Current Topics in Biophysics 2000, 24(2), 153–157

CHANGES OF PEROXIDASE ACTIVITY AND ASCORBATE CONTENT IN APOPLAST OF TOMATO LEAVES AFTER *BOTRYTIS CINEREA* INFECTION

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Changes in NADH oxidase/peroxidase, superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidase (PO) activities with guaiacol, ferulic acid, syringaldazine as well as ascorbate content in apoplast were studied after infection of tomato leaves with *Botrytis cinerea*. NADH oxidase/peroxidase activity increased more than twofold while SOD activity grew only slightly after infection. PO activities with all three studied phenol substrates were increasing starting from 24 hour after treatment with the fungus. However, the anionic apoplastic PO measured with ferulic acid and syringaldazine increased more markedly. The oxidized ascorbate content decreased but reduced ascorbate content did not change significantly.

INTRODUCTION

Lignification and cross-linking in the cellular wall are two of the reactions catalyzed by peroxidases (PO) that make pathogen fungus spread in tissues difficult. Those enzymes oxidize the monomeric lignin precursors to generate phenoxyl radicals (Pedreno, Ferrer, Gaspar, Munoz & Barcelo, 1995) and form dehydrodiferulic bridges between wall polysaccharides (Sanchez, Queijeiro, Revilla & Zarra, 1997). It is suggested that they are localized not only in cytosol but in apoplast as well. There is some evidence that apoplastic H_2O_2 necessary for PO action results from the dismutation of $O_2^{\cdot}$ generated by SOD or from the action of NAD(P)H oxidase/peroxidase or from both these processes (Bestwick, Brown & Mansfield, 1998; Frahry & Schopfer, 1998; Chen & Schopfer, 1999; Martinez, Montillet, Bresson, Agnel, Dai, Daniel, Geiger & Nicole, 1998). The apoplastic APX could contribute to defence reactions decreasing the excessive toxic H_2O_2 concentration. However, high concentration of ascorbate in apoplast inhibits PO activity to oxidation of phenols related to lignification and cross-linking (Otter & Polle 1994).

The object of this work was to study the activities of PO with different substrates and H_2O_2 generating enzymes in tomato leaf apoplast after infection.

MATERIALS AND METHODS

Tomato plants (*Lycopersicon esculentum* Mill.) cv. Perkoz were grown in a controlled environment chamber under a 16 h light/8 h dark photoperiod, with 350 $\mu E/m^{-2}s^{-1}$ light intensity at 23°C. At the age of 6 weeks the leaves were infected by application of droplets of *Botrytis cinerea* Pers.: Fr. conidial suspension (10^6 conidia/ml). Non-treated plants were used as control. The leaves were collected 5, 24, 48, 72 hours after inoculation.

Apoplastic extracts (intercellular washing fluid) were obtained from 1 g (fr. wt.) of the leaves. Sections of tomato leaves less than 10 mm long were washed in deionized water for 5 min, and subsequently vacuum-infiltrated for 5 min at –0.9 kPa at 4°C with 50 mM phosphate-Na buffer, pH 7.0. The sections were quickly dried and centrifuged in a 25 ml syringe barrel placed within a centrifuge at 900 × *g* for 5 min at 4°C. Contamination of apoplast extract by cytoplasm constituents, as monitored by the activity of glucose-6-phosphate dehydrogenase (Sigma diagnostic kit) (Creissen, Firmin, Freyer, Kular, Leyland, Reynolds, Pastori, Wellburn, Baker, Wellburn & Mullineaux, 1999), was always less than 1.5% in relation to the cytosolic fraction.

In these extracts of apoplast we measured: a) PO activity with guaiacol (Maehly & Chance, 1954), ferulic acid (Takahama, 1995), syringaldazine (Imberty, Goldberg & Catesson, 1985) and ascor-

bate (Nakano & Asada, 1981) b) SOD (Dhindsa, Plumb-Dhindsa & Thorpe, 1981) and NADH oxidase/peroxidase activities (Ishida, Ookubo & Ono, 1987) c) ascorbate content (Knörzer, Durner & Böger, 1996).

PO fractions were separated on DEAE Sepharose CL 6B in 50 mM phosphate buffer, pH 7.0.

RESULTS

Infection of tomato leaves with *B. cinerea* resulted in a quick and strong stimulation of apoplastic NADH oxidase/peroxidase activity (Fig. 1). The increase of this enzyme activity was well marked already 5 hours after treatment with the fungus and it reached the level of approximately 2.5 fold of that in control 48 hours after infection. SOD activity increased about 25% as compared with control only 24 hours after infection and this small activity increase was maintained up to 72 hours (Fig. 2). APX activity increased, 40% above control 24 hours after inoculation and then was gradually lowered to that of control (Fig. 3). Some increase in PO activities with guaiacol, ferulic acid and syringaldazine was noticed 24 hours after inoculation and was progressively enhanced with all these three substrates up to the 72nd hour (Fig. 4a, b, c). During chromatographic separation of apoplastic extracts on DEAE Sepharose two fractions were obtained: cationic and anionic one. The anionic fraction of PO activity with guaiacol was very small in case of both control and infected leaves (Fig. 5). In the non-infected leaves the activities of anionic PO fraction with both ferulic acid and syringaldazine were similar to those of cationic ones. After infection PO activity with syringaldazine increased in both anionic and cationic fractions, but in the case of ferulic acid substrate only anionic fraction increased (Fig. 6, 7). Total and oxidized ascorbate contents in apoplast of infected leaves gradually decreased but reduced ascorbate content did not change significantly (Fig. 8).

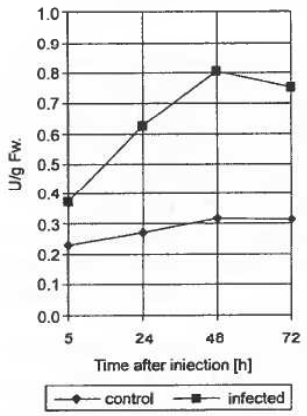

Fig. 1. NADH oxidase/peroxidase activity

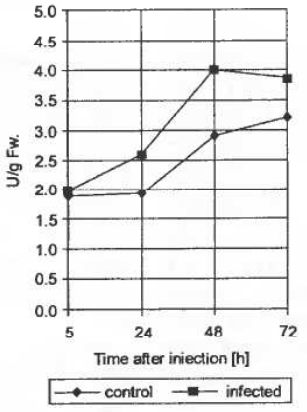

Fig. 2. SOD activity

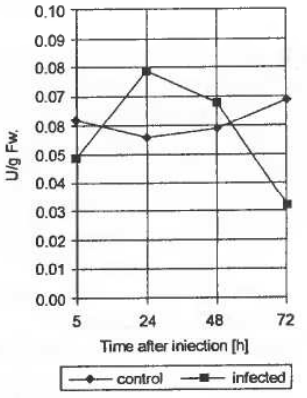

Fig. 3. APX activity

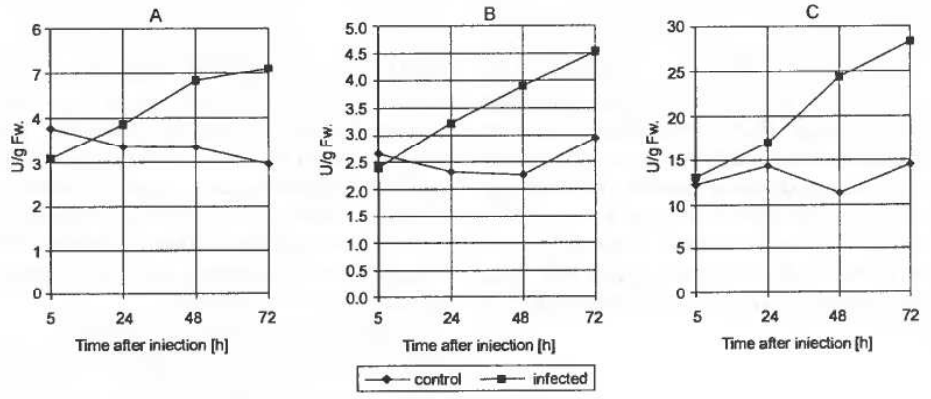

Fig. 4. Peroxidase activity. A – with guaiacol; B – with ferulic acid; C – with syringaldazine

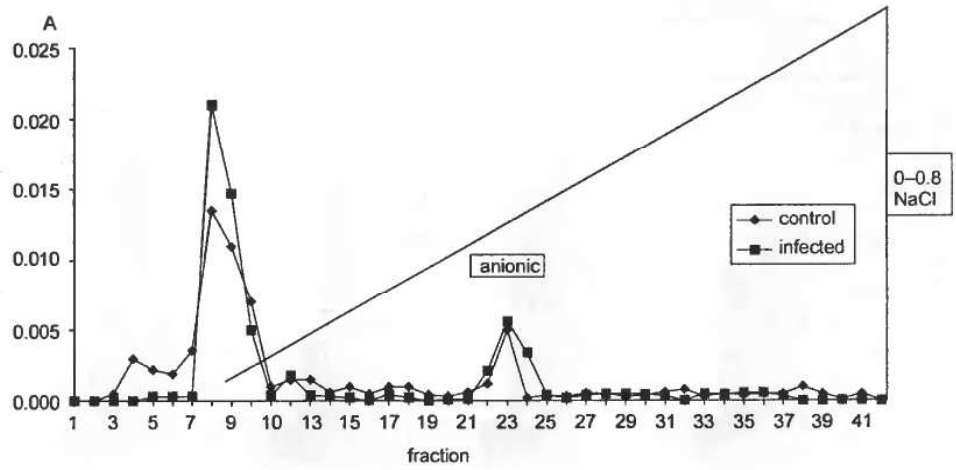

Fig. 5. Chromatographic separation of apoplastic PO on DEAE Sepharose CL6B. Guaiacol was used as a substrate

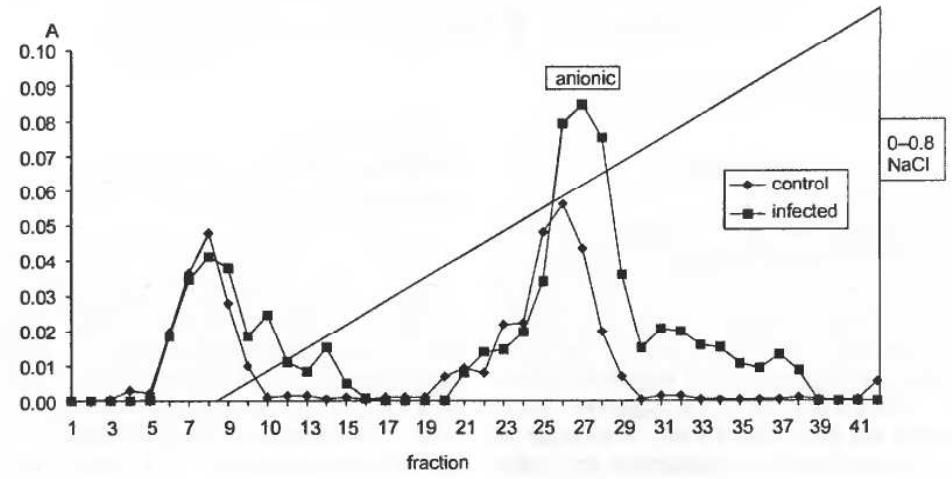

Fig. 6. Chromatographic separation of apoplastic PO on DEAE Sepharose CL6B. Ferulic acid was used as a substrate

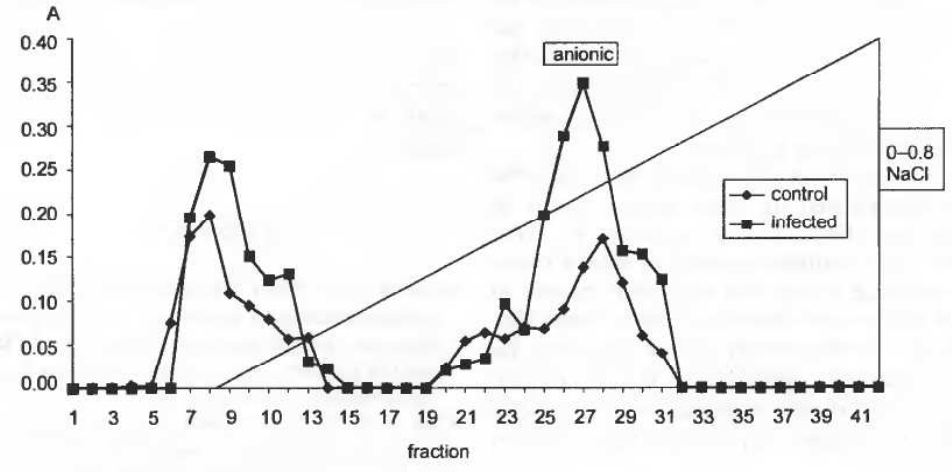

Fig. 7. Chromatographic separation of apoplastic PO on DEAE Sepharose CL6B. Syringaldazine was used as a substrate

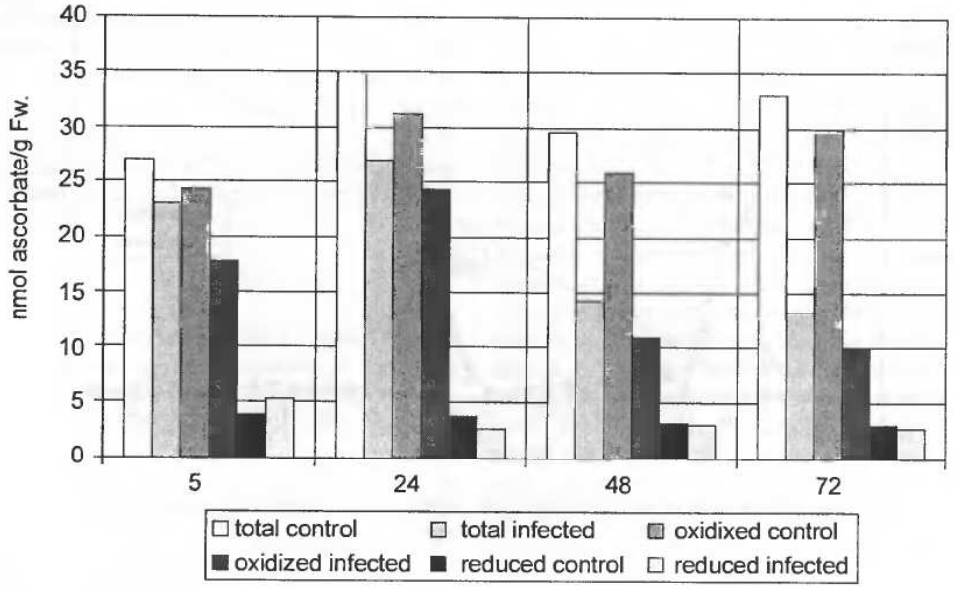

Fig. 8. Ascorbate content in the apoplast

DISCUSSION

In intercellular fluids from virus-infected cucumber fractionated on DEAE-Cellulose three anionic peroxidases were shown (Repka, Slovakova, Kollerova & Slovak, 1991). In our study, two fractions were obtained: cationic and anionic one during chromatographic separation of apoplastic extracts from tomato leaves on DEAE-Sepharose. Syringaldazine and ferulic acid are used as substrate for PO activities related to lignification and cross-linking, respectively (Imberty *et al.*, 1985; Ikegawa, Mayama, Nakayashiki & Kato, 1996; Takahama, 1995). In our work strong induction of PO anionic activity with these two substrates was observed. These results support the hypothesis that anionic apoplastic fraction plays an important role in cell wall strengthening as a defence reaction against the invading pathogen.

An important role of apoplast in plant resistance was demonstrated by some authors (Kerby & Somerville, 1998; Vanacker, Carver & Foyer, 1998). The significant increase in NADH oxidase/peroxidase activity and only slight increase in SOD activity after infection of leaves might indicate that the former enzyme was responsible for H_2O_2 generation. Generation of H_2O_2 in the apoplast by NADH oxidase/peroxidase was proved in spruce (*Picea abies* L.) needles (Otter & Polle, 1994).

Apoplastic ascorbic acid regulates the formation of dehydrodiferulic bridges in the cell wall inhibiting phenols oxidation by PO (Sanchez *et al.*, 1997, Otter & Polle, 1994). We found that total ascorbate and oxidized ascorbate content decreased but reduced ascorbate content did not significantly change after infection. The decrease in ascorbate content and lowering of APX activity after infection are favourable to support the appropriate concentration of H_2O_2 necessary for PO function related to creation of a cell wall defence barrier.

In summary, significant induction of NADH oxidase/peroxidase suggests that H_2O_2 in apoplast originates mostly from the activity of this enzyme. The obtained results favour the hypothesis that apoplastic enzymes and H_2O_2 take part in lignification and cross-linking in response to pathogen attack.

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