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Some biochemical reactions of strawberry plants to infection with *Botrytis cinerea* and salicylic acid treatment

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Abstract

The reactions of strawberry plants to infection with *B. cinerea* and treatment with salicylic acid has been studied. Infection of leaves with *B. cinerea* resulted in early increases in active oxygen species generation, superoxide dismutase and peroxidase activities and phenolic compounds content. Some increases of the above reactions were noticed in plants treated with salicylic acid but not in the plants treated with SA and then later infected with *B. cinerea*.

INTRODUCTION

Plants react to pathogen attack activating a variety of defence reactions. It is suggested that production of active oxygen species (AOS) and increase in some enzymes activities: superoxide dismutases (SOD), peroxidase (PO) and others, may be the early reaction to pathogen.

The predominant active oxygen species detected in plant-pathogen interactions are superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$). AOS may directly reduce pathogen viability, on the other hand the toxicity of AOS or AOS-derived compounds may contribute to host cell death during hypersensitivity reactions (M e h d y, 1994; S c a n d a l i o s, 1993). There is a possibility that AOS also serve as signal intermediates that induce phytoalexin biosynthesis (L a m b et al., 1989; M e h d y, 1994).

To protect plant cells against oxidative injury by AOS numerous defence mechanisms, both enzymatic and nonenzymatic, exist. The enzymatic antioxidant defences include enzymes capable of removing, neutralizing or scavenging active oxygen

species. SODs are among the most efficient antioxidant enzymes because of their capacity to scavenge $\cdot O_2^-$ (A l m a n s a et al., 1994; S e v i l l a et al., 1982). They play a significant role in protecting host cells in the vicinity of necrotized places in the hypersensitivity reaction against toxicity of active $\cdot O_2^-$.

Peroxidases might have a double role in both structural and physiological protection of the host. They take part in lignification of host cell walls. On the other hand peroxidases can take part in H_2O_2 production (C a n d e l a et al., 1994; P e n g et al., 1992; V a n c e et al., 1980).

The resistance reactions can be induced in plants by treatment with elicitors. According to current hypothesis, salicylic acid (SA) is assumed to be a systemic signal molecule that induces resistance reactions in plants (C h e n et al., 1993; V e r n o o i j et al., 1994). According to some authors exogenous application of SA can make a plant more resistant to a subsequent fungal infection. (W a l t e r s et al., 1993).

Our aim was to examine $\cdot O_2^-$ content, SOD, PO activities and phenolic compounds content in strawberry plants treated with salicylic acid and infected with *Botrytis cinerea*.

MATERIALS AND METHODS

Host: Strawberry plants cv. 'Dallas' were propagated "*in vitro*" on MS medium supplemented with BAP (2 mg/l), IBA (1 mg/l) and GA_3 (0.1 mg/l). Plants were rooted on 1/2 MS with IBA (0.5 mg/l), then plants were grown in soil in growth chamber, 16 h photoperiod at 25°C.

At the age of one month plants were treated with salicylic acid. Part of elicited plants was taken to examination 1, 3 and 24 hours after treatment. One week after SA treatment the second part of plants was challenged with *Botrytis cinerea* and examined 3, 24 and 48 h after challenge. Control plants were examined simultaneously with elicited and infected plants.

Pathogen: *Botrytis cinerea* in stock culture was maintained on potato dextrose agar in the dark at 24°C. The conidia suspension was obtained by washing potato dextrose agar slant cultures with sterylized distilled water. Conidia in suspension were counted in a hemocytometer. Conidia suspension containing 1 x 10^6 conidia in 1 ml was used to challenge the strawberry plants.

Elicitor: 5 mM water solution of salicylic acid was used as the elicitor.

Assay for generation of superoxide anion: The detection of $\cdot O_2^-$ was based on its ability to reduce nitroblue tetrazolium (NTB) according to D o k e (1983).

Five fresh leaf disc (1 cm Ø) were immersed in 3 ml 0.01 M potassium phosphate buffer pH 7.8 containing 0.05 % NTB and 10 mM NaN_3 for 1 hour. After that the mixture was heated at 85°C for 15 min and cooled. The reducing activity of NTB of the disc was expressed as increased absorbance at 580 nm per hour per disc.

Preparation of enzymes extract: Leaf tissue, 0.5 g was homogenized in 5 ml 50 mM phosphate buffer, pH 7.0 containing 1 % insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 15 000 g for 10 min and the supernatant obtained was used as enzyme extract to assay SOD and peroxidase activities.

Assay of superoxide dismutase activity: The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NTB) using method of Beauchamp and Fridovich (1971). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 μM NTB, 2 μM riboflavin, 0.1 mM EDTA and 20 μl enzyme extract. Riboflavin was added last and the reaction was initiated by placing the tubes under two 15 W fluorescent lamps (30 cm below light bank). The reaction was started by switching on the light and was allowed to run for 10 min. The reaction was stopped by switching off the light and the tubes were covered with black cloth. Noniluminated tubes served as control. The absorbance at 560 nm was read. The volume of enzyme extract corres-ponding to 50 % inhibition of the reaction was considered as one enzyme unit.

Assay of peroxidase activity: The peroxidase activity was assayed colorymetrically with guaiacol as substrate. To assay perioxidase activity 0.5 ml of diluted enzyme extract, 0.5 ml of 0.05 M acetate buffer pH 5.6, 0.5 ml 0.06 M H_2O_2 and 0.5 ml 0.02 M guaiacol were used. The linear increases in absorbance at 480 nm was monitored for 4 min at 30°C. The increase in absorbance equal to 1.0 in 1-min incubation was assumed as one activity unit.

Assay of phenolic compounds content: Leaf tissue, 1 g was homogenized in hot, 80 % ethanol (K n y p l and C h y l i ń s k a, 1974) then cooled and centrifuged at 15 000 g for 15 min. Supernatant was used as an extract to assay phenolic compounds. The total phenolic compounds content was assayed according to S w a i n and H i l l i s (1959).

RESULTS AND DISCUSSION

In strawberry plants treated with 5 mM salicylic acid we observed the increase in superoxide anions content (Fig. 1). They almost doubled as early as 1 h after treatment, the highest level of $\cdot O_2^-$ was observed 3 h after treatment and then the amount of $\cdot O_2^-$ decreased. The infection of strawberry plants with *B. cinerea* also caused the changes in superoxide anions content. The level of $\cdot O_2^-$ in plants 3 h after infection was over two times higher that in control (Fig. 2) then the level of $\cdot O_2^-$ in infected plants was decreasing during infection development and 46 hours after infection was similar to that at the control. In strawberry plants which were pretreated with salicylic acid the similar changes in $\cdot O_2^-$ content were observed. Researches conducted by some authors have previously demonstrated that one of the rapid processes in plant – pathogen or elicitor reaction is the release of active oxygen species (AOS). D o k e (1983) observed that inoculation of potato tuber tissue with *Phytophthora infestans* results in generation of $\cdot O_2^-$ which is involved in host cell hypersensivity. Elicitors of heypersensivity such as hyphal wall components also

had this effect. Similarly active oxygen species generation was associated with the hypersensitive reaction in interaction between tomato and *Cladosporium fulvum.*

Soybean cell suspension treated with nonspecific elicitors produced AOS within 1 to 2 min (A p o s t o l et al., 1989). In contrary M o r e a n and O s m a n (1989) did not observe any significant $HO_2^-/\cdot O_2^-$ generation by either potato tuber or leaf discs challenged with germination fluids from *P. infestnats.* As our results show generation of $\cdot O_2^-$ is an early reaction of strawberry plants to infection. The reaction to pure elicitor is even more rapid (1 h after treatment) then the reaction to contact with pathogen.

Paralelly with $\cdot O_2^-$ content we examined changes is SOD activity in strawberry plants treated with elicitor and infected with *B. cinerea.* In strawberry plants treated with salicylic acid the superoxide dismutase activity was similar to control plants (Fig. 1). Following the infection of strawberry plants with B. cinerea the SOD activity increased significantly. It was about 30 % higher than in control 3 h after infection then gradually increased and reached the level 80 % higher then control 48 h after infection (Fig. 3). The activity of this enzyme in strawberry plants pretreated with SA and then infected increased less visibly; reached the highest level 24 h after infection (about 30 % above control) and then the SOD activity decreased in these plants. Significant increase in SOD during the hypersensitive response of bean (*Phaseolus vulgaris*) to bean rust (*Uromyces phaseoli*) was observed by B u a n a u r i o et al. (1987). In tobacco leaves infected with tobacco mosaic virus, hypersensitively-reacting tissues showed higher levels of SOD than susceptible ones (M o r e a u et al., 1989). Hoever Z a c h e o and B l e v e Z a c h e o (1988) investigating the resistance of tomato varieties towards infection with nemotode *Meloidogyne incognita*, have reported a fall in SOD activity in hypersensitively-responsing tomato roots. Our results showing the reversed correlation between the $\cdot O_2^-$ content and SOD activity changes, especially in strawberry plants infected with *B. cinerea*, suggest that SOD probably counteract $\cdot O_2^-$ increase.

In strawberry plants treated with SA we observed some small increase in peroxidase activity and very significant increase in phenolic compounds content (Fig. 4), especially 24 and 48 hours after treatment. The peroxidase activity after infection of plants with pathogen increased visibly (Fig. 5). This enzyme activity was about 60 % higher in infected plants then in control 3 h after infection. The peroxidase activity was increasing during the whole studied period and was about 80 % higher than in control 48 h after infection. Paralelly with PO activity changes we observed the high increase in phenolic compounds content; they doubled 24 h after infection and 48 h after infection we observed that infected plants had three times more phenolic compounds then control ones (Fig. 6). In plants pretreated with SA and then infected with *B. cinerea* peroxidase activity and phenolic compounds content were on the level of control plants during the whole studied period.

Numerous investigations have shown that infection of plants or elicitor treatment may induce significant changes in peroxidase activity and phenolic compounds content (C a n d e l a et al., 1994; A m p o m a h and F r i e n d, 1988; B a s h o n

et al., 1987). The function of perioxidase in plant resistance reactions may be very different and has not been fully elucidated so far. Oxidation of phenol compounds and lignification are suggested to be one of the defence actions of PO. The early increase of PO activity after infection of strawberry leaves supports the hypothesis that the role of PO in desease resistance may be connected with active oxygen species generation.

It has been suggested that salicylic acid (SA) is the signal compound that induce systemic aquired resistance (H o r v a t h and C h u a, 1994; C h e n et al., 1993) because its exogenous application has been shown to induce resistance to a variety of bacterial, fungal and viral pathogens (W a l t e r s, 1993; R o s s, 1961; Y a l p a n i et al., 1991). When we applied SA as the elicitor of resistance reactions in strawberry plants we observed some increases in $\cdot O_2^-$ and phenolic compounds content, in SOD and PO activities (Fig. 1, 4). It is intriguing that when we compared the above reactions of nontreated and SA treated plants to subsequent infection we noticed that above reactions in pretreated plants were less visible or not visible at all. It may mean that SA elicit defence reaction as a nonspecific stress agent not as a signal and plants which previously responsed to its action become less sensitive to another stress e.g. infection. Induction of the defence reactions only at high concentration of SA confirms the hypothesis that it was caused by its stress action.

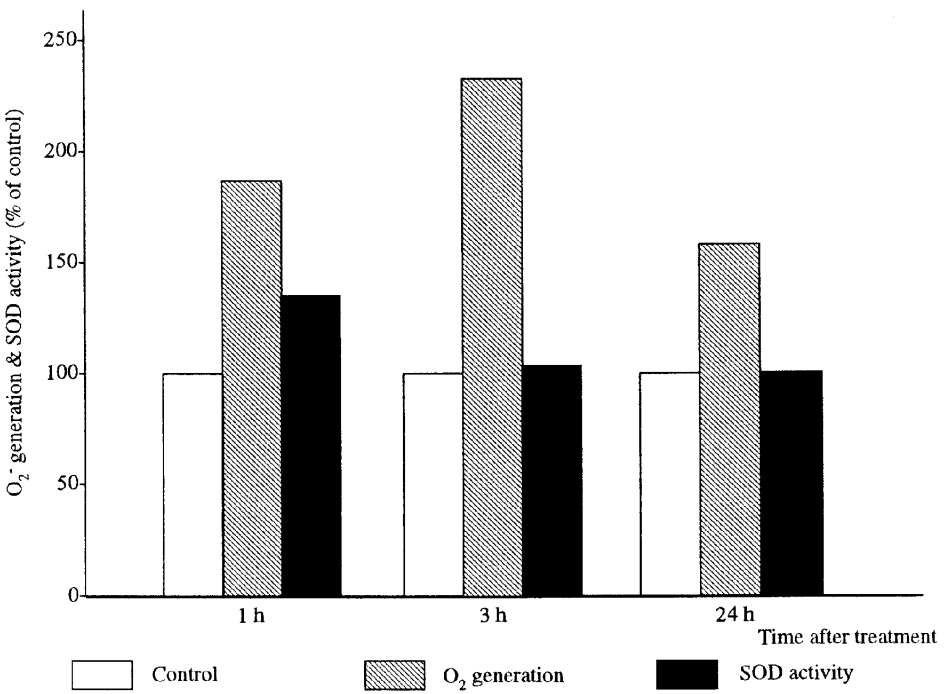

Fig. 1. Time course of $\cdot O_2^-$ generation and SOD activity changes in strawberry leaves after treatment with salicylic acid

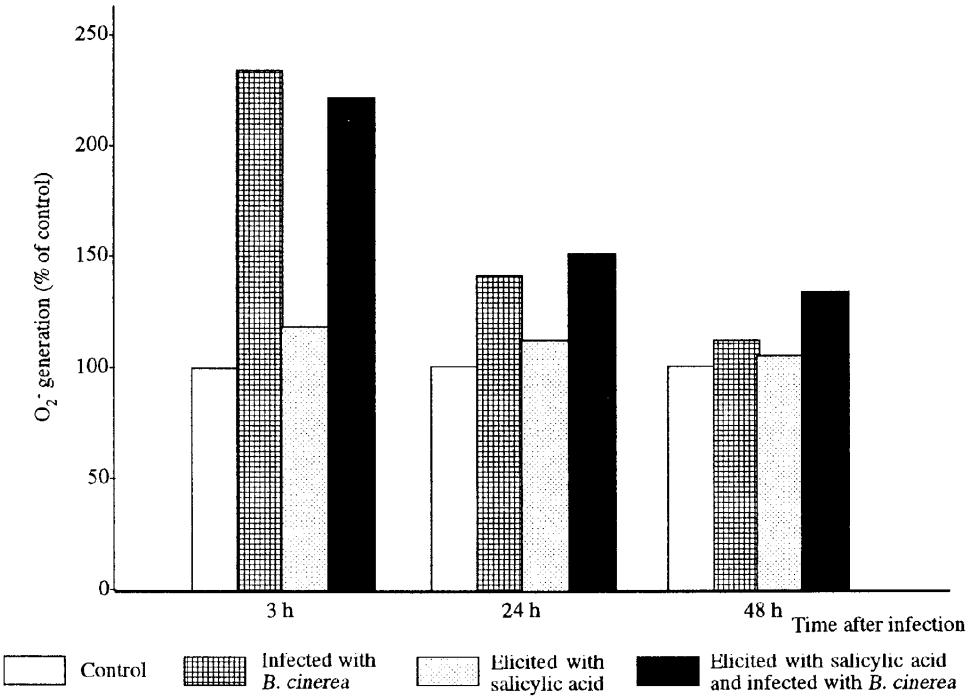

Fig. 2. Time course of $\cdot O_2^-$ generation in strawberry leaves after infection with *B. cinerea*

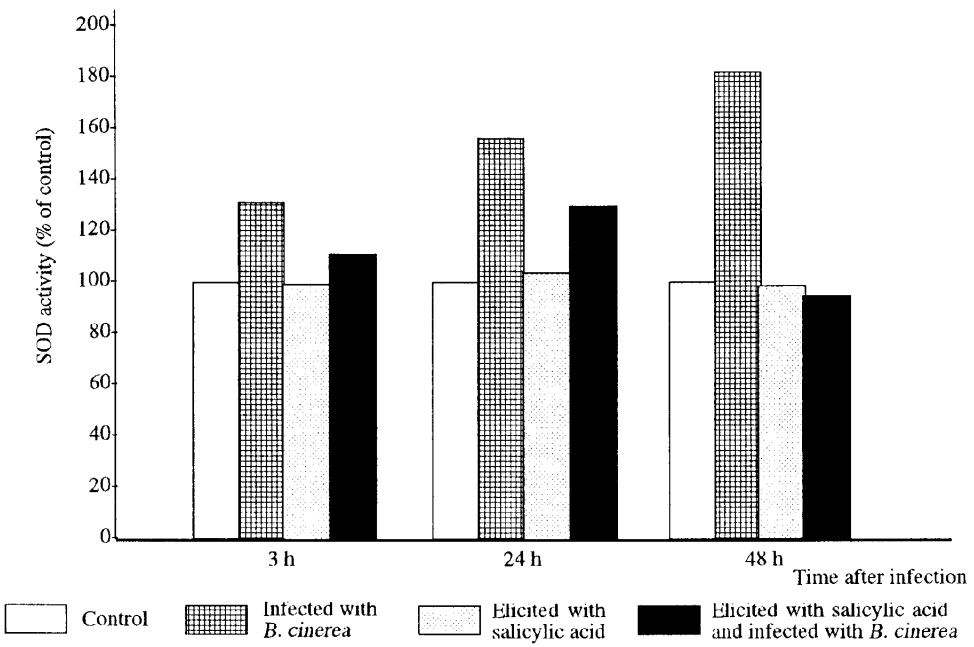

Fig. 3. Time course of SOD activity changes in strawberry leaves after infection with *B. cirenea*

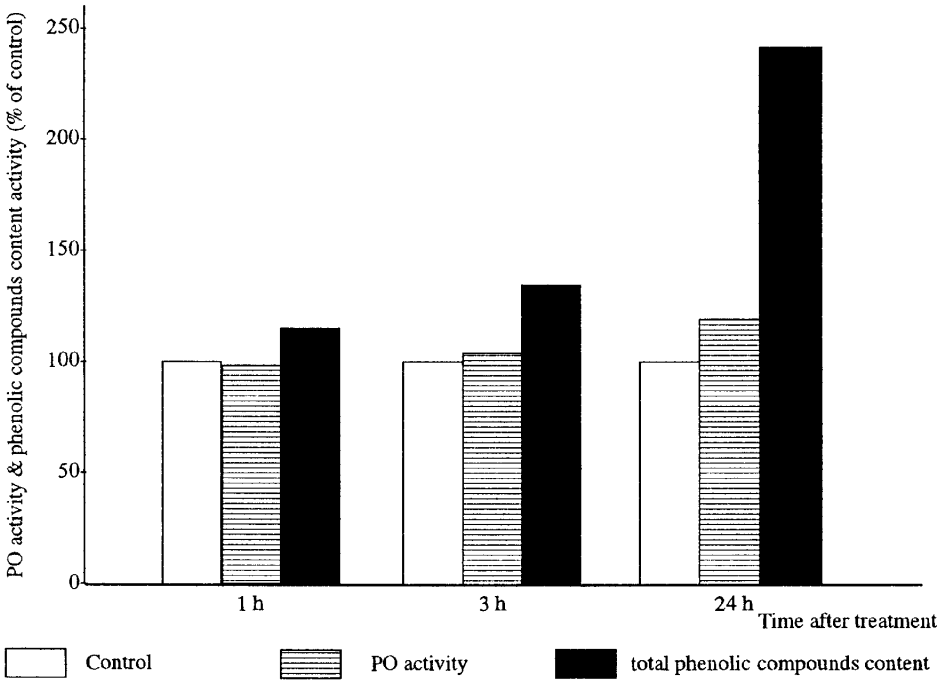

Fig. 4. Time course of peroxidase activity and phenolic compounds content changes in strawberry leaves after treatment with salicylic acid

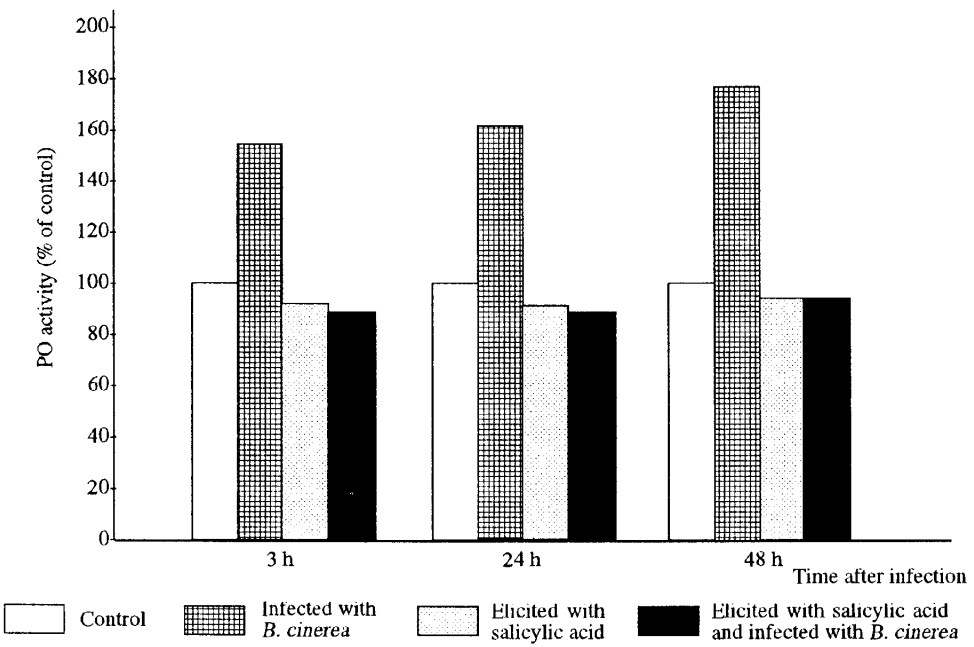

Fig. 5. Time course of PO activity changes in strawberry leaves after infection with *B. cirenea*

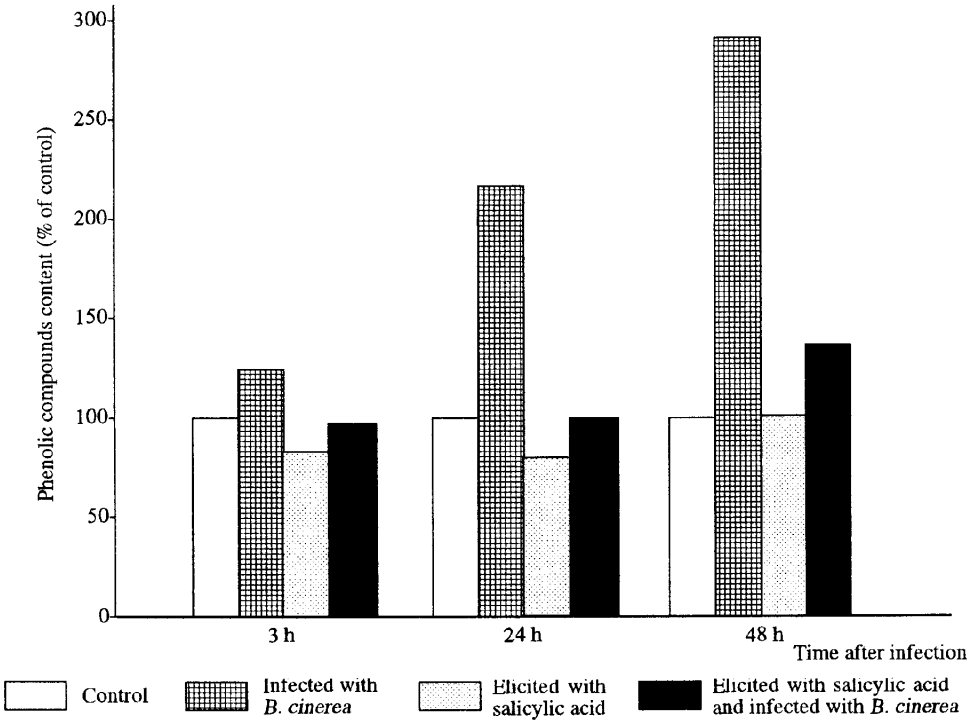

Fig. 6. Tim course of total phenolic compounds content changes in strawberry leaves after infection with *B. cirenea*

REFERENCES

Almansa M. S., del Rio L. A., Sevilla F., 1994. Characterization of an iron-containing superoxide dismutase from a higher plant, *Citrus limonium*. Physiol. Plantarum 90: 339-347.

Ampmah Y. A., Friend J., 1988. Insoluble phenolic compounds and resistance of potato tuber disc to *Phytophthora* and *Phoma*. Phytochem. 27: 2533-2541.

Apostol I., Heinstein P. F., Low P. S., 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Role in defense and signal transduction. Plant Physiol. 90: 109-116.

Bashan Y., Okon Y., Henish Y., 1987. Peroxidase, polyphenoloxidase, and phenols in relation to resistance against *Pseudomonas syringe* p.v. *tomato* in tomato plants. Can. J. Bot. 65; 366-372.

Beauchamp C., Fridovich I., 1971. Superoxide dismutase. Improved assays and assay applicable to acrylamide gels. Analyt. Biochem. 44: 276-287.

Buonaurio R., Torre G. D., Montalbini P., 1987. Soluble superoxide dismutase (SOD) in susceptible and resistant host-parasite complexes of *Phaseolus vulgaris* and *Uromyces phaseoli*. Physiol. Mol. Plant Pathol. 31: 173-184.

Candela M. E., Munoz R., Alcazar M. D., Espin A., 1994. Isoperoxidase involvment in the resistance of *Capsicum annum* to infection by cucumber mosaic virus. J. Plant Physiol. 143: 213-217.

Chen Z., Silva H., Klessig D. F., 1993. Active oxygen species in the induction of plant systemic aquired resistance by salicylic acid. Science 262: 1883-1886.

Doke N., 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Plant Pathol. 23: 345-357.

Horvath D. M., Chua N. H., 1994. The role of salicylic acid in systemic acquired resistance. Current Opinion in Biotechnol. 5: 131-136.
Knypl J. S., Chylińska K. M., 1974. Removal of IAA-oxidase inhibitors from carrot root extracts by griding in soluble polyvinypyrrolidone. Biochem. Physiol. Pflanzen 166: 333-343.
Lamb C. J., Lawton M. A., Dron M., Dixon R. A., 1989. Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell 56: 215-224.
Mehdy M. C., 1994. Active oxygen species in plant defence against pathogens. Plant Physiol. 105: 467-472.
Moreau R. A., Osman S. F., 1989. The properties of reducing agents released by treatment of *Solanum tuberosum* with elicitors from *Phytophthora infestus*. Physiol. Mol. Plant Pathol. 35: 1-10.
Peng M., Kuc J., 1992. Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf disc. Phytopathol. 82: 696-699.
Ross A. F., 1961. Systemic acquired resistance induced by localized virus infection in plants. Virology 14: 340-358.
Scandalios J. G., 1993. Oxygen stress and superoxide dismutases. Plant Physiol. 101: 7-12.
Sevilla F., Lopez-Gorge J., del Rio L. A., 1982. Characterization of manganase superoxide dismutase from the higher plant *Pisum sativum*. Plant Physiol. 70: 1321-1326.
Swain I., Hillis W. E., 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10: 63-68.
Vance C. P., Anderson J. O., Sherwood R. T., 1976. Soluble and cell wall peroxidases in reed canarygrass in relation to disease resistance and localized lignin formation. Plant Physiol. 57: 920-922.
Vernooij B., Uknes S., Ward E., Ryals J., 1994. Salicylic acid as a signal molecule in plant-pathogen interactions. Current Opinion in Cell Biol. 6: 275-279.
Vera-Estrella R., Blumwald E., Higgins V.J., 1992. Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. Plant Physiol. 99: 1208-1215.
Walters D. R., Mitchell A. F., Hampson J., McPherson A., 1993. The induction of systemic resistance in barely to powdery mildew infection using salicylates and various phenolic acids. Ann. appl. Biol. 122: 451-456.
Yalpani N., Silvermann P., Wilson T. M. A., Kleier D. A., Raskin I., 1991. Salicylic acis is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. The Plant Cell 3: 809-818.
Zacheo G., Bleve Zacheo T., 1988. Involvement of superoxide dismutases and superoxide radicals in the susceptibility and resistance of tomato plants to *Meloidogyne incognita* attack. Physiol. Mol. Plant Pathol. 32: 313-322.

Reakcje biochemiczne truskawek na infekcję *Botrytis cinerea* i traktowanie kwasem salicylowym

Streszczenie

W pracy badano reakcje biochemiczne roślin truskawek na infekcję *Botrytis cinerea* i traktowanie kwasem salicylowym.

Stwierdzono, że infekcja liści truskawek powodowała wzrost zawartości wolnych rodników tlenowych, aktywności dysmutazy ponadtlenkowej, peroksydazy, fenoli całkowitych. Wyraźny wzrost zawartości wolnych rodników tlenowych i związków fenolowych, a także pewien wzrost aktywności dysmutazy i peroksydazy obserwowano w roślinach traktowanych kwasem salicylowym. Po zakażeniu grzybem roślin wcześniej traktowanych kwasem salicylowym powyższe reakcje biochemiczne były mniej wyraźne bądź w ogóle nie ujawniały się.

Otrzymane wyniki potwierdzają hipotezę, że wytwarzanie wolnych rodników tlenowych, wzrost aktywności dysmutazy i peroksydazy są wczesnymi reakcjami roślin na infekcję i elicytację.

Kwas salicylowy wydaje się być niespecyficznym czynnikiem stresowym wywołującym w roślinach reakcje podobne do tych, stymulowanych przez infekcję.