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Rapid Hydrogen Peroxide Release in Cell Suspensions of *Capsicum* spp. Elicited by Fungal Preparations

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ABSTRACT

The release of H_2O_2 by plant cell suspensions elicited with crude hyphal wall preparations has been studied in a complex of plant genotypes (two cvs of *Capsicum annuum* and one of *C. frutescens*) and fungus species (*Phytophthora capsici, Ph. parasitica* and *Verticillium dahliae*), representing several combinations of compatibility and both host and nonhost resistance. Production of H_2O_2 was revealed as peroxidase-dependent and catalase-inhibited fluorescence quenching of an extracellular probe (Pyranine).

All the plant genotypes responded to at least one elicitor, but the cell sensitivity showed a great age-dependent variability. Riboflavine and Mn^{2+} added in the incubation medium acted to some extent as primers for activated cell response, as well as a high Na^+ concentration. Cell rest condition, however, was not removed. Some quantitative features of responsive plant/elicitor combinations (dose-response relation and lasting time) have been recorded.

The complex PO/H_2O_2 of elicited cells could perform detectable lignin-like polymerization of an exogenous natural substrate (coniferyl alcohol). The time-course of pyranine oxidation and lignin-like polymer formation could be recorded by adopting a fluorimetric procedure that allowed sequential observations on the same cell sample. In one instance, the cell reaction seemed associated with the *in planta* host/parasite incompatibility.

Index Entries: Cell suspensions; *Capsicum* spp.; *Phytophthora* spp.; *Verticillium* sp.; plant/pathogen interactions; H_2O_2 release; Pyranine oxidation; hyphal wall elicitors; lignin-like polymerization.

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INTRODUCTION

Plant cell suspensions have proved able to produce a flux of H_2O_2 within a few minutes of exposure to preparations from pathogenic fungi, also active in phytoalexin elicitation (*1,2*). This response, which mimics the respiratory burst triggered by various chemical stimuli in mammalian cells (*3,4*), seems to be one of the most rapid reactions following the contact with fungal constituents or products, presumably after recognition of elicitors and prior to any transcriptional event (*5*).

The interest of the subject arises both from the direct or indirect defense potential of the system peroxidase/H_2O_2 and from a proposed role of H_2O_2 as a second messenger in subsequent defense response, such as phytoalexin production (*1*). Moreover, the reaction could be of biotechnological interest as a tool for monitoring the eliciting activity of fungal components/products and possibly the plant resistance to selected microorganisms. These aspects are the ultimate concern of our investigation, which has been focused on a complex of plant genotypes of *Capsicum* and of fungus species, representing several combinations of compatibility and both host and nonhost resistance.

MATERIALS AND METHODS

Plant Cell Culture

Cell suspensions were initiated from 30-d-old cotiledonary callus and grown in MS basal medium, enriched with 2,4D (1 mg/L). The cultures were transferred to fresh medium every 7 d, when the growth curve was approximating to the plateau. For the assays, the suspension was filtered through a 500-μm nylon mesh, centrifuged for 5 min at 150*g* and resuspended in fresh medium to a final concentration of 0.08 mL PCV/mL.

Elicitors

For preparation of crude fungal wall elicitors, mycelium from 21-d-old cultures was disrupted in a Potter homogenizer and paper filtered. The nonfiltrable debris was repeatedly washed with double-distilled water. Ten milligrams of dried material were finely ground in 1 mL of water, then were autoclaved for 3 min at 120°C and centrifuged for 10 min at 13,000*g*. The supernatant was filter-sterilized and kept frozen until used. In general, 10 μL in a final suspension volume of 1 mL were used for fluorescence transition assay.

Fluorescence Transition Assay

The release of H_2O_2 in elicited suspensions was monitored by the fluorescence quenching of the exocellular probe 8-hydroxypiren-1,3,6-trisulfonic acid, pyranine (Molecular Probes, Inc.) as described by others,

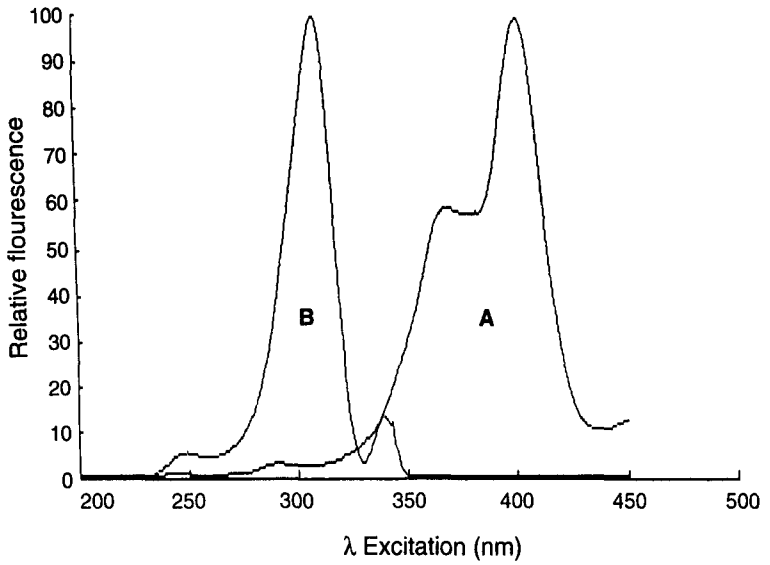

Fig. 1. Fluorescence excitation spectra of pyranine (**A**) and coniferyl alcohol (**B**). Emission wavelengths: 510 nm (**A**) and 680 nm (**B**).

with minor modifications (*1,5*). A stock solution of pyranine (0.2 mg/mL in double-distilled water) was diluted 1:5 just before use; 10 μL were added to 1 mL of the suspension into a 3-mL quartz cuvet, thermostatted at 25°C and mixed with a magnetic stirrer in the holder of a Kontron-SFM 25 spectrofluorometer. Excitation and emission wavelengths were set at λ_{ex} 405 nm and λ_{em} 510 nm: with 300 V high voltage level, the emission of the suspension attained relative fluorescence (RF) values of 1–3%; after adding the probe, the RF raised until 90–95%. Fluorescence was continuously recorded, starting 15 s after dispensing the elicitor.

Detection of Lignin-Like Polymers

In some experiments, after observing the elicitor effect, the spectrofluorometer was rapidly set at λ_{ex} 311 nm and λ_{em} 680 nm. In these circumstances, the fluorescence of the pyranine-enriched suspension was negligible. The supply of coniferyl alcohol at a final concentration of 30 μg/mL produced a strong light emission, corresponding to a peak, characteristic of this substance. In fact, the two compounds have quite distinct fluorescence parameters (Fig. 1).

A time-related decrement of this signal was assumed as index for the proceeding of lignin-like polymers formation. Because of the high turbidity of the cell suspension, the measurement of the increase in turbidity caused by the formation of polymerization products, as used by others with clean solutions (*6*), could not be adopted. The extent of coniferyl alcohol fluorescence decay seemed to represent a reliable indirect measure of polymerization, according to the results of experiments where pure horseradish

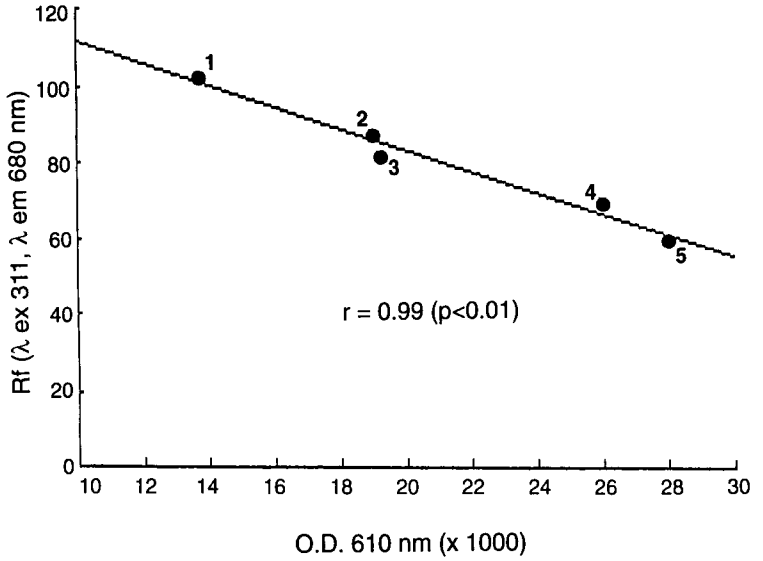

Fig. 2. Correlation between fluorescence and turbidity in water solutions of coniferyl alcohol plus horseradish peroxidase, after addition of H_2O_2. Fluorescence: λ_{ex} 311 nm, λ_{em} 680 nm. Turbidity as OD at λ 610 nm. 1 to 5 = 0, 12, 17.9, 34.7, 50.7 μM/mL H_2O_2 (final concentration). Each point represents the mean of five independent experiments.

peroxidase replaced the cell suspension and the fluorescence decay provoked by exogenous H_2O_2 correlated to a simultaneous increase of optical density at λ 610 nm (Fig. 2).

Plant and Fungus Species

Two genotypes of *Capsicum annuum*—the cv Quadrato d' Asti (QA) and a near isogenic line of the same cv (L23) (*7*)—plus one genotype of *Capsicum frutescens* (CF), were used. Elicitors were obtained from *Phytophthora capsici* (PC), *Phytophthora nicotianae* var. *parasitica* (PP), and *Verticillium dahliae* (VD). QA is susceptible to both PC and VD, whereas L23 and CF are resistant to PC and VD, respectively. All three *Capsicum* genotypes are resistant to PP (nonhost resistance).

RESULTS AND DISCUSSION

All the tested genotypes responded to at least one elicitor. When the cell response occurred, it was typically manifested through a decrease of fluorescence starting 2–4 min after the addition of fluorophore and proceeding at an increasing rate for a further 2–5 min (Figs. 3, 4). The dye was bleached instantaneously when a total of 15–20 μM H_2O_2 was dispensed to nonelicited cells, indicating that active oxidative enzymes were constitutively present. Both the elicited quenching and that caused by exogenous H_2O_2, were completely inhibited by previous addition of catalase

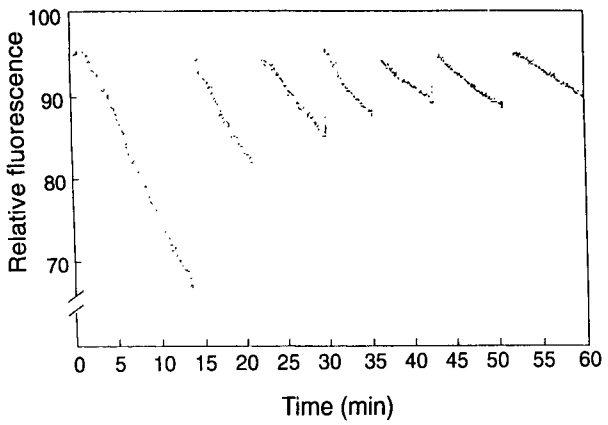

Fig. 3. Time-course of pyranine fluorescence decay in cell suspension of *Capsicum frutescens* elicited with a crude cell wall preparation of *Phytophthora nicotianae* var. *parasitica*. Elicitor concentration: 10 μL/mL. The successive linear traits were recorded after new probe addition (1/5 of the initial dose) and recalibration. Analogous figures were given by all the other responsive cell/elicitor combinations.

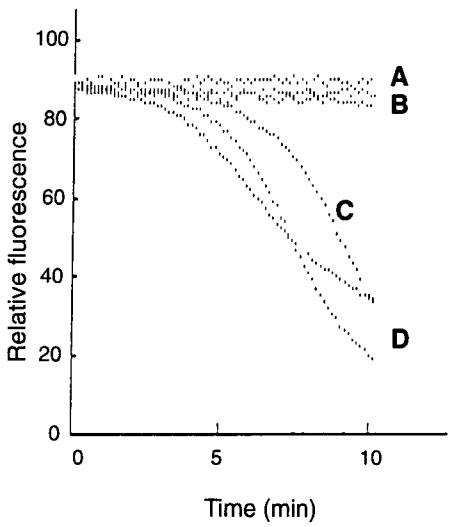

Fig. 4. Pyranine fluorescence decay in cell suspensions of the genotypes QA and L23 of *Capsicum annuum* treated with a cell wall preparation of *Phytophthora capsici*. (**A**) Nonelicited controls for both genotypes; (**B**) QA treated with elicitor 40 μL/mL; (**C,D**) L23 treated with elicitor 4 and 40 μL/mL.

(0.15 mg/mL, 50,000 U/mg protein). These observations confirm that the cell reaction was characterized by *de novo* generation of H_2O_2.

The cell sensitivity to the elicitor treatments showed great variability: Shortly after inoculation in fresh culture medium, cells were found insensitive; on the opposite, late in the growth curve the fluorescence transition occurred without addition of any elicitor (autoelicitation). A similar behavior has been described by others (*5,8*).

Table 1
Pyranine Fluorescence Decay in Elicited Cells of *Capsicum annuum* (L23): Effect of Priming Stimuli in Relation to the Cell Suspension Age

Priming stimulus[a]	Cell suspension age		
	24 h	48 h	72 h
None	—	+	++
Riboflavine, 40 m*M*	—	+	++
Mn^{2+}, 4 m*M*	—	++	++
Na^+ 50–100 m*M*	—	++	++
Nigericine, 0.7 m*M*	—	+	++
A-23187, 10 m*M*	—	+	++

[a]The priming stimulus was added 3–6 min before the elicitor (10 μL/mL of *Phytophthora capsici* cell wall extract). Without elicitor, no response was observed.

(—) Fluorescence decay never observed; (+) Fluorescence decay observed in 40–60% of the assays; (++) Fluorescence decay observed in more than 90% of the assays.

In our experience, the cells were susceptible to fungal elicitor not earlier than 48 h after subculture but, so far, selecting a suitable phase for reproducible results appeared problematic for all plant genotypes. Thus, efforts have been directed to the identification of molecules or conditions able to prime the cells for elicited probe transition. As summarized in Table 1, riboflavin and Mn^{2+} were effective to some extent, as well as a high Na^+ concentration in the incubation medium. Without effect appeared, instead, nigericyn and A-23187, ionophore of K^+/H^+ antiporter and of C^{2+} respectively, efficient primers for activation of mammalian cell systems (*9–11*).

The stimulating effect of pretreatments with riboflavin or Mn^{2+}, both known as cofactors of redox enzymes, is in agreement with the occurrence in plant of hydrogen peroxide generation brought about by either riboflavin or Mn^{2+}-dependent system (*12*). As for the priming obtained with high levels of Na^+, it parallels the effect of extracellular Na^+ on NADPH-oxidase activation in human neutrophils (*11*). The same enzyme system seems to be implicated in the generation of H_2O_2 in plant cells (*1,12*).

Anyhow, in our system priming seems not to remove the age-dependent cell rest condition, since it was clearly observed only close to the spontaneous appearance of cell sensitivity to elicitor (Table 1). As a matter of fact, the knowledge of treatments able to overcome the state of cell insensitivity to the elicitors, in addition to an improved understanding of the cell response, would represent a contribute to solve the problem of repeatable results that, as also documented in bibliography (*8*), is even more complex when several genotypes are studied. In spite of the limitations imposed by the age-dependent variability of cell sensitivity, we could observe some reliable features of the responsive cell/elicitor combinations,

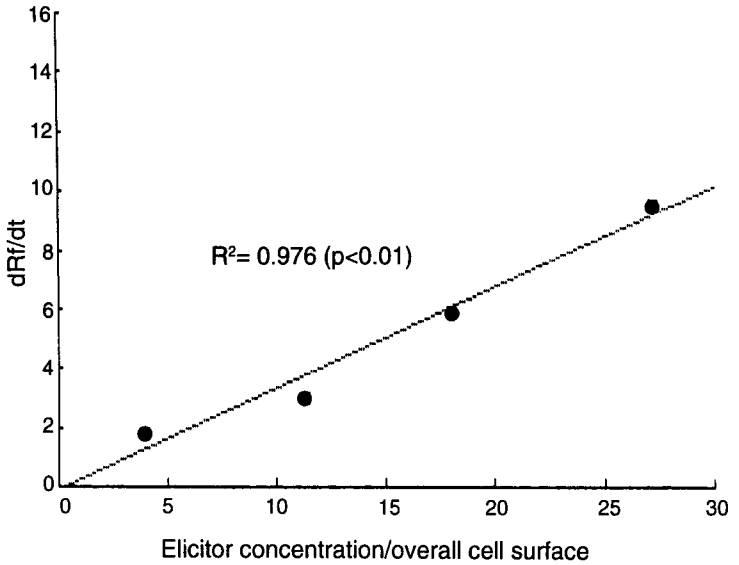

Fig. 5. Maximum speed of pyranine fluorescence decay in relation to the ratio elicitor concentration/overall cell surface. The data refers to cell subpopulations of L23 fractionated with 100–500 μm nylon filters, resuspended to the same concentration (0.08 mL PCV/mL) and treated with 25 μL/mL of *Ph. parasitica* cell wall elicitor. Theoretical values computed as function of the central values of filter fractionation classes. Each point represents the mean of four experiments.

relevant to the dose/response relationships and to the qualification of the observed response in the context of defense reactions, insofar as these are noticeable at cell suspension level.

In cell subpopulations obtained through filter fractionation of the suspension, the average cell (or cell aggregate) diameter influenced the kinetic of H_2O_2 release only quantitatively. Among several variables linked to the diameter, the ratio elicitor concentration/overall cell surface (theoretical values), resulted linearly correlated with the maximum speed of fluorescence decay, as it would be expected if the amount of response was determined by the number of stimulating molecules per exposed surface unit (or, perhaps, per receptor site) (Fig. 5).

By adding successive doses of the probe, the lasting time of hydrogen peroxide release was assessed: Once activated, probe oxidation progressed at slowly declining rate for over 60 min (Fig. 3). This seems to be a relatively long span in the timing of molecular events following elicitor treatment of plant cells (*13*) and could be of some interest in relation to the possibility that the released H_2O_2 itself or in combination with extracellular peroxidases, exerts defense actions such as plant cell death (commonly associated with the hypersensitive response) or restriction of growth and metabolism of microbes; or, also, the synthesis of products able to hinder the invasion of fungal pathogens *in planta*.

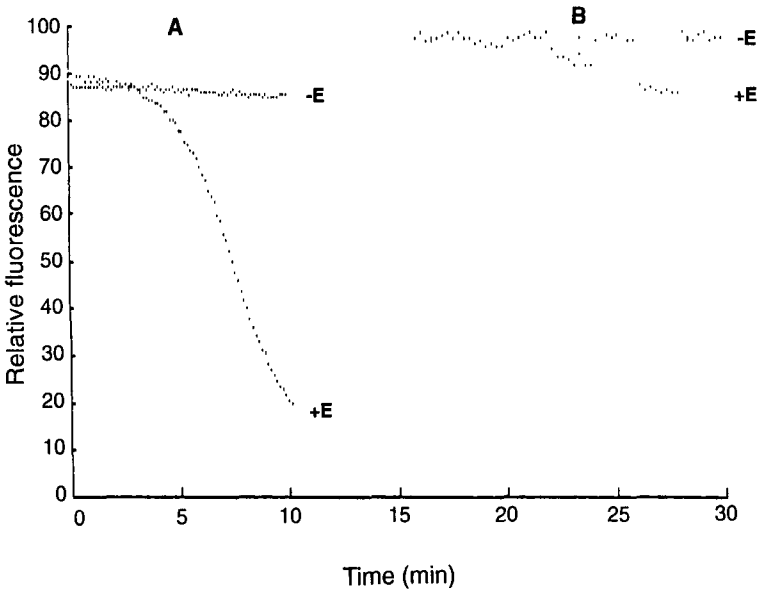

Fig. 6. Pyranine (**A**) and coniferyl alcohol (**B**) fluorescence decay in elicited (+E) and nonelicited (−E) cells of *Capsicum annuum*, line L23. −E: control; +E: 10 μL/mL of crude cell wall extract of *Phytophthora capsici*.

Induced lignification has been proposed as a mechanism of disease resistance (*14*). Lignin polymerization is initiated by peroxidase and hydrogen peroxide (*12*). In our experiments, the complex PO/H_2O_2 of elicited cells could perform detectable lignin-like polymerization of an exogenous natural substrate (coniferyl alcohol), as early as 10–15 min after elicitor addition (Fig. 6), whereas induced lignification, correlated with the increased peroxidase activity, has been reported to occur only some days after infection with different fungi (*6*). Our findings suggest that, at least in very early stages, lignin-like biosynthesis would be rather dependent on the secretion of hydrogen peroxide stimulated by the elicitor, in cooperation with the extracellular peroxidases normally present, possibly in the cell wall.

Finally, some data seem to indicate that the release of H_2O_2 might be associated with incompatible host/pathogen interactions. So, cells of the cv Quadrato d' Asti (susceptible to *Phytophthora capsici*) were unaffected when treated with doses of crude cell wall extract of the fungus, up to 10-fold higher than the threshold dose active on the cells of L23, a near isogenic line resistant to the same pathogen (Fig. 4).

As all the cells are likely to be supplied with the basic machinery for oxidative burst, this last result would implicate the recognition of eliciting molecules by the resistant plant genotype. It would contrast with the recent claiming of species-nonspecific activation of luminol-mediated chemiluminescence (a system for H_2O_2 detection, alternative to the pyranine fluorescence quenching), in cell suspensions of several *Solanaceae*, by hyphal wall components of different *Phytophthorae* (*15*). The compatibility would

be determined by the production of a fungus race-specific inhibitor of induced chemiluminescence and of the associated hypersensitive cell death. Coming back to our results, it cannot be ruled out that the differential response of the two plant genotypes to the same elicitor is merely indicative of a different state in cell responsivity.

CONCLUSIONS

In the present work, the quick release of hydrogen peroxide as response to treatments with cell wall preparations of both parenchimatic (*Phytophthora* spp.) and vascular (*Verticillium* spp.) fungi has been observed in cell suspensions of several genotypes of *Capsicum* spp. A quantitative relation linking the response to the elicitor dose and the cell surface, which could suggest the involvement of the ratio between stimulus molecules and receptor sites, has been established. Moreover, we have shown that the release of H_2O_2, as for intensity and lasting time, is able to drive processes typical of the defense response, such as the synthesis of lignin-like compounds, much earlier than so far described and prior to the rise of PO activity, previously associated to the incompatible interactions. In some instances, a relation between the kind of host/parasite interaction and cellular response have seemingly been outlined.

The system seems suitable to investigate the first molecular events in the plant/pathogen interactions; however, throughout the research, we have faced the major problem of a varying cell sensitivity. The assessment of age dependence for cell responsivity proved of limited help for its standardization, namely while handling different batches of several plant genotypes. It may be that the key lies in the increased understanding of the endogenous and exogenous factors that condition the cell suspension for a specific response. In this regard, the great deal of knowledge acquired on the regulation of respiratory burst in mammalian cell systems, whose analogy with plant cell suspension has emerged to some extent also during this work, should be useful.

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