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Differential Pathogenic Response in Strawberry Tissues and Organs by *Colletotrichum acutatum*

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Abstract: The susceptibility of different tissues and organs from strawberry plants, cv "*Camarosa*", to *Colletotrichum acutatum* was tested using a severity index based on infection response. Symptoms developed on inoculated tissues were characterized along 30 days. Flowers, except sepals, petioles and fruits were the most susceptible organs to the pathogen and they became necrotic tissues at 30 days post inoculation (dpi). Also, well-developed acervuli, which produced masses of orange-pink spores, were observed on these infected organs. An asymptomatic stage or latency phase was observed in green and white strawberry fruits. In spite of they were inoculated anthracnose symptoms were observed only when they became red fruits. On the other hand, strawberry leaves and sepals were resistant to infection by *C. acutatum* and only small flecks or light brown spots were observed reaching a size of 1 to 5 mm at 30 dpi. Likewise, the susceptibility of stolons and crowns to *C. acutatum* was evaluated as intermediate at 30 dpi. Finally, the infection process of the fungus on strawberry leaves and petioles was studied using light and electron microscopy. Pre-penetration events were similar on both, leaves and petioles: However, differences between colonization of strawberry leaves and petioles by *C. acutatum* were observed.

Key words: Anthracnose, *Colletotrichum acutatum*, *Fragaria* × *ananassa*, pathogenic response, host-pathogen interaction, ultrastructure.

1. Introduction

Several species of *Colletotrichum* have been described causing strawberry anthracnose [1] but only *C. acutatum* J.H. Simmonds and *C. gloeosporioides* (Penz.) Penz & Sacc. in Penz have been reported in Europe [2-4]. In fact, *C. acutatum* is the most common specie in this region and it is the casual agent of disease in strawberry production areas in Huelva, southwestern Spain [5]. Lesions can occur on all over the plant, including roots, leaves, flowers, stolons, and fruits but anthracnose crown rot is especially severe leading to wilt and plant death [6].

Host colonization and pathogenesis are well characterized for several species of *Colletotrichum* [7, 8]. The initial stages of host infection by *Colletotrichum* spp. include: conidial adhesion to the host surface, germination of conidia, production of germ tubes which differentiate to form melanized appressoria, and penetration of the host cuticle via appressoria [7]. According to Bailey et al. [9], *Colletotrichum* spp., uses primarily two infection strategies intracellular hemibiotrophic invasion and subcuticular invasion, which is used by *C. acutatum* and is characterized by the growth of the pathogen beneath the plant cuticle and within the periclinal walls of epidermal cells [10, 11]. Conidial development of *C. acutatum* on strawberry leaves have been described by several authors [10, 12, 13]. Curry et al. [14] reported the histopathology of *C. acutatum* and *C. fragariae* in strawberry stolons and petioles, showing at ultrastructural level the stages of invasion in these

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tissues. Furthermore, the ultrastructure of the early stages of infection of strawberry petioles by *C. acutatum* describing the infection structures during the penetration phase, such as the penetration peg and infection vesicle have been reported by Arroyo et al. [10]. However, few studies have investigated the invasion and colonization of *C. acutatum* in different tissues and organs from strawberry plants. Thus, it would be interesting to evaluate the susceptibility of different strawberry tissues to infection by *C. acutatum* and to know its strategy of colonization in susceptible and resistant strawberry tissues.

The purpose of this study was to evaluate the susceptibility of different aerial parts of strawberry plants to infection by *C. acutatum* by using a severity index modified from Delp and Milholland [15] and used to evaluate resistance of different strawberry plant cultivars to *C. fragariae*. Furthermore, a study of differential response of pathogenesis in strawberry leaves and petioles was carried out in order to relate the symptoms observed in these tissues with colonization of the fungus.

2. Material and Methods

2.1. Plant Material

Strawberry plants cv. 'Camarosa', susceptible to *C. acutatum* [16], were planted in 10.5 × 13.5 cm plastic pots containing sterilised peat (Klansmann-Deilmann, Geeste, Germany) and grown for 75 d, before inoculation with *C. acutatum*, in a greenhouse maintained at 25 ℃ d / 15 ℃ night ± 5 ℃

2.2. Inoculation

C. acutatum isolate CECT-20240 was used in this study. The fungus was grown on potato dextrose agar (PDA, DIFCO) for 7 days at 25 ℃ under continuous fluorescent light (Osram L 18 W/21-840 Hellweiss Lumilux Cool White, 75 μE m^{-2} s^{-1}). Conidial suspensions were prepared by flooding the culture plates with 4-5 mL of sterile distilled water, scraping the colony surface with a scalpel, and filtering the suspension through sterile cheesecloth. The concentration was adjusted to 1×10^6 conidia mL^{-1} using a haemocytometer [16].

A specific technique of inoculation was used for each tissue and organ as described by Arroyo [16]. Crowns, leaves, petioles, stolons, flowers, peduncle, and fruits in different ripening stage (green, white and red ripening stage) were inoculated by applying 50 μL droplets of conidial suspension [10]. Inoculated plants were enclosed in plastic bags for 48 h to maintain high relative humidity and were incubated in a growth chamber at 25 ℃, with a 16 h photoperiod beneath fluorescent light (Sylvania Luxline Plus F58W/840 Cool White de Luxe, Germany, 100.5 μE m^{-2} s^{-1}). Control plants were treated with 50 μL of sterile distilled water and incubated as described above.

2.3. Evaluation of the Susceptibility to C. Acutatum

The evaluation of symptoms in the strawberry inoculated tissues was made over 30 days, using a severity scale modified from Delp and Milholland [15] (Table 1). Strawberry tissues showing a severity index (SI) ≤ 2 were considered resistant, intermediate with a SI > 2-5 and susceptible if SI ≥ 5.

2.4. Ultrastructural Study of the Infection Process

To study the colonization of strawberry leaves and petioles by *C. acutatum*, samples of both tissues, leaves and petioles, were taken at 4, 8, 5, 24, 48, and 72 h after inoculation. The samples were processed as described by Arroyo et al. [10]. Slides with semithin sections (0.5 μm) were placed on a hotplate at 50 ℃, stained for 1

Table 1 Disease severity index (DSI) modified from Delp and Milholland [15] used for evaluating susceptibility of strawberry tissues to *C. acutatum*.

Lesions	DSI
No lesions	1
Flecks ≈ 1 mm size	2
Lesions > 1-2 mm	3
> 2-10 mm or 25% affected surface	4
> 10-20 mm or 50% affected surface	5
> 20 mm or 75% affected surface	6
Necrotic tissue/organ	7

min with 0.1% aqueous toluidine blue O, and examined using a light microscope (Leitz Aristoplan). Ultrathin 60-80 nm sections were made with a Reichert-Jung Ultracut E ultramicrotome and a diamond knife, and collected on 300-mesh copper grids [17]. Grids were stained with 7% aqueous uranyl acetate and lead citrate.

Sections were observed and images collected using a Philips CM-10 transmission electron microscope (TEM).

2.5. Statistical Analysis

The data were processed using the statistical package STATISTICA for Windows v.5 (StatSoft, Inc). The homogeneity of variance of data was checked with the Kruskal-Wallis's nonparametric test ($P < 0.05$).

3. Results and Discussion

Evaluation of susceptibility of different strawberry tissues to *C. acutatum* is presented in Table 2. Flowers were highly susceptible to the pathogen specially pistil, stamen, and petals, which became necrotic completely at 9 days post-inoculation (dpi). However, sepals were asymptomatic after 30 dpi. Flower peduncle developed lesions at 2 dpi, which were as orange-brown spots and 2-3 mm size. These lesions enlarged as infection progressed and eventually the peduncle became necrotic at 12 dpi. Lesions in crowns were orange brown and 1-2 mm size at 2 dpi. At 30 dpi, strawberry plants, which were infected in their crowns, showed symptoms in their aerial parts displaying wilting [6]. Inoculated leaves were asymptomatic and they showed resistant fleck-type lesions. However, inoculated petioles displayed numerous orange-brown, 1-2 mm size lesions at 2 dpi, which enlarged as infection progressed and they became sunken, dark brown, 15-20 mm-size lesions at 30 dpi. Also, lesions on stolons were brown, 10-15 mm size at 30 dpi. Likewise, anthracnose fruit rot was only visible on red fruit. Green and white strawberry fruits were asymptomatic until they turned red ripening stage. This asymptomatic stage or latency phase might be related to volatile compounds content of strawberry fruits [18]. Fruiting structures or acervuli of the fungus were observed on blossoms, petals, flower peduncle, red fruit and petioles from leaves (Table 2).

Table 2 Disease severity index (DSI) in different strawberry tissues and organs, acervuli production and evaluation of the susceptibility to C. acutatum at 30 dpi. Each data is the mean of 4 samples. Values with the same letter do not differ significantly, LSD test ($P = 0.05$). DSI ≤ 2 were considered resistant; 2 < DSI ≤ 5, intermediate; and DSI > 5, susceptible.

Inoculated tissue	DSI	Acervuli production	Evaluation
Blossoms	7.00 ± 0.00^{a}	+	Susceptible
Peduncle	6.25 ± 0.25^{b}	+	Susceptible
Petals	7.00 ± 0.00^{a}	+	Susceptible
Sepals	1.33 ± 0.33^{e}	–	Resistant
Green fruit	7.00 ± 0.00^{a}	–	Susceptible
White fruit	7.00 ± 0.00^{a}	–	Susceptible
Red fruit	7.00 ± 0.00^{a}	+	Susceptible
Adaxial surface leaf	1.25 ± 0.29^{e}	–	Resistant
Abaxial surface leaf	2.25 ± 0.25^{d}	–	Resistant
Petioles	6.50 ± 0.29^{ab}	+	Susceptible
Stolons	5.00 ± 0.00^{c}	–	Intermediate
Crowns	3.25 ± 0.25^{d}	–	Intermediate

In relation with the infection process of leaves and petioles strawberry the early stages of infection were similar to both tissues [10]. As reported by Arroyo et al. and Leandro et al. [10, 13], conidia germinated abundantly on both, petioles and leaves, at 4 hours post inoculation (hpi) by forming a germ tube from either end of the conidium, and occasionally from both ends. By 8 h after inoculation, globose and subglobose appressoria were detected as swellings of the germ tube tips. On leaves, especially on the adaxial surface, appressoria originated from short germ tubes. However, appressoria observed on petioles usually developed from elongated germ tubes, which often reached a length several times the conidium size. Microcyclic conidiation also was observed on either adaxial or abaxial surfaces of inoculated leaves, producing abundant secondary conidia from conidial and hyphal phialides. However, these conidiogenic structures were

not detected on petioles. Subsequently, the appressorium initiated the formation of a V-shaped penetration peg, which pushed the host cuticle. The peg was about 400 nm wide where it passed through the cuticle and eventually reached the upper epidermal wall, then a small infection vesicle was formed, which developed intramurally. Whereas other species of *Colletotrichum* produce a penetration peg and develop an intracellular infection vesicle [8, 19-21], *C. acutatum* produces a penetration peg that develops a subcuticular and intramural infection vesicle, which is related to species with a wide host range that are considered generalist invaders [9].

All these infection structures were developed by *C. acutatum* during infection process of strawberry petioles and leaves [10] so the initial infection strategy of the pathogen was similar to other *Colletotrichum* spp. that infect other hosts [8, 9, 19, 21-26].

However, because of cuticle of leaves has a greater thickness than cuticle of petioles (≅700-900 and 350-500 nm respectively) the penetration phase was more prolonged in leaves. At 24 hpi, the stage of pathogen in leaves was as peg penetration inside host cuticle but in this time it was as infection vesicle inside wall host cell in petioles (Table 3).

Table 3 Summary of colonization of *C. acutatum* in different strawberry tissues. Infection structures and location of the pathogen throughout time: Peg penetration (Pp), Infection vesicle (IV), H (hypha).

Strawberry tissues/organs		
Time (hpi)	Leaf	Petioles
24	Pp, plant cuticle	IV, wall cell
48	H, epidermal layer	H, epidermal layer and cortex
≥ 120	H, epidermal layer	H, epidermal layer and cortex

The pathogen developed abundant hyphae from the infection vesicle in the subcuticular and intramural spaces. Also, in further stages, at 2 days post inoculation (dpi) the pathogen was only observed in the subcuticular and intramural spaces and occasionally invaded several epidermal cells in leaves, specially in the adaxial surface (Figs. 1A-1B) but numerous hyphae invaded the epidermal layer and the second and third layer of cortex in petioles in this period (Figs. 1C-1D). At 10 dpi, the pathogen remained restricted in the wall

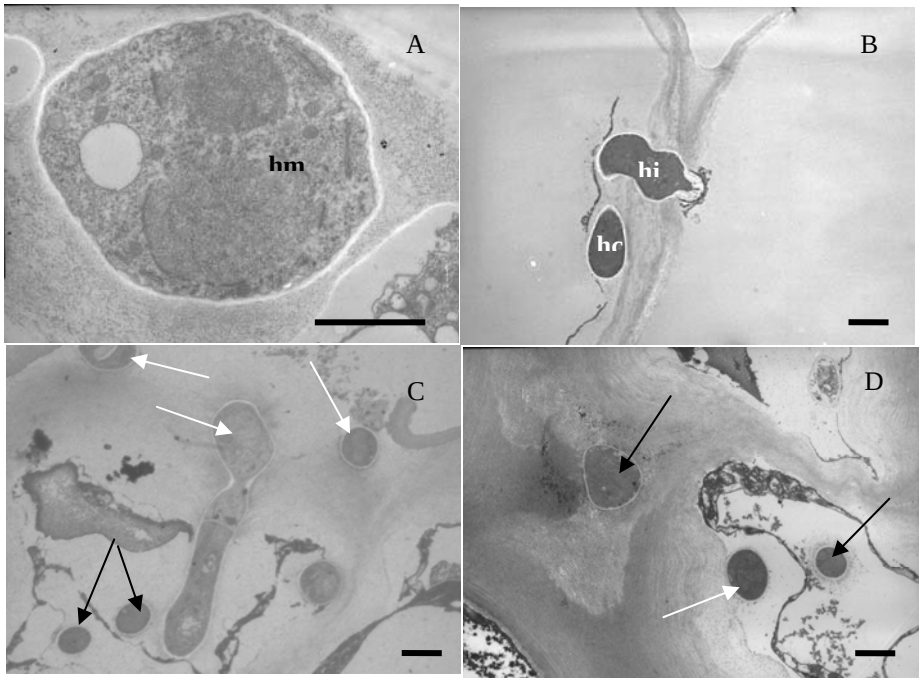

Fig. 1 Colonization of strawberry leaves and petioles by *C. acutatum* at 10 dpi (Transmission Electron Microscopy). A-B, intramural (hm), intra (hc) and intercellular (hi) hypha in the epidermal layer of leaves. C-D, subcuticular, intramural, and intercell hyphae (white arrow), and intracellular hyphae (black arrow) in the epidermis (C) and cortex (D) of petioles. Scale bar =1 μm.

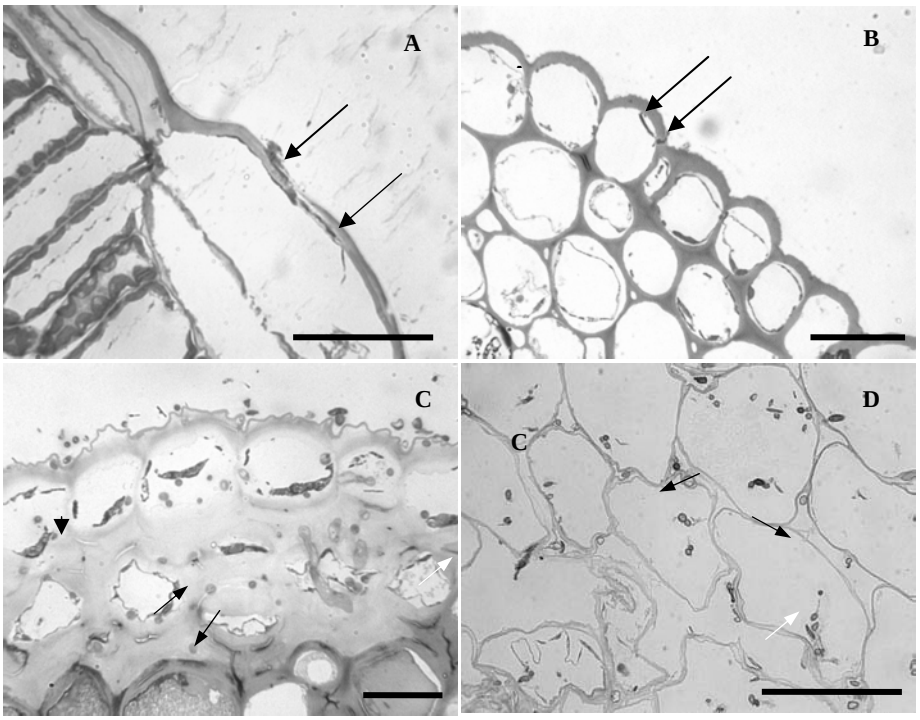

Fig. 2 Colonization of strawberry tissues by *C. acutatum* in leaves (A-B) and petioles (C-D) at 15 dpi (Light Microscopy). A-B, pathogen invading intramural spaces and epidermal cells of leaves (black arrows). C-D, pathogen invading epidermal and subepidermal layer (C) and cortex of petioles (D); subcuticular (headarrow), intramural, intercell (black arrows), and hyphae intracell (white arrows). Scale bar =10 μm.

of the epidermal cell of leaves (Figs. 2A-2B) whereas in petioles, the hyphae continued invading the cortex (Figs. 2C-2D). Table 3 summarizes the stages of colonization of the fungus in the studied tissues. Strawberry plants showed a different response to infection by *C. acutatum* from different tissues. There was a fungal progress constraint in the most photosynthetic tissues. The ultrastructural study of the infection process confirmed the differences in the colonization strategy of the fungus on leaves and petioles and also a different response of the tissues to the pathogen. It is unknown which mechanisms could be implicated for limiting the invasion and keep it in a symptomless stage in the foliar tissue.

4. Conclusions

The main feature of this work is the extension of knowledge about the infection process of *C. acutatum* and the specific response from different strawberry tissues to invasion. Furthermore, the ultrastructural study of the interaction strawberry-pathogen confirmed differences in the colonization strategy on leaves and petioles. Further works are needed to know the mechanisms that limit the invasion of the *C. acutatum* specially in the most photosynthetic tissues, which constrained fungal progress and kept it in a symptomless stage.

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