In Planta and *in vitro* Interactions between *Phomopsis (Diaporthe)* Isolates and Sunflower

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Abstract

In this study, we determined the amount of H_2O_2 released by sunflower callus cultures challenged by both crude hyphal wall extracts and culture filtrates of 26 *Phomopsis* isolates from sunflower of worldwide origin (Argentina, France, Italy, Yugoslavia, Rumania). The amount of H_2O_2 released by callus cultures and the production time-course response, however, did not correlate with both the amount of electrolytes released by sunflower leaf disks treated with crude culture filtrates and the results of pathogenicity tests on sunflower seedlings. Only few isolates induced a time-course response indicative of an oxidative burst. This would suggest that elicitors extracted from hyphal walls are not involved in this host-pathogen recognition system and toxic metabolites produced by *Phomopsis* in liquid cultures are not pathogenicity factors.

Keywords: *Phomopsis helianthi*; *Diaporthe helianthi*; *Phomopsis* stem canker; sunflower; pathogenicity; reactive oxygen species; hydrogen peroxide

INTRODUCTION

Phomopsis helianthi (perfect stage: *Diaporthe helianthi*) Munt.-Cvet. *et al*. (MUNTAÑOLA-CVETKOVIC *et al*. 1981) is the causal agent of leaf necrosis and stem canker of sunflower (*Helianthus annuus* L.). The most severe fungal disease of sunflower in many European countries (Yugoslavia, Hungary, Rumania, France and Austria), in Argentina and Brazil, but not in Italy where, however, climatic conditions are suitable for development of natural infections. Recently, a great genetic variability has been found among isolates of *Phomopsis*/*Diaporthe* from sunflower of worldwide origin using both molecular and biochemical methods (VANNACCI *et al*. 1996; VERGARA *et al*. 2001; PECCHIA *et al*. 2002; VANNACCI *et al*. 2002). The results of these studies indicate that no one of the *Phomopsis* biotypes found on sunflower in Italy can be refered to *Phomopsis (Dhiaporthe) helianthi sensu stricto*. The pathogenicity of these biotypes on sunflower is not known and pathogenicity test using conventional inoculation methods have given inconsistent results (PENNISI *et al*. unpubl. data). Resistance factors to the stem canker have been found in sunflower and the use of resistant cultivars has proven to be the most effective mean to control this disease, in France and other European countries (LANGAR *et al*. 1997). In this study we tested the *in vitro* interaction between different 26 *Phomopsis* isolates from sunflower and sunflower tissue explants in order to point out physiological events such as the production of reactive oxygen species (ROS) related to defense responses (DALY 1981; BAKER & ORLANDI 1995). To this purpose both crude hyphal wall extracts (CHWE) and culture filtrates of *Phomopsis* isolates were used to challenge sunflower tissues.

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MATERIALS AND METHODS

Isolates. Twenty six *Phomopsis* isolates from sunflower of different geographic origin (France, Italy, Argentina, Rumania and Yugoslavia) were characterized. Fungal were maintained as mass-transfer on potato-dextrose-agar (PDA, Oxoid), in Petri dishes.

Pathogenicity tests. Five sunflower cultivars (Laura, Solegen, EG11-2000, EG15-2000 and ALA) were used in each pathogenicity test. Four 6–8-week-old plants per isolate, grown under greenhouse in 30 cm-diameter pots, were inoculated with mycelial mats from 7 days old cultures grown on PDA. Inoculum was introduced on the stem, into a hole made with a sterile dissecting needle between the second and the third leaf node. In a separate test 50 μl of a mycelial suspension in sterile water, obtained by scraping superficially 7 days old cultures grown on PDA, were injected in the stem of 6-wk-old sunflower seedlings. Control plants were inoculated with sterile water. After inoculation wounds were wrapped with sterile moistened cotton wool and covered with parafilm foil to avoid desiccation. The length of brown lesion on the stem was measured 25 days after inoculation. In an additional test 10 days old sunflower seedlings grown in 30 cm-diameter pots (10 seedlings per pot) under greenhouse (25 $\pm$ 2°C, 12 h day/night, light intensity about 8000 lux) were inoculated by inserting a toothpick superficially colonized by fungal mycelium into the stem 1 cm below the cotyledon, as described by KEELING (1982). Control plants were inoculated using sterile toothpicks. Lesions developed on inoculated stems were measured 10 days after inoculation. Each test was performed at least twice.

Culture filtrates. Erlenmeyer flasks (500 ml) containing 200 ml of potato dextrose broth (PD, Oxoid), were inoculated with mycelial plugs of Phomopsis isolates. Flasks were incubated at 26 $\pm$ 1°C for 21 days on an orbital shaker at 60 rpm, in the dark. Culture filtrates were sterilized through the 0.22 μm Millipore filters.

Crude hyphal wall extracts (CHWE). CHWE were obtained from *Phomopsis* colonies grown in liquid culture for 21 days, according to the procedure described by MOZZETTI *et al*. (1997). Mycelium and filtrates were separated by vacuum filtration (Whatman No. 1). The mycelium was washed ten times with sterile distilled water and to each sample containing 1.5 g of mycelium 30 ml of sterile H_20_2 were added. Samples were homogenized and centrifuged (13 800 g for 20 min). After centrifugation the supernatant was discarded. The pellet was dried (30°C) overnight, ground in a mortar, suspended in distilled water (1:50 p/v) and clarified by centrifugation (1000 g for 10 min). The pellet was discarded and the supernatant, containing CHWE, was sterilized by filtration (1.2 μ, 0.45 and 0.22 Millipore filters).

Plant callus cultures. Achenes of the cv. Laura were washed with 70% EtOH, sterilized in 4% (w/v) sodium hypochlorite solution for 30 min and rinsed repeatedly with sterile distilled water. Seeds were aseptically germinated at 27°C in the dark in tubes containing B5 medium (GAMBORG *et al*. 1968) added of 0.5% (w/v) sucrose and 0.8% (w/v) agar (Difco). Epicotyl explants were isolated from 7 days-old sterile seedlings and micropropagated using HaR medium to produce callus (PATERSON-ROBISON & DOUGLAS 1987). The callus was subcultured every 4 weeks.

Fresh healthy leaves were collected from 30 day-old plants of *Daucus carota* L., sterilized in 20% sodium hypochlorite solution by immersion for 20 min and rinsed repeatedly in sterile distilled water. Leaf discs made with a cork-borer (5 mm diameter) were cultured in 0.8% w/v agarized Gamborg's medium (B5) (GAMBORG *et al*. 1968) pH 5.5–5.7 supplemented with 2,4-dichlorophenoxyacetic acid (2, 4 D), 6 benzylaminopurine (6, BAP) and 20 g/l sucrose. Explants, placed in plastic Petri dishes, were incubated in growth-chamber at 25°C in the dark to induce callus formation. The callus was maintained by subculturing at 4-weeks intervals on fresh multiplication medium.

Hydrogen peroxide detection and quantification. Hydrogen peroxide (H_2O_2) production was determined after incubation at 24°C of 0.5 g of host (sunflower) and non-host (carrot) callus in 1 ml of culture filtrate or CHWE of *Phomopsis* isolates, at 3, 5, 10, 30, 60, 120, 180, 240, 300, and 360 min intervals. Controls were treated with sterile distilled water. At the end of each time interval, callus was separated and suspension was weighed and centrifuged (6000 rpm for 5 min). To determine H_2O_2 concentration, 500 μl of xylenol orange solution were added to 500 μl of supernatant and the suspension incubated for 45 min in the dark, according to the method of JIANG *et al*. (1990). The increase in absorbance was determined spectrophotometrically (560 nm) and the amount of H_2O_2 produced (expressed as pmol/ml) was obtained using the following equation: $y = 0.265 + 0.005x - 4.475 \times 10^{-6}x^2 + 9.167 \times 10^{-10}x^3$, where x is the absorbance value and y is the H_2O_2 concentration (BADIANI pers. commun.).

Electrolyte leakage. Electrolyte leakage was determined using explants of sunflower leaves, according to the method of PENNISI and GRANITI (1987). Leaf

discs, about 5 mm in diameter, were immersed in the culture fluids and incubated at 24°C. The conductivity of the solution (expressed as μS/cm), was measured at 30 to 60 min intervals. All experiments were repeated three times, each with four replicates.

RESULTS

The results of pathogenicity tests were not reproducible (data not shown). Conversely, *in vitro* tests were significative and reproducible. Only the results of few experiments are reported as on example. Both culture filtrates and CHWE of all *D./P. helianthi* isolates induced significantly increase in the production of H_2O_2 by sunflower callus. The H_2O_2 production was significantly higher compared to the control 120–180 min after treatment with both the culture filtrates and the CHWE (Figure 1). Conversely, the amount of H_2O_2 produced by non-host (carrot) callus elicited with culture filtrates and CHWE was not significantly different from control. Crude culture fluids of all isolates affected cell membrane permeability on the of explants of sunflower leaves as shown by the increase of electrolytes leakage in conductivity tests presented (Figure 2). The results of conductivity tests on sunflower leaf explants treated with culture fluids of twenty six different *Phomopsis* isolates. Some isolates, such as the isolate 70/96 from Yugoslavia induced a significantly higher amount of electrolyte leakage. However this result did not correlate with pathogenicity tests or the amount of H_2O_2 produced by calli treated with culture filtrates or CHWE.

DISCUSSION

The methods of artificial inoculation with anamorph of *Diaporthe*/*Phomopsis* utilized for pathogenicity studies have not shown significative differences among *D./P. helianthi* isolates. The present results are in agreement with the variability of *D./P. helianthi* reported by VUKOJEVI *et al*. (1996). It is possible that the various techniques of artificial inoculation used to

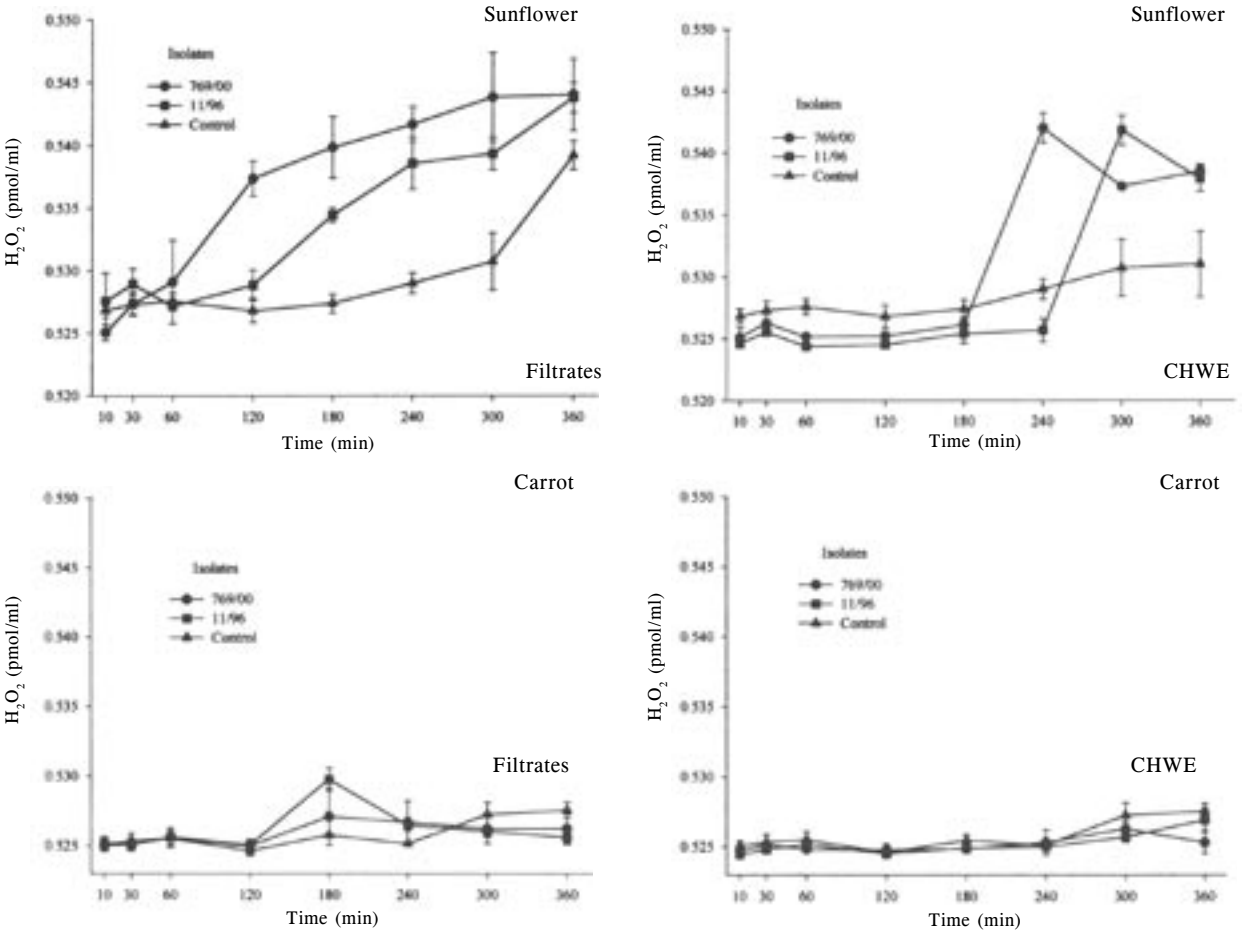

Figure 1. H_2O_2 released by host (sun ower) and non-host (carrot) callus elicited with culture filtrates or crude hyphal wall extracts (CHWE) of *D./P. helianthi* isolates. Error bars = standard deviation

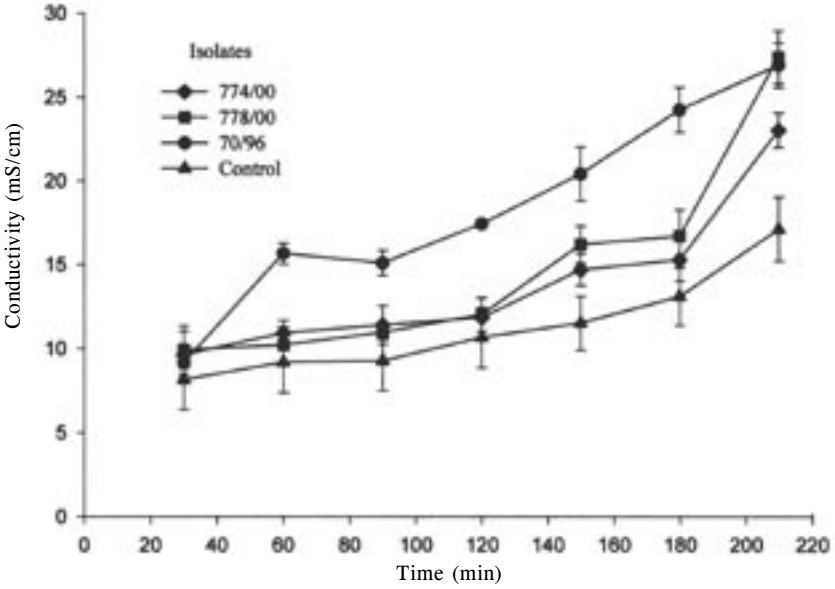

Figure 2. Electrolyte leakage from explants of sun ower leaves treated with culture filtrates of *D./P. helianthi* isolates. Error bars = standard deviation

pathogenicity tests measure different resistance factors (VIGUIÉ *et al*. 1999). The role of propagules of *Diaporthe/Phomopsis* (β-conidia, ascospores) regarding to tissue penetration and infection development is not yet clear. In the countries where stem canker of sunflower causes serious damages, the perithecia develop during autumn and winter on plant debris. At springtime, when the climatic conditions promote in the field increase of disease incidence, mature ascospores infect sunflower plants. Unfortunately perithecia and ascospores of *D. helianthi* are very difficult to produce *in vitro* to experimental purposes (VUKOJEVI *et al*. 1995). In conclusion, according to the results, no one of the artificial methods used in this studies for pathogenicity tests appears suitable for screening sunflower varieties for resistant to stem blight.

It is generally assumed that a host-pathogen system is incompatible if both the oxydative burst and the electrolyte leakage by plasmalemma peack 30–60 min after elicitation. In this study both H_2O_2 production and electrolyte leakage increased significantly 120–180 min after treatment, but both did not correlate with the pathogenicity on host plant. This results would suggest that in the *D./P. helianthi* sunflower-pathosystem the disease it is independent of H_2O_2 production and electrolyte leakage and that crude hyphal wall extracts do not contain elicitors involved into host-pathogen recognition mechanism. Moreover, it can be concluded that toxic metabolites produced by *D./P. helianthi* isolates in culture are not pathogenicity factors.

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