Effect of Combined Stress on the O_2 Scavenging Enzyme Activities in Bean and Poplar Leaves

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Wounding, Drought, Elicitation, Catalase, Peroxidase The activity of O_2-scavenging enzymes in bean leaves in different positions and poplar leaves in different leaf storeys was investigated. The effects of wounding, elicitation and drought and the combination of these treatments at various levels were studied.

Distal bean leaves showed higher responsiveness than those opposite the treated leaves. Poplar leaves above the treated ones proved to be more active than those in lower storeys. A comparison of the interaction of different stress treatments revealed both synergistic and antagonistic effects on the oxidant-generating rate and anti-oxidant synthesis of damaged and non-damaged plant tissues.

Introduction

Plant response to mechanical injury promotes gene activity changes both at the wounded site and systemically in intact, non-damaged parts. Well-known examples are the proteinase inhibitor genes of *Solanum tuberosum* (Pena-Cortés *et al.*, 1998) and *Lycopersicon esculentum* (Farmer and Ryan, 1990). Wound-inducible genes are activated by separate signal transduction pathways, including jasmonic acid-dependent and -independent steps (Damman *et al.*, 1997).

Wounding is known to induce different defence reactions, including lipid peroxidation and changes in the activity of O_2-scavenging enzymes, generated as a result of the oxidative burst (Rosahl, 1996). Many other stress factors proved to be related to the production of reactive oxygen species and the subsequent rise in the catalase and non-specific peroxidase levels. The proteins of the Haliwell-Asada cycle, functioning in many different compartments of the cell, are also important components of the overall defence machinery.

When different stressors are applied – wounding, fungal elicitors, drought, etc. – plants exhibit different enzymatic responses. The aim of the experiments presented here was to obtain information on the interactions between various stress factors and the resulting changes in the O_2-scavenging enzyme activities.

The transmission of the stress signal through the vascular system is mediated by systemin, a small octadecameric polypeptide with a central role in plant response to herbivore attacks and other forms of wounding (Pearce *et al.*, 1991). Systemin pre-treatment not only shortened the delay before the onset of H_2O_2 production, but also promoted oxidant biosynthesis at many times the rate observed in non-pre-treated cells in a tomato cell suspension culture (Stennis *et al.*, 1998). This high level of oxidants persisted for an extended period. In transgenic tomato plants, where the mobile precursor of systemin (prosystemin) is over-expressed, not only is the proteinase inhibitor synthesis enhanced but polyphenol oxidases – typical wound-induced enzymes – are also induced (Constabel *et al.*, 1995).

In the experiments reported here the defence reactions of the stressed leaves were compared to those of the neighbouring or distant leaves of a herbaceous and a woody plant species to obtain information on the spatial spread of the stress signals.

Abbreviations: BAP, benzylaminopurine; IBA, indolebutyric acid; SD, standard deviation; MS, MS medium (Murashige and Skoog, 1962).

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Materials and Methods

Plant material

In the experiments two types of plant material were used: 1. two-week old bean plants, 2. four-month old poplar plantlets. Seeds of bean (*Phaseolus vulgaris* cultivar 'Cherokee') were sterilised in 3% H_2O_2 for 15 min., continuously rinsed for 1 hour under running tap water and then germinated on wet filter paper (0.5 mM $CaSO_4$) at 27 °C in the dark in a controlled growth chamber. After 2 days the seedlings were transferred to hydroponics (Hoagland nutrient solution) and were grown at 22 °C with a 16-h photoperiod and a light intensity of approx. 4500 mW/m^2. Two-week old seedlings grown in a greenhouse were used for the experiments. Poplar (*Populus glauca* var. *Kopeczkii*) was cloned from apical bud meristems and micropropagated on MS medium supplemented with BAP. To avoid changes in the genetic material, 5–10 mm shoot cuts were transferred to shooting medium, incubated for 4 weeks, then transferred to IBA-containing rooting medium. Well-rooted plantlets were transferred to hydroponics with Hoagland medium and conditioned for 2 weeks in the greenhouse.

Stress treatments

Elicitation. Botrytis cinerea Persoon, a non-specific phytopathogenic fungus, was grown in liquid culture. The culture was purified after sterilisation to gain cell wall material, which was further hydrolysed with TFA to oligosaccharides. This fragmented cell wall preparation was used at a rate of 0.75% (w/w) as elicitor. The elicitation periods necessary to achieve a maximal induction rate of peroxidase activity in bean, poplar and in cell suspension cultures of *Rubia tinctorum* were determined in preliminary experiments.

Drought. Drought stress was applied to the roots by removing the plants from the nutrient solution, after which residual solution was removed by patting the roots with filter paper. Afterwards the plants were allowed to dry out for 1 hour in the controlled growth chamber. Drought treatment resulted in 4–6% loss of water content.

Wounding. As many small cuts as possible, 1 mm apart, were made with a scalpel parallel to the veins on the right side of given leaf blades. Leaves of intact plants of the same size were used as a control. The positions of the leaves are shown on Fig. 1. A, B and C refer to wounded, intact distal and intact opposite bean leaves, respectively. X, Y and Z refer to wounded, intact upper-storey and intact lower-storey poplar leaves, respectively. Usually, both halves of the wounded leaves were used for protein extraction. When the two halves were extracted separately, A refers to the wounded half and A* to the other, intact half of the leaf blade.

Preparation of crude extract

When isolating peroxidase (PER – EC 1.11.1.7) and catalase (CAT – EC 1.11.1.6) 0.5 g of leaf was extracted with 300 µl buffer (0.5 M phosphate buffer, 0.2 M EDTA with 50 µl/1000 ml Triton X-100 and 2 g/1000 ml PVP-25). Ascorbate peroxidase (APX – EC 1.11.1.11) was isolated with 300 µl buffer (0.5 M phosphate buffer pH 7.0, 2 mM EDTA, 1 mM ascorbic acid). The homogenates were centrifuged at 10,000×*g* for 15 min. The supernatant was stored in liquid nitrogen until used

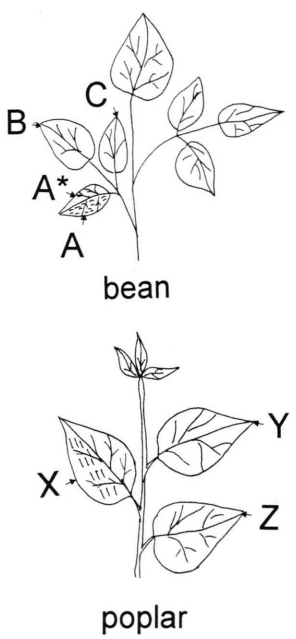

Fig. 1. Position of the leaves treated in the experiments. Bean: A-treated leaf; B-intact distal leaf; C-intact opposite leaf. Poplar: X-treated leaf; Y-intact upper storey leaf ; Z: intact lower storey leaf. A* refers to the intact half of the wounded leaf blade.

for electrophoresis. Fresh extracts were always used for spectrophotometric assays.

Native PAGE and activity staining

PER isoenzymes were separated with a Pharmacia™ Phast System apparatus on Phast gels (4–15% AA), using electrode buffer strips (LiOH 26 mM, pH 8.0). All electrophoretic separations were performed at 10 °C. After migration the gel was soaked in the staining solution (50 mg o-dianisidine dissolved in 5 ml N,N-dimethylformamide, 100 ml sodium acetate buffer (pH 4.4), 100 µl 2 M $CaCl_2$ solution and 200 µl 30% H_2O_2) for 10 minutes. Areas of PER activity showed up as light brown bands. After the bands developed the gels were scanned, then stored in sodium acetate buffer (pH 4.4). Only the active area of the scanned gel images is shown here.

Enzyme assays

The activity of CAT was determined by measuring the rate of decomposition of H_2O_2 at 240 nm during the 200-sec incubation time. Each 893 µl reaction mixture contained 67.2 mM sodium phosphate (pH 7.0), 13.4 mM H_2O_2 and 25 µl of crude extract.

APX activity was measured in a reaction mixture containing 100 mM sodium phosphate (pH 7.0), 100 mM H_2O_2, 10 mM ascorbic acid and 20 µl enzyme extract by monitoring the decrease in absorbance at 290 nm as the ascorbate was oxidised.

Each reaction mixture contained 5 mg protein in the above volumes. The protein concentration was determined by the method of Bradford, and the crude extracts were diluted with extraction buffer when necessary. All the data reported correspond to the linear phase of the reaction. The mean values and SD are taken from triplicate measurements from two separate experiments.

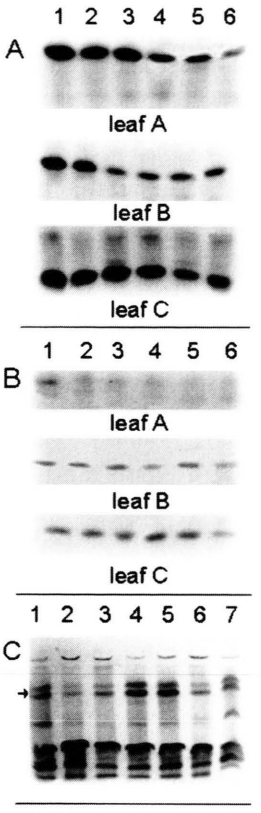

Fig. 2. (A) Peroxidase pattern from drought-stressed bean plants. Plants were drought-stressed for 60 minutes, then put back in nutrient solution for regeneration. Samples were extracted directly, 5, 15, 30, 40, and 60 minutes after the above treatment, and loaded on the gel in this order. Activity stained PER gels of leaf A, B, C protein extracts are figured from top to bottom respectively. (B) Peroxidase pattern of elicited and wounded bean leaves. Samples from left to right are: 3, 4, 5, 6, 7 and 8 hours of elicitation. The last three samples were wounded too for 1, 2 and 3 hours. Activity stained PER gels of leaf A, B, C protein extracts are figured from top to bottom respectively. (C) Electrophoretic pattern of wound treated poplar peroxidase isoenzymes. From left to right: control; leaf X 6, 12, 16 hours after treatment; intact leaf over the treated one (Y); intact leaf below the treated one (Z); control. Per isoformes affected by wounding are marked by arrows.

Results and Discussion

The PER activity in drought-stressed A leaves of bean was uniformly high in the first 15 minutes of the regeneration period (Fig. 2 A), after which it decreased. This effect – a decrease in PER activity as a function of the duration of the regeneration period – was faster, beginning after 15 min of regeneration and more pronounced in B leaves. The C leaves were not affected by the regeneration and rehydration process; the high peroxidase level induced by the 60-min drought stress period remained nearly constant throughout the regeneration. Using a similar drought stress and regeneration program, Mittler and Zilinskas (1994) also in-

duced a decrease in pea CAT activity following an approximately two-fold increase.

Wounding following a 5-h elicitation period resulted in a decrease in the peroxidase level (Fig. 2 B). This effect hardly reached the B leaves. The PER level also remained high in C leaves, decreasing only after 8 h of treatment. The peroxidase activity of wounded poplar leaves in different leaf storeys exhibited a considerable increase. In these experiments, a relatively long period (6–16 h) elapsed after wounding, so it is difficult to compare these patterns to that of the wounded bean leaves. In preliminary experiments, the maximum level of wound-induced PER activity was reached at 14–18 h after wounding. The electrophoretic pattern of poplar peroxidases showed a complex picture for the PER isoforms induced by wounding (see arrows on Fig. 2 C), while other isoforms were present at relatively constant levels. Leaves above the wound-treated one showed a many times higher induction rate in comparison to the PER activity of leaves beneath the treated one. Vander *et al.* (1998) have shown a very fast response of phenylalanine ammonia-lyase and a slower continuous induction of PER activity in chitosan elicitor-treated wheat during 24 h of elicitation.

CAT activity increased as a result of elicitor treatment and wounding, to the greatest extent in leaves A and C, while a decrease was shown in A* (Fig. 3 B). Drought stress also resulted in a decrease in CAT activity (Fig. 3 C). During a 30-minute period following the 1-h drought treatment, wounding seemed to suppress the effect of drought, again demonstrating the interaction of stressors (Fig. 3 D).

In wheat leaves R. Baisak *et al.* (1994) demonstrated a continuous rise in CAT activity during a 48-h drought stress period and a slow decrease during the following 24 h. R. Mittler *et al.* (1994) measured a two fold rise in CAT activity as a result of a 3-day drought treatment. In the latter experiment a 24-h regeneration period resulted in a CAT level similar to the control. During the short drought stress period used here the CAT induction was not unambiguous, while in 50–70 min after wounding a considerable increase in CAT activity could be observed in all the leaves.

DHAR levels also decreased when 1 h of drought stress was applied, followed by a rise at the end of a 1-h regeneration period. Wounding resulted in a similar picture, with a higher DHAR level in leaf B (results not shown). Elicitation itself and the combined treatments resulted in complex

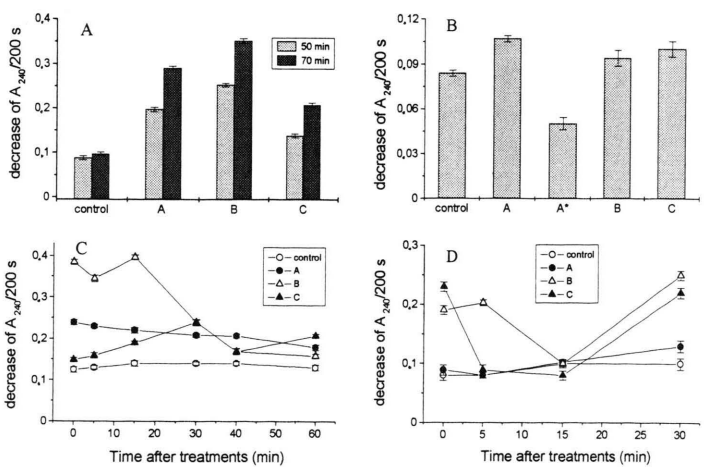

Fig. 3. (A) Effect of wounding on the CAT activity in bean leaves at different position. Samples were extracted and measured 50 and 70 minutes after wounding. (B) Combined effect of elicitation and wounding on the CAT activity in bean leaves at different position. Wound treatment was performed at the end of the 5th hour of elicitation. (C) Effect of drought on the CAT activity in bean leaves at different position. After 1 hour of drought treatment of roots, plants were put back to nutrient solution. CAT activity was measured during this regeneration period. (D) Combined effect of drought and wounding on the CAT activity in bean leaves at different position. Wounding was performed after 1 hour of drought stress period. Samples were extracted and measured directly after wounding, and 5, 15, 30 minutes after wounding.

profiles, which may be an indication of a side-reaction involving thioredoxins and trypsin inhibitors, as suggested by Morell *et al.*, (1997).

60 min of drought treatment resulted in a relatively high APX activity, decreasing to the control level at the end of the 1-h regeneration period (Fig. 4 B) in all three leaf types used. When wounding was applied after drought treatment just prior to the regeneration period, a further increase was detected (Fig. 5 C). The APX activity again reached the control level in C leaves after 30 min. Whereas wounding alone barely affected the APX level (Fig. 4 A), the combination of drought and

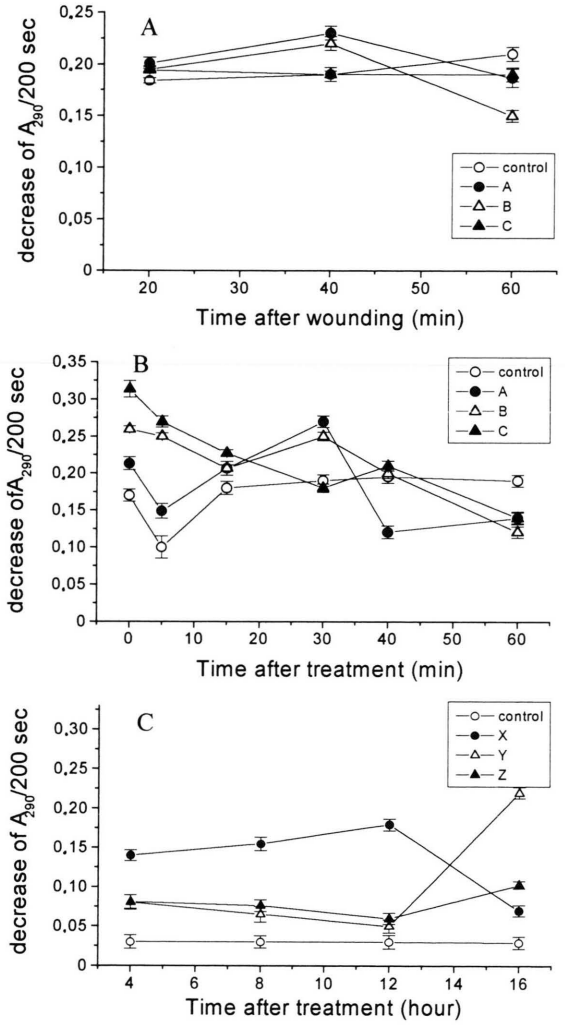

Fig. 4. (A) Effect of wounding on the APX activity in bean leaves at different position (B) Effect of drought on the APX activity in bean leaves at different position. After 1 hour of drought treatment of roots, plants were put back to nutrient solution. APX activity was measured during this regeneration period. (C) Effect of wounding on the APX activity in different poplar leaf storeys.

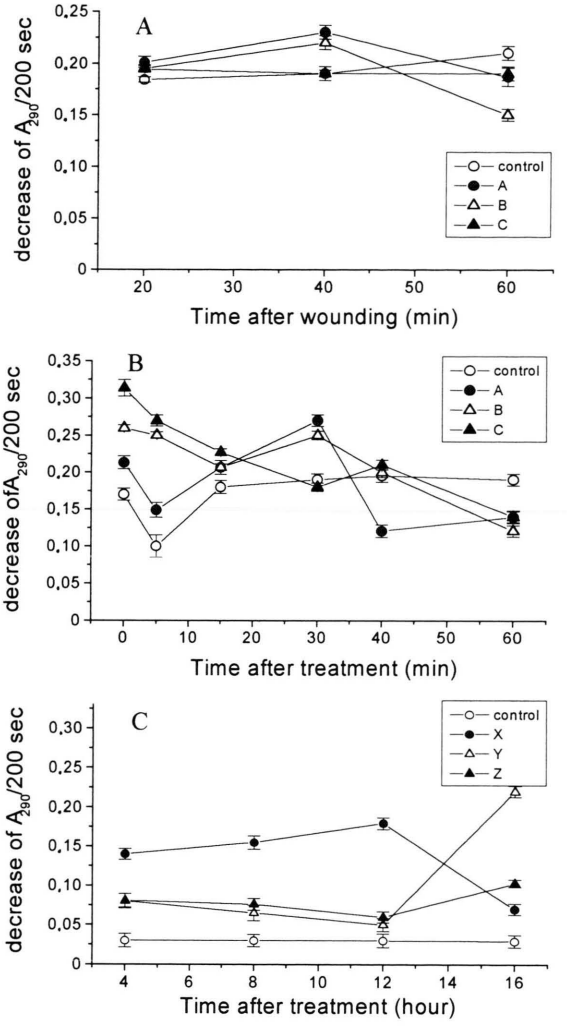

Fig. 5. (A) Combined effect of elicitation and wounding on the APX activity in bean leaves at different position. Wound treatment was performed at the end of the 5th hour of elicitation. Samples were extracted and measured every hour. (B) Combined effect of elicitation and wounding on the APX activity in bean leaves at different position. Wound treatment was performed at the end of the 5th hour of elicitation. Samples were extracted and measured every hour. (C) Combined effect of drought and wounding on the APX activity in bean leaves at different position. Wounding was performed after 1 hour of drought stress period and following 1 hour of regeneration. Samples were extracted and measured directly, 5, 15 and 30 minutes after wounding.

wounding resulted in a different picture. In the experiments of Mittler and Zilinskas (1994) mentioned above, the activity of pea cytosolic APX increased continuously during the 3-day drought stress and a 1-day regeneration period. On the other hand, the relative transcript rate of APX decreased during regeneration.

The elicitation and wounding of bean leaves induced an almost constant rise in the APX level and, in this respect, leaves A and B were the most active (Fig. 5 A, 1–5 h). In the case of this combined stress treatment the two stressors seems to act synergistically (Figs. 5 A and 5 B). The APX level in the wounded half of the treated leaf (A) remained below the activity of the intact half (A*).

The wounding of poplar leaves caused relatively small changes in APX activity (Fig. 4 C), similarly to that of the bean enzyme. It may be assumed that the stress signal induced by a local wound did not affect the neighbouring leaves during the 16 h elapsing after wounding, as the elevated levels of CAT and PER were able to balance the resulting oxidative burst. The time course of wound induction seems to be markedly different to that of elicitation. The transcripts of an anionic peroxidase (TAP1) promoter-GUS construct appeared 36–48 h after wounding in transgenic tobacco plants (Royce *et al.* 1993), while Fusarium infection elicited the transcription within 24 h.

When comparing the enzyme activities of bean leaves in different positions the B leaf (the intact distal one) generally showed higher responsiveness to stress treatments. In poplar the leaf above the stress-treated one was the most affected by wounding. The same is true in the case of elicitor treatment (results not shown). Howe *et al.* (1996) have shown that the accumulation of Proteinase Inhibitor I mRNA increases gradually along the leaf blade axis of apically wounded tomato leaves. This suggests that the signal is transmitted extravascularly (similar to the auxin transport) or by the xylem of the leaf veins. The present results suggest a different way of transmission: the wound signal seems to be transmitted most rapidly towards the distal leaves of bean, and towards the higher leaf storey of poplar. When comparing the enzyme activities of bean leaves in different positions the B leaf (the distal one) generally showed higher responsiveness to stress treatments. The leaf above the stress-treated one was the most affected by wounding. In the current experiments the spatial distribution of the wound-induced signal was studied within a leaf blade wounded at different and well-characterised sites.

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