Histopathology of the resistance to *colletotrichum gloeosporioides* of wild strawberries and species related to commercial strawberry

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Abstract

Three species of wild strawberries, as well as three related species, were tested against virulent isolate of *Colletotrichum gloeosporioides.* The purpose of this study was to assess genetic resources exhibiting resistance to anthracnose. The results of inoculation experiments showed that the species *Fragaria vesca*, *Fragaria virginiana* and *Fragaria chiloensis* were susceptible to fungi; whereas species *Duchesnea indica*, *Duchesnea chrysantha* and *Potentilla tucumanensis* showed higher levels of resistance. A histopathological study revealed a different response between susceptible and tolerant species. A significant thickening of the cell wall in epidermal cells, changes of stomata and mesophyll cells, the presence of idioblastic cells with unknown content and the accumulation of large starch grains in atypical tissues, namely, stomata and parenchyma cells, were observed in species of the genus *Duchesnea*. However, no morphological alteration was observed in *Fragaria* species or in *P. tucumanensis,* which did not show any apparent interaction with the isolate of *C. gloeosporioides*. Oxidative burst was evaluated in leaf tissues with the fluorescent probe DCFH-DA (2',7'-Dichlorofluorescein diacetate) in susceptible and tolerant genotypes. Results showed that tolerant species present a noticeable accumulation of H_2O_2 whereas susceptible species did not. These results have led us to the conclusion that the changes observed were correlated with the resistance to disease.

Keywords: *Duchesnea indica*; *Potentilla tucumanensis; Fragaria vesca; anthracnose; Colletotrichum*; disease resistance; wild material.
Abbreviations: DHCF - DA-2´,7´, Diclhorofluorescein diacetate, DSR - Disease Severity Rating, ROS - Reactive Species Oxygen dpi - day post infection, cv – cultivar, PGA - Potato Glucose Agar, co – conidia, gt - germ tube, ap – appresoria, ic - idioblastic cells sc - swollen epidermal cells, st – stomata, sg - starch grain, vs - vesicle of penetration, hf - necrotrophic hyphae, ac - acervulli

Introduction

Anthracnose is one of the most serious strawberry diseases causing major production losses (Mena et al., 1974; Howard et al., 1992). Three species of *Colletotrichum* are involved as the etiologic agents: *C. acutatum* J.H. Simmonds, *C. fragariae* Brooks and *C. gloeosporioides* (Penz.) Penz. and Sacc. In Penz. (teleomorpho *Glomerella cingulata* (Stonem.) Spauld. & Schrenk). A number of crops like maize, alpha, coffee, among others, are infected by pathogens belonging to the genus *Colletotrichum* (Cadena-Gómez & Nicholson, 1987; Martínez Carrillo & Zambrano, 1994; Shen et al., 2001; Salles et al., 2002; Alvarez et al., 2002; Cuoto & Menezes, 2004; Lugo de Cumare & Fuguet de Alvarado, 2004). Pathogens of this group of fungi, together with Ascomycetes (Alexopoulus and Mims, 1985), exhibit two main modes of nutrition and growth, defining thereby two groups of pathogens: biotrophic and necrotrophic (Thrower, 1966). In their attempt to colonize the hosts, these fungi develop many specialized infection structures, including germ tubes, appresoria, intracellular hyphae, and secondary necrotrophic hyphae. The initial stages of infection are similar in both groups; conidia adhere to, and germinate on plant surfaces, produce germ tubes, and then continue

forming appresoria, which penetrate the cuticle directly (Curry et al., 2002). Following penetration, pathogens grow beneath the cuticle by forming a subcuticular intramural network of hyphae before spreading throughout the tissue (with both inter- and intracellular hyphae). In the case of necrotrophic fungi, the process continues with the formation of secondary necrotrophic hyphae, which kill plant cells by using cellular debris as nutrients (Perfect et al., 1999). *C. gloeosporioides* is one of the few fungi that present both infection strategies (Bailey et al., 1992). The importance of wild material in breeding programs is well known. Wild germplasm is a valuable resource of new genes, including resistance to disease or to environmental stress (Lacadena, 1970; Hancock & Luby, 1993). For this reason, there are increasing numbers of phytopathological studies about the effect of pathogens on wild materials related to crops, such as peach, tomato, oat, avocado, etc. (Adaskaveg and Hartin 1997; Mieslerová et al., 2000; Lebeda and Mieslerová 2002; Sabri and Clarke 1996; Zamora-Magdaleno et al., 2001). However, little information is available about the behaviour of wild strawberries and related species against pathogens of genus *Colletotrichum* (Ramallo, 2002; Arias, 2005). Most of

the studies found in the literature are focused on interactions with different commercial varieties of *Fragaria x ananassa* (Sreenivasaprasad et al., 1992; Denoyes-Rothan et al., 1999; Arroyo et al., 2002; Curry et al., 2002; Garrido et al., 2002; Salazar et al., 2003; Mertely and Legard, 2004; Mackenzie and Legard, 2006). Arias (2005) evaluated the reaction of various accessions of *Fragaria vesca* L., *Potentilla Tucumanensis* Castagnaro & Arias and *Duchesnea Indica* (Andrews) Focke against *C. acutatum* y *C. fragariae*. She reported that the first genotype was susceptible, showing a Disease Severity Rating (DSR) of 4,9 on the 50th day post inoculation (dpi), whereas the genotypes of *D. indica*, the locally isolated albino *D. indica* and the species *P. tucumanensis* showed higher resistance (DSR < 2, 50 dpi) toward two pathogens evaluated. However, the behaviour of these wild species regarding *C. Gloesosporioides* has not received due attention. The value of the species above mentioned are remarkable; they are potential parents within the genetic improvement program of commercial strawberry in the area. These materials are currently stored at the Strawberry Active Germplasm Bank, which belongs to the local university, Universidad Nacional de Tucumán (UNT), Argentina. The generation of reactive oxygen species (ROS: H_2O_2, $O_2.-$, HO., etc) in living organisms have been studied extensively because they participate in important physiological and pathological processes (Griffiths et al., 2011). During plant-microbe interaction, they play a double role: first, they are involved as early signals of defensive responses, and second, they exert a toxic effect on possible aggressors. In plants, ROS species are rapidly generated due to the incomplete reduction of oxygen after the attack of certain pathogens (Bolwell et al., 2002; Grant and Loake, 2000; Wolfe et al., 2000; Salazar et al., 2007), producing a rapid and localized cell death known as HR (Hypersensitive Reaction) in most of the cases, It is assumed that this defensive reaction prevents the development of biotrophic pathogens at the infection site (Gilchrist, 1998; Pennell and Lam, 1997). However, ROS can also be produced in susceptible plants when they are attacked by necrotrophic pathogens that induce widespread cell death (Greenberg and Yao, 2004; Yao and Greenberg, 2006; Govrin and Levine, 2000). In our laboratory we have recently observed that some wild strawberries and species related exhibited different degrees of tolerance toward the virulent isolate L9 of *C. gloeosporioides*. The aim of this study is to evaluate the histopathology of the plant-pathogen interaction between wild strawberries and related species with the isolate L9 of *C. gloeosporioides* and the occurrence of biochemical markers associated to a defensive response as H_2O_2. We consider that the present study is important for the strawberry improvement programs. The results obtained can be used as a new approach toward the characterization of resistance sources to anthracnose.

Results

Phytopathological test

The interaction between the isolate L9 of *C. gloeosporioides* and different strawberry species were analyzed and the Disease Severity Rating (DSR) was estimated (Fig 1). All *Fragaria* species (*F. chiloensis*, *F. virgina* and *F. vesca*) and the commercial *Fragaria x ananassa* cultivar Pájaro, used as control, exhibited values of DSR close to 5 (very sensitive) on the 9th day post infection (dpi), whereas the red-fruit *D. indica*, white-fruit *D. indica*, *D. chrysantha* and *P.*

tucumanensis showed DSR < 2, on 9 dpi.

Histological analysis

After 9 dpi, the genotypes studied showed noticeable differences and were separated in two groups. One of them included the species of the genus *Duchesnea* and *Potentilla* that showed no evidence of fungal damage whereas the other, comprising the species of genus *Fragaria*, showed clear evidence of the fungal attack. In species of genus *Duchesnea*, further differences were observed in contrast to mock inoculated controls (Fig. 2A); the presence of germinated conidia, germ tubes with irregularly shaped appresoria and hyphal growth on the epidermis were observed on 9 dpi; however, penetration of pathogen into the host tissue was not observed (Fig. 2B). An evaluation of the density of the pathogen appresoria showed 20 appresoria per mm^2 (average). The occurrence of idioblastic cells with unknown content (Fig. 2C and 2D), changes in cell wall, such as thickening of the wall epidermal cells, stomata and mesophyll cells (Fig. 2E and 2F), starch accumulation on atypical places (Fig. 2G) and starch accumulation in stomata occlusive cells(Fig. 2H) were also observed. The results obtained in the red-fruit and the white-fruit forms of *D. indica* were similar. However, *D. chrysantha* presented clear differences in the profile of cell wall thickening and occurrence of idioblastic cells because these traits were less noticeable and fewer. In addition, the starch accumulation in stomata and mesophyll cell on 20 dpi covers almost all cell cytoplasm of these tissues (data not shown). *F. vesca* plants died on 20 dpi and showed differences in behavior in comparison with the other two species of the genus *Fragaria* analyzed. On 9 dpi many appesoria were observed on the surface of the leaves (Fig. 3A), most of which exhibit infection pegs and cuticle penetration; 80 appresoria per mm^2 (average) were counted. At that time, appesoria with infection pegs were also observed in petioles. Regarding *F. chiloensis*, on 9 dpi, the pathogen achieved an intracellular development (Fig. 3B) causing noticeable histological changes in leaves, such as swollen epidermal cells (similar to those observed in *Duchesnea*) and the presence of starch in occlusive stomata cells. In the petioles, the hyphae developed in parenchyma tissues without reaching the xylem. Plants died from 9 to 20 dpi. Average appressoria per mm^2 was 55. *F. virginiana* was the most susceptible of all the species studied, dying on 9 dpi; hence, it was impossible to evaluate histological changes. The extended necrosis was complete both in leaves and petioles. In leaves, the total destruction of mesophyll and epidermal cells was observed, with hyphae development and acervuli formation (Fig. 3C). *F. virginiana* and the cv Pájaro of *F. x ananassa* were the only species that exhibited acervuli formation on petioles in advanced anthracnose symptom stages. Average appressoria per mm^2 was 34. In petiole sections intracellular penetration of pathogen was observed, with a formation of a typical vesicle of penetration that characterizes this biotrophic phase (Fig. 3D). In the case of *P. tucumanensis*, despite all attempts, no noticeable histological changes were observed in either infected or not infected plants.

Oxidative burst detection

This experiment was carried out in order to find out whether the observed resistance observed in the phytopathological test
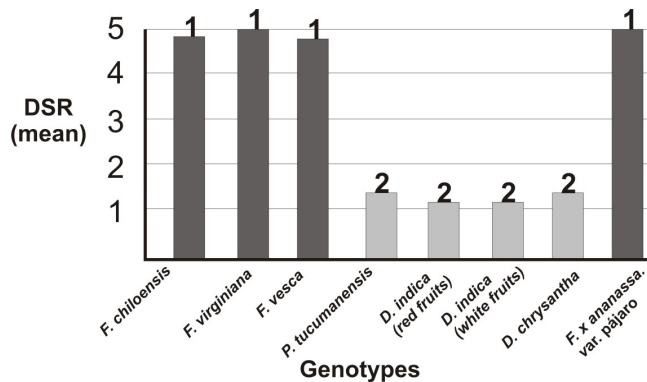
Fig 1. Average values of DSR in wild genotypes related with commercial strawberry and the commercial cultivar Pájaro used as a control species. DSR values with different numbers represent statistically different values (Tukey test, P = 0.05).

exhibited by certain genotypes against the virulent pathogen isolate L9 may be associated with an oxidative burst. In this way, the qualitative change of H_2O_2 in leaves was determined. The present results show that plants of the genera *Duchesnea* presented a strong oxidative burst as compared with plants treated with water (Fig. 4A and 4B) although the level of H_2O_2 depends on the species. Higher accumulation of H_2O_2 was observed in *D. chrysantha* than in red-fruit *D. indica* and white-fruit *D. indica* (data not shown). On the contrary, H_2O_2 accumulation was very weak or was absent in species of genera *Fragaria* analyzed (Fig. 4C, 4D, 4E and 4F).

Discussion

Changes of morphological structures and metabolism that can further be passive (innate) or active can be detected as the induction of a defense response in plants; this process involves a series of biochemical reactions (Schmelzer, 2002).The DSR values obtained agree with previous studies with Salazar et al. (2003) and Arias (2005). The results obtained here clearly show that plants exhibiting some degree of resistance are exposed to pathogens, and induce a defense response; however, a weak response, if any, takes place in susceptible plants. Accordingly, it is possible to speculate that the thickening of both the cuticle and cell wall observed in *Duchesnea* species is a passive resistance barrier to the pathogen and to the fungus enzymes endo-polygalacturonases (endo-PG) and pectin lyases (Perfect et al., 1999). Besides, the significant thickening of stomata cell wall , mesophyll and epidermal cells, the presence of idioblastic cells (with unknown content) and the accumulation of large starch grains in atypical tissues for this species confirmed that a physiological process was triggered by the plant/pathogen interaction (active resistance). The thickening of stomata cell wall, as well as mesophyll and epidermal cells, may be due to lignin deposits that hinder the entrance of the pathogen. Cell wall thickening is one of the most commonly observed phenomena in plants after a pathogen attack (Cadena Gomez and Nicholson, 1987; Dalisay and Kué, 1995; Schmelzer, 2002). These changes are expected for epidermal and mesophyll cells. In the case of stomata, however, cell wall thickening may take place due to their tendency to reflect any metabolic alteration, such as respiratory and photosynthetic
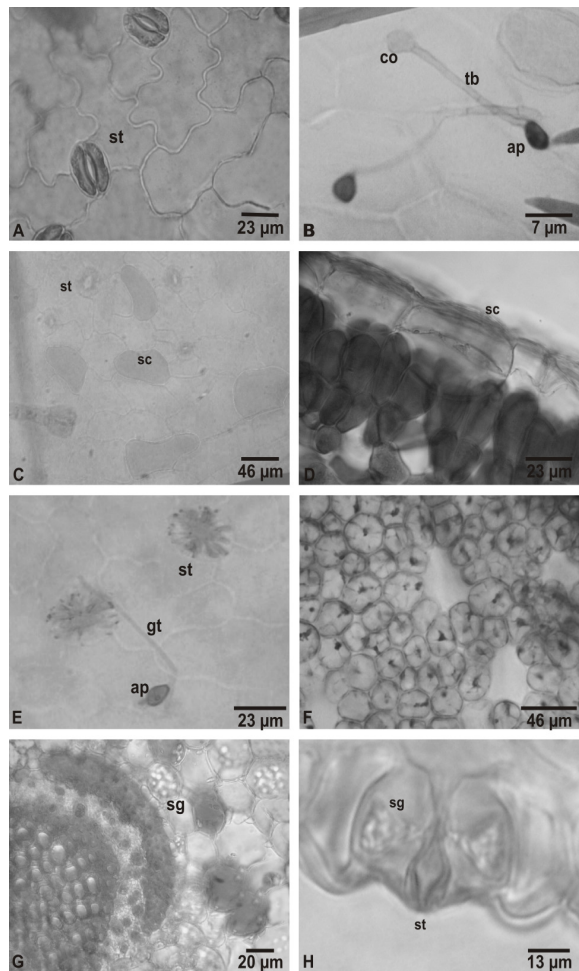

Fig 2. Morpho-anatomic analysis of *D. indica* leaves infected with conidia of the isolate L9 of *C. gloeosporioides*. A: abaxial epidermis of mock inoculated plants (control); B: conidia, germ tubes and appressoria on the leaf surface 9 dpi. No penetration was observed; C: abaxial epidermis showing idioblastic cells 9 dpi; D: swollen epidermal cell in transversal leaf cut 9 dpi; E: thickening of stomata cell wall 9 dpi; F: thickening of cell wall in mesophyll cells 9 dpi ; G: starch grain accumulation in petioles cells 20 dpi; H: starch grain accumulation in guard cells 20 dpi. References: co:conidia, gt: germ tube, ap: appresoria, ic. Idioblastic cells; sc: swollen epidermal cell, st: stomata, sg: starch grain, dpi: days post inoculation.

rate, regulation of gas exchange and transpiration; these processes are frequently affected during pathogen attack (Willmer, 1983). Plenty of studies attest that an earlier oxidative burst takes place in plants-pathogen incompatible interactions; however, in compatible interactions, the oxidative burst takes place at a very low level or it has been totally suppressed (Grant and Mansfield, 1999; Wolfe et Al., 2000; Harrach et al., 2008; Salazar et al., 2007). The toxicity of H_2O_2 and other reactive oxygen species have also been recorded for a great number of pathogens (Grant and Mansfield, 1999; Shetty et al., 2003; Mlicková et al., 2004). The latter has also been associated to lignin synthesis and accumulation (Dalisay and Kué, 1995). The interaction of
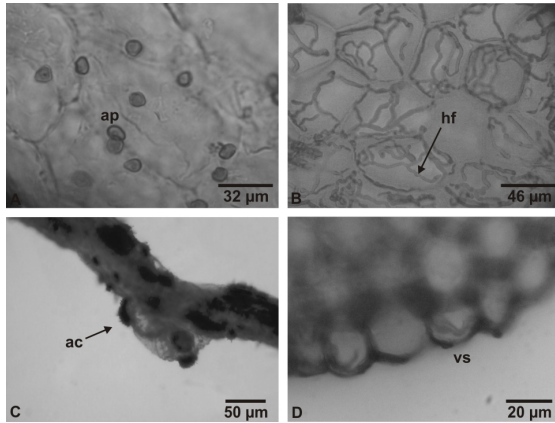
Fig 3. A: Leaf surface with many appessorias. 9 dpi (*F. vesca*); B: secondary necrotrophic hyphae developing into epidermal cells. 9 dpi (*F. chiloensis*); C: Leave transversal cuts showing acervules. 9 dpi (*F. virginiana*), D: Detail of the vesicle of penetration. (*F. vesca*). References: ap: appressoria; vs: vesicle of penetration; hf: necrotrophic hiphae; ac: acervuli; dpi: days post inoculation.
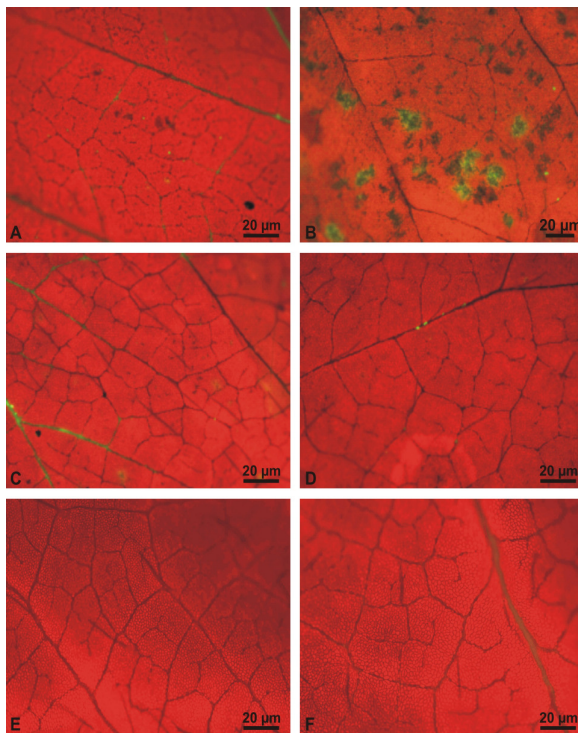

Fig 4. Accumulation of hydrogen peroxide in leaves 4 hpi of plants treated and not treated with the isolate L9 of *C. gloeosporioides*. A: oxidative burst in untreated plants of *D. chrysantha* (control); B: oxidative burst in infected plants of *D. chrysantha*. C: oxidative burst in untreated plants of *F. vesca* (control); D: oxidative burst in infected plants of *F. vesca*. E: oxidative burst in untreated plants of *F. ananassa* (control); F: oxidative burst in infected plants of *F. ananassa*.

plants belonging to genera *Fragaria* and certain isolates of *Colletotrichum* genera showed this type of reaction (Salazar et al., 2007), confirming that the oxidative burst would be an important signal of resistant response in plants. The present results clearly show that the wild species tested presenting resistance against the isolate L9 of *C. gloeosporioides* corresponded to those that exhibit the ROS accumulation; this is coincident with the resistance observed both in values of DSR and histological studies. Conversely, the *Fragaria* species assayed did not exhibit oxidative burst and was susceptible (showed susceptibility) to isolated L9 of *C. gloeosporioides*. It was also observed that as *F. ananassa* cv. Pájaro and *F. chiloensis* showed a low ROS accumulation, the level of H_2O_2 attained proved to be totally ineffective to protect plants against this pathogen. These results confirmed that an incompatible interaction between isolate L9 of *Colletotrichum* and species of genera *Duchesnea* do exist and, on the contrary, the interaction with species of genera *Fragaria* are compatible and, apparently, no interaction takes place with *P. tucumanensis*. Taken as ordinary phenotypic, the resistance/susceptibility character reinforces the taxonomic criterion analyzed by Arias (2005). Based on morphologic, anatomic and molecular studies, she suggests that *Fragaria*, *Duchesnea* and *Potentilla* should remain as separate taxonomic entities. The difference observed in the number of appresoria per mm^2 also showed a correlation between a number of appresoria and the susceptibility of species. The appresoria is a fungus structure that enables hyphae fixation onto the leaf surface, from which the penetration starts, involving mechanical forces and hydrolytic enzymes (Perfect et al., 1999). Apressoria formation is stimulated in *C. gloeosporioides* by contact with a hard surface, as it was observed in the case of red pepper infection (Il-Jung Ahn et al., 2004), and ethylene treatment in avocado (Liu and Kolattukudy, 1998) and banana (Podilla et al., 1993; Kolattukudy et al., 1995). Although the cuticle characteristic is not studied in this work, previous studies with scanning electron microscope showed structural differences between *Fragaria*, *Potentilla* and *Duchesnea* cuticles (Arias, 2005). The results obtained lead to the conclusion that *P. tucumanensis* present a non-host type of interaction with *C. gloeosporioides*; the plant did not present any symptoms of anthracnose disease after the infection with the isolate. It is also worth mentioning that it was not possible to observe any change in either appresoria formation, histological changes or H_2O_2 accumulation. However, previous reports have suggested that *P. tucumanensis* presents some interaction with virulent isolates of *C. acutatum* (Arias, 2005). Last but not least, the present analysis confirmed the outstanding potential of *Duchesnea*, among the other species studied, as resistance source against the anthracnose.

Materials and methods

Plant materials

The genotypes of *Duchesnea indica* (Andrews) Focke with red fruit and a white-fruit form of *D. indica*, and all other species used in this work, namely *Duchesnea chrysantha* (Zoll & Moritzi) Miq., *Fragaria vesca* L., *Fragraria chiloensis* Duch, *Fragaria virginiana* Duchesne and *Potentilla tucumanensis* Castagnaro & Arias were obtained from the Strawberry Active Germplasm Bank at Universidad Nacional de Tucumán (UNT), Argentina. They were asexually propagated in vitro from stolons tips, and then

taken to flower pots under controlled conditions to ensure plant health. Prior to experiments, plants were kept at 27 ºC, 70 % RH, photoperiod of 16 hours per day for 20 days to guarantee the optimal plant health. For phytopathological experiments, plants growing actively with at least 3 completely expanded leaves, without symptoms of disease or stress, were used per genotype. In our experiments, these conditions were achieved with 40-day-old plants.

Inoculations of plant materials

Experiments were carried out with the isolate L9 of *C. gloeosporioides* (Racedo, 2007), which was cultivated in Potato Glucose Agar (PGA) for 10 days at 28 ºC, under continuous white light. For inoculation experiments plants were sprayed with an aqueous suspension of conidial (1,5 x 10^6 conidia/ml-1) obtained by filtering through sterile gauze to remove mycelial debris under axenic conditions.

Phytopathological test

The experiment was randomized with six plants per genotype and experimental lot units, four infected, and two used as controls (mock inoculated). The Disease Severity Rating (DSR) was evaluated in petioles in scale from 1 (without disease) to 5 (maximum severity) (Delp and Milholland, 1980) on 9, 20, 30, 40 and 50 days post inoculation (dpi).
 Experimental data obtained from the phytopathological test were analyzed with the Statistix Program for Windows (Analytical Software, 1996). LSD test was used for determining the arithmetic mean of DSR value (significance level, 0.05) of plant inoculated and the Analysis of Variance test (ANOVA) was used for evaluating the data dispersion with respect to the mean value. Each experiment was performed twice and *F. x ananassa* cv. Pájaro was used as a positive control.

Histopathology

For histopathology studies, longitudinal and cross sections of petioles and leaves were carried out in control and inoculated plants, and at different times after inoculation. The plant-pathogen interaction at epidermal level was analyzed in leaves according to D`ambrogio de Argüeso(1986). Samples were mounted on glass slide with glycerin water and observed with a Zeiss light microscope and with an Enoza polarized light microscope. Photographs were taken with a Canon digital camera PowerShot A430 of 4.0 megapixels.

Detection of oxidative burst

Plant leaves of D. chrysantha, Duchesnea, D. indica f. albocaput, F. vesca, F. chiloensis, F. virginiana, F. ananassa cv. Pájaro, and P. tucumanensis species inoculated with L9 were used to detect the accumulation of H2O2 using the 2',7'-Dichlorofluorescein diacetate (DCFH-DA) fluorescent probe, according to Boszó et al. (2005). DCFH-DA (50µM) was freshly prepared in 10 mM phosphate buffer (pH 7.4) the day of the experiment from a 10 mM DCFH-DA dimethyl sulfoxide stock solution. Three young and fully expanded leaves per plant were used in these assays. Experiments were repeated two times using five plants for each treatment. Leaves from inoculated and mock-inoculated plants were detached from plants, immediately plunged into DCFH-DA solution, and two three-minute vacuum shockswere performed to ensure total infiltration of tissues. Samples were

then incubated for 15 minutes in darkness without vacuum, and then analyzed by fluorescent microscopy (Olympus-BX51 with U-LH100HG fluorescence system, and a U-MWB2 blue excitation filter). Images were captured by Olympus video/photo adapter.

Acknowledgements

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