Mycopathologia **157:** 291–302, 2004.
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Histopathology of *Sclerotinia sclerotiorum* attack on flower parts of *Helianthus annuus* heads in tolerant and susceptible varieties

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Received 20 August 2002; accepted in final form 19 June 2003

Abstract

Sunflower head rot is a major disease caused by *Sclerotinia sclerotiorum*. Sunflower varieties which are tolerant to the fungus have been developed. The changes occurring in flower parts at different times after inoculation with pathogen ascospores were studied for two sunflower varieties (tolerant HA 302 and susceptible HA 89). In variety HA 302 there was cell collapse, changes in cell wall composition, and an increase in phenolic compounds in the tissues of corolla and style, which prevented the pathogen from advancing. This response was weaker in susceptible variety HA 89, and occurred only in the style, so did not stop the pathogen from developing and reaching the ovary. Phenolic compounds were found in HA 302 corolla and style tissues only when the pathogen was present, constituted an induced response that prevented further development of the fungus. Principal component analysis (PCA) showed that at the beginning of the infection there was no difference in behavior between the two varieties. The difference arose during the final observation times, when in variety HA 89, the pathogen colonized ovary, style and base of filaments and produced noticeable colonization of the corolla.

Key words: histopathology, *Sclerotinia sclerotiorum*, susceptible and tolerant varieties of sunflower

Introduction

Head rot is an important disease of sunflower and causes heavy yield losses in Argentina. The infection begins when ascospores germinate on different parts of the flowers under conditions of high relative humidity and low temperatures, after which mycelium infects the flower parts [1].

Studies conducted by Says-Lesage and Tourvieille [2] showed that infection is favored at the time of flowering. Sunflowers undergo three phenological stages: (a) the corolla opens, (b) the anthers are projected beyond the corolla by increase turgidity of the basal cells of the filament, and (c) these basal cells become less turgid and the anthers descend, exposing the stigma [3]. To germinate, ascospores require water and an exogenous source of energy [4, 5]. This is related to the presence of saccharose-producing glands on leaves, petioles, stalks and heads, which may provide essential nutrients for germination and infection [5].

Two possible routes have been suggested for the attack of the lacunar parenchyma in the receptacle: (a) through the internal bracts and (b) through the ovary of the flowers [6]. Lumsden [7] suggests that there are three kinds of defense mechanism occurring in different hosts when attacked by different *Sclerotinia* species: (a) anatomical (tissue resistance to degradation), (b) pre-formed anti-fungal compounds and (c) post-infection anti-fungal compounds. One technique for studying the infection is to make cross sections of the tissue and to use differential staining (such as with safranine-fast green) to compare the anatomy and coloration of the infected tissue with that of the healthy control. The aim of this study was to compare the way the pathogen colonized the flower parts of tolerant and susceptible sunflower varieties, and to use safranine-fast green staining to compare how the tissues of the various flower parts responded.

Materials and methods

Field work

A field experiment was conducted in Balcarce, Buenos Aires Province (37° 52′ Lat. S and 58° 15′ Long. W) in experimental fields belonging to the ZENECA S.A.I.C company, during 1996/1997 to reproduce the disease cycle of *S. sclerotiorum*. The development of *S. sclerotiorum* infection was compared between a tolerant variety of sunflower (*Helianthus annuus*), HA 302 (TV), and a susceptible variety HA 89 (SV). Eighteen to twenty plants were sown in each of six rows, 6 m long, set 0.7 m apart, with plants set 0.3 m apart. Half the plants were TV and the other half SV. Within each row, half the plants were selected at random and inoculated, while the remaining plants (non-inoculated) were control plants. At each of the three sampling times, heads were picked randomly from each group. The total number of heads were designed randomly at each sampling time and picked in the corresponding moment.

A population of *Sclerotinia sclerotiorum* from Balcarce (Buenos Aires) was used. The esclerotia collected from the field were put in freezer (−20°C) for 15 days and then in pots with moistened sterile soil for one month to induce the carpogenic germination [8]. The spores obtained were collected in sterile conditions and preserved dry in Petri dishes at −20 °C for no more than six month. These standardized conditions resulted in a spore inoculum that caused 100% of disease in the susceptible control variety.

Inoculation and sampling

Ascospores were suspended in sterile distilled water with Tween 80 (0.05%) to a concentration of 5×10^3 spores/ml (10 ml per inflorescence). Plants were checked regularly for flowering. Sunflower heads were sprayed with inoculum when the anthesis of the two outer rows was complete [9], and so inoculation was carried out when the anthesis was produced. Control plants were sprayed with water and drops of detergent. The heads were covered to maintain humidity, and collected at the following times after inoculation: 24 h (t1), 6 days (t2) and 12 days (t3). A radial section comprising 1/6 of the head (including flowers and receptacle) was kept in a solution of formaldehyde: acetic acid: alcohol (37:5:50 (vol:vol:vol). For each sampling time, three flowers picked at random from the edge of a head from different plants were processed, because these were the flowers that had received the inoculum when anthesis occurred.

Anatomical studies

Flowers were embedded in paraffin and dehydrated in an increasing series of alcohol. A series of cross sections 15 μm thick was cut. After removing the paraffin, the sections were stained with safranine-fast green [10] and mounted in synthetic Canada balsam. This stain distinguishes secondary lignified walls (or any phenolic compounds deposited) from tissues with primary walls or non-lignified secondary walls, and from hyphae, which stain bright blue. Cross sections of all flower parts sampled were studied.

Observation and data analysis

A matrix was made to allow a principal component analysis. Each flower part was divided in portions. The portion was considered positive (+) when the pathogen appeared in one of the sections. The pathogen presence was recorded at the following sites: (a) corolla: adaxial and abaxial surfaces and inside the tissues in five different portions; (b) pappus (calyx): surface and inside the tissues; (c) anther: inner and outer surface, and inside the tissues of four portions; (d) filaments: surface and inside of two portions; (e) stigma: surface and inside, (f) style: surface and inside of five portions; (g) ovary: surface and inside the tissues. These observations were used to define the different behavior of the two sunflower varieties by principal component analysis (PCA), a method for ordering multivariate data, that sums the main trends in data variation [11]. A binary matrix of data was constructed where the variables (total 19) were the sites observed and the cases were the varieties, treatments and times. The value 1 was assigned to presence of mycelium and 0 to absence of mycelium. Only internal sections of tissues were used. The data were normalized by applying the following transformation: $x = \log(x + 0.5)$, where x is the variable considered.

Results

Morphological features and symptoms of heads

Head morphology differs between the two sunflower varieties according to the Knoules scale [12]. In variety HA 302 (TV), the head is convex (grade 3), while in variety HA 89 (SV) it is flat-concave (grade 2) so

that the involucral bracts and ligules of the peripheral flowers partly cover the inflorescence. The tubular corolla is more open in TV than in SV. The morphology of both varieties provides conditions suitable for ascospore germination by creating a microenvironment where there is greater relative humidity, particularly in SV. At t1 there was no difference in symptoms between the two varieties. At t2 the peripheral flowers in both varieties, particularly their anthers, were covered by mycelium, but TV flowers showed slight necrosis. At t3, TV had a large proportion of ripe achenes and necrotizing corollas. In flowers where the ovary had not ripened, necrosis had reached the ovary. In SV there were spots of wet rot on the corolla and ligules of peripheral flowers. The method of inoculation proved highly effective both by re-isolation of the pathogen, as well as by observation of the typical symptoms of head rot caused by *S. sclerotiorum* [13].

S. sclerotiorum attack on flower parts

At t1 in both varieties, there was a little mycelium development, mainly wherever there were pollen grains, and the papillae on the corolla were collapsed. When there was mycelium on the surface of TV, epidermal cells were flattened and phenols were present (Figure 1A, H). For both varieties, there was only a little superficial mycelium on the pistil (Figure 2A, B, C), covering up to 1/4 of total style length. The main difference was that in TV, on the upper section of the style, cells and walls were partly collapsed and there were phenols under the stigmatic branches (Figure 2A). On the lower section of the style, there was less collapse, and it mainly affected parenchymal tissue. In TV, style asymmetry and the presence of hyphae on their surface support the hypothesis that the collapse is related to pathogen presence. Mycelium did not develop much on stamens. The fungi grew around pollen grains, covering 1/4 of the length of the anthers in TV and 1/2 in SV. Filaments were never colonized.

At t2, pathogen development on the SV corolla was greater on the inner surface. The fungus penetrated and developed, disorganizing and slightly darkening 2/3 of the tissues. Infection of TV corolla did not progress; there was pathogen development only on the surface. There was collapse, especially in the papillaes (Figure 1B). Mycelium covered 1/3 of TV style, without penetration. There was greater colonization of SV style, where mycelium covered 2/3 of the surface and 1/2 of the interior. The differences described for t1 were again present. SV tissues were similar to control flower tissues, with primary walls intact, except for the parenchyma surrounding the style canal, where there were phenolic compounds, indicating a reaction to the presence of the fungus. The cells were partly collapsed (Figure 3A). In TV, this reaction occurred in a greater proportion of the tissues. Mycelium developed on the outer surface of the stamens in both varieties (Figure 4A, B), particularly in the connective area, where tissues were disorganized. There was inter- and intra-cellular colonization of epidermis and endothecium.

At t3, the colonization of the surface of the corolla was greater and more continuous on the interior for both varieties. However, there were differences in tissues invaded by the pathogen. In SV, there was inter- and intra-cellular development of mycelium (Figure 5A, B, F) and little cell collapse, while phenolic compounds were present in some cells (Figure 5G). There were hyphae in the parenchymal tissue, which was noticeably disorganized (Figure 5C, D), and in certain parts of the vascular bundle. In TV, tissues were less colonized, only the upper third of tissues and cells were very much collapsed (Figure 1C–F). Both varieties produced crystals (drusen) in the presence of mycelium, although there were more in SV (Figures 1D; 5: H). In both varieties, mycelium colonized the surface and interior of pistil tissues and there was cell collapse in part of the stigma: in SV, the upper half of the style partially collapses and there were crystals in the cells, whereas there was a more general collapse in TV, with increased phenolic content in the cells from the stigma (Figure 2D, E) to the middle of the style (Figure 2G, H), decreasing toward the base (Figure 2I). In SV the style was totally colonized, mycelium reached the top of the ovary (Figure 3D), whereas in TV it colonized the upper third of the style, with clearly visible invasion of the stigma canal (Figure 2E), but neither lower styles parts nor ovary were reached (Figure 2H, I). There was clear collapse in upper parts and they had phenols present. In both varieties there was considerable mycelium development on stamens on the surface and interior of tissues, mainly the anthers (Figure 4C, E). The filament were colonized partially in TV and totally in SV and certain collapse was observed (Figure 4D, F, I). The results are summarized in Table 1.

Order analysis

By using pathogen presence-absence data on different flower parts and at different times, the first 4 com-

Table 1. Description of the *S. sclerotiorum* growth throughout the flower parts in sunflower varieties: tolerant variety (TV) HA 302 and susceptible variety (ST) HA 89 over time (t1: 24 h, t2: 6 days, t3: 12 days)

Variety	VT			ST		
Flower part/ time	t1	t2	t3	t1	t2	t3
Corolla	Scarce, only on surface. Mycelium associated with pollen grains. Few phenolic compounds in epidermis in presence of hyphae.	Growth only on surface, mainly on inner side. More phenolic compounds in epidermis.	Growth on surface and inside upper third of tissues. Large proportion of tissues with phenolic compounds and cell collapse. Few crystals.	Scarce, only on surface, (with greater development on inner side). Mycelium associated with pollen grains.	Growth on the surface. Development on inside of upper 2/3. Some tissue disorganization in reaction to the pathogen.	Development on surface and inside upper 3/4 of tissues. Little collapse. Crystals.
Stigma	Development on surface, associated with pollen. Collapse cellular.	Only on surface. Reddish color with some papillae collapse.	Development on surface and inside tissues. Phenolic compounds.	Development on surface, associated to pollen grains.	Growth only on surface. Collapse in papillae.	Development on surface and inside. Few phenolic compounds. Crystals.
Style	Growth on surface often associated to pollen. Some collapse and phenolic compounds.	Growth on surface. Collapse and phenolic compounds present in certain zones.	Growth on surface and also inside (upper third). Collapse in upper 1/2. Phenolic compounds.	Growth on surface. No collapse or phenolic compounds.	Growth on surface. Penetration and development on upper half. Few phenolic compounds.	Partial collapse in upper 1/2. Development within tissues throughout the style.
Ovary	–	–	–	–	1/8 of its length.	
Anthers	Only on the surface. Development on both sides, greater on the inner side. Associated with presence of pollen.	Great development on surface. Broken connective associated with hyphae. Development inside tissues (upper 1/4).	Totally infected. Growth inside and on surface.	Development on both sides, greater on inner side. Associated with pollen grains.	Great development on the surface, particularly in the connective zone. Development inside tissues (upper 1/4).	Totally infected. Growth inside and on surface.
Filament	–	–	Development both outside and inside along the upper half. Cellular collapse.	–	Development on surface and inside along upper 1/3.	Development both outside and inside along the entire length. Cellular collapse.

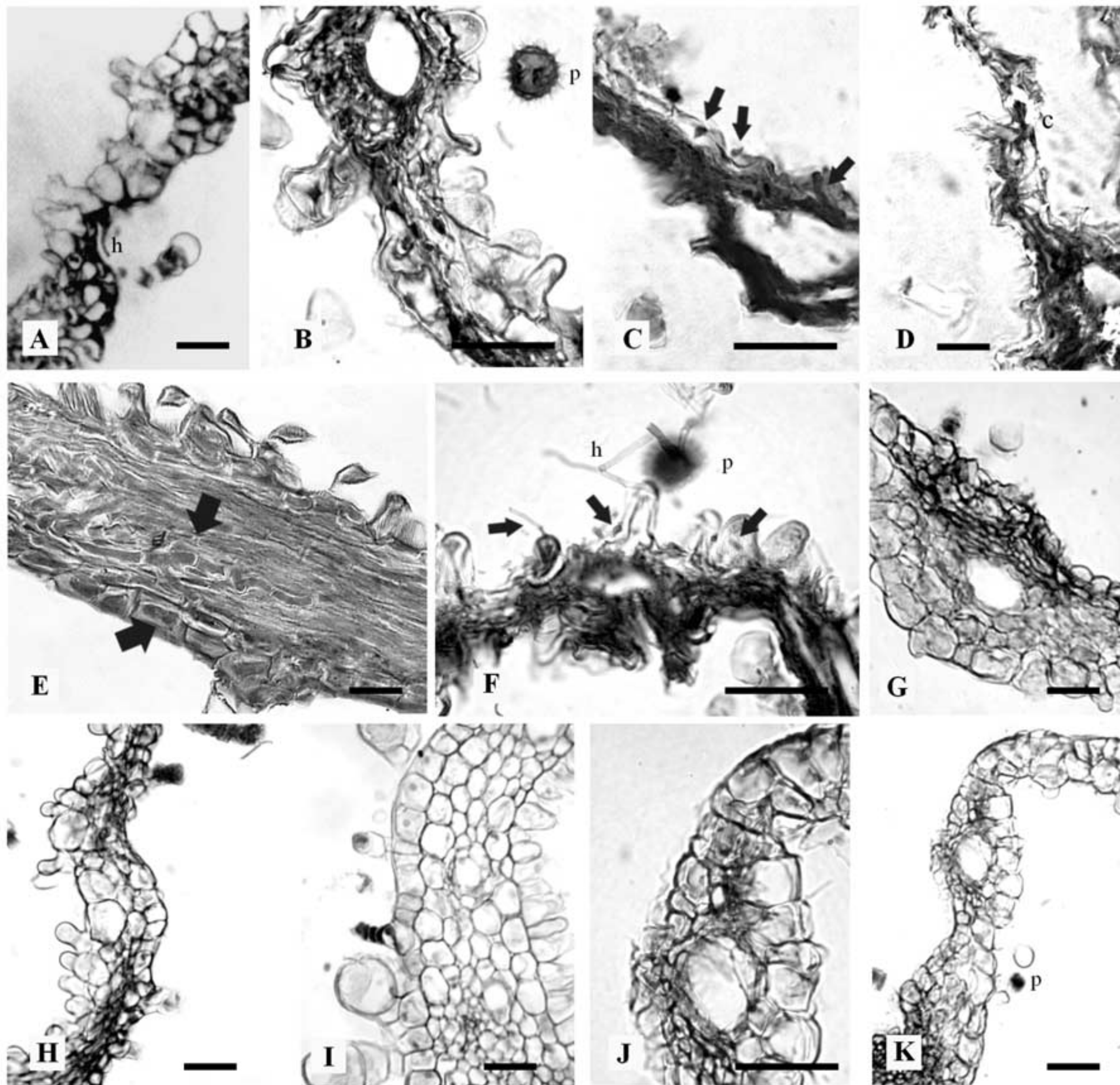

Figure 1. Inoculated (A–F) and non-inoculated (G–K) corolla of HA 302 variety (TV) in transversal section over time. A: t1, slightly darkness and collapse in epidermal cells when there are hyphae on the surface; B: t2, cellular collapse, hyphae only on surface; C–F: t3, C, E, F, upper third: E, sector tissue colonized by pathogen (arrow); C, F, tissue sector collapsed, hyphae only in epidermal cells (arrows); D, medium sector, tissues are very much collapsed, few crystals; G–K: non-inoculated corolla in different sectors at t3. The infected tissue is stained by safranine (red) except at t1 (A). c: crystal; h: hyphae; p: pollen grain. (Scale bar: 50 μm).

ponents from a principal component analysis (PCA) account for 88.95% of the variation in the samples (Figure 6).

The SV and TV samples taken at t1 and one of the TV samples taken at t2 were segregated in the negative sector of component 2, characterized by pathogen absence in the first anther and style portions. At t2, there was a greater separation between the two varieties, and there was colonization of the first anther and style portions in SV samples. Component 1 segregated SV samples at t3. They were placed in the positive sector and characterized by the presence of the pathogen in the last portions of the style (style 5), ovary and the lower section of filaments (fil 2) (Figure 6).

This ordination confirmed that the main differences in behavior between the two varieties occured

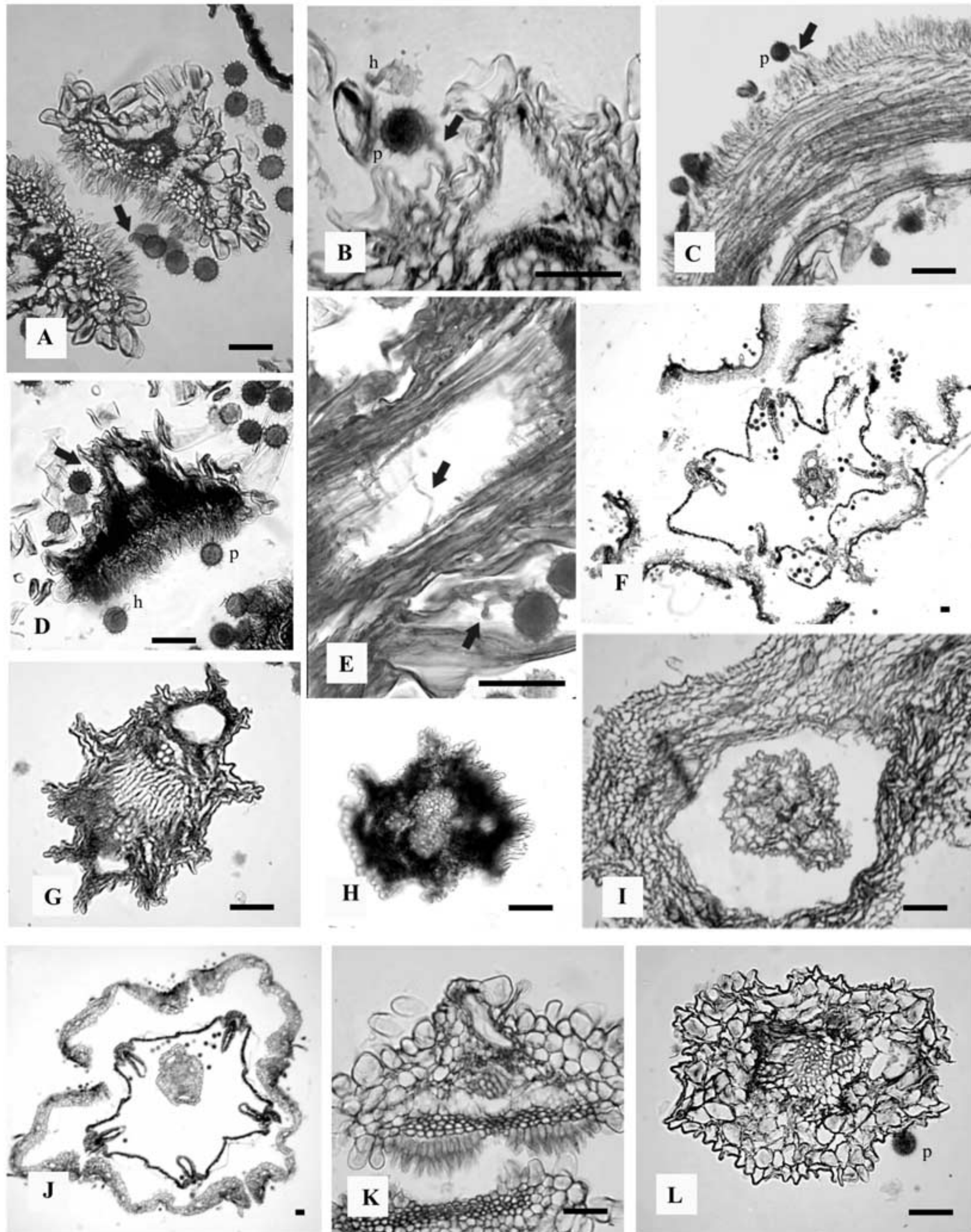

Figure 2. Inoculated (A–H) and non-inoculated (J–L) flowers in transversal section of HA 302 variety (TV). A–C: t1, slightly cellular collapse and phenolic content in parenchymal tissue of stigmatic branches, hyphae on surface (arrows); D–E: t3, stigmatic branches, abundant cellular collapse and phenolic compounds, hyphae on surface and inside the tissue (arrows); F: general aspect. G, H: medium sectors of style with increased cellular collapse and phenolic content, although there is no colonization; I: lower sector of style without colonization, there is less collapse and phenolic content. J–L: t3 non-inoculated flowers; J: general aspect; K: stigmatic branches; L: medium sectors of style. h: hyphae; p: pollen grain. (Scale bar: 50 μm).

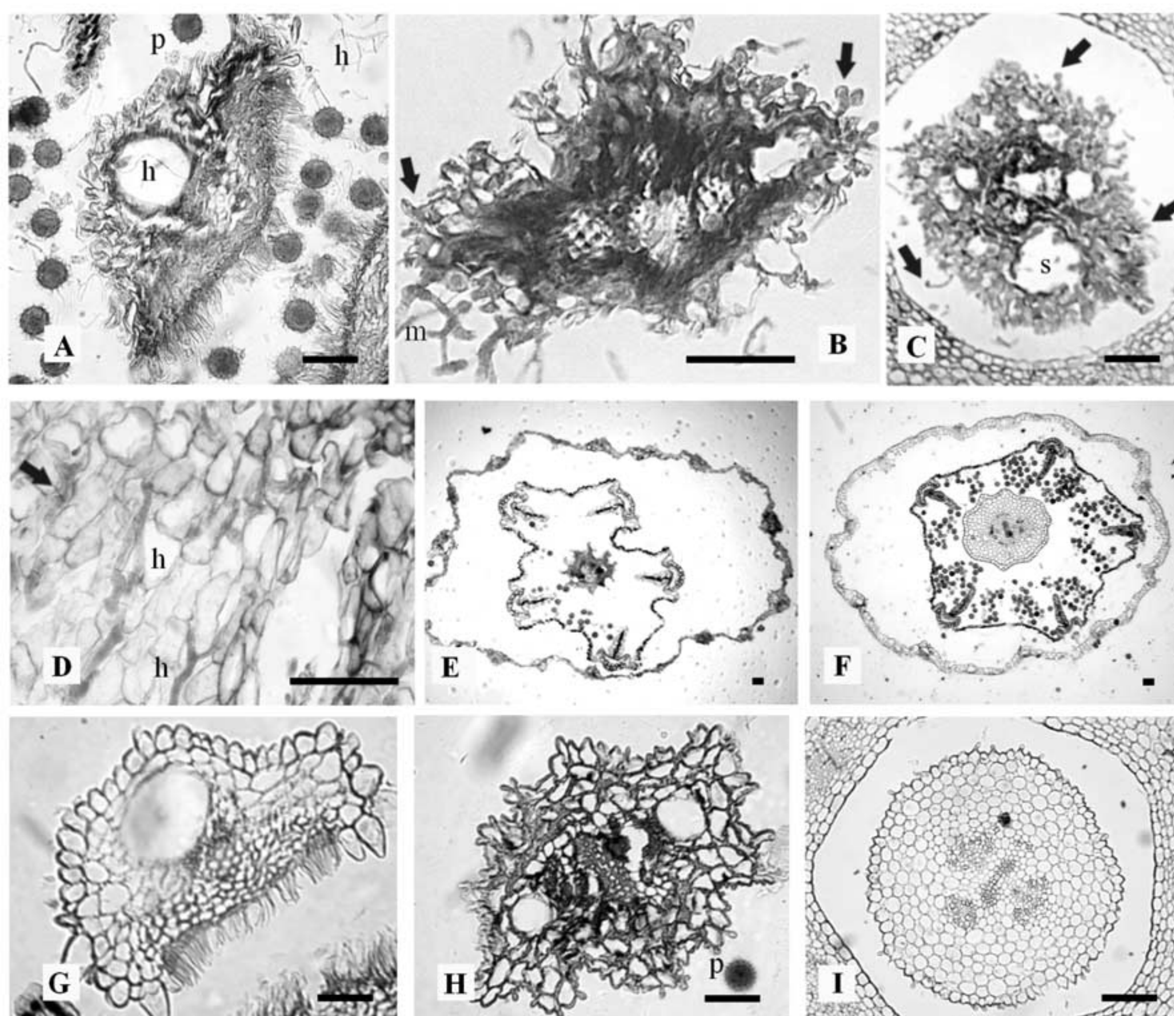

Figure 3. Inoculated (A–E) and non-inoculated (F-I) flowers at t3 in transversal sections of HA 89 variety (SV). A: cellular collapse and phenolic content in parenchymal tissue, abundant colonization in stigmatic branch; B: abundant pathogen growth (arrow) in medium sector of style; C: abundant colonization and degradation of the tissues in lower sector of style; D: top of ovary tissue colonized by the pathogen (arrow); E: general aspect of inoculated flower, there is colonization (arrows). F: general aspect of non-inoculated flower; G: stigma branch; H: medium sector of style; I: lower sector of style. h: hyphae; m: mycelium p: pollen grain; s: style. (Scale bar: 50 μm).

due to the fact that the pathogen managed to colonize the lower portions of filaments, style an ovary at t3, thus preventing fruit development.

Discussion

A plant species is disease-resistant when it has mechanisms to prevent pathogen penetration and development [14]. Penetration-preventing mechanisms include any structure that prevents the entry of the pathogen, and these were found in some tissues in the varieties we studied. In this study, the inoculum was applied to the upper surface of the sunflower heads, so the corolla, anther and pistil were the main floret parts that received the inoculum in our experimental conditions, similar to occurrences in natural conditions. The bracts head was also affected by the inoculum but preliminary tests concluded that the pathogen progressed along the longitudinal axis of the flower until it reachead the ovary, and it did not grow in the bract zone. Our experiments eliminated the route via internal branches suggested by Lamarque et al. [6] because at the final sampling time, only the upper part

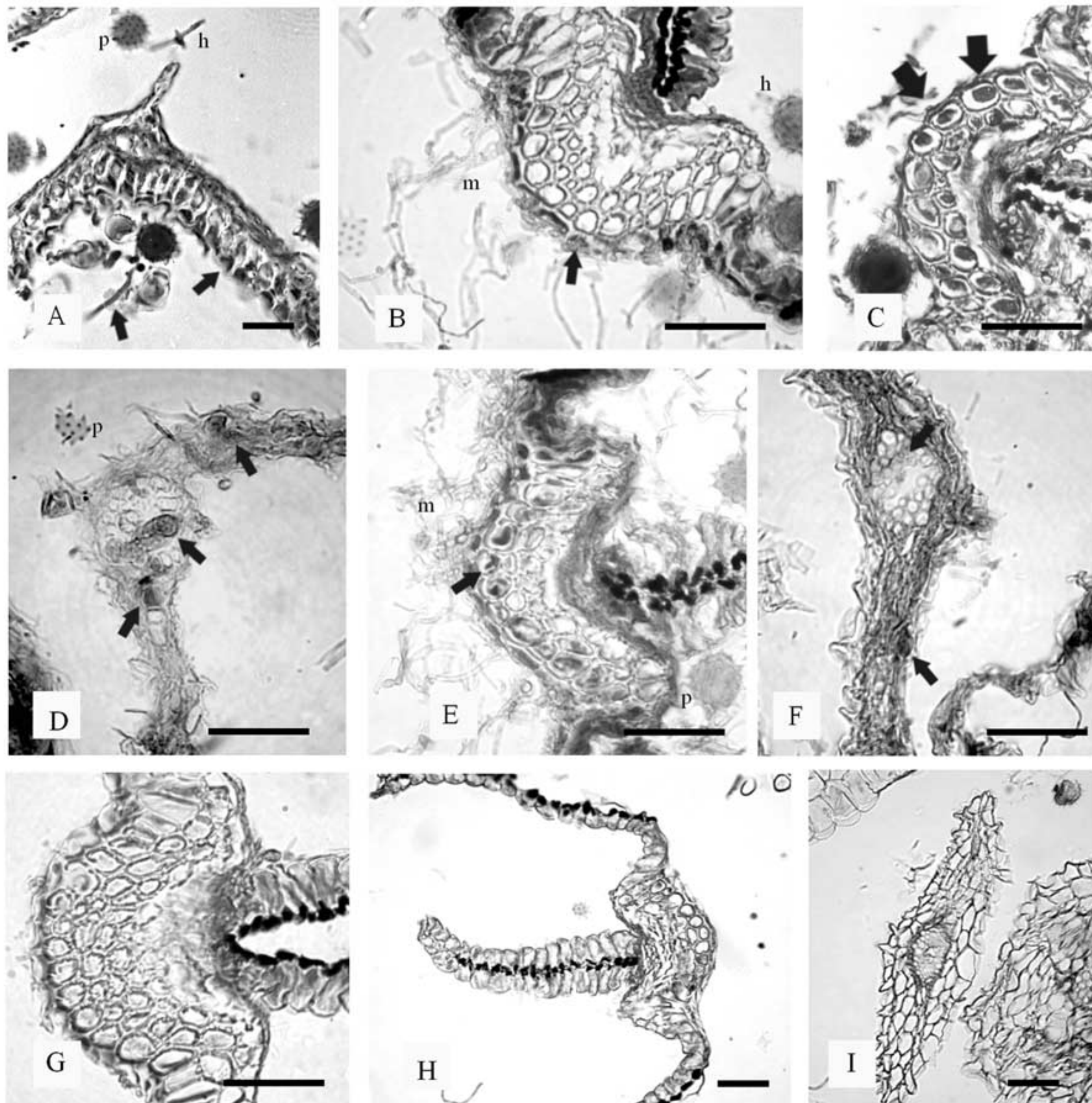

Figure 4. transversal section of inoculated (A-F) and non-inoculated (G-I) stamen. A–D, HA 89 variety (SV) A, B: t2 abundant surface growth and colonization of epidermic tissue of anthers; C, D: t3 abundant colonization of connective and filament respectively. E, F: anther and filament with pathogen colonization respectively of HA 302 variety (TV) at t3. G-I, non-inoculated at t3: G, anther of HA 89 variety (SV); H, I: anther and filament respectively of HA 302 variety (TV). Arrows show growth and colonization. h: hyphae, m: mycelium, p: pollen grain. (Scale bar: 50 μm).

of the ovary had been attacked, and there was no invasion of the receptacle through the bracts. The pathogen proliferation was successful through floret parts. Ascospores did not reach the pappus or the ovary, which were later infected by the mycelia growing from other colonized flower parts. The colonization of ovary occurred after the other floret parts, in agreement with Lamarque et al. [6] and the penetration of each flower part was different. There was a delay in colonization of the TV corolla, indicating greater resistance though not prevention of penetration. Lamarque et al. [6]

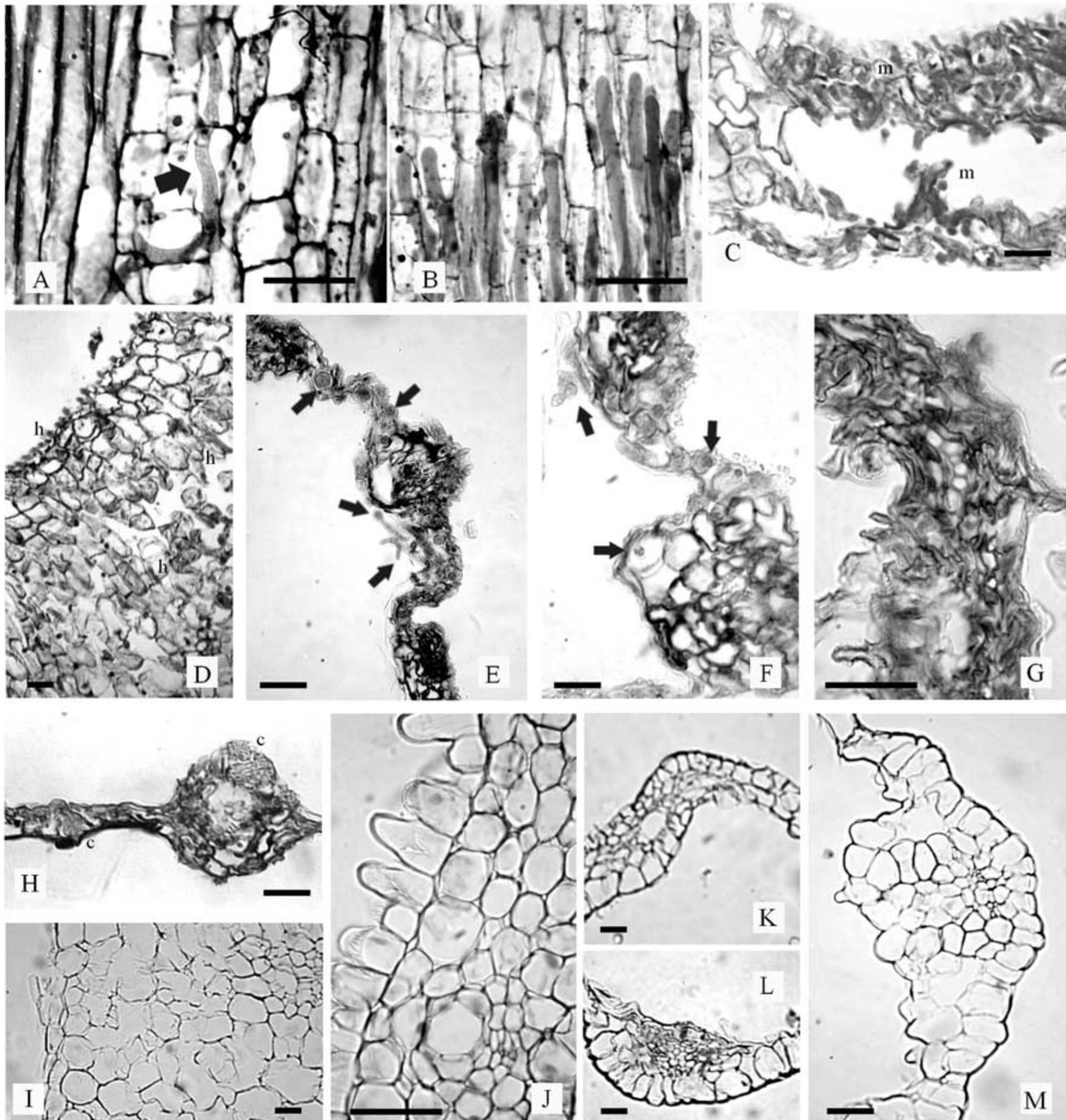

Figure 5. inoculated (A–H) and non-inoculated (I–M) corolla section of HA 89 variety (SV) at t3. A, B: longitudinal section with abundant colonization (arrow); C, D: lower sectors with disorganization and degradation of parenchymal tissue; E, F, H: medium sectors with colonized tissue; H: presence of crystals; G: upper sector with cellular collapse. I–M: non-inoculated corolla in different sectors. c: crystal; h: hyphae; m: mycelium. (Scale bar: 50 μm).

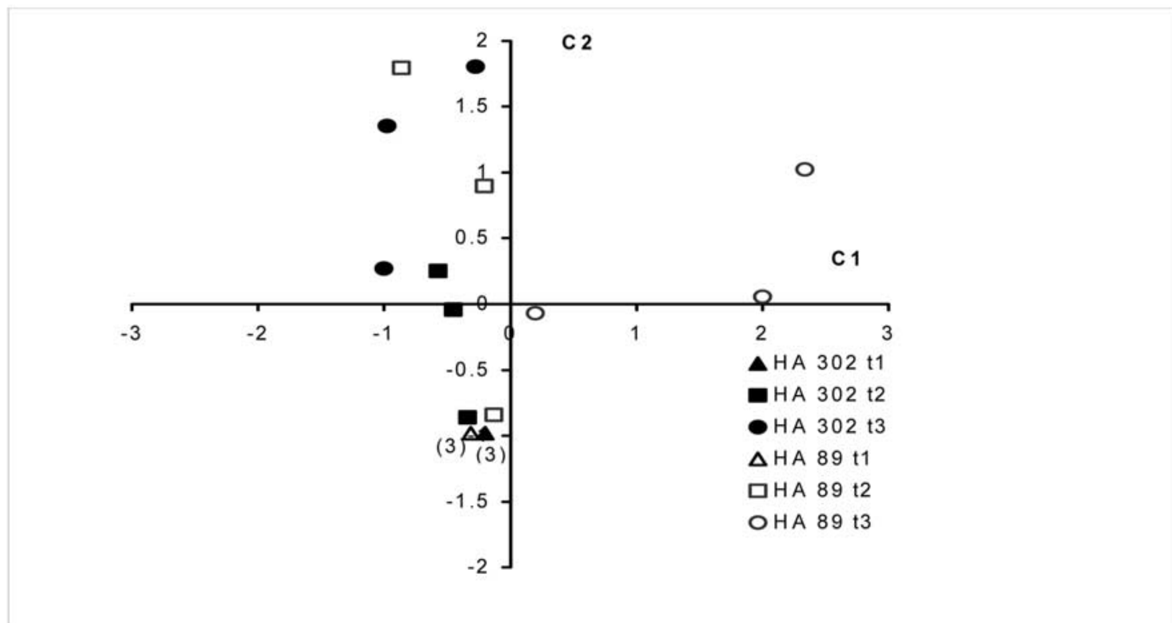

Figure 6. Principal Component Analysis. Distribution of the samples analyzed in the space of components C1 and C2. Component 1 segregated SV samples at t3 in the positive sector of the component characterized by the presence of the pathogen in the lower segments of the style, in the ovary and lower section of filaments. All samples at t1 and one of the TV and SV at t2 are segregated in the negative sector of component 2, characterized by the presence of the pathogen in the first anther and style segment.

found that corolla cuticle and hairs hindered ascospore germination on sunflower varieties Forsol and Boleró.

Gershenzon et al. [15] found terpenes in the glandular trichoma on anthers in "wild" sunflower varieties. This type of compound often has anti-fungal properties. Nevertheless, in our study, anthers were densely colonized, particularly on these trichoma (Figure 4A).

In both varieties there was a correlation between mycelium growth and presence of pollen grains at the first sampling time (t1). Abawi and Grogan [4] and Gulya et al. [5] showed that pathogen development requires water and an exogenous source of energy. During flowering, pollen favors the development of infectious hyphae from ascospores [2, 16]. In this study the greatest infection was found on stamens, in agreement with Says-Lesage and Tourvielle [2], who studied an experimental hybrid, cr2, in conditions of high relative humidity, finding high germination indices on the surface and greater penetration of stamens than other parts. In our experiments, the pathogen colonized the inside of pistil and corolla tissues in both varieties, although to a different extent, independently of the presence of pollen. These results were different to those of Says-Lesage and Tourvielle [2], who found that penetration only took place in the presence of pollen.

The reaction to the pathogen in the varieties we analyzed produced changes in the usual coloring. This might be a physical post-infection barrier involving synthesis and deposition of new material in cell walls, to prevent pathogen development. Bazzalo [17] found that there were changes in the cell walls of stems of these sunflower varieties, including the esterification of phenolic acids, which may hinder or prevent normal pathogen development. Many studies have described alterations in cell walls connected to disease resistance, such as deposition of oxidized phenol polymers, lignification, impregnation of walls with phenolic acids, etc. Jamaux et al. [18] and Sedún and Brown [19] studied different varieties of *Brassica napus* and found that there were collapsed corolla papillae in the presence of *S. sclerotiorum* spores. However, this did not seem to be a widespread response in SV at t1 but was shown by SV (at t2 and t3) and TV. It did not occur in either of the varieties Forsol and Boleró [6], or in the experimental hybrid cr2 [2].

Cell collapse in the style of both varieties and in the TV corolla may be considered a pathogen-induced metabolic defense reaction, since it does not occur in

control flowers of the same age. Cell collapse was very noticeable and probably involves cell dehydration. Where there was less cell collapse, phenolic compounds (safranine stained) were found. Many of these could have anti-fungal properties. Field observations showed necrosis of the corolla in TV. This might be connected to changes occurring in tissues in response to the pathogen, such as cell collapse and changes in cell wall composition. Cosson et al. [20] and Pratts-Pérez et al. [21] studied wild and cultivated varieties of sunflower heads and found that necrotized areas synthesize compounds which appear to be phytoalexins. Bazzalo et al. [22] found that there were more phenolic compounds, which are assumed to play a role in the plant's resistance to infection.

In both varieties, but particularly in SV, oxalate crystals were found near the pathogen. The crystals may have been produced by the synergic action of pectinolytic activity and oxalic acid [7]. Oxalic acid plays an essential role during the pathogenesis of several phytopathogenic fungi such as *Sclerotinia sclerotiorum* [23]. Smith et al. [24] suggested that the oxalic acid secreted by *Sclerotium rolfsii* precipitated calcium from the middle lamellae to form calcium oxalate crystals; so the pectic materials that remained were more susceptible to enzymatic degradation.

There were similar responses in the stamens of both varieties and no difference with control flowers, although infection in TV was less than in SV, where it was probably favored by the profuse colonization of the rest of the flower parts. The nectaries at the base of the style produce compounds that might favor mycelium development in SV. This could be another factor accounting for different behavior between the variety.

The resistance to development and spreading of the disease may be the main cause of the different response between varieties. We suggest that there was a post-infection or induced mechanism [25].

Acknowledgments

This work was supported by CONICET (PIP 812), University of Buenos Aires (TYO2) and Zeneca SAIC. We thank C. Connon for help with the English manuscript.

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