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Activities of Antioxidant Enzymes in Cucumber Plants Infected with Cucumber Mosaic Virus

By

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Summary

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Reactive oxygen species are produced in numerous plant diseases and their role in hypersensitive reactions to several plant viruses has been intensively investigated. Little, however, is known about the role of reactive oxygen species in systemic virus infections and about enzymatic defense reactions of the plant to this oxidative stress.

Cucumber plants (varieties Gemini F7 and Slice Master) were artificially inoculated with cucumber mosaic virus strain DSM 0187. Peroxidases, superoxide dismutases and catalases in healthy and virus-infected leaves of both varieties were investigated by non-denaturating polyacrylamide gel electrophoresis. Virus spread was monitored by blotting entire leaves onto nitrocellulose membranes followed by a serological detection.

Inoculated cotyledons of both varieties showed a significant rise in activity of peroxidases within three days after inoculation. In developing true leaves of Slice Master, changes in enzyme activity correlated with the appearance of external symptoms. Both the development of symptoms and the rise in enzyme activity within a growing leaf occurred several days after a systemic virus spread had taken place. The variety Gemini F7 developed no or only weak virus symptoms and peroxidase activity remained at the starting level. At the beginning of the experiment both varieties contained equal levels of peroxidase, consequently the higher tolerance of Gemini F7 cannot be attributed to enhanced peroxidase activity.

Peroxidase probably also interfered with the superoxide dismutase assay and may account for several isozyme forms on gels stained for superoxide dismutase activity. No indication for virus-induced increases of superoxide dismutase activity was found. In true leaves, however, the total superoxide dismutase activity was very low, hence a slight increase of activity due to virus infection might have been masked by peroxidase.

No variation in catalase activity was observed.

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Introduction

Cucumber mosaic cucumovirus (CMV) is a widely distributed virus infecting 1241 species in 101 families (EDWARDSON & CHRISTIE 1991). In cucumber (*Cucumis sativus*) it is distributed systemically causing yellow mosaic and curling of the leaves, stunting of the plants and mosaic patterns on the fruits. The severity of the virus symptoms that are expressed depends on the variety.

Different works have been carried out on the involvement of antioxidant enzymes and reactive oxygen species in virus-host plant interactions (LAGRIMI & ROTHSTEIN 1987, ELSTNER & HOCK 1988, MONTALBINI & al. 1991, VISEDO & al. 1991, EL MOSHATY & al. 1993, CANDELA & al. 1994). However, these investigations were mainly focused on hypersentitive reactions and little is known about compatible plant-virus interactions.

Hence the activities of peroxidases, superoxide dismutases (SODs) and catalases in systemically virus-infected cucumber plants (*Cucumis sativus*) may be of interest and were the objective of the present study. In order to obtain more information on susceptible or tolerant plant-pathogen interactions two differing varieties of *Cucumis sativus* were investigated.

The relations between changes in enzyme activity, spread of the virus particles and development of external virus symptoms were examined.

Materials and Methods

Preparation of samples: *Cucumis sativus* plants (varieties Slice Master and Gemini F7) were artificially inoculated with cucumber mosaic virus strain DSM 0187 and cultivated in the greenhouse. The growth stages of the plants were determined as described previously (FELLER & al. 1995). Plants from growth stage one (leaf development on main shoot) to growth stage five (inflorescence emergence) were included in the trial. In periodical intervals, leaves were harvested, inspected for external symptoms and blotted onto nitrocellulose membranes (pore size 0.45µm).

External symptoms were graduated in five classes. In class one the leaves were symptomless and in class five more than 50% of the surface exhibited mosaic and curling symptoms.

For electrophoresis, leaves were ground 1:10 (w/v) in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 4 mM ß-mercaptoethanol and 0.16 mM cysteine, pH 7,0) at 4°C and were centrifuged for 2 min at 15,500 g.

Electrophoresis and visualization of enzyme activty: The separation of the isoenzymes was performed by non-denaturing discontinuous electrophoresis on basic or acidic (separation at pH 9,5 or pH 3,8) 11% polyacrylamide gels as described previously (RICKWOOD 1993). Gels were stained for peroxidase activity by incubating in 0.3% (w/v) 3,3',5,5'-tetramethylbenzidine, 0.3 M H_2O_2, 1.5 M acetic acid (SCHOPFER 1989). SOD activitiy was assayed photochemically. The reaction mixture was composed of 0.03 mM nitroblue tetrazolium, 0.065 mM phenazine methosulfate, 0.033 mM ßNADP, 5 mM $MgCl_2$, 1 mM DETAPAC, 0.2 M Tris/HCl pH 8.0 (SICILIANO & SHAW 1976). For the catalase assay, gels were washed in double distilled water, incubated in 0.6 mM H_2O_2 and stained with 1% (w/v) ferric chloride-potassium ferricyanide (KLOTZ & HUTCHESON 1992).

Activity analysis was carried out by densitometry of the gels (RFLPScan, MWG Biotech). The integrated optical density of each band was taken as measure for its relative enzyme activity. Between the classes of symptoms and the total integrated densities regressions were calculated.

Leaf blotting: Several previously described methods (MANSKY & al. 1990, HOLT & BEACHY 1992, HSU & al. 1992) were modified. Leaves were pressed on a piece of dry nitrocellulose membrane (pore size 0,45µm) with a Pollähne-Meku leaf press. The nitrocellulose membranes were air dried, blocked with 2% (w/v) skimmed milk, washed and incubated with CMV specific polyclonal rabbit antiserum (1:1000, DLO Research Institute for Plant Protection, Wageningen, The Netherlands). The rabbit IgG was coupled to alkaline phosphatase labeled goat anti rabbit serum (1:50000, Sigma) and the blots were developed as described by HSU & al. 1992.

Results

Development of symptoms, spread of the virus-particles: The variety Slice Master showed distinct symptoms which became apparent as increasing yellow spots near the margins of enfolding true leaves. Growth of virus-infected plants was reduced. The tissue blots proved that symptom development had happened several days after a systemic infection of the leaf. The variety Gemini F7 developed no or only weak virus symptoms. Nevertheless tissue prints indicated that a systemic virus spread had also occurred in this variety. No differences between leaves collected during distinct growth stages were detected.

Peroxidase: In CMV infected leaves four isozymes became apparent both in acidic and basic buffer systems. Healthy plants contained only one detectable isozyme (Fig. 1). Inoculated cotyledons of both varieties showed a definite increase of enzyme activity within three days after inoculation. In true leaves the rise in activity correlated with the development of mosaic symptoms and symptomless

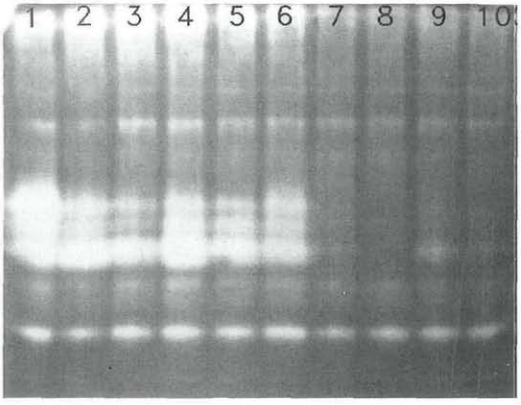

Fig. 1. Peroxidase activity in true leaves. The electrophoresis was carried out on acidic gels. Lanes 1-4, healthy control plants; lanes 5-9, virus infected plants; Pa1-Pa4, peroxidase isoforms.

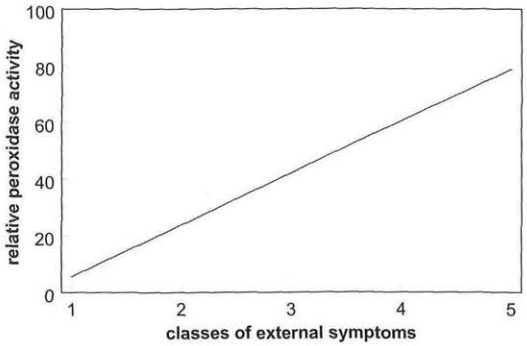

Fig. 2. Correlation between external symptoms and peroxidase activity.

leaves did not show enhanced peroxidase activity. For the variety Slice Master the regression lines are illustrated in Fig. 2. The average correlation coefficient was r=0,58***. No distinction was detected between true leaves that had been collected during different growth stages.

Superoxide dismutase: Separations of cotyledon extracts on basic gels yielded eight bands. Quantitative differences between healthy and infected plants were observed for three bands whereas the other isoforms remained nearly unchanged (Fig. 3). The locations of these three bands (Sb3-Sb5) on the gels were identical to the peroxidase isoforms mentioned above.

Extracts from virus-infected true leaves produced three bands on basic gels and four bands on acidic gels. In both buffer systems all bands coincided with the peroxidase isoforms (Fig. 1, Fig. 4). In true leaves no independent SOD isozymes were observed.

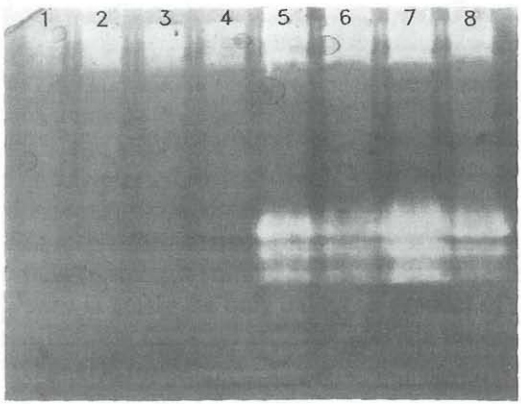

Fig. 3. SOD activity of cotyledons on basic gels. Lanes 1-6, virus infected plants; lanes 7-10, healthy control plants; Sb1-Sb8, SOD isoforms; Pb1-Pb3, peroxidase isoforms.

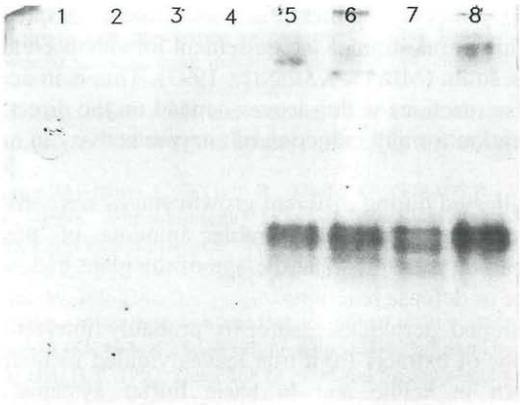

Fig. 4. Visualization of SOD activity of true leaves on acidic gels. Lanes 1-4, healthy control plants; lanes 5-9, virus-infected plants; Sa1-Sa4, SOD isoforms; Pa1-Pa4, peroxidase isoforms.

Catalase: Staining of gels for catalase activity only showed one isozyme. No quantitative differences between the two varieties or between healthy and infected plants were detected.

Discussion

CMV infections were closely correlated to increases of peroxidase activity in virus infected plants. These findings agree with results reported for other systemic host-virus interactions (SHARMA & al. 1984, YE & al. 1990, MONTALBINI & al. 1991, AVDIUSHKO & al. 1993, CANDELA & al. 1994). In cotyledons, peroxidase levels rised within three days after inoculation as a reaction to the presence of the virus. This increase was detected in all inoculated cotyledons although no differences from healthy plants were visible, regardless whether later on mosaic symptoms developed in the true leaves.

In true leaves invaded by the virus, enzyme activity coincided with the emergence of virus symptoms. Both, the appearance of virus symptoms and the rise of peroxidase activity within a leaf seems to be a reaction versus the systemic virus spread that was already detectable 2-3 days earlier.

Isoperoxidases were reported to be involved in resistance of *Capsicum annuum* to CMV (CANDELA & al. 1994). In this study healthy plants of both varieties showed comparable peroxidase activities. Hence the CMV-tolerance of Gemini F7 is likely not due to enhanced enzyme levels. This is in agreement with reports that in tobacco peroxidases do not have an important role for induced resistance to Tobacco Mosaic Virus (YE & al. 1990).

From cross protection studies it is known that a complete systemic spread of the protecting mild virus strain is a requirement for enhanced tolerance of a plant to the severe virus strain (MEYER-KAHSNITZ 1993). This is in accordance with the finding that defense reactions within leaves depend on the direct presence of virus particles. No indication for any induction of enzyme activity in non invaded leaves was observed.

Leaves collected during different growth stages were invaded by the virus to the same extent and had comparable amounts of peroxidase activity. Consequently, it can be presumed that the age of the plant had no influence on the degree of tolerance or defense reactions.

The mentioned peroxidase isoforms probably interfered with the SOD assay as separations of extracts from true leaves yielded identical patterns for the two enzymes both in acidic and in basic buffer systems. Interferences of peroxidases in superoxide dismutase assays were described previously (GIANNOPOLITIS & RIES 1977). This suggests that the bands of SOD activity detected in gels could be artifacts due to peroxidases.

Several SOD isoforms discernible from peroxidases, however, were observed in cotyledons, though it must be stressed that they were not affected by virus infection. These data indicate that SOD is not an important factor in the interaction of *Cucumis sativus* and CMV though it can not be excluded that a slight increase of activity due to the virus infection might have been masked by peroxidase. Since these data are in contrast to previous reports that SOD activity is enhanced by virus infections (MONTALBINI & al. 1991, EL MOSHATY & al. 1993), the role of SODs in systemic virus infections needs to be further investigated.

Catalase activity of *Cucumis sativus* was not influenced by CMV infections. These findings coincide with investigations on Southern Bean Mosaic Virus infected cowpea plants (EL MOSHATY & al. 1993).

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