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The role of proline and hydroxyproline in the sensitivity of *Weigela* to the action of sulphur dioxide*

Abstract

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It was found that the treatment of *Weigela* shoots with solutions of 2,2′-dipyridyl, benzimidazole, proline and hydroxyproline have induced changes in the degree of injury to leaves inflicted by SO_2. The treatment with 2,2′-dipyridyl and proline in most of the concentrations used has caused a significant reduction of injury. It is suggested that the compounds used have affected the degree of leaf injury by SO_2 through their influence on the content of protein bound proline and hydroxyproline.

Additional key words: air pollution, amino acids, 2,2′-dipyridyl, benzimidazole.

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INTRODUCTION

The action of SO_2 on plants intensifies ageing processes in them. Similarily as it happens in ageing plants, the action of sulphur dioxide causes a lowering of the content of chlorophyll (Williams et al. 1971, Malhotra and Hocking 1976, Beckerson and Hofstra 1979), appearance of chloroses and necroses (Hindawi 1970, Taylor 1973, Białobok et al. 1980), plasmolysis of cells and drying of tissues (Taylor 1973, Werner 1981), dying of tissues (Thomas et al. 1950, Werner 1981), premature leaf fall (Paprzycki 1962, Knabe 1971, Taylor 1973) and eventually the death of plants.

The first reactions of plants to SO_2 that it is possible to measure are disturbances in the physiological and biochemical processes. Determination of changes, directions and intensity of these processes taking place in plants after action of a toxic dose of SO_2 may be used as an indicator of the degree of plant injury, that is of the reaction of the plant to this gas. In plants subjected to the action of SO_2 besides several other

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processes there is observable a release of ethylene (Guttenberger et al. 1978, Bressan et al. 1979, Mudd 1979). From investigations that have been conducted by Osborne et al. (1970), Ridge and Osborne (1971) it appears that ethylene, the natural hormonal inhibitor of cell growth, causes a reduction in the plasticity of the cell walls. This phenomenon is being explained as an influence of ethylene on the increased hydroxylation of proline bound in peptides. The hydroxyproline that results from this reaction is bound with the proteins of the cell walls and conditions changes in the maturation of the tissues, disrupts cell growth and reduces plasticity of the cells walls. Earlier (Karolewski 1984 a, b) observed in the genus *Weigela* an increase in the content of protein bound hydroxyproline as a consequence of SO_2 action, coupled with a reduction in the content of proline. Intensity of the process was higher in species and varieties that were more sensitive to this gas.

The possibility of manipulation of the process of proline hydroxylation with the help of substances that affect the proline-hydroxyproline balance was the subject of the investigations reported here. Influence of these substances on the changes in the degree of plant sensitivity to the action of SO_2 indirectly suggests that proline and hydroxyproline play an important role in the reaction of plants to this gas.

MATERIALS AND METHODS

PLANT MATERIAL

The plant material used consisted of one-year-old shoots (with 4-5 pairs of leaves) cut from 15-years-old shrubs from the genus *Weigela* (Thunb.), namely *Weigela florida* A. DC and *Weigela* × Bailey cv. 'Van Houtte', growing in the Arboretum of the Institute of Dendrology of the Polish Academy of Sciences in Kórnik. Experiments conducted earlier have shown that the first one is characterized by a higher sensitivity and the latter by a lower one to the action of SO_2 (Karolewski 1984a).

The shoots have been cut from the shrubs choosing a similar location in the shrub and stage of development. Immediately after detaching the shoots were placed with the cut end into containers with water. In each of the two experiments conducted individual variants have been represented by 5 shoots.

EXPOSITION OF PLANTS TO SO_2

The experiments were conducted under controlled laboratory conditions. Shoots were exposed to the action of SO_2 using an equipment consisting of chambers located in a climatized greenhouse and having a dosing-analysing system for sulphur dioxide (Białobok et al. 1978).

In the first of the experiments conducted shoots of *Weigela florida*, 48 h after cutting them, have been subjected to the action of 1 ppm SO_2 for 4 successive days, 6 h a day. The experiment was conducted in August, with a relative air humidity of 65-70%, and a temperature of 20-23°C and a natural illumination of 30-45 $W \cdot m^{-2}$. The same number of shoots have been placed in an identical chamber without SO_2 (control).

In the second experiment the shoots of *Weigela florida* and *Weigela* 'Van Houtte', 48 h after cutting them, have been subjected to the action of 2 ppm SO_2 for 12 h (2 days, 6 h per day). The exposition of plants to SO_2 was performed in July with similar humidity, temperature and light conditions.

In each experiment 24 h after termination of the last exposition to SO_2 the degree of injury to leaves by the gas has been estimated. For the purpose a 6 point scale of leaf injury has been used, as proposed by Schönbach et al. (1964), where 0 indicates lack of visible injuries and 5 an injury covering more than 70% of the leaf surface. From the data obtained mean values of injury estimated on 5 shoots have been calculated. The mean degree of injury has been assumed to be a measure of the sensitivity of plants.

TREATMENT OF PLANTS WITH COMPOUNDS

Shoots detached from shrubs have been treated with a solution of one of the following compounds: 2,2'-dipyridyl, benzimidazole, proline and hydroxyproline or with water (control). In both experiments the shoots have been subjected to the action of these substances in two different ways. The first (A) consisted of a twofold spraying of leaves with the given solution using 10 cm^3 for each shoot, immediately after detaching from the shrub and 24 hours later. The second way (B) consisted of placing the detached shoot with the cut end in the solution immediately after cutting and keeping in there till the termination of the expositions to SO_2.

In the first experiment 0.01, 0.1, and 1.0 mM solutions of 2,2'-dipyridyl or benzimidazole and 0.001, 0.01 and 0.1 mM solutions of proline and hydroxyproline have been used. In the second one 2.5, 5.0 and 7.5 mM solutions of 2,2'-dipyridyl and benzimidazole have been used.

ANALYTICAL METHODS

The content of proline and hydroxyproline has been determined in whole leave of plants that have not been subjected to the action of SO_2 and in the case of plant exposed to the action of SO_2 only in the parts of leaves that have not been visibily injured. In view of the large amount of plant material (15 g) that was necessary for the determinations of the amino acids using the method described below only one measurement was made for each experimental variant. The analyses were made on combined leaf samples collected evently from five shoots.

The leaf samples were homogenized in 100% acetone at a temperature of −15°C. The homogenate was filtered, washing the residue with acetone until all pigments were gone. The residue was used for the determination of bound amino acids. After prior hydrolysis of the samples using 12 N HCl from sealed glass ampoules for 18 h at a temperature of 107°C and after evaporation of the HCl under vacuum, the bound proline was determined with ninhydrine by the method described by Bergman and Loxley (1970). The absorption of the solutions was determined at 512 nm wavelength with the help of a Spekol (GDR) spectrophotometer. The results are presented in mg of proline per g of fresh weight. To determine the level of bound hydroxyproline

use was made of the method of Stegeman and Stalder (1967) based on the colorimetric absorption of the complex of the amino acid with p-dimethylaminobenzaldehyde. The content of the bound amino acids is presented in $mg \times 10\ mg^{-1}$ of sample.

STATISTICAL METHODS

Results of the degree of injury to leaves has been verified statistically using the new multiple range test *D* (Oktaba 1976). Significance of differences between the values of the degree of injury have been calculated at a confidence level of $\alpha = 0.05$. For the evaluation of the interrelationships of traits the correlation coefficients were calculated between injury of plants by SO_2, and the content of proline and hydroxyproline and changes of these contents in control plants and those subjected to the action of SO_2. Their significance was designated as r^* when r was significant at a level of confidence $\alpha = 0.1$, r^{**} for $\alpha = 0.05$ and r^{***} for $\alpha = 0.01$.

RESUL TS

The treatment of cut shoots of *Weigela florida* with solutions of 2,2'-dipyridyl and bezimidazole as well as with proline and hydroxyproline, depending on the concentrations of the solutions used and the mode of treating plants, has had an influence on the level of bound proline and hydroxyproline in the leaves as well as on their ratio (PRO : HYPRO) (Tab. 1 and 2).

Treatment with these compounds has also affected the degree of leaf injury by SO_2 (Tab. 1 and 2). The treatment of plants with the compounds only, that is without SO_2, has not caused any visible changes on the leaves relative to the controls. After SO_2 treatment less injured were plants treated by spraying (A) with all the compounds and concentrations except benzimidazole at 1.0 mM and only two variants, those sprayed with benzimidazole at 0.01 and 0.1 mM, were not significantly less injured than the water control. Benzimidazole at 1.0 mM was the only variant that had a higher injury estimate than the control. though not significantly different from it (Tab. 1). In the experiment with dipping cut ends into solutions of the compounds (B) the picture was similar. All variants were less injured than the water control but those with the highest concentrations, 0.1 mM for hydroxyproline, 1.0 mM for 2,2'-dipyridyl and benzimidazol not significantly so (Tab. 2).

A comparison of the content of bound proline in the leaves of *Weigela florida* treated with solutions of the above mentioned compounds, either by spraying (A) or by dipping (B), but not subjected to the action of SO_2 with the degree of injury of shoots exposed to SO_2 has shown a significant negative correlation ($r = -0{,}596^{**}$ for A and $r = -0{,}527^{**}$ for B). A significant negative correlation was also observed between the PRO : HYPRO ratio in the leaves treated by spraing (A) and the degree of their injury by SO_2 $r = -0{,}465^{*}$. When the shoots were treated by dipping (B) also a negative correlation was observed, but this was not significant $r = -0{,}417$, ($\alpha > 0.1$).

Table 1

Influence of solutions of chemical compounds supplied by spraying (method A) on the changes in the levels of bound proline and hydroxyproline in the leaves of *Weigela florida*, and also the degree of leaf injury inflicted by SO_2

Compound	Conc. (mM)	SO_2 conc. (ppm)						Degree of injury
		0			1			
		PRO mg/100 mg	HYPRO mg/100 mg	PRO : HYPRO	PRO mg/100 mg	HYPRO mg/100 mg	PRO : HYPRO	
Proline	0.001	0.450	0.235	1.9	0.870	0.130	6.7	0.40a
	0.01	0.435	0.255	1.7	0.815	0.153	5.3	0.48a
	0.1	0.525	0.235	2.2	0.605	0.168	3.6	0.10a
Hydroxyproline	0.001	0.485	0.240	2.0	0.555	0.255	2.2	0.33a
	0.01	0.760	0.255	3.0	0.800	0.235	3.4	0.26a
	0.1	0.385	0.168	2.3	0.555	0.140	4.0	0.73a
2,2'-dipyridyl	0.01	0.605	0.143	4.2	1.045	0.123	8.5	0.37a
	0.1	0.755	0.225	3.3	0.815	0.227	3.6	0.17a
	1.0	1.030	0.217	4.7	0.680	0.158	4.3	0.00a
Benzimidazole	0.01	0.485	0.161	3.0	0.420	0.133	3.1	0.90ab
	0.1	0.500	0.255	2.0	0.485	0.222	2.2	0.81ab
	1.0	0.385	0.228	1.7	0.585	0.136	4.5	1.80 c
Water control	–	0.410	0.190	2.1	0.365	0.128	2.8	1.68 bc

Table 2

Influence of solutions of chemical compounds supplied by dipping shoots with the cut end (method B) on the changes in the levels of bound proline and hydroxyproline in the leaves of *Weigela florida*, and also the degree of leaf injury inflicted by SO_2

Compound	Conc. (mM)	SO_2 conc. (ppm)						Degree of injury
		0			1			
		PRO mg/100 mg	HYPRO mg/100 mg	PRO : HYPRO	PRO mg/100 mg	HYPRO mg/100 mgg	PRO : HYPRO	
Proline	0.001	0.605	0.242	2.5	0.815	0.145	5.5	0.14a
	0.01	0.670	0.256	2.6	1.160	0.145	8.0	0.14a
	0.1	0.460	0.234	2.0	0.555	0.166	3.3	0.52abc
Hydroxyproline	0.001	0.670	0.186	3.6	0,520	0.186	2.8	0.13a
	0.01	0.460	0.203	2.3	0.510	0.220	2.3	0.29a
	0.1	0.535	0.198	2.7	0.680	0.185	2.7	1.16 cd
2,2′-dipyridyl	0.01	0.670	0.166	4.0	0.645	0.124	5.2	0.27a
	0.1	1.070	0.185	5.8	0.555	0.166	3.3	0.23a
	1.0	0.425	0.144	2.9	0.605	0.180	3.4	1.45 d
Benzimidazole	0.01	0.535	0.198	2.7	0.485	0.145	3.3	1.09 bcd
	0.1	0.585	0.245	2.4	0.705	0.172	4.1	0.59abc
	1.0	0.480	0.235	2.0	0.390	0.160	2.4	1.31 cd
Water control	–	0.410	0.190	2.1	0.365	0.128	2.8	1.68 d

After treatment with SO_2 of shoots that were dipped (B) a higher level of proline was observed in the leaves of variants that were less injured by the gas ($r = -0{,}438$*). More injured leaves contained less bound proline. When the leaves were treated by spraying (A) also a negative correlation was obtained ($r = -0{,}344$) but this was not significant ($\alpha > 0.1$).

The results obtained indicate that the higher was the level of proline and hydroxyproline in the leaves of control plants the greater was also their content after SO_2 treatment. However the correlation coefficient is significant ($r = 0{,}419$*) only when the shoots were sprayed (A). When dipping (B) was employed also a positive correlation was obtained but the correlation coefficient was low ($r = 0{,}219$).

The levels of proline and hydroxyproline in the leaves of shoots that were not subjected to the action of SO_2 are negatively correlated with the changes in the levels of these amino acids following the action of this gas. This would be indicated by the obtained significant correlation coefficients in the case of proline after dipping (B) ($r = -0{,}528$**) and of hydroxyproline regardless of treatment method (A and B) ($r = -0{,}419$* and $r = -0{,}714$*** respectively).

Table 3

Influence of the action of 2,2′-dipyridyl and benzimidazole solutions on the degree of leaf injury by SO_2 in *Weigela* 'Van Houtte' and *W. florida*

Compound	Method of treatment	Conc. (mM)	*Weigela*	
			'Van Houtte'	*florida*
			Degree of injury	
2,2′-dipyridyl	A*	2.5	1.90 abc	1.78 bc
		5.0	2.10 abc	0.71 ab
		7.5	1.05 a	1.58 abc
	B**	2.5	2.60 cd	0.48 a
		5.0	3.35 d	1.59 abc
		7.5	1.01 a	3.00 de
Benzimidazole	A	2.5	1.78 abc	2.64 cd
		5.0	1.39 ab	3.07 de
		7.5	2.27 bcd	3.74 def
	B	2.5	2.74 cd	3,10 def
		5.0	2.85 cd	4.20 f
		7.5	2.70 cd	3.65 def
Water control	–	–	2.01 abc	3.18 def

A* – spray
B** – dip

The relationships between these two amino acids are better illustrated by their relative ratio (PRO : HYPRO). The difference of the PRO : HYPRO ratio between the SO_2 treated and control plants decreases the larger is the value of this ratio in control plants PRO : HYPRO. However the correlation coefficients is significant only when the shoots were dipped (B) ($r = 0.531$*) and not after spraying.

The influence of the solutions of 2,2′-dipyridyl and benzimidazole on the change in the degree of leaf injury by SO_2 was also observed when two varieties of *Weigela*, a more sensitive one (*W. florida*) and a less sensitive one (*W.* 'Van Houtte') were used. The comparison of mean injury values in both varieties has shown that the action of

benzimidazole caused an increase in the degree of leaf injury by SO_2 when the compound was introduced by dipping (B), regardless of concentration and by spraying (A) at the highest concentration of 7.5 mM (Tab. 3).

The action 2,2′-dipyridyl, regardless of the method of treating with it (A or B) did not cause any lesser injury by SO_2 in the less sensitive variety *W.* 'Van Houtte'. On the other hand in the more sensitive variety *W. florida* the injuries caused by this gas were lower than in control plants following treatment with the compound regardless of concentration when spraying (A) and at 2.5 and 5.0 mM when dipping (B).

DISCUSSION

One of the important injurious physiological effects of SO_2 on plants is the increase in the rate of ageing. One of the elements of these changes as observed in earlier studies is the increase in the content of protein bound hydroxyproline (Karolewski 1984 a, b). The observed lowering of protein bound hydroxyproline content reported here may have been caused by proteolysis which could have started as a consequence of the high dose of SO_2 used here. The influence of SO_2 on the hydrolysis of proteins has been observed among others by Constantinidou and Kozlowski (1979) and by Malhotra and Sarkar (1979). Also our earlier studies on the kinetics of changes in the level of bound hydroxyproline in leaves of plants from the genus *Weigela* subjected to the action of SO_2 indicate that after an initial increase in the level of this amino acid there follows a reduction as the duration of exposition to SO_2 is extended (Karolewski 1984 b).

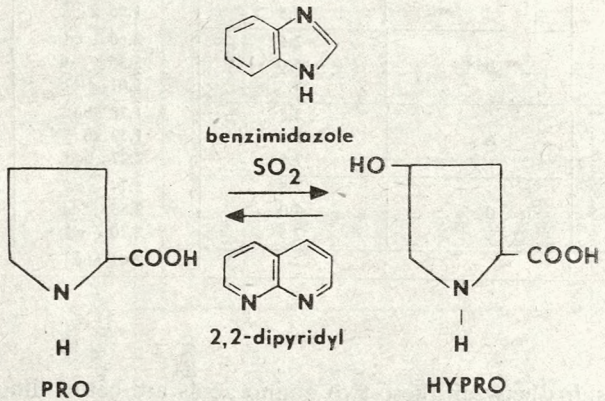

Fig. 1. Mechanism of action of sulphur dioxide and 2,2′-dipyridyl and benzimidazole on the proline-hydroxyproline balance

If the action of sulphur dioxide on plants causes specific changes in leaves in the content of protein bound proline and hydroxyproline, then induction of these same changes by a different method than fumigation with SO_2 should sensitive the plants and when the induced changes are in an opposite direction this should increase the resistance of the plants to the action of SO_2 (Fig. 1). The results presented in this

study on the influence of some chemical compounds on plants confirm this hypothesis.

Plants in the leaves of which following treatment with the compounds a lower level of bound proline and a lower proline to hydroxyproline ratio was found were more sensitive to SO_2. Treatment of plants with benzimidazole causes an increase in the synthesis of protein bound hydroxyproline (Giebel and Krenz 1975) which leads to changes in the ratio of proline to hydroxyproline. A treefold increase in the level of hydroxyproline in cell walls of epicotyls of soya beans has been found by Sadava and Chrispeels (1973) after incubating them in a benzimidazole solution. An increase in the sensitivity of plants to the action of SO_2 may be caused by the stimulating action of benzimidazole on the photosynthetic assimilation of CO_2 by leaves, which was found in lucerne and maize by Hortwáth and Pozsšar (1974). At the same time this would confirm the hypothesis that plants which photosynthesize more intensively are more sensitive to the action of SO_2 (Vogl and Börtitz 1969, Oleksyn 1981, Oleksyn i Bialobok 1986).

A reduction in the injury of plants by SO_2 has been achieved by treatment of them with compounds that induce an increase in the content of proline and ratio of the two amino acids. A reduction in the sensitivity of plants to the action of SO_2 has been achieved by treatment with solutions of 2,2′-dipyridyl, which is an inhibitor of the biosynthesis of hydroxyproline in the cell walls (Barnet 1970). This compound binds into complexes ions of Fe^{+2} essential for the hydroxylation of proline which inhibits the formation of protein bound hydroxyproline. At the same time it does not affect the synthesis of proteins (Chrispeels 1970). A reduction in the hydroxylation of proline which takes place under the influence of SO_2 may be the cause of the reduced sensitivity of plants to this gas. A reduction in the degree of sensitivity has been achieved by treating plants with proline, even though the action of this gas causes an increase in the content of free proline (Karolewski 1984 a, b, 1985). This can be explained by the high rate of proline metablism which has been demonstrated by Durzan and Ramaiah (1971) in seedlings of *Pinus banksiana*. These authors have shown that after treating seedlings with ^{14}C-proline already after a short time (6 h) there was observable a rapid decline in total radioactivity of the free amino acid pool, including proline and an increase in the radioactivity of bound proline, while at the same time the level of hydroxyproline bound in proteins was lowered. These changes can explain the decrease in sensitivity of plants treated with proline solution.

In leaves of plants treated by solutions of proline, hydroxyproline, 2,2′-dipyridyl and benzimidazole, there occured changes in the levels of amino acids following SO_2 treatment similar to those observed following SO_2 treatment only.

The possibility of reducing the degree of plant injury by SO_2 by treating them with solutions of 2,2′-dipyridyl and proline or of increasing it by the treatment with benzimidazole and hydroxyproline has been confirmed also in an experiment with two varieties of *Weigela*, *W. florida* and *Weigela* 'Van Houtte' differing in sensitivity to SO_2.

As can be seen from the results presented in this paper both amino acids, proline and hydroxyproline, play a significant role in the mechanism of plant sensitivity to the action of sulphur dioxide.

SUMMARY

In order to confirm the role of proline and hydroxyproline in the sensitivity of plants to the action of SO_2 detached shoots of shrubs from the genus *Weigela* have been treated with compounds that induce changes in the levels of these amino acids in plants, 2,2′-dipyridyl, a compound that inhibits the hydroxylation of proline and benzimidazole, that promotes the synthesis of hydroxyproline, as well as by proline and hydroxyproline themselves. It was found that depending on the concentration and mode of treating the plants with solutions of 2,2′-dipyridyl and proline there resulted a lowering of injury by SO_2 relative to the water treated controls. The use of benzimidazole at 1.0-7.5 mM contributed to an increase in the extent of injury inflicated by the gas.

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ПЕТР КАРОЛЕВСКИ

Роль пролина и гидроксипролина в чувствительности Weigela к действию сернистого ангидрида

Резюме

Для подтверждения роли пролина и гидроксипролина в чувствительности растений к действию SO_2 срезанные побеги из вида *Weigela* обрабатывали соединениями влияющими на изменение уровня этих аминокислот 2,2'-дипиридилом — соединением тормозящим

гидроксилацию пролина, и бензимидазолом — активатором синтеза гидроксипролина а также пролином и гидроксипролином.

Отмечено, что в зависимости от концентрации и способа обработки побегов растворами соединений, 2,2'-дипиридил и пролин вызывали уменьшение повреждений вызванных SO_2 по сравнению с контролем которым были побеги обработанные водой.

Применение бензимидазола в концентрациях 1.0 - 7.5 мМ способствовало увеличению повреждений вызванных этим газом.

Rola proliny i hydroksyproliny we wrażliwości krzewów z rodzaju Weigela na działanie dwutlenku siarki

Streszczenie

W celu potwierdzenia roli proliny i hydroksyproliny we wrażliwości roślin na działanie SO_2, odcięte pędy z rodzaju *Weigela* traktowano związkami wpływającymi na zmiany poziomów tych aminokwasów, 2,2′-dwupirydylem – związkiem hamującym hydroksylację proliny i benzimidazolem — promotorem syntezy hydroksyproliny oraz proliną i hydroksyproliną.

Stwierdzono, że w zależności od stężenia i sposobu traktowania pędów roztworami związków, 2,2′-dwupirydyl i prolina powodowały obniżenie uszkodzeń przez SO_2 w porównaniu z grupą kontrolną (pędy traktowane wodą). Stosowanie benzimidazolu w stężeniach 1,0 - 7,5 mM przyczyniało się do zwiększenia uszkodzeń spowodowanych przez ten gaz.