Plant Physiol. (1996) 111: 885–891

Relationship between Active Oxygen Species, Lipid Peroxidation, Necrosis, and Phytoalexin Production Induced by Elicitins in *Nicotiana*[1]

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Excised leaves of *Nicotiana tabacum* var Xanthi and *Nicotiana rustica* were treated with cryptogein and capsicein, basic and acidic elicitins, respectively. Both compounds induced leaf necrosis, the intensity of which depended on concentration and duration of treatment. *N. tabacum* var Xanthi was the most sensitive species and cryptogein was the most active elicitin. Lipid peroxidation in elicitin-treated *Nicotiana* leaves was closely correlated with the appearance of necrosis. Elicitin treatments induced a rapid and transient burst of active oxygen species (AOS) in cell cultures of both *Nicotiana* species, with the production by Xanthi cells being 6-fold greater than that by *N. rustica*. Similar maximum AOS production levels were observed with both elicitins, but capsicein required 10-fold higher concentrations than those of cryptogein. Phytoalexin production was lower in response to both elicitins in *N. tabacum* var Xanthi cells than in *N. rustica* cells, and capsicein was the most efficient elicitor of this response. In cryptogein-treated cell suspensions, phytoalexin synthesis was unaffected by diphenyleneiodonium, which inhibited AOS generation, nor was it affected by tiron or catalase, which suppressed AOS accumulation in the extracellular medium. These results suggest that AOS production, lipid peroxidation, and necrosis are directly related, whereas phytoalexin production depends on neither the presence nor the intensity of these responses.

The hypersensitive reaction is a common response of a plant to an incompatible pathogen. It initiates a number of metabolic changes in the host cells. Among these responses, oxidative processes can play a crucial role during the early steps of elicitation. Although molecular oxygen, which is an essential element in aerobic metabolism, is relatively unreactive, it is a potential source of reactive forms, such as free radicals or H_2O_2. AOS are present at low levels in plant cells; they can be generated during electron transport or enzymatic processes involved in redox systems (Elstner, 1982; Crane et al., 1985). The production of AOS such as H_2O_2, superoxide anions, and hydroxyl radicals in plant-microbe interactions has been recently reviewed (Mehdy, 1994). The generation of superoxide anions can induce lipid peroxidation, leading to loss of membrane integrity and finally to tissue necrosis development (Adam et al., 1989). Moreover, the possibility that AOS could act as a signal that triggers phytoalexin production has also been proposed (Epperlein et al., 1986; Apostol et al., 1989; Ellis et al., 1993; Degousée et al., 1994).

The interaction between the phytopathogenic fungus *Phytophthora cryptogea* and the nonhost tobacco (*Nicotiana tabacum*) leads to a hypersensitive reaction characterized by plant tissue necrosis and restricted growth of the fungus (Bonnet, 1985; Bonnet et al., 1995). Cryptogein, a protein secreted by this fungus, causes necrosis in tobacco plants at the site of application and also in distant leaves. It induces physiological and structural changes in tobacco leaves (Bonnet et al., 1986; Milat et al., 1991). When added to tobacco cell suspensions, cryptogein induces a rapid increase in extracellular pH and conductivity (Blein et al., 1991), and later the production of ethylene and phytoalexins (Milat et al., 1990). Recently, we reported that production of AOS (Viard et al., 1994), lipoxygenase induction (Bottin et al., 1994), a change in lipid composition (Tavernier et al., 1995a), and uptake of calcium (Tavernier et al., 1995b) also occur. All of these responses are sensitive to staurosporine, indicating the involvement of protein phosphorylation in the elicitation process (Viard et al., 1994; Tavernier et al., 1995). Evidence for specific, high-affinity binding sites has also been published (Wendehenne et al., 1995). For a general review of elicitin effects, see Ricci et al. (1993) and Yu (1995).

In this work, we compare the effect of two elicitins: cryptogein, a basic holoprotein from *P. cryptogea*, and capsicein, an acidic holoprotein secreted by *Phytophthora capsici*, on two *Nicotiana* species (*N. tabacum* var Xanthi and *Nicotiana rustica*). Their necrotizing activity and ability to induce lipid peroxidation were studied as were their potential to trigger AOS and phytoalexin accumulation in *Nicotiana* cell suspensions. The aim of this work was

[1] This work was supported by Institut National de la Recherche Agronomique, Société Nationale d'Exploitation Industrielle des Tabacs et Allumettes Association pour la Recherche sur les Nicotianées, and the Conseil Régional de Bourgogne.

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Abbreviations: AOS, active oxygen species; MDA, malondialdehyde; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TL, thermoluminescence.

to determine the interdependency of these different responses.

MATERIALS AND METHODS

Elicitins were prepared according to Ricci et al. (1989) and used as aqueous solutions. Each experiment was carried out at least twice.

Leaf Assays

Nicotiana plants (*Nicotiana tabacum* var Xanthi and *Nicotiana rustica*) were grown in a greenhouse for 9 weeks. Young leaves weighing about 3 g were selected, detached, and treated with elicitins. A 10-μL drop of an aqueous solution containing different amounts of elicitins (10 μg to 1 mg mL^{-1}) was put on the cut petiole of excised leaves and followed three times with 10 μL of water to ensure full absorption of the elicitor. Control leaves were treated in the same way with water only. Leaf petioles were placed in water and kept in the dark at room temperature. Necrotizing effects were assessed from changes in leaf fresh weights and expressed as percentages of initial leaf weights.

Cell Assays

Nicotiana cell-suspension cultures were grown in Chandler's medium (Chandler et al., 1972) on a rotary shaker (150 rpm, 25°C) under continuous light. Cells in exponential growth phase were filtered and resuspended in a 2 mM Mes buffer, pH 5.75, containing 175 mM mannitol, 0.5 mM $CaCl_2$, and 0.5 mM K_2SO_4 (Keppler and Baker, 1989). After a 30-min equilibration period on a rotary shaker, cells were filtered and resuspended again in the same buffer and then aliquoted (20 mL, 0.1 mg fresh weight mL^{-1}) into 50-mL Erlenmeyer flasks. After a 4-h equilibration period (24°C in darkness, 150 rpm), cells were treated with elicitins, added to buffer directly, and used for the determination of AOS and phytoalexin production.

AOS Quantitation

The production of AOS was determined by chemiluminescence using luminol as reagent and the single photon mode of a Beckman LS 600 TA scintillation counter as previously described (Viard et al., 1994).

Briefly, aliquots of cell suspensions (250 μL) were transferred to scintillation vials during the 180-min treatment period and every 10 min after the elicitin treatment. Then, 350 μL of a 10 mM Mes buffer, pH 6.5, containing 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and luminol (final concentration 25 μM) were added. Counts were recorded at 3-s intervals for 40 s. Results represent total cpm at 40 s, corresponding to the sum of the values obtained for each measurement expressed as H_2O_2 equivalents (μM). Assays for monitoring H_2O_2 production were performed by adding different known amounts of H_2O_2 to cell suspensions in the range of concentrations measured in experiments with cryptogein-treated cells. The increase in chemiluminescence was linearly proportional to the amount of H_2O_2 added in the range 0 to 230 μM.

Capsidiol Determination

Aliquots (10 mL) of cell suspensions were treated with the elicitor for 18 h in the incubating medium (Milat et al., 1990). Cells were then removed by filtration and the capsidiol was extracted from the extracellular medium with dichloromethane (3 × 15 mL). The organic extracts were pooled and concentrated to dryness and the residue was dissolved in ethanol (200 μL). Aliquots (5 μL) were analyzed directly by GC (packed column [3% OV-225 on Chromosorb (Delsi, Sinesnes, France) W100–120, 1.5 m × 3 mm]; N_2 carrier gas 30 mL min^{-1}; injector at 235°C; detector at 250°C; column at 180°C). Capsidiol concentrations were calculated from a standard curve obtained with an authentic sample of capsidiol (Blein et al., 1991).

Lipid Peroxidation Measurements

TL measurements and signal analysis were performed according to Ducruet and Miranda (1992). Briefly, a piece of leaf was inserted into the cuvette of the sample holder of a laboratory-made set-up. The sample was cooled to −5°C and then illuminated by single-turnover flashes. This was followed by fast refrigeration to −40°C. After reaching this temperature, the sample was then heated to 100°C at a rate of 30°C min^{-1}. TL was recorded at wavelengths higher than 670 nm by a cooled photomultiplier, linked to a photon-counting system (Stallaert et al., 1995).

Lipid peroxidation was also estimated from the accumulation of TBARS (Stallaert et al., 1995). Leaves were ground in liquid nitrogen for 40 s. Ice-cold 0.2 M sodium-phosphate buffer (2.5 mL buffer g^{-1} fresh weight) containing 1% Triton X-100 (Sigma) and 0.01% butylhydroxytoluene were added and the mixture was homogenized. The mixture was centrifuged for 20 min at 10,000*g*, and the pellet was discarded. A 150-μL aliquot of the leaf extract was mixed with 300 μL of TCA (10%) and 450 μL of TBA (0.67%) and heated in a boiling water bath for 15 min. After cooling, the A_{532} was measured and corrected for nonspecific dissipation at 600 nm. The concentration of lipid peroxides was expressed as the amount of MDA formed (molar extinction coefficient = 156×10^3 M^{-1} cm^{-1}).

Data were analyzed using unpaired *t* tests and significance assigned if $P \leq 0.05$. Pearson's product moment-correlation coefficients were calculated to measure linear association.

RESULTS

Effects of Cryptogein

Leaf Necrosis Induction

When excised leaves of *N. tabacum* var Xanthi and *N. rustica* were treated with cryptogein, leaves partly dried up and necrosis developed first in the apical part of the leaves, leading to dramatic structural modifications. Cells from the upper epidermis, palisade parenchyma, and spongy parenchyma were disorganized; they lost their turgidity and collapsed, giving rise to a small, translucent depression in the mesophyll. Their vacuolar volume decreased drastically, and the original cell shape was no longer maintained

(Milat et al., 1991). An important decrease in leaf fresh weight resulted from these modifications. This response was time and dose dependent. For a given cryptogein treatment (e.g. 10 μg), necrosis developed faster in Xanthi than in *N. rustica* leaves (Fig. 1). Symptoms were visible in Xanthi leaves for 24 h after treatment with 0.1 μg of cryptogein, whereas no effects were noticeable in *N. rustica* leaves 48 h after treatment with 1 μg of cryptogein (Fig. 1). These observations did not result from differences in leaf size, since the mean of their initial weights was 3.1 ± 0.4 g.

Lipid Peroxidation in N. tabacum *var Xanthi*

Evidence for lipid peroxidation in cryptogein-treated tobacco leaves was provided by the emergence of a high-temperature thermoluminescent peak and also by accumulation of TBARS.

After illumination of frozen tobacco leaves with one flash and subsequent heating, the TL signal showed peaks at 32 and at 95°C (Stallaert et al., 1995). The first band was the well-characterized B band, and the second band, originating from a heat-induced transfer of energy from excited forms, was that involved in lipid peroxidation toward chlorophyll. This band was similar to the 75°C band observed in spinach and mung bean chloroplasts (Hideg and Vass, 1993).

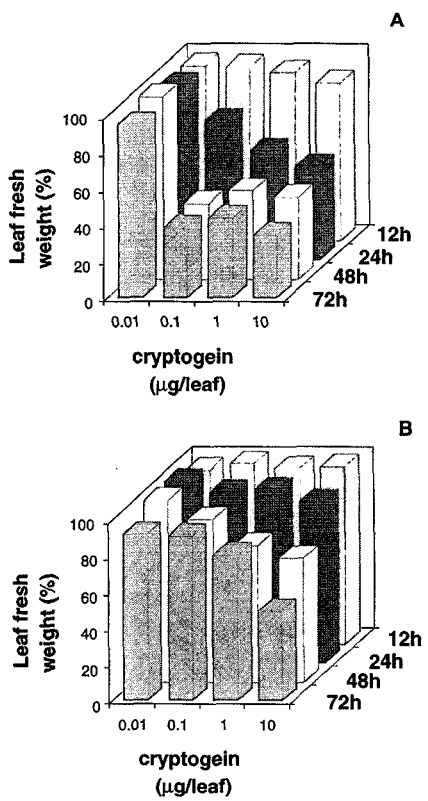

Figure 1. Induction of leaf necrosis by cryptogein in *N. tabacum* var Xanthi (A) and *N. rustica* (B). A 10-μL drop of an aqueous solution of cryptogein was put on the petiole of an excised leaf. At various times leaf fresh weights were measured. Results are expressed as percentage of the initial values of the fresh weight (two repetitions, SE, 8%).

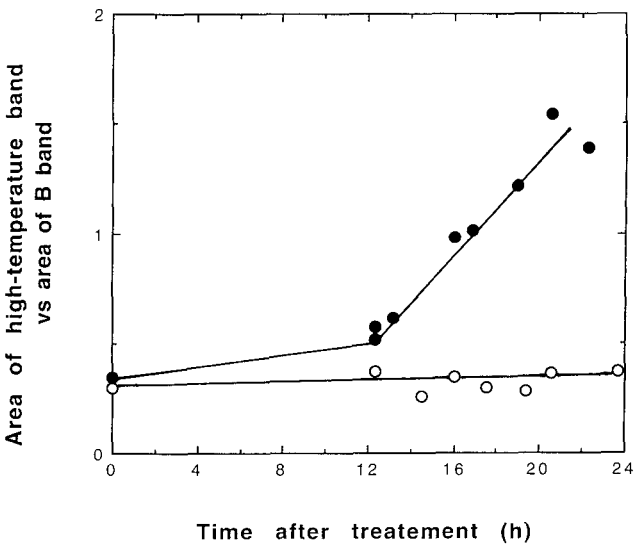

Figure 2. Time course of signal area of the high-temperature TL band versus the area of the TL B band. Leaves of *N. tabacum* var Xanthi were treated with 2.5 μg of cryptogein (●) or with H_2O (○).

To reduce variability between leaves (resulting from heterogeneous dispersion of the cryptogein molecule, leaf size, leaf age, and variations in optical properties between leaf samples), the ratio of the high-temperature band to the B band was calculated (Fig. 2). It remained low in control leaves and increased about five times over a 24-h period after treatment with 2.5 μg cryptogein $leaf^{-1}$. This increase was due only to a progressive elevation of the high-temperature band, since no significant changes of the B band area were recorded.

The accumulation of TBARS showed a good linear correlation with the TL data (Stallaert et al., 1995). Isolation of the TBA-MDA complex from the total TBARS mixture, through purification on a Sep-Pak C_{18} cartridge (Waters) and HPLC, revealed that the observed increase in TBARS resulted from the increased TBA-MDA complex. Therefore, preference was given to quantify spectrophotometrically the overall TBARS content rather than employing more elaborate methods. TBARS in cryptogein-treated tobacco leaves (2.5 μg per leaf over a 24-h period) increased from 20.9 ± 0.7 to 55.3 ± 6.2 nmol g^{-1} leaf fresh weight. These values were compared to the corresponding fresh weight changes. Figure 3 (open symbols) revealed that the accumulation of lipid peroxides is linearly related to a decrease in leaf fresh weight. This correlation was also observed for leaves treated with different amounts of cryptogein (0.1–10 μg) after 24 h of treatment (Fig. 3, filled symbols). The TBARS values for control leaves were 21.8 ± 0.5 nmol g^{-1} leaf fresh weight. The linear equations for the two series of data presented were not found to be significantly different at $P \leq 0.05$, which allowed us to trace only one line satisfying both conditions. Apparently, this tight coupling between TBARS and leaf fresh weight was not disturbed, regardless of the amount of cryptogein used.

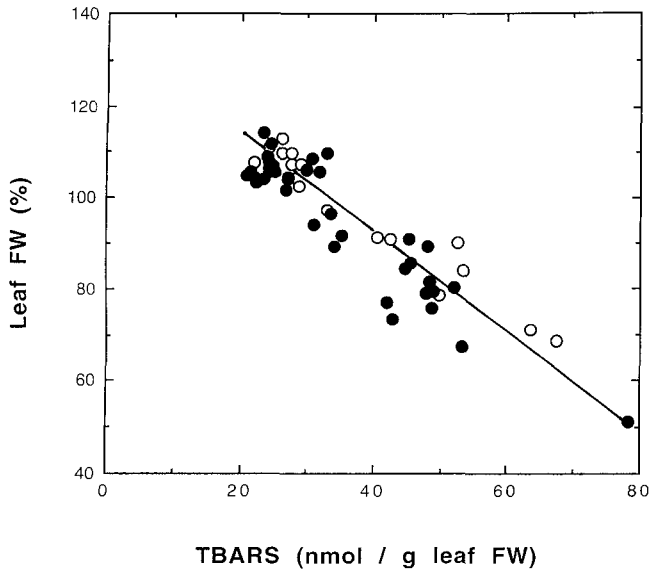

Figure 3. Relationship between lipid peroxidation and necrosis intensity in *N. tabacum* var Xanthi leaves. Necrosis intensity is expressed as a percentage of the variation of fresh weight after 24 h of treatment with different amounts (0.1–10 μg) of cryptogein (●) and after 12, 16, 20, or 24 h of treatment with 2.5 μg of cryptogein (○). Lipid peroxidation is expressed as nmol per g leaf fresh weight of TBARS generated. FW, Fresh weight.

AOS Production

Cell-suspension cultures were established from leaves of *N. tabacum* var Xanthi and *N. rustica*. Cryptogein treatment of *Nicotiana* cells induced a rapid and transient burst of AOS (Viard et al., 1994), as shown in Figure 4. Up to 10 nM, cryptogein induced a strong increase in AOS production, particularly for Xanthi cells, which appeared 6-fold more reactive than *N. rustica* cells. Higher elicitin concentrations (10–100 nM) also increased AOS production, but to a lesser extent.

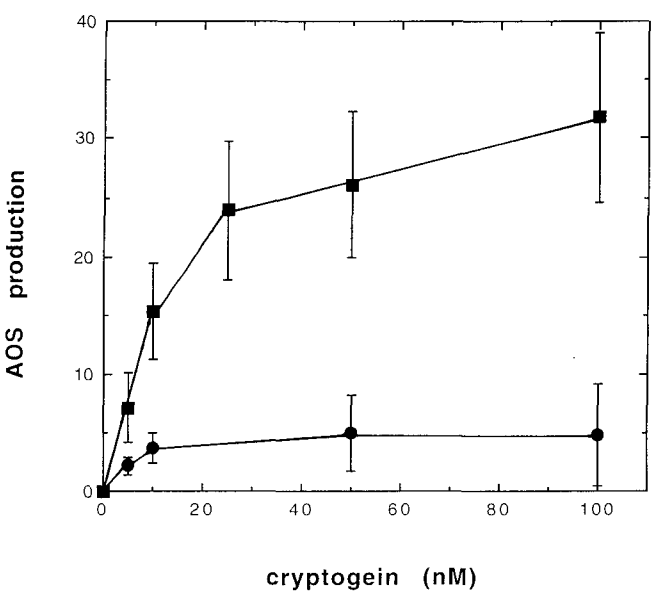

Figure 4. Effects of cryptogein concentration on AOS production by *Nicotiana* cells. Results are expressed as H_2O_2 equivalents (μM) as indicated in "Materials and Methods" (three repetitions). Control values have been subtracted: ●, *N. rustica* (9.3 ± 0.2); ■, *N. tabacum* var Xanthi (9.1 ± 0.1).

Capsidiol Production

Capsidiol accumulated mainly in the extracellular medium of treated cell suspensions (Blein et al., 1991). Cryptogein induced the production of capsidiol in both cell suspensions (Table I). The highest amount of capsidiol was obtained with cryptogein concentrations from 10 to 100 nM. Nevertheless, with the same cryptogein concentration, e.g. 5 nM, capsidiol production was much higher in *N. rustica* (76 ± 3 μg g^{-1} fresh weight) than in *N. tabacum* var Xanthi cells (8 ± 7 μg g^{-1} fresh weight). The background of phytoalexin production by *N. rustica* cells could likely result from a higher sensitivity of these cells to the experimental conditions (cell filtration and equilibration in another medium).

Diphenyleneiodonium (10 μM), tiron (1 mM), or catalase (100 μg mL^{-1}), added at time 0, fully abolished cryptogein-induced AOS accumulation in the extracellular medium, whereas none of them was able to reduce capsidiol production (Table II).

Effects of Capsicein

Leaf Necrosis Induction

After 72 h, capsicein did not significantly affect *N. rustica* leaf fresh weight (Fig. 5). On the contrary, Xanthi leaves developed some necrosis, but less than it developed with cryptogein, as described above (Fig. 1).

AOS Production

Capsicein induced AOS production in both cell suspensions, but Xanthi cells appeared much more responsive than *N. rustica* cells (Fig. 6). This production was much lower than that induced by cryptogein; a 10-fold higher concentration of capsicein was needed to obtain the same AOS production.

Table I. *Capsidiol production by Nicotiana cells treated for 18 h with cryptogein*

Each capsidiol quantitation was performed twice. Results correspond to the mean of three different experiments ± SE.

Cryptogein Concentration	Capsidiol Production	
	N. tabacum var Xanthi	*N. rustica*
nM	μg g^{-1} fresh weight	
0	0.2 ± 0.4	11 ± 6
5	8 ± 7	76 ± 3
10	15 ± 5	97 ± 7
50	26 ± 4	69 ± 9
100	26 ± 4	46 ± 2

Table II. *Effect of tiron, catalase, and diphenyleneidonium (DPI) on AOS and capsidiol production induced in N. tabacum var Xanthi cells treated by cryptogein*

AOS production induced by cryptogein was 12.7 ± 2.9 μM H_2O_2 equivalents and capsidiol accumulation was 15 ± 5 μg g^{-1} fresh weight. Control values have been subtracted: AOS, 9.1 ± 0.1 μM; capsidiol production, 0.2 ± 0.38 μg g^{-1} fresh weight. Results represent the mean of three independent experiments ± SD. Tiron (1 mM), catalase (100 μg mL^{-1}), and DPI (10 μM), added alone to tobacco cell suspensions, did not induce any AOS production or capsidiol accumulation.

Compound	AOS Production	Capsidiol Production
	%	%
Cryptogein (10 nM)	100 ± 23	100 ± 15
Cryptogein + tiron	0.5 ± 0.5	87 ± 9
Cryptogein + catalase	1.20 ± 0.01	84 ± 4
Cryptogein + DPI	1.2 ± 0.2	78 ± 9

Capsidiol Production

Both cell suspensions assayed produced capsidiol in response to a capsicein treatment (Table III). Nevertheless, with 5 nM capsicein the production of capsidiol was much lower with Xanthi (11 ± 3 μg g^{-1} fresh weight) than with *N. rustica* (93 ± 8 μg g^{-1} fresh weight). At 50 to 1000 nM capsicein, capsidiol production in Xanthi and *N. rustica* cell suspensions was similar (Table III).

DISCUSSION

Relationship between Necrosis, Lipid Peroxidation, and AOS

When excised leaves of *N. tabacum* var Xanthi and *N. rustica* were treated with elicitins, the development of necrosis was strongly delayed when leaves were exposed to continuous light (7000 lux of white light 24 h before elicitin treatment). Under these conditions, a cryptogein treatment (at 1 μg g^{-1} fresh weight for 18 h) induced only about 10% of the necrosis developed in the dark (data not shown). This light effect could be related to a lower induction of PR1 protein because (a) its presence seems to be associated with hypersensitive necrosis formation (H. Keller, P. Bonnet, L. Pruvot, L. Friedrich, J. Ryals, and P. Ricci, unpublished results), (b) it is dark inducible (Sessa et al., 1995), and (c) its accumulation is mediated by AOS (Green and Fluhr, 1995). Further experiments will be developed in an attempt to confirm this hypothesis.

The AOS burst is a general response of elicited cells (Sutherland, 1991; Baker et al., 1993; Sanchez et al., 1993; Elstner and Osswald, 1994; Mehdy, 1994). It has been related to lipid peroxidation (Keppler and Novacky, 1986, 1987; Rogers et al., 1988; Adam et al., 1989; Keppler and

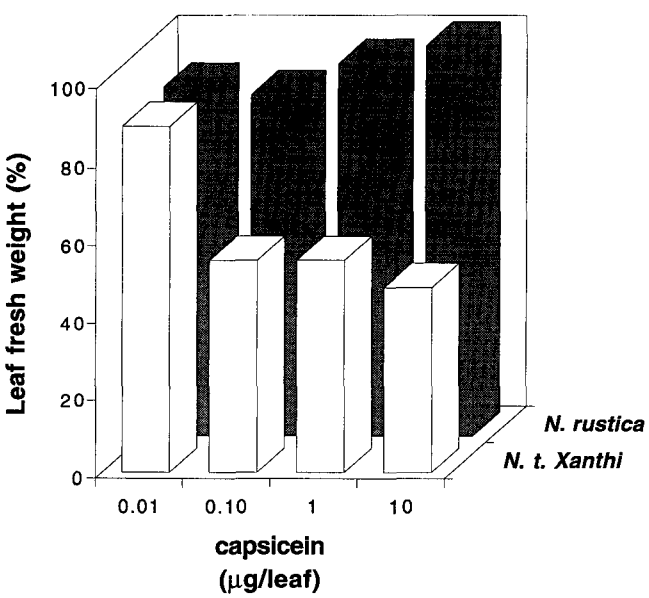

Figure 5. Induction of leaf necrosis by capsicein in *N. tabacum* var Xanthi and in *N. rustica*. A 10-μL drop of an aqueous solution of capsicein was put on the petiole of an excised leaf. At 72 h, leaf fresh weights were determined. Results are expressed as percentages of the initial values of fresh weight.

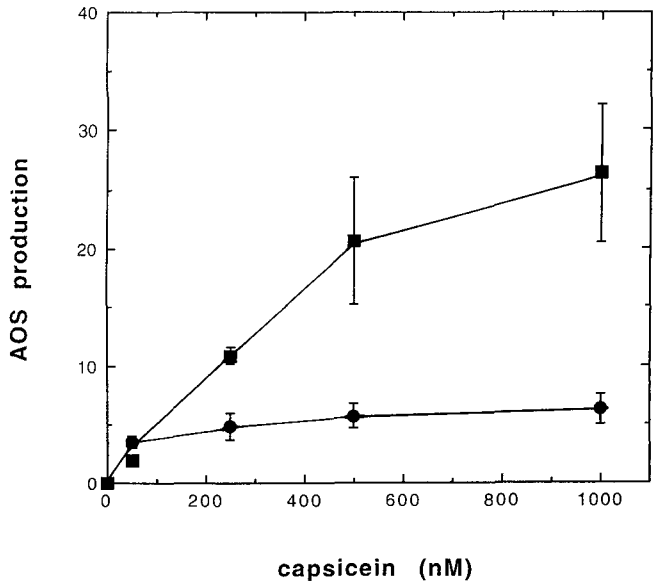

Figure 6. Effects of capsicein concentration on AOS production by *Nicotiana* cells. Results are expressed as H_2O_2 equivalents (μM) as indicated in "Materials and Methods" (three repetitions). Control values have been subtracted: ●, *N. rustica* (9.3 ± 0.2); ■, *N. tabacum* var Xanthi (9.1 ± 0.1).

Table III. *Capsidiol production by Nicotiana cells treated for 18 h with capsicein*

Each capsidiol quantitation was performed twice. Results correspond to the mean of three different experiments ± SE.

Capsicein Concentration	Capsidiol Production	
	N. tabacum var Xanthi	*N. rustica*
nM	μg g^{-1} fresh weight	
0	0.2 ± 0.4	11 ± 6
5	11 ± 3	93 ± 8
50	35 ± 9	40 ± 3
250	34 ± 5	39 ± 2
500	31 ± 6	37 ± 2
1000	30 ± 3	34 ± 8

Baker, 1989; Vera-Estrella et al., 1992; El-moshaty et al., 1993). Doke and Ohashi (1988) suggested that a NADPH-dependent $O_2^{\cdot-}$-generating reaction in leaves of tobacco mosaic virus-infected tobacco cultivars is involved in the induction of necrotic lesions, and the involvement of the redox system associated with plasma membranes in lipid peroxidation has been reported (Qiu and Liang, 1995). Evidence for lipid peroxidation in cryptogein-treated *Nicotiana* leaves was seen by a high-temperature TL peak as well as an accumulation of TBARS. In *N. tabacum* var Xanthi, the intensity of lipid peroxidation was closely correlated with extensive necrosis, suggesting that necrosis appearance, lipid peroxidation, and AOS formation are related. Elicitor-induced peroxidative breakdown of cellular membranes is consistent with the hypothesis that changes in membrane permeabilities could be caused by various forms of activated oxygen. In this way, it has been reported that formation of hydroperoxides might be the chemical process leading to changes in membrane permeability (Maccarrone et al., 1995). Changes in lipid peroxidation are also observed during aging or senescence of tissues (Kumar and Knowles, 1993) and could be associated with apoptosis (Sandstrom et al., 1995), showing that AOS production and lipid peroxidation are involved in mechanisms leading to cell death.

In the present study, there was a close correlation between the intensity of necrosis and the production of AOS. The species developing the most dramatic necrosis showed the highest AOS production, and the most toxic elicitin induced the greatest generation of AOS.

Independence of AOS and Phytoalexin Production

Tiron and catalase, which suppress the extracellular accumulation of AOS, or an inhibitor of plasma membrane NADPH oxidase (diphenyleneiodonium) did not change the amount of elicitin-induced phytoalexin production. This is in accordance with other recent work reporting that AOS generation is not a signaling element triggering phytoalexin production. For example, degradation of AOS by catalase or SOD does not affect elicitor-stimulated phytoalexins (Devlin and Gustine, 1992; Nürnberger et al., 1994). Moreover, AOS production without phytoalexin induction or phytoalexin production without AOS detection has been reported using abiotic elicitors or live and autoclaved bacteria (Devlin and Gustine, 1992) or biotic elicitors such as oligogalacturonides with different degrees of polymerization and glycoproteins (Davis et al., 1993; Legendre et al., 1993). In the same way, although polyunsaturated fatty acids are able to induce aspecific lipid peroxidation, only some of them, such as eicosanoic acids (arachidonic acid and eicosapentaenoic acid), lead to cell browning and rishitin accumulation in potato tissue, likely via their peroxidation by specific lipoxygenase activities (Davis and Currier, 1986; Ricker and Bostock, 1994). The elicitor activities of these polyunsaturated fatty acids are highly dependent on the lengths of their carbon chains and the numbers and positions of their double bonds (Preisig and Kuc, 1985).

Furthermore, phytoalexin accumulation was not strictly related to the intensity of necroses or AOS production in the two *Nicotiana* species investigated here. The most necrotizing elicitin did not induce the highest capsidiol accumulation. In the same way, the *Nicotiana* species that most readily developed necroses or generated AOS produced a lower amount of phytoalexin. Since living cells are required for phytoalexin synthesis, it is possible that the differences observed in phytoalexin production are a consequence of the cytotoxicity induced by elicitin treatment. However, at 5 nM cryptogein, AOS production is quite different in the two *Nicotiana* species, whereas there is a great change in the amount of phytoalexin produced.

In conclusion, AOS and phytoalexin production seem to be independent, whereas necrosis appearance could be related to lipid peroxidation, which probably results from AOS induction. The different susceptibility of *Nicotiana* species to elicitins could be due to a balance between accumulation of phytoalexin and enzymatic activities involved in generation and metabolism of AOS.

ACKNOWLEDGMENTS

We thank V. Houot for her excellent technical assistance, P. Bonnet for helpful discussions, and V. Gianinazzi Pearson for critical reading of the manuscript and for correcting the English.

Received December 4, 1995; accepted April 4, 1996.

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