Host-Pathogen Interaction in Dual Cultures of *Helianthus annuus* L.-*Sclerotinia sclerotiorum* (Lib.) De Bary.[1]

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ABSTRACT

A new system for *in vitro* dual culture of the pertotroph fungus *Sclerotinia sclerotiorum* and a callus derived from cotyledons of *Helianthus annuus* is described. The dual culture was accomplished by surrounding a piece of mycelium with a plastic ring. As in the case of an intact plant stem, the lack of a mechanical barrier did not restrict fungal invasion, causing the complete deterioration of the callus after 72 hours. However, an increase of phenolic compounds was detected both in the inoculated zone and the rest of the callus. This biochemical reaction to the infection is similar to that described for the whole plant. Isochlorogenic acid showed the highest increase. The increment in phenolic concentrations was lower than in the whole plant, never attaining a level that could inhibit fungal invasion.

RESUMEN

En este trabajo se describe un nuevo sistema para el cultivo dual *in vitro* de un hongo pertótrofo *Sclerotinia sclerotiorum* y un callo derivado de cotiledón de *Helianthus annuus*. Dicho cultivo dual se logró circunscribiendo el crecimiento del micelio del hongo mediante un anillo de plástico. A diferencia de lo que sucede en la planta entera, la ausencia de una barrera mecánica no detuvo la invasión fúngica causando el completo deterioro del callo a las 72 h de la inoculación. Sin embargo, tanto en la zona inoculada como en la adyacente se produjo un incremento de los compuestos fenólicos, en especial de ácido isoclorogénico. Esta reacción bioquímica es similar a la descrita para la planta entera. De todos modos, el incremento en la concentración fenólica fue menor al producido en la planta entera.

INTRODUCTION

The stem rot disease of sunflower (*Helianthus annuus* L.) caused by the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary has widespread distribution in Argentina and other sunflower-producing countries (20, 21, 23). Complete resistance among lines and hybrids does not exist, buy rather only different levels of susceptibility (9).

Previous research has shown that in less-susceptible sunflower plants there are two defense mechanisms: a biochemical one, related to the metabolism of phenols (1), and a mechanical one, connected to the histology of the stem (2).

In vitro cell and tissue techniques have been used by different authors in studies of host-pathogen interactions (5, 10). The use of such simplified systems allowed the study of the interaction without complicating of correlative phenomena. In this way, cell lines with resistance to a determined pathogen have been isolated (3, 13), the products of the interaction characterized (8) and the regeneration of resistant individuals obtained from toxin-resistant cell lines (4).

Due to the special characteristics of the dual cultures of the host tissues and any of their pathogens, these kind of cultures have been carried out with obligate parasites (13, 14). However, the development of this technique with another kind of pathogen could improve the knowledge of host-pathogen interaction. This paper describes the achievement of *in vitro* dual culture of *H. annuus* L. tissues and the facultative fungus *S. sclerotiorum*. The existence of the same biochemical reaction for the infected whole plant is also established (1).

MATERIALS AND METHODS

Callus cultures were derived from cotyledons of the sunflower line 500. These calli have been subcultured over four years. The medium composition, procedure and growth conditions were presented in an earlier paper (21).

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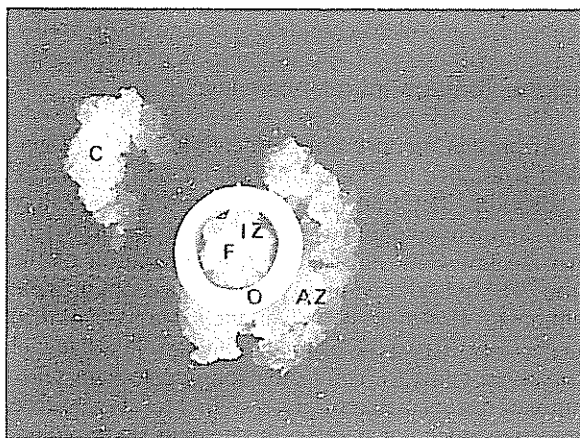
The virulent line no. 16 of the fungus *S. sclerotiorum*, kept at the *Centro de Ecofisiología Vegetal (Buenos Aires, Arg.)*, was employed in the inoculation. The pathogen was grown on semisolid Hancock medium (11). The inoculum were discs 0.5 cm in diameter of the medium plus the fungal mycelium, cut from the external portion of a seven-day-old colony. The pathogen growth conditions were 24+1°C and a photoperiod of 16 under low light intensity (0.95 $W.m.^{-2}$).

In order to restrict fungal growth, the inoculation was carried out in the center of a 1 cm diameter plastic ring, in such a way as to keep the mycelium in close contact with the callus (Fig. 1). Both the inoculated and the control calli were grown in the same flask (160 ml capacity), with 30 ml of nutrient medium. The extraction of the total soluble phenols was performed 72 h after inoculation. This was carried out by crushing the calli in a pot with 80% methanol (5 ml/g fresh weight of callus) and the boiling the extracts for 2 min, and then centrifuging (3000 rpm) them for 15 minutes. The total phenols of the supernatants were colorimetrically determined at 660 nm after reacting with Folin-Ciocal tea reagent (18).



C: control; IZ: inoculated zone; AZ: adjacent zone; O: plastic ring; F: fungus.

Fig. 1. *H. annuus* callus inoculated with *S. sclerotiorum* mycelium

A standard curve of chlorogenic acid was run for the calculation of the phenol concentrations. The methanolic extracts of both control and inoculated calli were analyzed by two-dimensional descending paper chromatography for the qualitative determination of the phenols. Samples were spotted on Whatman no. 1 paper and the solvent systems were:

- n-butyl-alcohol/glacial-acetic acid/water (6:1:2) and
- a 2% aqueous solution of acetic acid.

Phenols were detected under a UV light (366 nm) before and after fuming the chromatograms with ammonia and characterizing them by the Rf values, UV spectral analysis and co-chromatography with authentic markers (22).

Finally, extracts from both inoculated stem portions of sunflower plants and from calli were co-chromatographed. The experiments were repeated twice and each treatment consisted of at least 30 replicates. Results of the quantitative analysis were submitted to an analysis of variance test.

RESULTS

The inoculation of the callus with the pathogen mycelium provoked changes in its aspect and structure. Color changed from whitish-grey to brown, and the callus acquired a soft and watery consistency. These changes allowed the separation of two neat zones: the inoculated one (IZ) and the rest of the callus (AZ). Due to the virulence of the line, a prolonged dual culture period of more than 72 h caused a complete destruction of the callus. The presence of the fungus caused a significant increase of soluble phenols (F=24.6) in both the IZ and AZ zones (Table 1). The chromatographies of the methanolic IZ extracts revealed the presence of several phenolic compounds (Table 2).

In the chromatograms of the controls, some of the spots were sporadically detected. However, those present were the same as those that appeared in the IZ chromatograms. This difference could be due to the small amount of some compounds in the controls just below the resolution of the detection methods employed in the analysis. Spots were arranged in two groups: 1 and 2; 3, 3'and 4. Spots 1 and 2 gave a light

Table 1. Phenols concentration in the different zones of *H. annuus* calli inoculated with *S. sclerotiorum*: Results are expressed as phenols (μ g) per fresh weight: of callus (g).

Callus zone	(Phenols)
Z	1 367[a]
AZ	787[b]
Control*	470[c]

* (Phenols) of controls were determined in the non-inoculated callus grown in the same flask.
Means followed by different letters are statistically significant.

Table 2. Rf values of the different phenolic compounds detected in the IZ of *H. annuus* callus inoculated with *S. sclerotiorum* (for solvent systems, see Materials and Methods).

Spot	Rf value A	Rf value B
1	0.45	0.61
2	0.41	0.61
3	0.58	0.12
3'	0.62	0.24
4	0.64	0.34

blue color under UV light, which changed to yellowish when the chromatograms were exposed to NH_4 vapors. The absorption spectra of these compounds in 80% methanol gave a maximum peak at 328 nm. According to Koeppe *et al.* (15, 16, 17), this may correspond to chlorogenic acid. The reactions of spots 3,3' and 4 were similar to those of chlorogenic acid, although the Rf values were similar to those of the isochlorogenic acid (7, 12).

When the calli extracts were co-chromatographied with extracts of an inoculated zone of a whole plant, the same distribution of spots and reactions was revealed. However, phenol concentrations were quite different. While the IZ extracts contained a mean of initial fresh weight (FW) of 1367 ug/g, extracts from susceptible and tolerant plants had initial FWs of 5000 and 2200 ug/g respectively.

DISCUSSION

Although extensive research has been carried out with *in vitro* dual cultures of obligate parasite-host tissues (5), it is thought that this is the first work with a successful culture of a pertotroph parasite and tissues of one of its hosts. As opposed to *in vitro* cultures of obligate pathogens, the dual cultures of sunflower *S. sclerotiorum* presented the problem of parasite virulence and its readiness to grow in a synthetic medium. For these reasons, was necessary to restrain the fungal growth by inoculating it inside a plastic ring so as showed to isolate the IZ from the rest of the callus.

In spite of the short duration of the dual culture (72 h), the inoculated calli showed the same response to the attack as was observed in the whole plant: activation of the phenolic metabolism, especially of the isochlorogenic acids. Bazzalo *et al.* (1) considered the isochlorogenic acid as a strong inhibitor of mycelial growth, with a ED50 value of 0.3 mg per milliliter.

However, the increment of these compounds was lower compared with that determined in the infected areas of an intact plant, up to a susceptible zone. At the same time, the response was extended to the AZ, as it happened in the adjacent areas of organized tissue, although lacking the organization of a stem. The aliquots of the extracts of the different zones and controls that were chromatographied were chosen on the basis of the phenol concentration in the inoculated zone. This could explain why only some compounds were sporadically present in the chromatograms of control calli. This means that although phenolic compounds were present before inoculation, their concentrations were increased as a consequence of the infection. This is mainly true for the isochlorogenic acid. According to Lutrell (19), *S. sclerotiorum* being a pertotroph pathogen, it kills host tissues before penetration. As a consequence, the increment of the same phenolic compounds in the AZ must be an effect of the presence of the fungus in the inoculated zone. These substances must therefore be considered as anti-fungal (6).

Bazzalo (2) pointed out that the presence of lignin in the cortical tissues of the infected stem could mean a mechanical barrier to the fungal penetration, since the pathogen does not have lignins. As the mechanical barrier is not present in the *in vitro* tissues, fungal penetration provoked a rapid deterioration of the callus. This could explain the lower increment of the phenolic compounds below the inhibitory level.

At present, neither lines nor hybrids having complete resistance to *S. sclerotiorum* are in commercial production. Different levels of susceptibility of any cultivar are related to the environmental conditions (9) that are prevalent during the growing season of the crop. The hybrid used in this work is susceptible to the pathogen. However, it showed the same biochemical mechanism that was determined at the whole plant level in another cultivar (1). New experiments with other cultivars having different susceptibility levels would be needed in order to generalize this response to the attack of the fungus as well as for a better understanding of the basic aspects of the interaction between sunflower and the pathogen.

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