control	UV-B	Cu in media	Cu-sprayed
Quercetin-3-O-rutinoside	4.820±0.433 ^b	2.768±0.328 ^{ab}	1.180±0.186 ^a	1.076±0.103 ^a
Quercetin glycoside 1	1.723±0.237 ^b	0.795±0.118 ^{ab}	0.407±0.059 ^a	0.345±0.030 ^a
Quercetin glycoside 2	0.764±0.083 ^b	0.399±0.105 ^{ab}	0.332±0.030 ^a	0.548±0.118 ^{ab}
Quercetin glycoside 3	1.007±0.091 ^b	0.437±0.061 ^a	0.512±0.043 ^a	0.550±0.098 ^a
Quercetin glycoside 4	2.063±0.187 ^b	1.086±0.147 ^b	0.306±0.045 ^a	0.301±0.030 ^a
Quercetin glycoside 5	0.890±0.082 ^b	0.376±0.067 ^a	0.320±0.028 ^a	0.369±0.056 ^a
Quercetin glycoside 6	1.343±0.163 ^b	0.701±0.110 ^a	0.423±0.017 ^a	0.715±0.066 ^a
Total quercetin	11.701±0.838^b	6.602±0.929^a	3.478±0.373^a	4.228±0.158^a
Kaempferol-3-O-rutinoside	2.309±0.198 ^b	2.076±0.298 ^{ab}	1.309±0.090 ^a	1.748±0.069 ^{ab}
Kaempferol glycoside 1	1.612±0.124 ^a	1.103±0.135 ^a	1.301±0.107 ^a	1.311±0.211 ^a
Kaempferol glycoside 2	1.456±0.108 ^c	1.416±0.110 ^{bc}	0.809±0.053 ^a	0.955±0.109 ^{ab}
Total kaempferol	5.524±0.405^b	4.594±0.542^{ab}	3.472±0.153^a	4.157±0.343^{ab}

Total quercetin and total kaempferol levels (bold) were calculated as a sum of all glycosides in the tissue. Numbers are the means [μmol/g DM] ± SE (n=3). Different letters indicate significant differences among treatments (ANOVA + Tukey's test, *P*-value < 0.05)

Note: UV-B – ultraviolet radiation between 280–315 nm; DM – dry mass; SE – standard error; ANOVA – analysis of variance; *P* – probability value of no difference; n – number of repetitions.

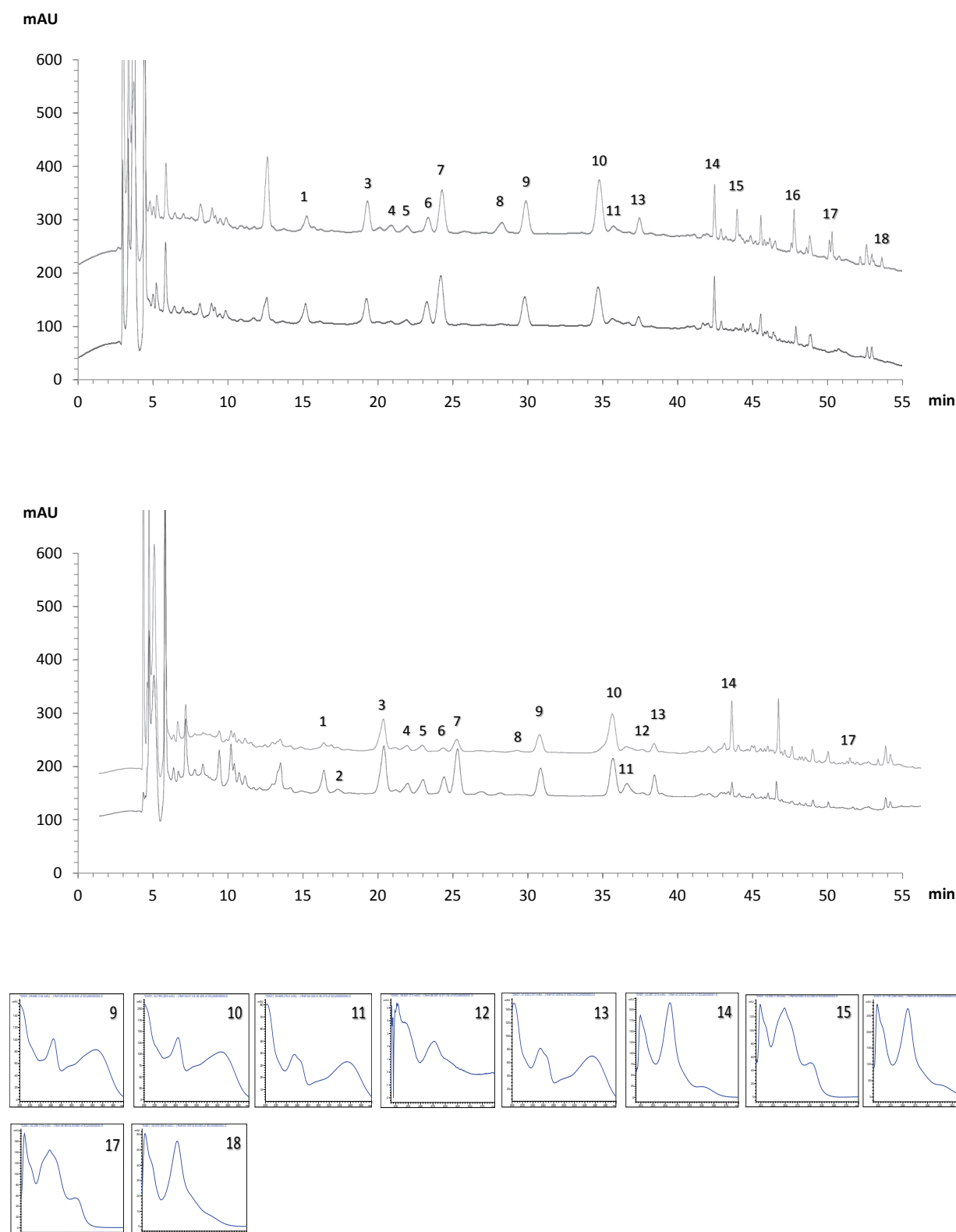


Figure 1. High-performance liquid chromatography (HPLC) elution profile of the soybean leaves irradiated by UV-B (280-315 nm) (A) or sprayed by 20 mM Cu (B). Retention time (s) is plotted against miliabsorbance units (mAU). 1 quercetine glycoside 1; 2 daidzin; 3 kaempferol glycoside 1; 4 quercetin glycoside 2; 5 quercetin glycoside 3; 6 quercetin glycoside 4; 7 quercetin-3-O-rutinoside; 8 genistin; 9 kaempferol-3-O-robinoside; 10 kaempferol-3-O-rutinoside; 11 quercetin glycoside 5; 12 malonyldaidzin; 13 quercetin glycoside 6; 14 malonyl genistin; 15 daidzein; 16 genistein; 17 formononetin; 18 biochanin A.
 Note: UV-B – Ultraviolet B Radiation; mAU – miliabsorbance unit.

present as aglycones, but also glycosides and malonyl-glycosides (Table 2). The HPLC elution profiles of the leaves treated with UV-B irradiation and 20 mM Cu by spraying are presented in Figure 1, as these treatments showed the strongest induction of isoflavonoids. In control conditions the leaves of the variety Williams 82 contained traces of daidzein and formononetin. Most of the isoflavonoids were present in their glycosylated form, as malonyl-daidzin, genistin, and malonyl-genistin (Table 2). Glycitein and glycitin were not detected in leaves. UV irradiation (treatment 1) induced the accumulation of isoflavonoids, mainly the aglycones of genistein, daidzein and formononetin, and some biochanin A that were not detectable in the control conditions (except for low levels of daidzein). The accumulation of isoflavonoid glycosides was generally less affected. Genistin (genistein-7-O-glycoside) and daidzin (daidzein-7-O-glycoside) increased significantly, whereas the accumulation of their malonylated forms was unchanged (Table 2).

Following the exposure to UV irradiation, an increase in genistein content was also described in 7-day-old soybean sprouts together with malonyl-genistin and daidzin (Lim *et al.* 2020), and increased

levels of daidzein, daidzin, genistein, and genistin were observed in soybean pods (Zavala *et al.* 2015).

Copper treatment 2 via root media had little effect on the isoflavonoid accumulation in leaves, the only exception being a moderate accumulation of daidzin. Such treatment likely affected more the roots than the leaves. Directly spraying the leaves with Cu (treatment 3) was more efficient; it induced the accumulation of isoflavonoid glycosides, mainly an increase in genistin and malonyl-genistin but also a moderate accumulation of daidzin. Aglycones were only slightly affected (Table 2).

In summary, both types of stress factors induced the accumulation of soybean-isoflavonoids, but in a different manner. On UV-B irradiation mainly aglycones accumulated, while the direct copper treatment, resulted in the strong accumulation of genistin and its malonylated form and aglycones were only slightly affected. Previously, genistein showed a better Cu-chelating ability than daidzein due to the presence of the 5-hydroxyl position. Glycosylation occurring at position 7 in genistin and daidzin does not negatively affect the chelation properties of these compounds (Karlíčková *et al.* 2015). Those two findings described above suggest

T a b l e 2

Effect of UV-B and Cu treatments on the isoflavonoid content in soybean leaves

Compound [$\mu\text{mol/g DM}$]	Treatment			
	Control	UV-B (treatment 1)	Cu in media (treatment 2)	Cu-sprayed (treatment 3)
Aglycones:				
daidzein	0.021 ± 0.021^a	3.554 ± 0.814^b	0.299 ± 0.150^a	n.d. ^a
genistein	n.d. ^a	6.005 ± 1.228^b	n.d. ^a	0.985 ± 0.247^a
formononetin	0.051 ± 0.035^a	3.324 ± 0.061^b	0.706 ± 0.129^a	0.441 ± 0.073^a
Biochanin A	n.d. ^a	0.549 ± 0.011^b	n.d. ^a	n.d. ^a
Glycosides:				
daidzin	n.d. ^a	0.342 ± 0.098^b	0.521 ± 0.048^b	0.359 ± 0.191^b
genistin	0.827 ± 0.186^a	3.116 ± 0.407^c	0.717 ± 0.072^a	2.138 ± 0.324^b
malonyl daidzin	0.311 ± 0.056^a	0.196 ± 0.028^a	0.333 ± 0.025^a	0.543 ± 0.165^a
malonyl genistin	3.202 ± 0.752^a	3.657 ± 0.907^a	5.412 ± 0.280^a	9.083 ± 0.771^b

Numbers are the means [$\mu\text{mol/g DM}$] $\pm$ SE (n=3). Different letters indicate significant differences among treatments (ANOVA + Tukey's test, $P < 0.05$)

Note: n.d. – not detected; UV-B – ultraviolet radiation between 280–315 nm; DM – dry mass; SE – standard error; ANOVA – analysis of variance; P – probability value of no difference; n – number of repetitions.

that genistein and genistin might be more effective in response to copper in comparison to daidzein and daidzin.

Genistein and daidzein are potent antioxidant compounds that can be involved in the inhibitory effect on lipid peroxidation in plant membranes (Dwiecki *et al.* 2009). As phytoalexins, they can affect the response of plants to biotic stress by altering their accumulation (Jeandet *et al.* 2013). UV-B-induced isoflavonoid accumulation correlated with soybean resistance to stink bugs (*Nezara viridula* and *Piezodorus guildinii*) under field conditions (Zavala *et al.* 2015). Thus, abiotic induction of isoflavonoids in soybean may not only act as a defense against a specific type of stress factor but may also improve plant resistance and health status.

CONCLUSIONS

In contrast to other, mostly non-legume species, the antioxidant quercetin accumulation in soybean was not induced either by UV-B irradiation or copper treatment. On the other hand, the strong increase in isoflavone content suggests that these compounds are related to the plant response to both UV-B irradiation and copper toxicity. The results of the study suggest that in soybeans, the production of isoflavonoids can serve as an alternative to the production of flavonols under conditions of abiotic stress. Moreover, the different degree of glycosylation of isoflavonoids may indicate their different function. While in the case of UV-B irradiation, the primary function of isoflavonoids may be their antioxidant activity, in the case of heavy metal exposure, their chelation activity may be at least equally important. Soybean isoflavonoids, phytoalexins with antioxidant and chelating properties, are at the cross point of the response to abiotic and biotic stress. Therefore, understanding their accumulation, regulation and functions is an important milestone in improving of crop resistance.

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