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Merocyanine 540 as an optical probe to monitor the effects of culture filtrates of *Phytophthora cactorum* on apple cell membranes

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The response of apple (*Malus domestica*) cells to valinomycin, gramicidin and culture filtrates (CF) from an avirulent and from virulent strains of *Phytophthora cactorum* was monitored by measuring changes in fluorescence of cells stained with Merocyanine 540, an optical probe for changes in transmembrane electrical potential (PD). Cells of MM.106 rootstock (susceptible to *P. cactorum*) exposed to either valinomycin or to CF from virulent strains of *P. cactorum* showed a decrease in fluorescence, whereas cells exposed to gramicidin showed an increase in fluorescence. The response of cells to fungal culture medium (FCM) was similar to the response to CF from an avirulent strain of the fungus. These results support the feasibility of using Merocyanine 540 as a probe of PD changes in apple cells and suggest a role for *P. cactorum* toxin in disease development. This research opens up the possibility of using fluorescence measurements as a screening system for identifying resistance in apple germplasm to *P. cactorum* and for using the toxin as an in vitro selective agent.

Key words: Malus domestica; fluorochrome; cytofluorometry; *Phytophthora cactorum*; culture filtrate

Introduction

Crown or collar rot caused by *Phytophthora cactorum* (Leb. et Cohn) Schroet. is considered one of the most important diseases of apple [1]. The increased incidence and severity of this disease, observed during the last 30 years in commercial fruit tree orchards, coincides with profound changes in horticultural practices [2] and with the development of several apple rootstock breeding programs for disease control [3]. The relative resistance of various apple rootstocks can be influenced by different factors such as the *Phytophthora* spp. to which the rootstocks are exposed, the virulence of the strain of the pathogen [4], the season of the year [5,6] and the different environmental conditions in which apples are grown [7]. Progress has been made in developing inoculation techniques that give a close evaluation of plant resistance [8,9]. Recent studies evaluated apple rootstock resistance to *P. cactorum* by incubating in vitro proliferating shoots on media supplemented with the fungal culture filtrate [10]. The reduction in shoot proliferation and shoot fresh weight observed with culture filtrates of strains MM.106 and M.26 paralleled the field behavior of the two rootstocks, which suggest involvement of a toxin in disease development.

A rapid system for evaluating resistance in peach to *Xanthomonas campestris* pv. *pruni* was described by Hammerschlag [11]. In this system, peach mesophyll cells, stained with the optical probe Merocyanine 540 (MC-540) [12,13], were monitored for changes in membrane potential (PD) following exposure to the culture filtrate of the bacterium. This system proved to be useful for

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screening purposes and helped to establish a role for a toxic metabolite (present in culture filtrate of *X. campestris* pv. *pruni*) in bacterial leaf spot of peach.

The objectives of this study were to determine if the leaf mesophyll cell-MC-540 system mentioned above could be adapted for apple cells and if so, to utilize this system to provide additional evidence for the role of a toxic metabolite(s) in culture filtrates of *P. cactorum* in crown rot development, as suggested by the study of Rosati et al. [10] and to determine if the toxic metabolite acts at the membrane level.

Materials and Methods

Culture filtrate (CF)

Phytophthora cactorum virulent strains P39 (highly virulent, isolated from MM.106), NY 007, NY 184 and NY 120 (highly virulent, isolated from MM.106, MM.26 and MM.111) were obtained from Dr. H.S. Aldwinckle, NYSAES, Geneva, NY and Dr. J.L. Maas, USDA-ARS-PSI, Beltsville, MD. One avirulent strain on apple (Ph84) was obtained from Dr. G. Cristinzio, Plant Pathology Department, University of Naples, Italy. The various strains were grown on a solid medium (containing Difco Bacto-agar 15 g/l, Campbell V8 Juice 200 g/l and $CaCO_3$ 3 g/l) for 10 days in darkness at 25°C. A standard inoculum was prepared by adding 10 ml of sterile double-distilled water to a Petri dish containing the fungus; after brushing lightly with a scalpel, 1 ml of suspension was withdrawn and used to inoculate 150 ml of liquid culture medium (containing KH_2PO_4, 0.5 g/l; $MgSO_4 \cdot 7H_2O$, 2.0 g/l; asparagine, 1.0 g/l; thiamine HCl, 0.01 g/l; Difco Yeast extract, 5 g/l and glucose, 25 g/l) [14] with a final concentration of approximately 5×10^5 spores/ml. After 15–20 days of incubation with shaking in darkness at 25°C, the culture medium was filter-separated from the fungal mat using Whatman No. 3 paper. The culture filtrates (CF) obtained from P39 and Ph84 were tested separately, while the other virulent strains were combined in equal parts. Filtrates so prepared were freeze-dried and stored at −20°C until used.

Apple shoot proliferation

Shoots of MM.106 apple rootstock, highly susceptible to *P. cactorum* and of MM.111, resistant to the disease [8,7] were grown in vitro on a standard proliferation medium containing Murashige and Skoog (MS) salts [15], 4.44 μM 6-benzyladenine (BA), 0.49 μM indolebutyric acid (IBA), 1.3 μM gibberellic acid (GA_3), 1.2 μM thiamine-HCl, 0.56 mM myo-inositol, 87.6 mM sucrose and 0.65% (w/v) Difco Bacto-agar. The pH was adjusted to 5.8 with 0.1 N KOH prior to autoclaving for 20 min at 121°C and 1.1 $kg \cdot cm^{-2}$. Cultures were maintained at a constant temperature of 25°C ± 2 C, under a 16-h per day photoperiod provided by warm white fluorescent lamps at a photosyntetic photon flux (PPF) of 37.5 $\mu mol\ s^{-1} \cdot m^{-2}$.

Apple mesophyll cell isolation and staining

Leaf tissue (20 mg) from 25- to 30-day-old proliferating shoots was chopped into small pieces and incubated overnight in 10 ml of filter-sterilized enzyme solution. Three concentrations of cellulysin (0.1, 0.5 and 1.0% w/v) in factorial combination with three concentrations of macerase (0.1, 0.5 and 1.0% w/v) (both Calbiochem-Boehring Corporation, La Jolla, CA) and with three osmotic levels (0.25, 0.45 and 0.65 M) of sucrose (pH 5.7) were tested for MM.106 tissue digestion. Digested cells were filtered through a 94-μm and then a 43-μm mesh stainless steel screen and centrifuged at 100 × *g* for 3 min. The pellet was resuspended in 5 ml of washing solution containing half-strength MS salts (MS/2) and the same sucrose concentration as the digesting solution (pH 5.7) and recentrifuged as described above. The washed cells were incubated for 10 min in 1 ml of 5.7 μM merocyanine 540 (MC-540; Eastman Kodak Company, Rochester, NY) dissolved in 0.01 M MES (2[*N*-Morpholino]ethanesulfonic acid) buffer (pH 6.0) containing 0.6 M sorbitol [11]. The cells were washed twice with the same washing solution and adjusted to 1×10^5 cells/ml for the different measurements.

Fluorescence measurements of MM.106 apple cells, incubated over a 24-h period in the different washing solutions, were made to determine the sucrose concentration needed to maintain a stable

Table I. Response of apple cells (as Merocyanine 540 fluorescence emission) to three osmotic sucrose concentrations. Twenty-five cells per treatment were measured with four replications (values ± S.E.).

Sucrose (M)	5 min	3 h	24 h
0.25	56.6 ± 6.2	62.6 ± 6.4	51.8 ± 2.1
0.45	53.1 ± 3.2	52.2 ± 1.9	54.1 ± 3.9
0.65	46.7 ± 4.5	42.0 ± 3.8	33.4 ± 1.3

fluorescence emission. Membrane fluorescence changes induced by 1 mM gramicidin S (Sigma, St. Louis, MO) and 1.0 mM valinomycin (Sigma) compounds known to alter PD at this concentration [16–20], were observed to verify the capacity of MC-540 to detect apple cell membrane alteration and to aid in the interpretation of the results obtained using the CF.

Cytofluorometry

Fluorescence measurements were performed with a Zeiss Photomicroscope III equipped with epifluorescence and containing an HBO 100 mercury source for excitation, a BP 546/10 exciter filter and an LP 590 barrier filter. Fluorescence emission was measured with a Hamamatzu photomultiplier tube type R777. The first fluorescence measurement on each cell was made 5 min after the treatment started, then repeated after 3 h and after 24 h. Twenty-five single cells per treatment were measured with four replications. Data are expressed as fluorescence emissions of treated cells compared with controls (cells in MS/2 + 0.45 M sucrose).

Results

Enzyme and washing solution

The combination 0.5% cellulysin and 0.5% macerase resulted in the highest single cell yield from MM.106 leaf tissue (Table I). MM.106 cells incubated in 0.45M sucrose did not exhibit changes in fluorescence over time. The same cells exposed to other concentrations of sucrose exhibited changes in fluorescence.

Response to valinomycin and gramicidin

Fluorescence emission of MM.106 cells decreased within 5 min after exposure to 0.1 mM valinomycin and continued to decrease after 3 and 24 h (Table II). NM.106 cells showed an increase in fluorescence 5 min and 3 h after exposure to 1.0 mM gramicidin S and this increase leveled off after 24 h.

Response to CF of P. cactorum and to fungal culture medium (FCM)

MM.106 cells (highly susceptible to *P. cactorum*), incubated in CF of P39 (virulent strain of *P. cactorum*), showed a sharp increase in fluorescence within 5 min after exposure and then a decrease after 3 and 24 h (Table II). Similar cells, when treated with CF of Ph84 (an avirulent strain of the fungus) showed a slight increase in fluorescence after 5 min followed by a small decrease in fluorescence after 3 and 24 h. The FCM induced only slight changes in fluorescence at 5 min, 3 h and 24 h. The response to FCM was similar to the response to CF of the avirulent strain of the fungus.

Table II. Response of apple (MM.106) cells (as Merocyanine 540 fluorescence emission) to valinomycin, gramicidin, culture filtrates (CF) of *Phytophthora cactorum* and fungal culture medium (FCM). Twenty-five cells per treatment were measured with four replications (% of control ± S.E.).

Treatment	5 min	3 h	24 h
Valinomycin (0.1 mM)	81.0 ± 5.4	73.0 ± 5.8	81.2 ± 6.6
Gramicidin (1.0 mM)	141.5 ± 9.6	161.2 ± 10.7	168.7 ± 8.5
CF-virulent strain P39	125.5 ± 3.4	83.0 ± 5.4	67.7 ± 10.6
CF-avirulent strain Ph38	104.0 ± 4.2	101.7 ± 5.6	91.5 ± 5.3
FCM	101.0 ± 6.9	93.0 ± 4.8	94.2 ± 8.9

Table III. Response of apple (MM.106 and MM.111) cells (as Merocyanine 540 fluorescence emission) to combined culture filtrates of several virulent strains of *Phytophthora cactorum*. Twenty-five cells per treatment were measured with 4 replications (% of control ± S.E.).

Rootstock	5 min	3 h	24 h
MM.106-susceptible to *P. cactorum*	123.2 ± 3.5	78.7 ± 6.6	75.0 ± 7.6
MM.111-resistant to *P. cactorum*	98.7 ± 3.7	96.2 ± 6.2	95.2 ± 4.5

Response of MM.106 and MM.111 cells to combined CF from P. cactorum

MM.106 cells, incubated in combined CF of 3 virulent strains of *P. cactorum* responded with similar changes in fluorescence as cells exposed to CF of virulent strain P39 (Tables II and III). MM.111 (resistant to *P. cactorum*) cells did not show changes in fluorescence when exposed to the combined CF.

Discussion

Fluorochromes are being used more frequently to check cell viability and membrane polarization [11,21]. The present study suggests the possibility of detecting PD changes in apple cells by monitoring fluorescence emission of cells stained with MC-540 [13].

Cell preparation and culture conditions were defined for fluorescence measurements. Changes in fluorescence emission induced by different ionophores were monitored to verify the MC-540 sensitivity in detecting apple cell membrane alteration. Addition of 0.1 mM valinomycin induced a decrease in cell fluorescence with respect to the control solution. Valinomycin has been reported to enhance the K^+ permeability of the plasmalemma, inducing an increase in PD, followed by a stimulation of ATPase activity [22,23]. Changes in PD alter some of the structural and electrical properties of the membrane, that in turn affect the orientation of the dye in the membrane [13]. The optical changes of MC-540-stained apple cells induced by valinomycin can be related to an increase of dipolar chromophore molecules oriented parallel to the bilayer lipid membrane and the formation of dimer non-fluorescent molecules.

Gramicidin S induces a decrease in PD (20). The alteration induced by this compound in apple cell membranes resulted in an increase of fluorescence emission probably linked with an increase of dye monomer molecules oriented perpendicular to the membrane.

The same approach was used to test the effect of FCM and CF from virulent and avirulent strains of *P. cactorum* on cells of MM.106, an apple rootstock susceptible to *P. cactorum*. The CF from the virulent strain of the fungus was the only treatment able to induce a significant change in fluorescence emission (a rapid membrane depolarization followed by a hyperpolarization reaction). The same response was obtained using combined CF from three additional virulent strains of *P. cactorum*, which confirmed the hypothesis that other virulent strains of the fungus could induce similar membrane changes. The observed changes in fluorescence emission can be explained by the presence in the CF of a particular metabolite(s) able to induce changes in PD. The hypothesis that this metabolite is involved in disease development can be supported by the limited cell membrane fluorescence alterations induced by the CF of the avirulent strain of *P. cactorum* and by the FCM alone.

Compounds toxic to apple and tomato leaves have already been extracted from *P. cactorum* CF [14], but no specific compound has been isolated and studied for its specific action on membrane polarization. Additional studies are now in progress to verify this possible host-pathogen interaction in the apple *P. cactorum* system. MC-540 has proven to be a useful fluorochrome to study apple cell-membrane alterations and we are currently extending the application of this technique to disease resistance screening.

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