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# REGULATION OF THE SUPEROXIDE ANION GENERATION IN TOMATO ROOTS SUSCEPTIBLE AND RESISTANT TO NEMATODES

by
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**Summary**. Tomato roots susceptible and resistant to root-knot nematodes were tested for their ability to produce superoxide anions measured as the NADPH-cytochrome *c* reductase activity that was inhibited by superoxide dismutase (SOD). NADPH oxidase activity of isolated microsomes was completely inhibited by the addition of SOD. Microsomal and cell extract activities, measured with high NADPH concentrations, were higher in resistant than in susceptible roots. Microsomal activity was inhibited by $Ca^{2+}$ and acetylcholine. The nature of the $Ca^{2+}$ inhibition and the extent of acetylcholine inhibition were different between the two tomato cvs tested. Nematode attack did not significatively change microsomal activity in both cvs.

Doke (1985) first reported the production of superoxides ($O_2^-$) in membrane-rich fractions from potato tubers when provided with NADPH; this was associated with the invasion of the tubers by incompatible races of *Phytophthora infestans*. Since then, the enzymic activity involved in superoxide production has been purified from membrane-rich fractions of plants and characterized (Rich and Lamb, 1977; Benveniste *et al.*, 1986; Cakmak and Marschner, 1988). This activity has been defined as NADPH-cytochrome P-450 (cytochrome *c*) reductase but other enzymic sources of oxygen free radicals, such as lipoxygenases, peroxidases and xanthine oxidase, occur in plant tissues.

Although oxygen reactive species are supposed to be involved in the hypersensitive reaction (HR) to pathogens (Kauss, 1990) and wound response (Bostock and Stermer, 1989), very little is known about the regulation of the enzymes generating these toxic radicals. For instance, the attack to potato protoplasts by fungi causes an activation of $O_2^-$ generating NADPH oxidase but how it is activated remains unclear (Doke *et al.*, 1987).

Nematode attack of resistant tomato roots has been reported to result in a large increase in $O_2^-$ production but this did not occur in compatible interactions (Zacheo and Bleve-Zacheo, 1988). As well as $O_2^-$ production, different metabolic responses have been observed between the resistant tomato cv. Rossol and the susceptible cv. Roma VF when attacked by nematodes (Molinari, 1991a, 1991b; Molinari *et al.*, 1990). On the other hand, recent studies have shown that these two cultivars differ in their ability to neutralize superoxides as demonstrated either provided by incubating roots, cultured *in vitro* with the superoxide-generator paraquat, or induced by wounding (Molinari, 1991c); these findings suggest that such a difference may be crucial, *in vivo*, at the earlier stages of nematode infestation when the production of oxygen radicals is highly induced.

However, the control of oxygen active species in root cells inolves not only the rate of their disruption but also the rate of $O_2^-$ generation, mainly by the specific membranous NADPH oxidase. Once produced, $O_2^-$ rapidly provides the origin for even more toxic compounds, $OH^\bullet$ and $H_2O_2$ in the cells. The experiments described in this paper were carried out to identify some regulative elements of this production in resistant and susceptible tomato roots, in relation to their particular response to nematode infestation. Therefore, the influence of two widespread components of plant cells, $Ca^{2+}$ and acetylcholine, on $O_2^-$ generating activity of tomato roots was tested. The role of $Ca^{2+}$ in sensing and transducing stress signals across the plasma membrane has been generally recognized (Gilvoy and Trewavas, 1990). Acetylcholine has been reported to influence ion permeability and increase oxygen uptake in mung bean roots (Yunghans and Jaffe, 1972); moreover, it shares a similar molecular structure with orphenadrine and tofenacine which are well-known inhibitors of NADPH-cytochrome P-450 reductase in rat liver microsomes (Bast *et al.*, 1988).

Finally, the observed different regulation on the $O_2^-$ generating activity associated with the reported different ability in scavenging oxygen radicals, between the resistant and susceptible tomato roots tested, has been proposed as to be involved in determining such different responses of these roots to the nematode attack.

## Materials and methods

Seeds of the tomato (*Lycopersicon esculentum*, Mill.) cvs Roma VF and Rossol, respectively susceptible and resistant to root-knot nematodes, were germinated in sterilized quartz sand and 10 days later the seedlings were transplanted into 3-cm diam. clay pots containing quartz sand. Fifteen days later, the roots were used for the preparation of the cellular fractions and detection of the enzyme activities. Plants were maintained in a growth chamber at 26 °C and 65% relative humidity, illuminated for 12 h per day and watered with Hoagland's solution twice daily.

In nematode inoculation experiments plants were grown fo 15 days and then inoculated with 80 active second-stage juveniles of the root-knot nematode *Meloidogyne incognita* (Kofoid *et* White) Chitw. race 2 per seedling. Roots were used for microsome preparation (see below) 5 days after inoculation.

Seedlings were thoroughly rinsed in distilled water and then roots (approx. 200 g per batch) were separated from shoots and kept in an ice-bath. Then they were placed in ice-cold 0.05 M potassium phosphate buffer, pH 6.0, containing 0.55 M mannitol. Roots were cut with scissors and then ground with a Polytron PT-10.35 (Kinematica GmbH-Switzerland). The homogenate was filtered through four layers of gauze and centrifuged twice for 10 min at 500 x *g*. Pellets were discarded and one aliquot (4-6 ml) of the supernatant (crude fraction) was saved and ultrafiltered at 4 °C through a YM ultrafiltration membrane (10,000 molecular weight cut off, Amicon Co.) in Centricon-10 microconcentrators. Both retentate and ultrafiltrate were put on ice. This retentate was used as the Cytoplasmic Fraction (CF). A small aliquot of the ultrafiltrate was immersed in a boiling water bath for few minutes and used as a source of quinones (Maine, 1960). The crude fraction was centrifuged for 15 min at 12,000 x *g* and the pellet was discarded. The supernatant of this last centrifugation was spun at 100,000 x *g* for 90 min to obtain the Microsomal Fraction (MF). Pellets were suspended with the grinding medium to yield suspensions containing 1.2-1.7 mg protein $ml^{-1}$.

The enzyme activity of the samples was tested spectrophotometrically (using a Perkin-Elmer 557 spectrophotometer with a double wavelength mode) by monitoring at room temperature the increase in absorbance at 550 nm, with the reference wavelength set at 540 nm, due to the reduction of externally added oxidized cytochrome *c* (20 µM, horse heart, Sigma type III) by NADPH at different concentrations (10-100 µM). Reaction mixture (1 ml final volume) consisted of 0.01 M potassium phosphate buffer, pH 7.8, approx. 0.20 mg protein of sample; 10 mM $NaN_3$ or 0.5 mM KCN and 20 mM $NaN_3$ were used for Microsomal and Cytoplasmic Fractions, respectively. $NaN_3$ or KCN was used to inhibit cytochrome *c* oxidase. Reduction of cytochrome *c* in the absence of appropriate amounts of such inhibitors was non-linear and slower. Each measurement of the enzyme activity, expressed as nmoles Cyt. *c* reduced $min^{-1}$ $mg^{-1}$ protein, with an $\varepsilon_{550/540}$ = 19 $mM^{-1}$ $cm^{-1}$, was replicated at least twice per each experiment. Lineweaver-Burk (double reciprocal) plots were used to calculate the kinetic parameters of the microsomal enzyme activity. SOD (from bovine erythrocytes, Sigma Co.) was added at the concentration of 15 µg $ml^{-1}$ to evaluate the SOD-sensitive, superoxide generating activity of the samples. Addition of higher amounts of the enzyme did not increase the extent of its inhibition, and thus, such concentration was suitable to reach the maximum inhibition for measurements on cellular fractions in my conditions.

TABLE I - *Comparison of NADPH-cytochrome* c *reductase activity (nmoles Cyt.* c *reduced* $min^{-1}$ $mg^{-1}$ *protein) of cytoplasmic fractions (CF) and isolated microsomes (MF) from susceptible (Roma VF) and resistamt (Rossol) tomato roots.*

| Additions | Roma VF | | Rossol | |
|---|---|---|---|---|
| | C F | M F | C F | M F |
| Controls | 50.0 | 3.1 | 68.4 | 3.7 |
| + SOD | 30.1 | 0.0 | 37.3 | 0.0 |
| + ultrafiltrate | 39.8 | n.d. | 58.5 | n.d. |
| + boiled ultrafiltrate | 175.2 | n.d. | 226.7 | n.d. |
| + $Ca^{2+}$ | n.d. | 1.9 | n.d. | 2.7 |
| + Acetylcholine | n.d. | 2.2 | n.d. | 1.7 |

Assay medium contained 20 µM oxidized Cyt. *c* and 20 mM $NaN_3$. Reactions were started by 100 µM NADPH. Separate additions of 15 µg $ml^{-1}$ SOD, 50 µl ultrafiltrate, 50 µl boiled ultrafiltrate (see Materials and methods), 1 mM $Ca(NO_3)_2$, and 20 mM Acetylcholine were carried out. n.d. = not determined.

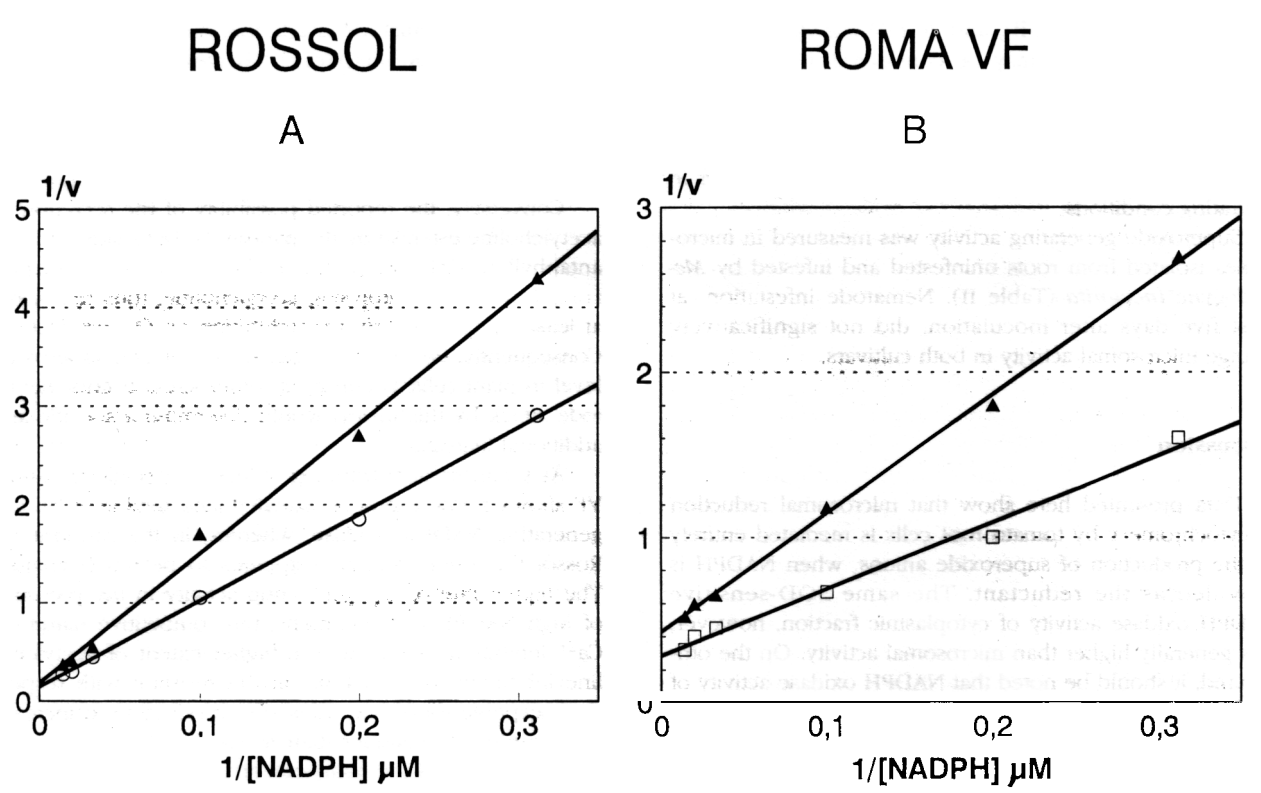


Fig. 1 - Double reciprocal plots of the initial rate (v) of Cyt. *c* reduction by various amount of NADPH and its inhibition by $Ca^{2+}$. Control activity (o, regression line y=8.2*x* + 0.17, r=0.99) and activity in presence of 1 mM $Ca(NO_3)_2$ (▲, regression line y=13.1*x* + 0.18, r=0.99) of resistant roots is shown in A. Control activity (□, regression line y=4.0*x* + 0.28, r=0.99) and activity in presence of 1 mM $Ca(NO_3)_2$ (▲, regression line y=7.2*x* + 0.42, r=0.99) of susceptible roots is shown in B. NADPH-Cyt. *c* reductase activity was measured at 25 °C in the presence of 10 mM potassium phosphate buffer, pH 7.8, containing 10 mM $NaN_3$, approx. 0.2 mg protein, 20 µM Cyt. *c* and various concentrations of NADPH in a final volume of 1 ml.

The protein content of each samle was determined by the Lowry procedure (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

## Results

The NADPH-cytochrome *c* reductase activity of cytoplasmic fractions and isolated microsomes from excised roots was detected in both resistant (Rossol) and susceptible (Roma VF) tomato cvs (Table I). Microsomal activity has been found to be completely abolished by the addition of SOD, whereas, the activity of cytoplasmic fractions was only partially inhibited by the enzyme. Furthermore, isolated microsomes showed a much lower activity with respect to cytoplasmic fractions.

It should be noted that only the amount of the NADPH oxidase activity inhibited by SOD is actually indicative of the generation of $O_2^-$ by the samples. Since the SOD-sensitive activity of cell extracts from resistant roots was found to be about 50% higher than that from susceptible roots, the ability in producing $O_2^-$ may be considered generally higher in resistant than in susceptible cv. The addition of the ultrafiltrate of the homogenate (see Materials and methods), which included most of the ions and small molecules, inhibited the activity of cytoplasmic fractions; on the contrary, when the ultrafiltrate was boiled and provided as a source of quinones the activity was greatly enhanced. Therefore, the effect of both $Ca^{2+}$ and a small molecule, such as acetylcholine, was tested on NADPH-cytochrome *c* reductase activity of isolated microsomes since, as mentioned above, this was the activity specifically related to the generation of $O_2^-$ . Both compounds were found to have an inhibitory effect on the activity.

Lineweaver-Burk (double reciprocal) plots of microsomal activity and the inhibitory effect of $Ca^{2+}$, provided as 1

mM $Ca(NO_3)_2$, (Fig. 1A-B), and 20 mM acetylcholine (Fig. 2A-B) show that $Ca^{2+}$ may be considered a competitive-type inhibitor of the activity in cv. Rossol, whereas acetylcholine had almost halved the activity in saturating conditions with NADPH as substrate. In cv. Roma VF, either $Ca^{2+}$ or acetylcholine are, apparently, effective inhibitors in saturating conditions.

Superoxide generating activity was measured in microsomes isolated from roots uninfested and infested by *Meloidogyne incognita* (Table II). Nematode infestation, at least five days after inoculation, did not significatively change microsomal activity in both cultivars.

## Discussion

Data presented here show that microsomal reduction of cytochrome *c* by tomato root cells is mediated entirely by the production of superoxide anions, when NADPH is provided as the reductant. The same SOD-sensitive NADPH oxidase activity of cytoplasmic fraction, however, was generally higher than microsomal activity. On the other hand, it should be noted that NADPH oxidase activity of the samples tested was found to be heat-labile and rapidly deteriorated as the suspensions aged; moreover, samples could not be stored frozen because freezing and thawing them completely abolished the enzymic activity. Also the use of KCN rather than $NaN_3$ in the assay consistently changed the range of the rate of activity. Therefore, comparisons between activities of the two tomato cultivars tested or between uninfested and infested roots were made with the same fresh experimental preparations, kept and assayed under identical conditions. Moreover, the relatively higher activity found in cytoplasmic with respect to the particulate fraction may be explained by the reported presence of activating factors in the cytosol, which were found to be heat-labile and non-dialysable in Jerusalem artichoke (Benveniste and Durst, 1974); in my experiments, such presumed cytosolic factors would be retained in the cytoplasmic fraction and lost during particulate fraction preparation.

Cytoplasmic fractions of tomato roots from the resistant cv. Rossol had a higher NADPH oxidase activity than the susceptible counterparts. This was particularly evident with the activity inhibited by SOD, representing superoxide generating activity. Also, this major rate of superoxide production by the resistant cv. was retained in isolated microsomes only when high concentrations of NADPH were used.

$Ca^{2+}$ and acetylcholine were effective inhibitors of microsomal NADPH oxidase in both cultivars. Interestingly, $Ca^{2+}$ apparently showed a competitive type of inhibition only in the resistant cv., thus suggesting that in the case of a consistent rise of NADPH level in the cell, which can be determined by nematode attack, this particular inhibition is likely to be inconsistent. However, increase of $NAD(P)H/NAD(P)^+$ ratio as well as $Ca^{2+}$ fluxes across cell membranes are events normally occurring in general stress conditions of plant cells (Gilvoy and Trewavas, 1990; Kauss, 1990).

Conversely, the reported possibility of the secretion of acetylcholine esterase by the parasite (Esbenshade and Triantaphyllou, 1986) suggests that this enzyme may be operating *in vivo* by hydrolysing acetylcholine, thus removing, at least partially, the relative inhibition on $O_2^-$ production. Consequently, besides the aspecific increase of superoxide level in plant cells, occurring in other stress events, nematode attack by this means would determine a specific and additional increase.

As seems evident from Fig. 2, the susceptible cv. Roma VF shows a low extent of acetylcholine inhibition on $O_2^-$ generating NADPH oxidase, whereas, in the resistant cv. Rossol this same inhibition appears to be much greater. The higher rate of $O_2^-$ generating activity in the presence of high NADPH concentrations, the competitive nature of $Ca^{2+}$ inhibition as well as the higher extent of acetylcholine inhibition, observed *in vitro* in resistant with respect to susceptible roots, may cause $O_2^-$ production to increase more rapidly in resistant than in susceptible roots during the early stages of the nematode attack.

The major effect of this rapid $O_2^-$ overproduction would be the peroxidation of membrane lipids, liberation of free fatty acids, and production of endogenous $Ca^{2+}$ ionophores with increased $Ca^{2+}$ entry (Leshem, 1987). If the level of oxygen active species in the cell, which is increased by this overproduction, is not controlled and lowered, cell death would rapidly occur. Normally, the synthesis of specific scavenging enzymes, such as SOD and some peroxidase isoforms and catalase, is highly induced in cells to avoid this danger. On the contrary, when cultured resistant roots were treated with the superoxide-generator paraquat, which greatly increases oxygen active species in

TABLE II - *NADPH-cytochrome* c *reductase activity (nmoles Cyt.* c *reduced* $min^{-1}$ $mg^{-1}$ *protein* ± *standard deviation of three replicates) of microsomes from Roma VF and Rossol tomato roots uninfested and infested by the nematode* Meloidogyne incognita.

| | Roma VF | Rossol |
|---|---|---|
| Uninfested | 13.8±2.4 | 17.1±2.5 |
| Infested | 9.2±4.2 | 17.7±1.0 |

Measurements were made in presence of 20 µM oxidized Cyt. *c*, 0.5 mM KCN and approx. 0.5 mg protein; reactions were started by 10 µM NADPH.

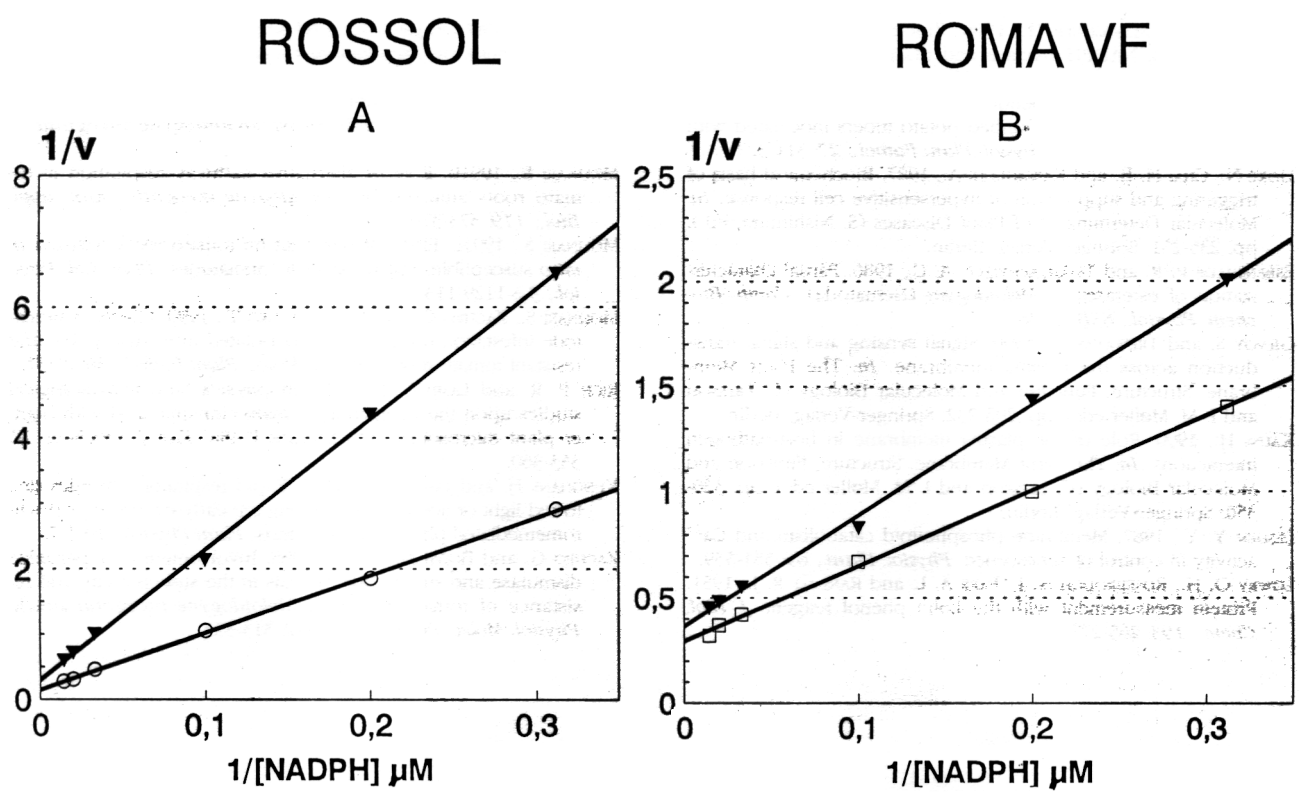


Fig. 2 - Double reciprocal plots of the initial rate (v) of Cyt. *c* reduction by various amount of NADPH and its inhibition by acetylcholine. Control activity (o, regression line $y=8.8x + 0.14$, $r=0.99$) and activity in presence of 20 mM acetylcholine (▼, regression line $y=19.9x + 0.29$, $r=0.99$) for resistant roots is shown in A. Control activity (□, regression line $y=3.5x + 0.29$, $r=0.99$) and activity in presence of 20 mM acetylcholine (▼, regression line $y=5.2x + 0.36$, $r=0.99$) of susceptible roots is shown in B. Conditions as described in Fig. 1.

cells, the activity of SOD, ascorbate and glutathione peroxidase, was surprisingly found to be lower than in control roots, also if catalase activity was enhanced (Molinari, 1991c).

Importantly, the hypersensitivity of the tested resistant roots towards nematodes may be interpreted as the effect of the unusual response of these roots to events that rapidly raise oxygen active species in cells. A detoxification system impaired as toxic compounds are dispersing in cells is likely to cause cell death and tissue necrosis, which is what occurs in an incompatible root-nematode interaction. It is reasonable to believe that this unusual response is genetically determined in the genes for nematode resistance.

Finally, since superoxide level also increases in other plant stress conditions, there must be some additional specific actions by the nematode, other than the presumed removal of acetylcholine inhibition on superoxide generation, on catalase level and on phenol metabolism, for example, that trigger the browning reaction commonly observed after nematode invasion of resistant roots. This major visible damage to roots, in fact, was not observed with aspecific oxidative stress like paraquat treatment or wounding.

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