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THE EFFECT OF TROPOSPHERIC OZONE ON FLAVONOIDS AND PIGMENTS CONTENT IN COMMON BUCKWHEAT COTYLEDONS

WPŁYW OZONU TROPOSFERYCZNEGO NA ZAWARTOŚĆ FLAWONOIDÓW I BARWNIKÓW W LIŚCIENIACH GRYKI ZWYCZAJNEJ

Abstract: Tropospheric ozone forms in photochemical reactions or by refuse burning and combustion of exhaust gases from engines, and during some industrial processes. The mean ambient ozone concentration doubled during the last century, and in many urban areas has reached the phytotoxic level. In the present study, there was determined the effect of ozone fumigation on levels of individual flavonoids, chlorophylls, carotenoids and total phenols in the cotyledons of four common buckwheat cultivars (Hruszowska, Panda, Kora and Red Corolla). Six-day-old buckwheat seedlings were grown in controlled conditions and treated with an elevated dose of ozone (391 $\mu g \cdot m^{-3}$) during 5 days for 1 h each day. After the experiment, the cotyledons of the seedlings were analysed for individual flavonoids, chlorophylls, carotenoids and total phenols. Shoot elongation was also measured. Individual types of flavonoids in buckwheat cotyledons were found to respond to an elevated ozone dose in various ways. The response was also dependent on the cultivar evaluated. In the cotyledons of ozonized buckwheat seedlings, contents of C-glucosides of luteolin and apigenin decreased or did not change depending on the cultivar examined. In the case of flavonols, the contents of quercetin-3-O-rhamnosyl-galactoside and rutin (quercetin-3-O-rhamnosyl-glucoside) were markedly reduced in most cultivars. O_3 had no effect on the level of anthocyanins and chlorophylls but it decreased carotenoids, and tended to inhibit buckwheat growth. In conclusion, a thesis can be formulated that, due to high reduction in important flavonoids, an elevated level of ambient ozone decreases the nutritional value of common buckwheat seedlings.

Keywords: common buckwheat, seedling, ozone, flavone, flavonol, chlorophyll, carotenoid

Introduction

Ozone is brought down from the stratosphere by turbulence during severe electrical storms but, more importantly, it is formed in photochemical reactions involving nitrogen oxides, carbon monoxide and volatile organic compounds or by refuse burning and combustion of exhaust gases from engines, and during some industrial processes [1].

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Therefore ozone concentrations are usually highest in urban and rural areas in developed countries. The mean tropospheric ozone concentration doubled during the last century, and in some parts of Poland has reached the level of 100-200 $\mu g \cdot m^{-3}$, which indicates that O_3 is at phytotoxic levels [2].

Ozone is the main phytotoxic air pollutant to the plant since it causes more damage to plants than all other air pollutants combined [3]. The available scientific literature on the topic has shown that exposure to even small concentrations of O_3 decreases the photosynthesis and favours premature aging of the plant, which results in an inhibition of growth and yield [4, 5]. Generally, young plants are the most sensitive to ozone whereas mature plants are relatively resistant.

The type of injury is dependent on the concentration of ambient O_3, length of exposure, weather conditions and plant genetics. An elevated level of ozone can cause damage to plants by imposing conditions of oxidative stress and injury [6]. Production of antioxidant metabolites is one of the defense responses to the impact of O_3 which increases total phenols contents, but variable patterns were also observed, especially when considering single metabolites [7-9].

It has been found that ozone treatment induces synthesis of flavonoids by enhancing activities of flavonoid pathway enzymes and phenylpropanoids accumulation [4, 10-13]. Elevated O_3 increased the contents of flavonoids in the leaves of birch [12, 14] and maize [15]. Contrary to this, elevated ozone caused a significant reduction in total phenolic content in soybean leaves [8]. Similarly, ozone decreased total phenolics and did not display any significant effects on kaempferol biosynthesis while steeply enhancing the biosynthesis of quercetin derivatives in *Ginkgo biloba* leaves [16]. On the other hand, Haikio et al. [17] found that elevated ozone did not affect the level of total flavonol glycosides in hybrid aspen. These results may suggest that ozone can in various ways affect individual types of phenolic compounds. Recently, it has also been observed that rice plants [18] and mung bean [19] have a cultivar specific response to ozone stress. This means that in response to such stress a genetic factor is also important.

Many studies have shown that ozone treatment reduces the levels of pigments, such as chlorophylls *and* carotenoids, involved in photosynthesis [20-24]. The decrease of chlorophyll concentration is considered to be a secondary response to ozone exposure [25].

Common buckwheat (*Fagopyrum esculentum* Moench) is a fast-growing dicotyledonous plant. Tissues of buckwheat sprouts and seedlings accumulate large concentrations of various flavonoids [26]. Three of the main classes of flavonoids have been found in buckwheat: flavonols, flavones and anthocyanins. Buckwheat tissues contain rutin (quercetin-3-O-rhamnosyl-glucoside), the major flavonol, and recently identified quercetin-3-O-rhamnosyl-galactoside. The flavones present in buckwheat seedlings are C-glucosides of apigenin (vitexin, *iso*-vitexin) and luteolin (orientin, *iso*-orientin) [27]. Flavonoids biosynthesis is regulated by a complex interaction between internal and external stimuli, such as temperature, type and intensity of light, abiotic stressors and plant hormones [7]. These compounds can serve as antioxidants and/or as substrates of phenol-consuming antioxidant enzymes. Due to high rutin content, buckwheat sprouts are a valuable component of human diet. The consumption of sprouts, including buckwheat sprouts, is becoming more and more popular [27].

The contents of rutin and other flavonoids are affected by many abiotic factors, including air pollution. Buckwheat seedlings provide a convenient model plant for investigating of the influence of such factors on an accumulation of phenylpropanoids [26,

28, 29]. The aim of the present study was to determine the effect of ozone fumigation on individual flavonoid glycosides, chlorophylls, carotenoids and total phenols in the cotyledons of seedlings of common buckwheat cultivars.

Materials and methods

Plant material and growth conditions

Seedlings of common buckwheat (*Fagopyrum esculentum* Moench) cv. Hruszowska, Luba, Kora and Red Corolla were used in this study. Hruszowska is the oldest and widely grown Polish cultivar. Kora and Panda were obtained as a results of breeding from Hruszowska and introduced to cultivation in the 90s. Red Corolla was established by crossing Buriatskaja and Hruszowska cultivars. The Buriatskaja population is grown in steppe conditions in Russia, and it is distinguished by the red color of the leaves and sepals.

Four-day buckwheat seedlings were obtained as described earlier [26]. Next, the buckwheat seedlings were transferred to controlled-environment conditions with a 16/8 h 24/18°C day/night scheme and light intensity of 100-120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ which was provided by 400 W high pressure sodium lamps, and grown in one fifth Hoagland solution. After two days of incubation in these conditions, the buckwheat seedlings were treated with ozone during 5 days for 1 h each day, beginning at 10 am.

The seedlings were fumigated with a stream of ozone (1 $dm^3 \cdot min^{-1}$; 391 $\mu g \cdot m^{-3}$) in a closed glass chamber, volume 30 dm^3. Ozone obtained in a generator was mixed in a mixing vessel with the air and supplied to the bottom of a glass jar with a polypropylene pipe, and excess gases were discharged through another tube from the upper part of the jar. After the fumigation, plants were left in a stream of ambient air (1 $dm^3 \cdot min^{-1}$). Control seedlings were exposed only to ambient air. O_3 was produced in a generator Korona 01/05 (Ekotech, Poland) in which the negative corona discharges are applied. To measure ozone concentration, a GasHunter Ozone Meter (Alter, Poland) was applied. The experiments were repeated three times.

After the experiment, part of fresh material (cotyledons of 10-20 seedlings) was analysed for chlorophylls, carotenoids and total phenols, and the rest (cotyledons of 10 seedlings per replicate) was used to conduct HPLC analyses of individual flavonoids. Before flavonoids were determined, plant samples were frozen, then freeze dried in a laboratory freeze drier (Christ, Germany) and pulverized in Tube Mill (IKA, Germany).

Determination of flavonoids

Details of the flavonoids analysis were described previously [26]. Briefly, freeze-dried samples of buckwheat cotyledons were extracted five times by sonication with a mixture of 60% methanol and 0.4% trifluoroacetic acid. Pooled extracts were centrifuged and used for HPLC analysis. A HPLC system was equipped with a 250×2.0 mm i.d. Cadenza CD-C_{18} 3 μm column, and UV detector set at 350 nm (flavones and flavonols) and 520 nm (anthocyanins). The analysed flavonoids were eluted in a gradient system composed of solvent A (water/acetonitrile/formic acid, 89 : 6 : 5) and solvent B (water/acetonitrile/formic acid, 15 : 80 : 5). The flavonoids were identified by comparing the retention times to the available standards. Commercial standards were used to calculate flavonoids contents, except for quercetin-3-galactosyl-rhamnoside, the contents of which were calculated based on the standard for rutin.

Total phenolics assay

Total phenolics were determined spectrophotometrically using Folin-Ciocalteau reagent (Sigma). Reagent mixtures were allowed to react at ambient temperature in darkness for 2 hours, and the absorbance of supernatants was measured at 725 nm. Chlorogenic acid was used for a standard curve preparation (Sigma).

Determination of chlorophylls and total carotenoids

Chlorophylls and total carotenoids contents were quantified with a spectrophotometer and extinction coefficient published by Lichtenthaler and Welburn [30].

Elongation of shoots

Differences between aerial parts (shoots) before and after 5-day treatment with ozone were treated as elongation of shoots. Mean results of elongation were obtained from 20-30 seedlings.

Statistics

Fumigation with ozone and analyses of metabolites were performed in triplicate. Statistical analyses of results were achieved with the *t*-test, and significant differences in relation to the control plants were indicated with *, $p < 0.05$.

Results and discussion

The biosynthesis of flavonoids is regulated by various internal and external stimuli such as temperature, type and intensity of light, abiotic stress and plant hormones [7]. The mean tropospheric ozone content in Siedlce by 2013 was 50 $\mu g \cdot m^{-3}$, and the maximum levels reached 135 $\mu g \cdot m^{-3}$ [31]. In the growth room where experiments were conducted, the background level of ozone was below 40 $\mu g \cdot m^{-3}$ (below the limit of determination of the meter used). The ozone dose used (391 $\mu g \cdot m^{-3}$ during 5 days for 1 h each day) did not lead to any necrotic changes, even though it exceeds about 8 times the mean for Siedlce and approximately 3 times the maximum ozone content in the year 2013. According to reports of environmental services, concentrations of ground-level ozone higher than 300 $\mu g \cdot m^{-3}$ occur in Poland incidentally.

Particular types of flavonoids were found to respond to an elevated level of ambient ozone in different ways (Tables 1 and 2). The response was also dependent on cultivar evaluated. In the cotyledons of cultivar Hruszowska, the contents of all flavones (C-glucosides of luteolin and apigenin) decreased under the influence of the applied ozone dose. For Panda, the content of flavones did not change under the O_3 level, but had a slight tendency to decrease (Table 1). Similarly, in the case of Red Corolla, the O_3 dose used had no significant influence on the level of flavonoids. However, the level of all flavones in Panda significantly increased under the influence of O_3.

In the case of flavonols, the quercetin-3-O-rhamnosyl-galactoside (QRGal) was markedly reduced in the ozonized cotyledons of Hruszowska, Panda and Kora, but only slightly in Red Corolla. Ozone caused a large decrease in the level of rutin in the cotyledons of Panda, Kora and Red Corolla. This decrease reached about 30% in Red Corolla and Panda. A different response to O_3 exposure of the buckwheat cultivars may be due to genetic factors. It has been established that common buckwheat is characterized by high

intraspecific genetic variability due to self-incompatibility and a large number of genes self-infertility [32]. Recently, rice plants [18] and mung bean [19] have been found to desplay an intraspecific response to ozone stress.

Table 1

Effect of ozone treatment on content of flavones and flavonols [mg·g^{-1} d.w.] in cotyledons of common buckwheat seedlings

Object	Cultivar analysed			
	Hruszowska	**Panda**	**Kora**	**Red Corolla**
Orientin (Luteolin-8-C-glucoside)				
Control	3.29±0.13	3.02±0.06	2.97±0.14	2.89±0.16
Ozone treated	2.77±0.06*	2.83±0.05	3.59±0.08*	3.16±0.02
iso-Orientin (Luteolin-6-C-glucoside)				
Control	5.65±0.21	5.25±0.07	5.25±0.09	5.18±0.14
Ozone treated	4.90±0.07*	4.97±0.07	6.21±0.20*	5.59±0.03
Vitexin (Apigenin-8-C-glucoside)				
Control	4.01±0.15	3.03±0.06	3.11±0.09	3.23±0.11
Ozone treated	3.16±0.07*	3.07±0.05	3.85±0.12*	3.56±0.04
iso-Vitexin (Apigenin-6-C-glucoside)				
Control	8.02±0.31	6.37±0.10	6.04±0.08	7.05±0.34
Ozone treated	6.66±0.09*	5.98±0.13	7.61±0.25*	7.43±0.07
Quercetin-3-O-rhamnosyl-galactoside (QRGal)				
Control	1.85±0.07	1.06±0.03	1.74±0.04	2.15±0.09
Ozone treated	0.58±0.02*	0.67±0.02*	1.44±0.04*	2.00±0.11
Rutin (Quercetin-3-O-rhamnosyl-glucoside)				
Control	12.65±0.49	15.16±0.28	11.59±0.16	25.49±0.87
Ozone treated	12.44±0.27	10.62±0.12*	10.30±0.16*	18.36±0.24*
Total flavones and flavonols				
Control	35.47±1.35	33.89±0.58	30.71±0.56	45.98±1.46
Ozone treated	30.51±0.52*	28.13±0.42*	33.01±0.51	40.11±0.47*

Means marked with an asterisk were considered statistically significant in comparison to control at $p < 0.05$. Lack of the asterisk means no significant difference in comparison to control.

Table 2

Effect of ozone treatment on content of anthocyanins [mg·g^{-1} d.w.] in cotyledons of common buckwheat seedlings

Object	Cultivar analysed			
	Hruszowska	**Panda**	**Kora**	**Red Corolla**
Cyanidin-3-O-rhamnosyl-galactoside				
Control	0.92±0.02	0.66±0.01	0.65±0.02	2.54±0.12
Ozone treated	0.76±0.01*	0.64±0.02	0.89±0.02*	2.59±0.10
Cyanidin-3-O-rhamnosyl-glucoside				
Control	0.19±0.01	0.10±0.01	0.14±0.01	0.74±0.02
Ozone treated	0.15±0.01*	0.11±0.01	0.14±0.01	0.71±0.03
Total				
Control	1.11±0.02	0.76±0.02	0.79±0.03	3.27±0.14
Ozone treated	0.92±0.01*	0.75±0.03	1.03±0.02*	3.36±0.05

Means marked with an asterisk were considered statistically significant in comparison to control at $p < 0.05$. Lack of the asterisk means no significant difference in comparison to control.

The much higher impact of O_3 on flavonols may be explained by the fact that quercetin derivatives have a higher antioxidant capacity compared to flavones [33]. Therefore, quercetin glycosides can be more quickly oxidised by enzymes involved in oxidative stress.

Ozone damages the integrity of cell membranes, which can lead to oxidation of flavonoids by oxidative enzymes.

Considerably higher levels of anthocyanins: cyanidin-3-O-rhamnosyl-galactoside and cyanidin-3-O-rhamnosyl-glucoside, were found in Red Corolla than in the cotyledons of the other cultivars (Table 2). However, most cultivars (Red Corolla, Panda and Kora) showed no significant effect of the applied elevated dose of O_3 on the level of anthocyanins. Ozone contributed to reduction in the anthocyanins content only in the cotyledons of Hruszowska.

The results of the present study do not confirm the findings of earlier reports about the effects of tropospheric O_3 on the level of flavonoids. The results of most of these reports show an increase in the content of phenol compounds in ozonized plant tissues [11-14]. However, these investigations relate to chronic elevated ozone exposure on metabolic woody plants. On the other hand, elevated O_3 increased the contents of flavonoids in the leaves of maize [15] and soybean [34].

Our findings do confirm a significant reduction in phenolics content in soybean leaves due to an elevated level of ozone reported by Betzelberger [8]. Similarly, O_3 decreased total phenolics, had no effect on kaempferol biosynthesis but enhanced the biosynthesis of quercetin glycosides in *Ginkgo biloba* leaves [16]. In studies on red clover, it was found that individual isoflavones responded differently to ozone compared to other flavonoids [9]. On the other hand, Haikio et al. [17] found that elevated O_3 did not affect the level of total flavonol glycosides in aspen. According to these authors, flavonol glycosides do not play a role in defense against oxidative stress, despite their antioxidative capacity. In the biosynthesis of flavones and flavonols, there is the same precursor - trihydroxyflavanone (THF) [7]. It is possible that ozone inhibits conversion of THF to flavonols more preferably than the biosynthesis of flavones. However, this far-reaching speculation requires examination of the activity of relevant enzymes.

The applied elevated dose of O_3 had no significant effect on the contents of chlorophyll *a* or *b* in the cotyledons of buckwheat cultivars (Table 3). It was reported previously that a decrease in chlorophylls concentration is considered to be a secondary response to ozone exposure [25]. Probably, O_3 used for 1 h per day for five days is not sufficient for a larger loss of pigments. It has been shown that chronic ozone exposures can cause a significant decline in pigment content, but acute treatment may not be sufficient to induce any modification in the constitutive levels of chlorophylls [35-37]. Also, there was found no effect of the concentration and time of O_3 use on the level of total phenols analyzed by the Folin assay. The Folin method is not specific and, therefore, the results obtained can be imprecise.

The elevated ozone dose applied had a much greater impact on carotenoids. In all the examined cultivars, significant reduction of total carotenoids content was found (Table 3). The main role of carotenoids is the detoxification of reactive oxygen species in chloroplasts. The antioxidant activity of carotenoids is based on their singlet oxygen quenching and an ability to trap peroxyl radicals [38]. Regarding antioxidant properties, carotenoids play a crucial role as photoprotectors in photosynthetic units preventing self-oxidation of the photosystems [38]. It seems that this dual function of carotenoids in plant cells is one of the reasons for reduction of their level caused by ozone [20-22].

The use of an elevated dose of ozone for 1 hour during consecutive five days resulted in growth inhibition of buckwheat shoots (Table 3). However, this inhibitory effect has not been statistically proven in three of the four cultivars due to the large discrepancy between measurements. Only in Red Corolla, an inhibitory effect of O_3 on shoot elongation was

demonstrated. The inhibition of plant growth caused by elevated tropospheric ozone is commonly known [6, 22, 39]. It has been found that exposure to O_3 decreases the photosynthesis and favours premature aging of the plant, which results in growth inhibition [4].

Table 3

Effect of ozone treatment on contents of chlorophylls, total carotenoids and total phenolics in cotyledons of common buckwheat seedlings, as well as shoot elongation

Object	Cultivar analysed			
	Hruszowska	**Panda**	**Kora**	**Red Corolla**
Chlorophyll *a* [mg·g^{-1} f.w.]				
Control	1.31±0.08	1.39 ±0.08	1.30±0.06	1.43±0.12
Ozone treated	1.27±0.10	1.23±0.07	1.20±0.14	1.26±0.13
Chlorophyll *b* [mg·g^{-1} f.w.]				
Control	0.46±0.03	0.51±0.05	0.46±0.08	0.51±0.06
Ozone treated	0.48±0.07	0.46±0.05	0.42±0.05	0.44±0.03
Total carotenoids [mg·g^{-1} f.w.]				
Control	0.289±0.024	0.296±0.024	0.281±0.023	0.329±0.038
Ozone treated	0.252±0.026*	0.249±0.024*	0.210 ±0.047*	0.244±0.045*
Total phenolics [mg·g^{-1} d.w.]				
Control	56.81±1.33	52.42±3.16	57.47±4.62	57.47±0.91
Ozone treated	55.59±2.33	53.99±2.93	54.96±2.58	61.88±1.56
Shoot elongation [mm]				
Control	31.1±14.0	40.9±15.0	35.4±12.2	33.9±5.8
Ozone treated	25.3±9.4	42.7±13.1	29.3±8.1	27.6±6.4*

Means marked with an asterisk were considered statistically significant in comparison to control at $p < 0.05$. Lack of the asterisk means no significant difference in comparison to control.

Conclusions

Particular types of flavonoids present in buckwheat cotyledons were found to respond to an elevated dose of ambient ozone in different ways. The response was also dependent on cultivar evaluated. In the cotyledons of ozonized buckwheat seedlings, contents of apigenin C-glucosides decreased or did not depend on the cultivar examined. In the case of flavonols, the contents of quercetin-3-O-rhamnosyl-galactoside and rutin (quercetin-3-O-rhamnosyl-glucoside) were markedly reduced in most cultivars. However, the elevated dose of O_3 applied showed no effect on the level of anthocyanins. O_3 also had no effect on the contents of chlorophylls but decreased total carotenoids, and simultaneously had a tendency to inhibit seedling growth. In conclusion, a thesis that an elevated level of tropospheric ozone in various ways affects the content of particular flavonoids in the cotyledons of common buckwheat seedlings can be formulated. Due to high reduction in important flavonoids, tropospheric ozone decreases the nutritional value of common buckwheat sprouts.

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