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Effect of SO_2 on changes in proline and hydroxyproline content in leaves of eight species and varieties from the genus *Weigela**.

INTRODUCTION

Changes in the levels of free amino acids are a good indication of the reaction of plants to the action of sulphur dioxide. The action of SO_2 causes an increase in the general pool of free amino acids in such plants as clover (Arndt 1970), beans (Arndt 1970, Godzik and Linskens 1974), spruce (Jäger and Grill 1975), pine (Malhotra and Sarkar 1979, Lorenc-Plucińska 1983) and birch (Nikolaevskij et al. 1975).

A similar response of increase in free amino acids, particularily of proline is observed in plants subjected to a water stress (Kemble and Macpherson 1954; Thompson et al. 1966; Procenko et al. 1968, Hsiao 1973; Britikov 1975). Procenko et al. (1968) underline that when proline is formed toxic ammonia is being bound which normally is formed in plants during drought periods. This amino acid can also perform some other protective functions in plants subjected to stress from environmental factors.

An increase in the level of free proline is also observed under the influence of simultaneous action of several stress factors. This has been demonstrated among others by Tesche (1979) on Norway spruce trees exposed to sulphur dioxide, low temperature and drought. A positive correlation between the degree of tolerance of spruce grafts to the action of SO_2 and their tolerance to a water deficit has been demonstrated by Klein (1980). This would suggest the presence of common mechanisms in the reaction of plants to these factors.

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The action of SO_2 on plants causes an increase in the intensity of ageing processes within them. Thus a hydroxylation of peptide bound proline that conditions changes in the maturation of tissues (Ridge and Osborne 1971) may play a certain role in the sensitivity of plants to the action of this gas.

The changes in the content of free proline under the influence of SO_2 action and the hydroxylation of the proline bound in peptides in various species and varieties from the genus *Weigela* differing in sensitivity, were the subject of the present investigation.

MATERIALS AND METHODS

PLANT MATERIAL

The experimental material consisted of one year old shoots (with 4 - 5 pairs of leaves) detached from 15-year old *Weigela* Thunb. shrubs growing in the Kórnik Arboretum of the Polish Academy of Sciences. The plants represented eight species and varieties, namely: *W. coraeensis, W. florida, W.* 'Gustave Mallet', *W.*×*wagneri, W. japonica, W.* 'Van Houtte', *W.* 'Mme Couturier' and *W.* sp., seedling of 1930.

The shoots were cut from the shrubs choosing as far as possible a similar location on the shrub and a similar stage of development. Immediately after detaching the shoots they were placed with the detached end in containers with water.

For the amino acid analyse use was made of 36 shoots from each of the eight species and varieties of *Weigela*. Four shoots constituted one replicate. Twelve shoots (3 replicates 4 shoots each) were placed in a chamber with SO_2, 12 in a chamber without SO_2 and 12 were used for immediate analysis of proline and hydroxyproline content.

EXPOSITION OF PLANTS TO SO_2

The experiments were performed in controlled laboratory conditions. The shoots were exposed to the action of SO_2 using the equippment described earlier and consisting of chambers within a climatized greenhouse and a dosing-analysing unit for sulphur dioxide (Białobok et al. 1978).

The plants were exposed to the action of sulphur dioxide at a concentration of 2 ppm (about 5.34 mg$\times$m^{-3}) for 12 h (10^{00} - 22^{00}) in June. The relative air humidity was maintained at 60 - 70%, light illumination at 10 - 20 klx and temperature at 17 - 21°C. Additional shoots were exposed to SO_2 for estimation of their sensitivity. After 12 h from the moment of termination of exposition the plants were estimated for leaf injury which was considered a measure of their sensitivity. When estimating the injury use was made of a 6 point scale described earlier (B i a ł o b o k et al. 1980).

Proline and hydroxyproline have been determined in leaves of control plants and exposed to the action of SO_2. Whole leaves of the plants not subjected to the fumigation treatment were analysed, and in the case of fumigated plants with SO_2 only those parts of leaves that were not visibly injured.

ANALYTICAL METHODS

Leaf samples have been homogenized in 100% acetone at a temperature of −15°C. The homogenate was filtered through a S c h o t t filter washing the residue with acetone to a complete washing out of the pigments. The residue was used to determine the bound amino acids proline and hydroxyproline.

Free proline was determined with the help of ninhydrin in water solutions after previous evaporation of the acetone under vacuum according to the method described by B e r g m a n and L o x l e y (1970). Absorption of the solutions at a wavelength of 512 nm has been determined with the help of a Spekol (GDR) spectrophotometr. The results were presented in mg of proline$\times g^{-1}$ fresh weight.

After hydrolysis of the residue samples, using 12 N HCl in glass viles for 18 h at a temperature of 107° C and evaporation of the HCl under vacuum, bound proline has been determined identically as free proline.

In order to determine the content of bound hydroxyproline use was made of the S t e g e m a n n and S t a l d e r (1967) method based on a colorimetric measurement of the absorption complex of the amino acid with p-dimethylaminobenzaldehyde.

The content of bound amino acids has been given in mg$\times$100 mg^{-1} of the sample.

The content of free and bound proline and hydroxyproline has been determined directly after detaching the shoots (Z_{K_0}), after a time equal to the exposition time (Z_K) and after treatment with SO_2 (Z_{SO_2}).

STATISTICAL METHODS

Results of the estimate of the degree of leaf injury was verified statistically using the new multiple range test D (**O k t a b a 1976**). The significance of differences between degrees of injury to the plants was determined at a confidence level of $\alpha=0.05$. For the mean values of injury degree a standard deviation was calculated. For an estimate of the interrelationships between traits, namely injury due to SO_2 treatment, level of proline and hydroxyproline and changes in the levels of these amino acids, in control plants and in those subjected to fumigation with sulphur dioxide, the significance of correlation coefficients was estimated. They were given r^* when the coefficient was significant at $\alpha=0.1$, r^{**} at $\alpha=0.05$ and r^{***} at $\alpha=0.01$.

RESULTS AND DISCUSSION

Results of the selection performed and grouping of mean values according to the degree of injury of leaves by SO_2 allowed the conclusion that the species and varieties of *Weigela* which are less sensitive to this gas are *W. coraeensis* and *W. japonica* and the most sensitive ones were the *W. sp.* seedling of 1930, *W. florida*, *W. 'Gustave Mallet'* and *W×wagneri* (Tab. 1).

The results presented in the paper indicate (Tab. 2) that in leaves of plants subjected to the action of SO_2 there results an accumulation of free proline. It is larger when the degree of injury of the plants is greater (Tab. 3). Accumulation of large quantities of free proline in leaves after the action of SO_2 on the plants may be the consequence of proline synthesis (Mudd 1979) or its proteolysis (Fischer 1971; Malhotra and Sarkar 1979).

In the experiments conducted by me an increase in the content of free proline was observed also in leaves from detached shoots 12 hours after their detachment (Tab. 2). This increase was significantly greater in the more sensitive plants than in the less sensitive ones to SO_2 (Tab. 3).

The action of SO_2 has also caused an increase in the content of free proline in leaves of both the more and less sensitive sepcies and varieties of *Weigela*. This may suggest that SO_2 causes a further enhencement of

Table 1

Mean values of the degree of injury by SO_2 of leaf surfaces of 8 species and varieties from the genus *Weigela*

Weigela	Degree of injury $a \pm \sigma$
coraeensis	0,50±0,20 a*
japonica	1,33±0,33 ab
'Van Houtte'	1,83±0,44 bc
'Mme Couturier'	2,17±0,29 bc
× *wagneri*	2,67±0,28 cd
'Gustave Mallet'	3,33±0,53 d
florida	3,33±0,35 d
sp. 1930 seedling	3,58±0,54 d

* When the same latter is next to a mean injury level it indicates that the values are not significantly different at a confidence level of $\alpha=0.05$.

Table 2

Content of proline and hydroxyproline in leaves of 8 species and varieties from the genus *Weigela* in control (K_o-before exposition to SO_2; *K* – after a time equal to that in the treated plants) and SO_2 treated plants (SO_2)

Weigela variety	Free proline mg × g⁻¹ fresh wt.			Bound proline mg × 100 mg⁻¹ sample			Bound hydroxyproline mg × 100 mg⁻¹ sample			Bound proline / Bound hydroxyproline		
	K_o	K	SO_2	K_o	K	SO_2	K_o	K	SO_2	K_o	K	SO_2
coraeensis	0,145 ± 0,018	0,167 ± 0,010	0,270 ± 0,054	0,700 ± 0,019	0,850 ± 0,057	1,167 ± 0,032	0,153 ± 0,024	0,144 ± 0,019	0,165 ± 0,006	4,6	5,9	7,1
japonica	0,119 ± 0,019	0,178 ± 0,011	0,359 ± 0,040	0,767 ± 0,081	0,850 ± 0,086	0,825 ± 0,041	0,137 ± 0,047	0,139 ± 0,017	0,159 ± 0,018	5,6	6,1	5,2
'Van Houtte'	0,084 ± 0,011	0,142 ± 0,021	0,450 ± 0,041	1,063 ± 0,205	0,917 ± 0,130	0,850 ± 0,162	0,155 ± 0,016	0,150 ± 0,014	0,196 ± 0,020	6,8	6,1	4,3
'Mme Couturier'	0,130 ± 0,004	0,199 ± 0,016	0,523 ± 0,047	0,958 ± 0,105	1,000 ± 0,054	1,017 ± 0,097	0,157 ± 0,009	0,150 ± 0,017	0,159 ± 0,027	6,1	6,7	6,4
wagneri	0,128 ± 0,026	0,187 ± 0,014	0,481 ± 0,050	1,150 ± 0,214	1,200 ± 0,114	0,925 ± 0,071	0,167 ± 0,009	0,147 ± 0,017	0,137 ± 0,023	6,9	8,2	6,7
'Gustaw Mallet'	0,104 ± 0,004	0,160 ± 0,010	0,594 ± 0,061	1,092 ± 0,083	0,958 ± 0,225	0,883 ± 0,099	0,159 ± 0,024	0,153 ± 0,024	0,149 ± 0,045	6,9	6,3	5,9
florida	0,124 ± 0,029	0,208 ± 0,026	0,767 ± 0,022	1,107 ± 0,158	1,058 ± 0,113	0,633 ± 0,099	0,150 ± 0,006	0,185 ± 0,011	0,145 ± 0,019	7,4	5,7	4,4
sp. 1930 seedling	0,095 ± 0,013	0,204 ± 0,004	0,688 ± 0,064	1,392 ± 0,047	1,150 ± 0,220	0,767 ± 0,142	0,160 ± 0,010	0,219 ± 0,034	0,125 ± 0,012	8,7	5,2	6,1

Table 3

A matrix of correlation coefficients between the degree of plant injury by SO_2 (a) and the content or changes in content of free proline (PROf), bound proline (PROb) and bound hydroxyproline (HYPROb) in leaves of 8 species and varieties of *Weigela* immediately after detaching shoots from plants (Z_{K_0}), after a time equal to treatment (Z_K) and after treatment with SO_2 (Z_{SO_2}) and changes in the content of these amino acids (Z_{K-K_0} and Z_{SO_2-K})

		a	K_0				K			
			Z_{PROf}	Z_{PROb}	Z_{HYPROb}	$Z_{PRO:HYPRO}$	Z_{PROf}	Z_{PROb}	Z_{HYPROb}	$Z_{PRO:HYPRO}$
K_0	Z_{PROf}	−0,527								
	Z_{PROb}	0,890***								
	Z_{HYPROb}	0,352								
	$Z_{PRO:HYPRO}$	0,906***								
K	Z_{PROf}	0,407	0,316							
	Z_{PROb}	0,744**		0,831***						
	Z_{HYPROb}	0,646*			0,500					
	$Z_{PRO:HYPRO}$	−0,053				−0,190				
SO_2	Z_{PROf}	0,948***					0,519			
	Z_{PROb}	−0,692*						−0,376		
	Z_{HYPROb}	−0,791**							−0,719**	
	$Z_{PRO:HYPRO}$	−0,220								0,358
$K-K_0$	Z_{PROf}	0,949***	−0,817**							
	Z_{PROb}	−0,728**		−0,845**						
	Z_{HYPROb}	0,565			−0,091					
	$Z_{PRO:HYPRO}$	−0,702**				−0,847***				
SO_2-K	Z_{PROf}	0,940***					0,387			
	Z_{PROb}	−0,842***						−0,803**		
	Z_{HYPROb}	−0,743**							−0,889***	
	$Z_{PRO:HYPRO}$	−0,165								−0,471

the process of free proline accumulation over and above the accumulation caused by detaching the shoot from the parent plant. After treating with sulphur dioxide in leaves of the more sensitive species and varieties of *Weigela* the increase in the content of the amino acid was greater compared to that in the less sensitive varieties. Similar relationships have been observed by Hanson et al. (1977), when studying the sensitivity of rye seedlings to a water stress. They have found that the more sensitive the plant is the greater was the increase in their leaves of free proline.

The increase in free proline observed here following action with SO_2 probably takes place along a series of changes: glutamic acid→glutamicγ-semialdehyde→proline-5-carbocylic acid→proline. The possibility of proline synthesis from glutamic acid along this pathway has been shown by Durzan and Ramaiah (1971) and Durzan (1973).

Studying the possible pathways for free proline synthesis when plants wilt several authors have found (Stewart et al. 1966; Palfi, 1968; Nylor, 1972) that the synthesis is associated with an accumulation of sugars and the formation of α-ketoglutaric acid on their oxidation. Increase in the content of mono- and poly-saccharides occurs also under the influence of SO_2. This was observed also by Börtitz (1967, 1968) in field conditions following the action of SO_2 on larch and pine and it has resulted in a complete hydrolysis of starch in needles of these trees. Also Mudd (1979) has reported that the action of SO_2 at a concentration of 0.77 ppm on bean seedlings has caused an increased incorporation of ^{14}C to soluble sugars and a decline in the level of labelled starch. The increase in sugar level may be the result of rapid starch hydrolysis caused by SO_2 or a decline in its synthesis.

Proline together with hexoses is an effective activator of the Krebs cycle (Britikov et al. 1970; Britikov 1975). This may contribute to the stimulation of respiration of plants following SO_2 as observed by some investigators (Lorenc-Plucińska 1978).

The results of experiments conducted indicate that there is a decline in the level of bound proline and hydroxyproline in leaves of various species and varieties of *Weigela* following the action with SO_2 (Tab. 2). This decline was proportional to the degree of sensitivity of the studied varieties (Table 3). It is most probably caused by a hydrolysis of proteins after SO_2 action. This was also observed by Fischer (1971), Malhotra and Sarkar (1979) and Constantinidou and Kozlowski (1979).

Comparing the changes in the levels of bound hydroxyproline in leaves of plants after SO_2 action a decline in its level was observed only in the most sensitive species and varieties (Tab. 2). In the less sensitive species and varieties an increase in bound hydroxyproline was observed. This is confirmed by investigations of the kinetics of changes in the levels of proline and hydroxyproline under the influence of SO_2 in the leaves of rooted cuttings of two *Weigela* varieties differing in sensitivity to SO_2 (Karolewski 1984). This would suggest that when the reaction of plants to SO_2 treatment is not sufficiently intense to cause a proteolysis the gas causes an increase in the process of hydrolysis of peptide bound proline.

After detachment of the shoots a tendency was observed for a decline in the content of bound proline in leaves of plants and an increase in the level of bound hydroxyproline. This has resulted in a lowering of the PRO:HYPRO ratio. The magnitude of this ratio was greater when the plants were more sensitive to the action of the gas (Tab. 3). Sulphur dioxide intensified the changes, with the difference however that in the more sensitive plants there occured not an increase but a decline of the level of bound hydroxyproline. This may indicate that protein hydrolysis was intensified in the case of the most sensitive species and varieties used in the experiment. Thus SO_2, similarily as was observed by Chrispeels et. al. (1974), in ageing plant tissues, intensifies the process of proline hydroxylation.

The results presented in this paper indicate that the differences in the degree of sensitivity of plants may be the consequence of differences in the intensity of ageing processes in cells that are induced by the action of SO_2. Results of this investigation suggest also that the content of proline and hydroxyproline in leaves may be one of the indicators useful in determining the negative influence of SO_2 on plants.

A clear evaluation of the role of proline and hydroxyproline in the reaction of plants to SO_2 action is however very difficult now. Sulphur dioxide is a non-specific compound interfering in several metabolic processes. Thus the changes occurring under the influence of this gas in the level of proline and hydroxyproline may occur as a result of direct SO_2 action or indirectly through an earlier disturbance in some more sensitive metabolic chain.

SUMMARY

The role of proline and hydroxyproline in the leaves of 8 species and varieties from the genus *Weigela* was investigated in relation to sensitivity to SO_2. Changes were also observed in the levels of these amino acids following action with this gas. It was found that the greater was the sensitivity of the plants to this gas the greater was the level of proline bound in proteins in leaves and the proline/hydroxyproline ratio. The action of SO_2 has caused an accumulation of free proline in leaves

and it was greater in the more sensitive species and varieties, After treatment with SO_2 there was a decline in the level of both the studied amino acids bound in proteins. This decline was proportional to the sensitivity of the studied species and varieties.

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Wpływ SO_2 na zmiany zawartości proliny i hydroksyproliny w liściach ośmiu gatunków i odmian z rodzaju Weigela

Streszczenie

Badano wpływ zawartości proliny i hydroksyproliny w liściach ośmiu gatunków i odmian z rodzaju *Weigela* na ich wrażliwość na działanie SO_2. Określano również zmiany zawartości badanych aminokwasów po działaniu gazu. Stwierdzono, że wrażliwość roślin na działanie SO_2 była tym większa, im większa była zawartość w liściach związanej z białkiem proliny i stosunek związanych proliny do hydroksyproliny. Działanie SO_2 powodowało akumulację wolnej proliny w liściach, przy czym była ona większa u gatunków i odmian bardziej wrażliwych na ten gaz. Po działaniu SO_2 stwierdzono spadek zawartości obydwu badanych aminokwasów związanych z białkiem. Był on proporcjonalny do wrażliwości badanych gatunków i odmian.

Влияние SO_2 на изменение содержания пролина и гидроксипролина в листьях восьми видов и разновидностей с рода Weigela

Резюме

Исследовалось влияние содержания пролина и гидроксипролина в листьях восьми видов и разновидностей с рода *Weigela* на их чувствительность к действию SO_2. Определялись также изменения в содержании исследуемых аминокислот под влиянием этого газа. Найдено, что чувствительность растений к SO_2 была тем выше, чем больше было содержание в листьях связанного с белком пролина и соотношение связанных пролина и гидроксипролина. Сернистый ангидрид вызывал аккумуляцию свободного пролина в листьях, причем его накопление было большим у более чувствительных разновидностей. После воздействия SO_2 отмечено уменьшение содержания обеих связанных с белком аминокислот. Оно было пропорционально к степени чувствительности исследуемых видов и разновидностей.