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Effect of urban pollution on 4-coumarate:CoA ligase and flavonoid accumulation in *Berberis thunbergii*

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Abstract: Flavonoids are polyphenolic compounds commonly found in plants and they play an important role in stress tolerance. They have the capacity to chelate heavy metals and scavenge free radicals. Urban pollution causes oxidative stress in plants and flavonoids may protect cells against the negative effect of free radicals. In this study levels of anthocyanins and flavonols and 4-coumarate:CoA ligase (4CL) activity were determined in *Berberis thunbergii* (DC.) plants grown in polluted and residential areas in the city of Poznań. The results showed significantly higher accumulation of anthocyanins and stimulation of 4CL activity in plants from the contaminated sites in comparison to the control plants. Probably the activation of the phenylpropanoid pathway was a response to stress caused by urban pollution.

Additional key words: anthocyanins, flavonols, 4-coumarate:CoA ligase, environmental stresses

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Introduction

Rapid developments of human civilization and industrialisation have resulted in an increase in the number of automobiles, which are responsible for 60 to 70% pollution in the urban environment (Singh et al. 1995). Composition of urban pollution has changed significantly over the recent decades. Diesel and petrol fuelled vehicles are responsible for the generation of a wide range of pollutants, with concentrations and relative proportions of pollutants depending on vehicle technology and operating conditions (Colvile et al. 2001). Vehicle exhaust, as well as several industrial activities, emit heavy metals and air pollution and as a consequence soils, plants and residents along roads with heavy traffic loads are subjected to increasing level of contamination (Lagerwerff and Specht 1970, Solomon and Hartford 1976, Bower et al. 1978, Reeves and Brooks 1983, Turner 2005, Guan Dong-Sheng and Pert 2006). However, plants play the role of a major absorbent of atmospheric dust. Pollution may affect plant tissues directly or indirectly (acid rain), but tolerant plants possess defense mechanisms which enables them to survive under critical conditions.

Observation of the response of living organism exposed to urban pollution is very important and some compounds formed in these reactions may be used as bioindicators. Bioindication of air pollution has been described in many studies on plants and some biochemicals may indicate the status of the environment (Zobel and Nighswander 1991, Rautio et al. 1998, Rossbach et al. 1999, Rahman et al. 2000, Vassileva et al. 2000, Conti and Cacchetti 2001, Godefroid 2001). Studies conducted under natural conditions are rare and sometimes concern only as one pollutant (Karl-

sson et al. 1995, Oleksyn at al. 1999, Chappelka et al. 1999), but validation bioindicators require such experiments.

Important role in response to environmental stresses, such as wounding, pathogen infection, ultraviolet irradiation or air pollution, is played by the synthesis of phenolic compounds such as low molecular phenolic acids and flavonoids (Hahlbrock and Scheel 1989, Dixon and Paiva 1995, Peńuelas et al. 1996, Pisani and Distel 1998). Many researchers focused on examining the impact of air pollution on total phenol contents (Karolewski 1990, Giertych and Karolewski 1993, Karolewski and Giertych 1995, Peńuelas et al. 1996, Giertych et al. 1999). Moreover, flavonoids were studied as bioindicators of air pollution (Loponen et al. 2001, Robles et al. 2003, Qayoom Mir et al. 2009, Rezende and Furlan 2009, Furlan et al. 2010). Loponen et al. (2001) showed an increase in (+)–catechin content and a decrease in flavonols with high levels of heavy metal pollution in *Betula*. Flavonoids are involved in an array of processes, including plant-pathogen interactions, pollination, light screening, seed development and allellopathy (Winkel-Shirley 2001). Their levels increased during exposure to biotic and abiotic stresses, *e.g*. wounding or metal toxicity (Dixon and Paiva 1995, Winkel-Shirley 2002).

Under stress conditions an accumulation of reactive oxygen species (ROS) occurs, which can damage cellular components, such as DNA, lipids, proteins and sugars (Asada 2006, Van Breusegem and Dat 2006). Flavonoids have been suggested to act as antioxidants, protecting plants from oxidants through the number and location of phenolic hydroxyl groups, especially the presence of the 3',4'-dihydroxy system in the B ring and 3-OH on the C ring, that act as radical targets (Amic et al. 2003). Carotenoids are other plant compounds reacting with free radicals. Matysiak (2001) observed that the level of carotenoids in needles of *Pinus sylvestris* was modified in plants growing in a polluted area.

4-coumarate:CoA ligase (4CL, EC 6.2.1.12) is a key enzyme of the phenylpropanoid pathway and it catalyzes the formation of CoA esters of hydroxycinnamic acids, while these activated intermediates are used in the biosynthesis of diverse compounds *via* specific branch pathways, such as those leading to the biosynthesis of flavonoids and lignins (Costa et al. 2005, Yun et al. 2007). In our studies we analyzed the activity of this major phenylpropanoid pathway enzyme (4-coumarate:CoA ligase) and determined the content of flavonoids (flavonols and anthocyanins) during vegetation period and correlation between control and polluted sites in *Berberis thunbergii* growing in polluted and residential area positions within the city of Poznań.

Thus it was assumed that the activation level of the phenylpropanoid pathway may be an indicator of the contamination level and at the same time evidence for the triggering of the mechanism of environmental pollution resistance.

Material and methods

Plant material

Berberis thunbergii (DC.) is a decorative plant grown in gardens, parks and in the immediate vicinity of streets. *Berberis* (barberry) shrubs in general are tolerant to adverse environmental factors such as drought, and urban pollution (Borowski and Latocha 2006). Barberry leaves contain large amounts of flavonoid pigments.

The experimental material of *Berberis thunbergii* was collected from selected sites located in the city of Poznań. There were sites directly exposed to pollution near roads, tram tracks, and a petrol station, and control sites in residential areas, not surrounded by transport routes (Chadzinikolau et al. 2010). Leaves from from at least 4 randomly selected shrubs were collected, at the beginning of May, July and September. After being delivered to the laboratory, leaves were surface-washed, and 3 weighted samples of each position were frozen in liquid nitrogen and stored at –18°C. Analyses for each parameter were made for 3 analytical replicates, which were averaged. The mean values for each term involve 6 analyses for polluted sites and 2 for the control ones.

4-coumarate: CoA ligase activity

Quantification of 4CL was performed according to Knobloch and Hahlbrock (1977). Leaves samples (0.5 g) were ground in liquid nitrogen. Frozen powder was mixed with 100 mM Tris-HCl buffer at pH 7.8 containing 5 mM 2-mercaptoethanol and 5% glycerol. Next, 0.1 g ml^{-1} of Dowex AG 1-X2 was added, stirred for 15 min at 4°C and centrifuged at 23 000 g for 30 min. The reaction mixture in the final volume of 0.2 ml contained 100 mM Tris-HCl (pH 7.8), 100 mM p-coumaric acid, 0.5 mM ATP, 0.3 mM CoA, 5 mM $MgCl_2$ and 100 μl supernatant. Activity was determined spectrophotometrically at room temperature (Jasco V-530 UV-VIS Spectrophotometer). The formation of CoA esters of coumaric acid derivatives was measured as an increase in A_{333} nm according to the absorption maxima for 4-coumaroyl:CoA (Stöckigt and Zenk 1975). Molar absorption coefficient values for 4-coumaroyl:CoA (21 mM^{-1} cm^{-1}) were used to calculate the enzyme activity. The specific activity was expressed in picokatals per mg of total extractable protein determined according to Bradford (1976), using bovine serine albumin as a standard.

Flavonol contents

Leaf samples (0.5 g) were cut into pieces and homogenized with 5 ml of a mixture of methanol, HCl and distilled H_2O (90:1:1, v/v/v). Homogenates were stirred and heated (60°C) for 10 min, cooled at room temperature for 15 min and centrifuged at 23 000 g for 30 min (Day 1993). The level of flavonols was determined by measuring absorbance of the supernatant at 254 nm with a UV/visible spectrophotometer (Jasco V-530 UV-VIS Spectrophotometer). Flavonol contents were calculated using the calibration curve of quercetin (Stefova et al. 2001) and expressed in $\mu g\ g^{-1}$ of fresh weight (FW).

Anthocyanin contents

Level of anthocyanins was measured according to Arakawa (1991). Plant material (0.5 g) was homogenized with 0.5 N HCl, and centrifuged at 23 000 g for 30 min. Absorbance of the supernatant was measured at 530 nm with a UV/visible spectrophotometer (Jasco V-530 UV-VIS Spectrophotometer). Anthocyanin contents were calculated with a calibration curve of cyaninchlorid and expressed in $\mu g\ g^{-1}$ of FW.

Statistical analysis

Results were subjected to Anova statistical analysis and the Tukey's HSD multiple range test, using Statistica 9.0 software. Moreover, the correlation coefficient and linear regression for the relationship between activity of 4-coumarate:CoA ligase, and flavonols and anthocyanins accumulation were determined.

Result and Discussion

Developments of industry and urbanization have resulted in increased environmental pollution. Plants have created mechanisms minimizing the destructive effect of such stressors. Phenolic compounds, including flavonoids, can act as metal chelators and directly scavenge molecular species of active oxygen. They are important in UV radiation, heavy metal and ozone stress.

In *Berberis thunbergii* leaves a successive increase of anthocyanins and flavonols, in the period from May to September was observed (Fig. 1). Independently of the plant locality, the lowest values of anthocyanins were recorded in May (7.3 and 30.1 $\mu g\ g^{-1}$ FW at control and polluted sites, respectively) and the highest in September (62.9 and 104.5 $\mu g\ g^{-1}$ FW, respectively).

The flavonol levels in May were similar at control and polluted sites and did not exceed 1 mg g^{-1} FW. Their accumulation was observed at the successive dates of determinations. In September at the control site this amount was 1.6 $\mu g\ g^{-1}$ FW, but at the contaminated sites the level of flavonols was statistically higher, reaching 2.2 μg (Fig. 1).

The content of anthocyanins, flavonols and 4CL activity were depend on plant growth site and term of material collection (Table 1).

An increase in total flavonoid contents was observed under the influence of vehicle pollution also in *Ocimum sanctum* L. and *Catharanthus roseus* L. (Qayoom Mir et al. 2009), whereas in *Artemisia vulgaris* L. and *Veronica chamaedrys* L. it was in relation to air pollution stress (Nikolova and Ivancheva 2005). Enhancement of total phenolics and flavonoids as a result of pollution impact has been observed in *Betula pubescens* leaves and *Betula papyrifera* (Loponen et al. 1997, 1998). Robles et al. (2003) showed that total flavonol concentration was positively correlated with the O_3 and negatively with the SO_2 level. They also studied total proanthocyanidin concentration and it was negatively correlated with the content of O_3 and SO_2. Similar investigations were conducted on flavonoid accumulation in guava tree (*Psidium guajava*), commonly grown in tropical countries; however, the response was dependent on the type of the pollutant (Rezende and Furlan 2009, Furlan et al. 2010). Loponen et al. (2001) showed that (+)-catechin content increased and flavonols decreased with increase of heavy metals in air pollution. In leaves of bilberry plants growing near a Zn-Pb smelter Bialonska et al. (2007) observed higher contents of these compounds

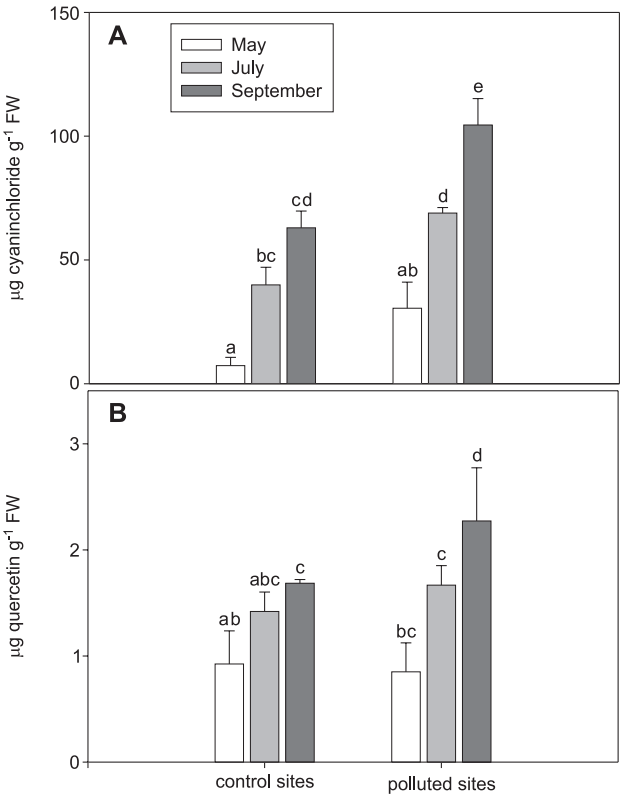

Fig. 1. Anthocyanins (A) and flavonols (B) content in *Berberis thunbergii* (DC.) leaves growing in green area located in the city of Poznań. Different letters indicate significant differences between all means (mean ±SE, $P < 0.05$)

Table 1. Summary of ANOVA results for 4CL activity, anthocyanins and flavonols content by site of plant growth and term of material collection

Source of variation	4CL activity			Anthocyanins content			Flavonols content		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Site (S)	1	4.8	0.041	1	44.8	<0.0001	1	5.41	0.0318
Term (T)	2	6.86	0.006	2	65.29	<0.0001	2	32.75	<0.0001
S × T	2	3.43	NS	2	1.39	NS	2	3.36	NS
Error	18			18			18		

NS – non significant.

measured by absorbance at 280 and 325 nm. These authors correlated the results to scavenging of free radicals produced under the influence of metal ions. According to Pieta (2000), flavonoids have been described as inhibitors of some enzymes involved in physiological processes that generate reactive oxygen species, such as xanthine oxidase, lipoxygenase, glutathione S-transferase and NADH-oxidase. Thus in each of these capacities they contribute to a reduction of oxidative stress.

Flavonoids are products of the phenylpropanoid pathway. 4-coumarate:CoA ligase is a key enzyme of this pathway. This enzyme catalyses the synthesis of intermediate compounds, e.g. 4-coumaroyl:CoA used as a substrate for flavonoid synthesis.

An increase in 4CL activity was observed in *Berberis* leaves during the vegetation period (Fig. 2). In May at the control location its activity was 10.3 pkat · mg^{-1} protein and it increased to 25.8 pkat mg^{-1} protein in September. The activity of 4CL increased dramatically in September in the polluted area and was 119.3 pkat mg^{-1} protein, i.e. approximately four times higher than at to the control site. The analysis of correlation between activity of 4-coumarate:CoA ligase and anthocyanins and flavonols level in barberry leaves revealed a positive and statistically significant relationship (Fig. 3).

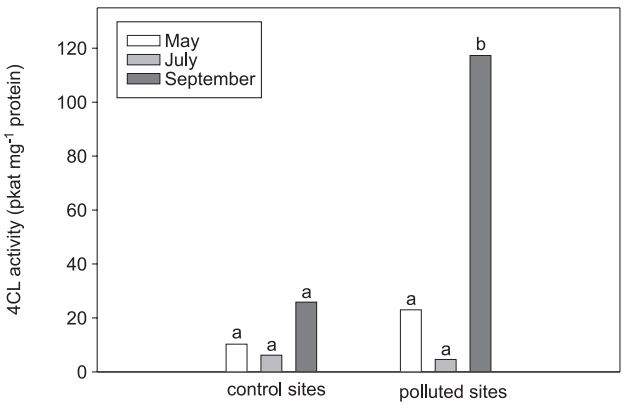

Fig. 2. 4-coumarate:CoA ligase activity in *Berberis thunbergii* (DC.) leaves growing in green area located in the city of Poznań. Different letters indicate significant differences between means ($P < 0.05$)

The observed activation of 4CL indicates sensitivity of phenylpropanoid pathway enzymes to air pollutants. The native enzyme can contain different amounts of metals, originating from the growth environment of the plant. Heavy metals probably have an effect on the structure and catalytic level of the enzyme (Herrmann and Weaver 1999). Moreover, according to the report on the status of the environment in the Wielkopolska region in 2008 of the Provincial Inspectorate of Environmental Protection in Poznań (Kaczmarek et al. 2009) air pollution levels in the city was caused by sulfur and nitrogen oxides and ozone.

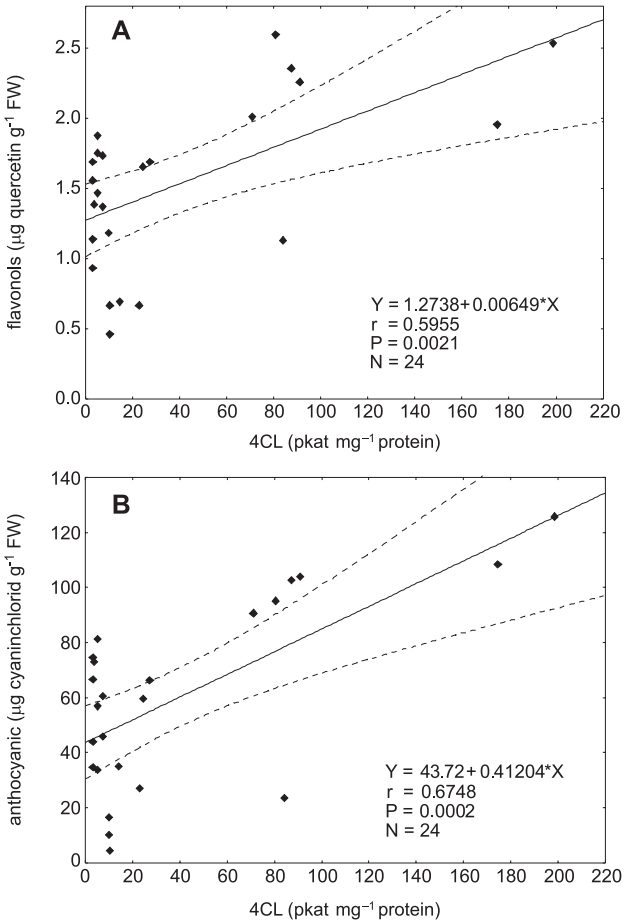

Fig. 3. The relationship between flavonols (A) and anthocyanins (B) accumulation and 4-coumarate:CoA ligase activity in leaves of *Berberis thunbergii* (DC.)

We suppose that the accumulation of anthocyanins and the increase of 4CL activity can be a response to stress caused by heavy metals and other air pollutants.

The results indicate that although *Berberis thunbergii* is a species commonly planted and growing well in urban areas, chemical contamination modifies secondary metabolism of this plant. Accumulated flavonoids as free radical scavengers may be involved in the reduction of oxidative stress and due to their metal chelating properties they may be engaged in the mechanism of tolerance to heavy metals. Moreover, heavy metals may be activators of phenylpropanoid pathway enzymes.

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