Oxidative stress and some antioxidant systems in acid rain-treated bean plants
Protective role of exogenous polyamines

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Received 10 March 1999; accepted 27 September 1999

Abstract

The effect of simulated acid rain (AR) (pH 1.8) on H$_2$O$_2$ and malonyldialdehyde (MDA) levels and activities of peroxidase and catalase in bean plants were investigated. The influence of exogenous polyamines spermidine and spermine on these parameters was also studied. AR treatment induced lipid peroxidation and increased level of H$_2$O$_2$ in leaves. Pretreatment with spermidine and spermine prevented these changes. The protective effect of spermine was higher than that of spermidine. The impact of polyamines could be attributed to their acid neutralizing and antioxidant effects, as well as to their ability to stabilize membranes by associating with negatively charged phospholipids. It was also found that AR significantly increased peroxidase and decreased catalase activities at the first hours after treatment. Later, both enzyme activities were enhanced that could contribute to the scavenging and detoxification of active oxygen species. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Phaseolus vulgaris; Acid rain; Hydrogen peroxide; Lipid peroxidation; Catalase; Peroxidase; Polyamines

1. Introduction

Acid rain (AR) induces changes in the cellular biochemistry and physiology of the whole plant. Biological effects of acid deposition on plants are numerous and complex, and include visible symptoms of injury (chlorosis and/or necrosis), and invisible effects such as reduced photosynthesis, nutrient loss from leaves, altered water balance, variation of several enzyme activities [1,2]. Previous data indicated that photosynthetic CO$_2$ fixation and photochemical activity of bean plants were decreased following pH 2.2–1.8 AR [3].

The ability of plant to overcome the effect of the AR stress and to sustain its productivity may be related to the scavenging of stress-induced toxic oxygen species, such as H$_2$O$_2$ (hydrogen peroxide), OH$^*$ (hydroxyl radical) and O$_2$ $^*$ (superoxide radical). Catalases and peroxidases are two major systems for the enzymatic removal of H$_2$O$_2$ in plants [4]. Polyamines (PAs) are now regarded as a new class of growth substances [5] and are also well known for their anti-senescence and anti-stress effects due to their acid neutralizing and antioxidant properties, as well as to their membrane and cell wall stabilizing abilities [6–8]. Scarce data, however, are available on the production of active oxygen species and antioxidant systems in plants subjected to AR, as well as on the involvement of PAs as protectants against this type of stress.

The aim of the present paper was to investigate the effect of simulated AR (pH 1.8) on lipid peroxidation, H$_2$O$_2$ content, peroxidase and cata-
lase activities, as well as the possibility of exogenous polyamines (spermidine and spermine) to inhibit the accumulation of the above oxidative species in AR-treated bean plants.

2. Material and methods

2.1. Plant material and treatments

Experiments were carried out with 10-day-old bean plants (*Phaseolus vulgaris* L. cv. Cheren Starozagorsky) after the emergence of the first composite bean leaf. Plants were grown in a climatic chamber at photon flux density about 120 \( \mu \text{mol m}^{-1} \text{s}^{-1} \), temperature 25 ± 2°C and 12 h photoperiod.

The following variants were experimented, applying single spraying treatments:

- Plants sprayed with cocktail pH 5.6-control;
- Plants sprayed with simulated AR pH 1.8 (AR);
- Plants sprayed with 1 mM spermidine·3 HCl (Spd pH 5.6);
- Plants sprayed with 1 mM spermidine·3 HCl + AR pH 1.8 (Spd + AR);
- Plants sprayed with 1 mM spermine·4 HCl (Spm pH 5.6);
- Plants sprayed with 1 mM spermine·4 HCl + AR pH 1.8 (Spm + AR).

The AR was prepared according to Seufert et al. [9] and contained the following components: \( \text{NH}_4\text{NO}_3 \) (1.3 g l\(^{-1} \)), \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \) (3.1 g l\(^{-1} \)), \( \text{Na}_2\text{SO}_4 \) (2.5 g l\(^{-1} \)), \( \text{KHCO}_3 \) (1.3 g l\(^{-1} \)), \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \) (3.1 g l\(^{-1} \)). After dilution of initial solution 1:100, pH value was adjusted to 1.8 with 1NH\(_3\)PO\(_4\) and 1NH\(_2\)SO\(_4\). The cocktail used to spray the control plants has the same composition as the AR but the pH value is 5.6. Tween 80 (0.5%, v/v) was used as surfactant. Each plant was sprayed with 2 ml simulated AR or cocktail pH 5.6. The vessels were covered with lids, which did not permit the AR to contaminate the nutrient solution.

Polyamines were used as foliar sprays applied to plants 24 h before spraying with pH 5.6 cocktail and before the AR treatment. Each plant was sprayed with 2 ml polyamine solution.

The measurements were carried out at different intervals after AR treatment: at the 3rd, 5th, 24th and 168th h.

2.2. Determination of \( \text{H}_2\text{O}_2 \) content

Hydrogen peroxide levels were determined according to Sergeev et al. [10]. Leaf tissues (500 mg) were homogenized in ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was centrifuged at 12000 \( \times g \) for 15 min and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The absorbancy of supernatant was read at 390 nm. The content of \( \text{H}_2\text{O}_2 \) was given on a standard curve.

2.3. Determination of the malonyldialdehyde (MDA) content

For the measurement of lipid peroxidation in leaves, the thiobarbituric acid (TBA) test, which determines MDA as an end product of lipid peroxidation [11], was used. Leaf material (500 mg) was homogenized in 5 ml 0.1% (w/v) TCA solution. The homogenate was centrifuged at 10000 \( \times g \) for 20 min and 0.5 ml of the supernatant was added to 1 ml 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30 min, and the reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10000 \( \times g \) for 5 min, and the absorbancy of supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA–TBA complex (red pigment) was calculated from the extinction coefficient 155 mM\(^{-1} \) cm\(^{-1} \).

2.4. Enzyme analysis

For the measurement of enzyme activities, leaf tissue (500 mg) was homogenized in 5 ml 10 mM potassium phosphate buffer (pH 7.0) containing 4% (w/v) polyvinylpyrrolidon (\( M_r \) 25 000). The homogenate was centrifuged at 12000 \( \times g \) for 30 min and supernatant obtained was used as enzyme extract. All steps in the preparation of the enzyme extract were carried out at 0–4°C. An aliquot of the extract was used to determine its protein content by the method of Bradford [12] utilizing bovine serum albumin as standard.

The activity of peroxidase (EC 1.11.1.7) was assayed according to the method of Dias and Costa [13] with some modifications. The following reaction mixture (3 ml) was used: 10 mM potas-
sium phosphate buffer, pH 7.0, 0.02–0.06 ml enzyme and 0.6 ml guaiacol 1% (w/v) aqueous solution. The reaction was started by adding 0.15 ml of 100 mM H$_2$O$_2$ and the optical density at 470 nm was recorded in a spectrophotometer Shimadzu against an identical mixture to which no H$_2$O$_2$ was added. The linear initial reaction rate was used to estimate the activity, which was expressed in µmol of guaiacol dehydrogenation product (GDHP) formed per milligram protein per minute, using the extinction coefficient of 26.6 mM$^{-1}$ cm$^{-1}$.

The activity of catalase (EC 1.11.1.6) was assayed by measuring the initial rate of disappearance of H$_2$O$_2$ by the method of Kato and Shimizu [14]. Three ml of catalase assay reaction mixture contained 10 mM potassium phosphate buffer, pH 7.0, 0.1 ml enzyme extract and 0.035 ml H$_2$O$_2$, 3%. The decrease in H$_2$O$_2$ was followed as decline in optical density at 240 nm, and the activity was calculated using the extinction coefficient (40 mM$^{-1}$ cm$^{-1}$) for H$_2$O$_2$ [14].

2.5. Reagents

Chemicals were purchased from Serva and were of analytical grade.

2.6. Statistics

All results were represented as means ± S.E. from at least five independent series of experiments (3–5 measurements each). The significant differences were determined by the Student’s t-test.

3. Results

3.1. H$_2$O$_2$ content

Changes in H$_2$O$_2$ content are shown in Table 1. An increase was observed after AR spraying. The response was more pronounced early after treatment, reaching 161% of the control (pH 5.6) at the 3rd h. Later, the level of H$_2$O$_2$ was relatively decreased but remained higher than in the control.

To evaluate the effect of PAs in AR-treated plants, data for the H$_2$O$_2$ content in the variants Spd + AR and Spm + AR were compared with data for AR. It appeared that pretreatment with PAs interfered with H$_2$O$_2$ accumulation, particularly early after acid application (at the 3rd h): H$_2$O$_2$ was reduced to 82 (Spd + AR) and 69% (Spm + AR) of its level in the variant AR. In the later intervals the effect of PAs was less pronounced (except for Spd + AR and Spm + AR, 168th h). The supply of PAs to plants at pH 5.6 (control) had no significant impact on H$_2$O$_2$ content.

3.2. Lipid peroxidation

Variations in the content of MDA are presented in Table 2. An increase of MDA following acid application was scored having a maximum at the 5th h. The response amounted to 122, 155, 153 and 135% of the control (pH 5.6) at the 3rd, 5th, 24th and 168th h, respectively. Preliminary supply of Spm attenuated the effect of AR during the whole period studied: the MDA content in variant Spm + AR was reduced to 74 (3rd h), 80 (5th and 24th h) and 79% (168th h) of its value in acid sprayed plants (AR). The reduction caused by Spd was 88% in the first two intervals but later it was less expressed. The level of MDA was not significantly influenced by polyamine application at pH 5.6 (except for Spm, 3rd and 168th h).

3.3. Peroxidase and catalase activities

Fig. 1 represents the changes of peroxidase and catalase activities. The results obtained showed that peroxidase was higher in acid-treated plants, particularly in the first hours after spraying: at the 3rd h peroxidase increased to 133% and at the 5th h it was more than 2-fold higher (253%) as compared with the control plants (pH 5.6). At the 24th, 72nd and 168th h peroxidase activity gradually decreased but remained higher than control (202, 187 and 140%, respectively, of the control pH 5.6).

In contrast to peroxidase, activity of catalase tended to decrease at the first hours after AR application: it was 71 and 53% of the control pH 5.6 at the 3rd and 5th h, respectively. At the end of experimental period catalase activity increased to 142 (72nd h) and 114% (168th h). Pretreatment with PAs had no significant effect on peroxidase and catalase activity at pH 5.6. Application of Spd and Spm led to a reduction of peroxidase activity in AR-treated plants amount-
Table 1
Effects of simulated acid rain (AR, pH 1.8) applied independently or in combination with 1 mM spermidine·3 HCl (Spd) and 1 mM spermine·4 HCl (Spm) on H₂O₂ content in bean leaves 3, 5, 24 and 168 h after acid treatment.

<table>
<thead>
<tr>
<th>Variants</th>
<th>Time after acid rain treatment</th>
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<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td></td>
<td>µmol g⁻¹ FW</td>
</tr>
<tr>
<td>Control</td>
<td>pH 5.6</td>
</tr>
<tr>
<td>pH 3.4</td>
<td>–</td>
</tr>
<tr>
<td>AR pH 1.8</td>
<td>5.06 ± 0.16</td>
</tr>
<tr>
<td>Spd+pH 5.6</td>
<td>2.95 ± 0.17</td>
</tr>
<tr>
<td>Spd+AR 5.6</td>
<td>4.14 ± 0.23</td>
</tr>
<tr>
<td>Spm+pH 5.6</td>
<td>2.75 ± 0.18</td>
</tr>
<tr>
<td>Spm+AR 5.6</td>
<td>3.49 ± 0.28</td>
</tr>
</tbody>
</table>

* Data are presented as µmol g⁻¹ FW, as % of the control (pH 5.6) and as % of the AR (pH 1.8) values.
* P<0.05;
** P<0.01;
*** P<0.001 (statistical differences with control, pH 5.6).
+ P<0.05;
++ P<0.01;
+++ P<0.001 (statistical differences with AR, pH 1.8).
### Table 2
Effects of simulated acid rain (pH 1.8) applied independently or in combination with 1 mM spermidine·3 HCl (Spd) and 1 mM spermine·4 HCl (Spm) on malonyldialdehyde (MDA) level in bean leaves 3, 5, 24 and 168 h after acid treatment

<table>
<thead>
<tr>
<th>Variants</th>
<th>Time after acid rain treatment</th>
<th>3 h</th>
<th>5 h</th>
<th>24 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmol g⁻¹ FW</td>
<td>% of control</td>
<td>μmol g⁻¹ FW</td>
<td>% of control</td>
</tr>
<tr>
<td>Control</td>
<td>pH 5.6</td>
<td>0.092 ± 0.006</td>
<td>100 –</td>
<td>0.078 ± 0.004</td>
<td>100 –</td>
</tr>
<tr>
<td>AR pH 1.8</td>
<td></td>
<td>0.112 ± 0.001</td>
<td>122**</td>
<td>0.121 ± 0.003</td>
<td>155***</td>
</tr>
<tr>
<td>Spd + pH</td>
<td>pH 5.6</td>
<td>0.080 ± 0.007</td>
<td>87 –</td>
<td>0.076 ± 0.007</td>
<td>97 –</td>
</tr>
<tr>
<td>Spd + AR</td>
<td></td>
<td>0.099 ± 0.002</td>
<td>88+++</td>
<td>0.106 ± 0.001</td>
<td>136***</td>
</tr>
<tr>
<td>Spm + pH</td>
<td>pH 5.6</td>
<td>0.071 ± 0.005</td>
<td>77**</td>
<td>0.068 ± 0.005</td>
<td>87 –</td>
</tr>
<tr>
<td>Spm + AR</td>
<td></td>
<td>0.083 ± 0.001</td>
<td>90 74+++</td>
<td>0.097 ± 0.001</td>
<td>124***</td>
</tr>
</tbody>
</table>

* Data are presented as μmol g⁻¹ FW, as % of the control (pH 5.6) and as % of the AR (pH 1.8) values.

* P < 0.05;
** P < 0.01;
*** P < 0.001 (statistical differences with control, pH 5.6).
+ P < 0.05;
++ P < 0.01;
+++ P < 0.001 (statistical differences with AR, pH 1.8).
ing to 92% (Spd + AR, 3rd h), 84 and 88% (Spm + AR, 3rd and 5th h), respectively, of the variant AR.

4. Discussion

The results obtained from the present study show that after single AR spraying an increased H₂O₂ content and MDA accumulation were observed, i.e. a state of oxidative stress is induced related to membrane damage. The effect is diminished after the 5th h that pointed to the development of recovery processes. As Foyer et al. [15] stated H₂O₂ is a strong oxidant that can initiate localized oxidative damage leading to disruption of metabolic function and losses of cellular integrity at sites where it accumulates. H₂O₂ and other active oxygen species, such as OH⁺, O₂⁻ and O₂⁻ can be expected to be responsible for the lipid peroxidation [16,17].

H₂O₂ can diffuse relatively long distances causing changes in the redox status of surrounding cells and tissues where at relatively low concentrations it initiates an antioxidative response [15]. Rather than just the scavenging capacity, a fine-tuning of H₂O₂ levels is expected to be determining for efficient stress control. The rationale of this assumption is that H₂O₂, whilst deleterious to some cellular components, is essential to plants in various biosynthetic reactions and, as suggested by some studies, possibly also in signal transduction pathways that could contribute to plant defense [18]. It may be supposed that the increased level of H₂O₂ observed by us in AR-treated plants (Table 1) may also have signal functions; the reduced catalase activity (Fig. 1) may be one of the factors of H₂O₂ accumulation. Chen et al. [19] and Du and Klessig [20] proposed that catalase may be inactivated by binding to salicylic acid or by other cellular components, such as semidehydroascorbate [21], reduced glutathione [22], superoxide and hydroxyl radicals [23] and H₂O₂ [24], but the relevance of these data towards physiological conditions is difficult to assess. In this study, during the later terms after AR treatment the H₂O₂ content was relatively reduced (Table 1), which could be explained by the increased catalase activity (Fig. 1). In this way the oxidative stress in plants may be overcome. H₂O₂ may also be involved in peroxidase-mediated reactions of oxidative polymerisation resulting in cell wall strengthening and formation of barrier anti-stress structures [25]. The increased activity of peroxidase in AR-treated plants (Fig. 1) suggests the protective role of the enzyme in this system.

The results are in accordance with other authors reporting similar patterns of peroxidase and catalase activities in different stress situations, such as Fe [26] and Al toxicity [27]. Catalase activity in spinach levels decreased by about 30% after 8 h treatment with 0.5 ppm O₃ [28]. Visible symptoms of injury and a significant activation of peroxidase during pH 2.2 treatment were found in two tobacco cultivars [29].

In these experiments pretreatment with Spd or Spm prevented the enhancement of H₂O₂ and MDA caused by AR. Multiple properties of PAs may underly this protective effect. As bases PAs can neutralize the acid supplied to plants, ‘buffer-
ing' cell pH. According to Tadolini [30] PAs act as antioxidants by inhibiting lipid peroxidation; this is accounted for by the ability of PAs to form a ternary complex with iron and the phospholipid polar heads that may change the susceptibility of Fe$^{2+}$ to auto-oxidation and thus its ability to generate free oxygen radicals. The results of Borrell et al. [7] suggest that inhibition of lipid peroxidation may be one of the mechanisms responsible for the anti-senescent effects of the PAs. Recently, an antioxidant effect of PAs was demonstrated in paraquat-treated plants [31]. Moreover, PAs are shown to stabilize membranes by associating (as organic polyations) with negatively charged phospholipids. It was also demonstrated that treatment with Spd or Spm prevented the loss of chlorophyll, stabilized the molecular composition of the thylakoid membranes and delayed senescence [7,32]. Ultrastructural observations showed that the integrity of the thylakoid membrane system is preserved with Spm [33]. Protection of photosynthetic functions under acid stress and heat shock by PAs was demonstrated in earlier papers [34,35]. It was suggested that PAs kept a significant part of thylakoid membranes native by binding with them. The interaction of PAs with thylakoid membrane surface led to their stacking and to the association of light harvesting complex 2 with the PS 2 core complex. The assumption of the anti-stress effect of PAs is also supported by the reduced defensive peroxidase response to AR in the presence of Spm (3rd and 5th h) reported in the present work.

The results presented in this paper clearly indicate that single AR treatment induced oxidative stress related to membrane damage but did not cause irreversible changes. Catalase and peroxidase are involved in overcoming of oxidative stress. Exogenous PAs (Spd and Spm) applied before AR are suggested to prevent bean plants in this stress situation. For the period (24 h) between PA and AR supply, i.e. before AR stress, PAs may ‘prepare’ the cell to meet and combat stress by stabilizing membranes and forming a potential of higher ‘buffering’ and antioxidant capacity. After the stress, all protective properties of PAs may be involved in plant responses; the ‘buffering’ ability would be probably of primary importance. The more pronounced protective effect of Spm in comparison with Spd could be accounted for by its longer chain and greater number of positive charges which allows more important neutralizing and membrane stabilizing ability.

Acknowledgements

This research was supported by the National Fund of ‘Scientific Investigations’ (Project MUBAV-7/1995) at the Ministry of Education, Science and Technology.

References


