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Events Occurring at *Colletotrichum orbiculare* Infection Sites on Watermelon

V. M. Russo[1], B. M. Russo[2], and B. Cartwright[2]

[1]United States Department of Agriculture, Agricultural Research Service, South Central Agricultural Research Laboratory, Lane, OK 74555

[2]Wes Watkins Agricultural Research and Extension Center, Oklahoma State University, Lane, OK 74555

3Currently Merck & Co., 180 Ashwood Circle, Plano, TX 75075

ABSTRACT

There is insufficient information concerning infection and subsequent colonization of watermelon by the anthracnose fungus (*Colletotrichum orbiculare*). Since control methods are, in part, developed based on the biology of the pathogen it is important to know how that organism colonizes tissues. Observations of this interaction were made with bright-field, phase-contrast, and reflected-fluorescence microscopy on inoculated living and dead host and dead non-host (onion) tissue. Cytochemical tests were employed to interpret fungus and host responses. Spore germination on dead tissues was observed by 18 h, but was delayed an additional 6 h on living tissue. There was no evidence of direct penetration of the fungus through the cuticle, cell wall, or stomata using primary infection mycelia originating from appressoria on any tissues. There were no autofluorescent deposits below appressoria, but the hypersensitive response was observed in plant cells in living tissue. Phenolics and tannins were present in non-colonized host tissues. The colonization method used by *C. orbiculare*, race 6, in this study was different from that previously reported for the watermelon anthracnose fungus (*C. lagenarium*, syn. *C. orbiculare*). This suggests that different physiological processes were used during infection, and may be related to the race of *C. orbiculare*. It remains to be determined how other *C. orbiculare* races colonize watermelon, and ascertain how those methods are related to resistance.

RESUMEN

No existe suficiente información sobre los procesos de infección y subsecuente colonización de la sandía por el hongo agente causal de la antracnosis (*Colletotrichum orbiculare*). Es importante conocer come el patógeno coloniza los tejidos ya que los métodos de control son, en parte, desarrollados en base a la biología del patógeno. Se realizaron observaciones de esta interacción utilizando microscopía de campo brillante, de contraste de fase y de fluorescencia-refleja sobre hospederos inoculados, vivos y muertos, y sobre tejido no hospedero inoculado (cebolla). Se utilizaron pruebas citoquímicas para estudiar las respuestas del hongo del hospedero. La germinación de las esporas sobre tejidos muertos se observó a las 18 horas, pero se retrasó seis horas más sobre el tejido vivo. No hubo evidencia de penetración derecta del hongo a través de la cutícula, la pared celular, o los estomas cuando se usó el micelio de la infección primaria originado de apresorios sobre cualquiera de los tejidos. No hubo depósitos de autoflorescencia bajo los apresorios, pero se observó la respuesta hipersensitiva en células vegetales en tejido vivo. Se presentaron compuestos fenólicos y taninos en los tejidos hospederos no colonizados. El método de colonización usado por *C. orbiculare,* raza 6, en este estudio fue diferente del reportado previamente para el hongo de la antracnosis de la sandía (*C. lagenarium*, syn. C. *orbiculare*). Esto sugiere que diferentes procesos fisiológicos fueron usados durante la infección y pueden estar relacionados a la raza de *C. orbiculare*. Falta ser determinado como otras razas de *C. orbiculare* colonizan la sandía, y descubrir como estos métodos estan relacionados con la resistencia.

Additional index words: Citrullus lanatus, Onion, Allium cepa, cytochemistry, infection process

Gardner (1918), stated that the first authentic report of anthracnose, caused by *Colletotrichum lagenarium* (Pass.) Ell and Hals., on a cucurbit dates to 1867. Lesions formed by *C. lagenarium* on the foliage reduce the canopy, expose fruit to sunburn, and affect photosynthesis (Peregrine and bin Ahmand, 1983). Lesions also form on stems and fruit, and combined with foliar and fruit lesions, result in severe yield losses in watermelon (Amin and Ullasa, 1981; Damicone et al., 1994; Sitterly and Keinath, 1996).

Since Gardner's report, *C. lagenarium* has come to be considered to be synonymous with *C. orbiculare* (Berk. & Mont.) v. Arx [anamorph of *Glomerella cingulata* var. *orbiculare* Jenkins et Winstead], and as many as 7 physiological races of the fungus have been proposed (Jenkins et al., 1964). More recently Wasilaw et al. (1993) determined that instead of 7 races there are 3 vegetative compatibility groups and 2 races. They also stated that their results on differentials agreed with previous reports (Goode, 1958;

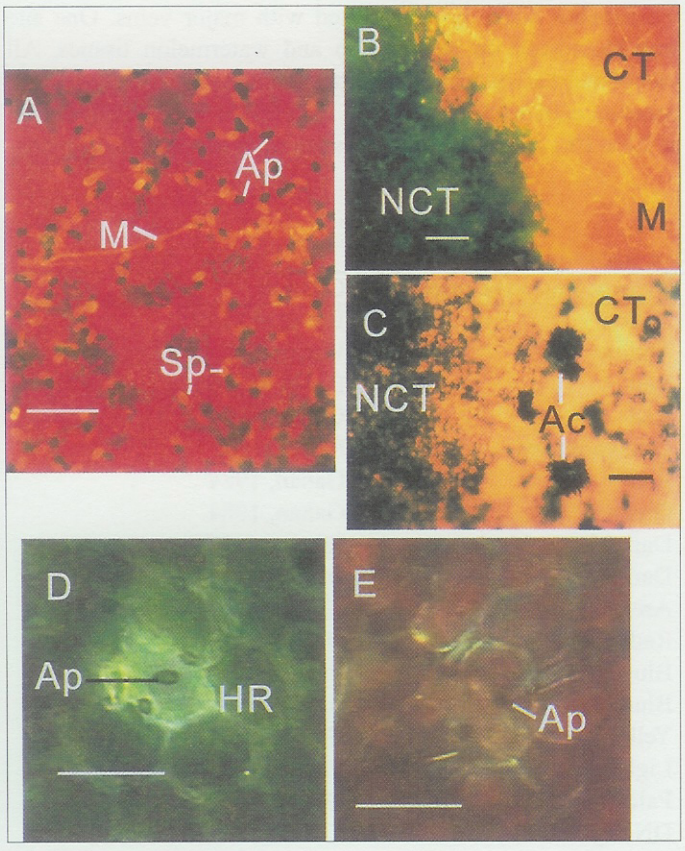

Fig. 1. Germination of *Colletotrichum orbiculare* and colonization of living watermelon tissue. A, Germinated spores (Sp) with appressoria (Ap) and small amounts of mycelia (M) 40 to 48 h after inoculation stained with acridine orange and observed with reflected fluorescence microscopy. B, Mycelia (M), colored yellow, on colonized tissue (CT) as opposed to green colored non-colonized tissue (NCT) stained with acridine orange observed with reflected-fluorescence microscopy at 120 to 168 h. C, Acervuli (Ac) on CT stained with lactophenol-cotton blue at 120 to 168 h. D, Appressorium (Ap) on the lighter colored host cell undergoing the hypersensitive response (HR) at 40 to 48 h viewed with reflected-fluorescence microscopy. E, Dying cells around the infected cell at 72 to 96 h viewed with reflected-fluorescence microscopy. Bar=100 μm in A to C and 50 μm in D and E.

Winstead et al., 1959; Jenkins et al., 1964). These reports were based on symptoms visible to the eye.

In disease development infection occurs between inoculation and the manifestation of visible symptoms, and consists of an interaction between the host and pathogen. Possible reactions that can occur are: completely susceptible - where the disease organism becomes established and severe visible symptoms occur on the plant; completely resistant - where the disease organism does not become established and there are no visible symptoms on the plant; or an intermediate reaction - where the disease organism becomes established, but does not progress as it would if the plant was completely susceptible.

To be a successful pathogen, a fungus must overcome plant defenses (Russo and Bushnell, 1989; Johal et al., 1995; Kobayashi et al., 1995; Matern et al., 1995; Schmelzer et al., 1995). This can occur by physical penetration (Howard et al., 1991), through the use of enzymes to breakdown plant tissues, or by a combination of both methods (Xuei et al., 1988). Sometimes penetration of the plant can be slowed, or defeated, by physical and/or chemical means (Daniels, 1988; Gabriel et al., 1988; Johal and Rahe, 1990; Egley, 1994; Goodman and Novacky, 1994; Dickman et al., 1995; Hwang and Kolattukudy, 1995).

Gardner (1918) stated that *C. lagenarium* (syn. *C. orbiculare*) penetrates the adaxial epidermis during the infection process, but that the exact method used to penetrate the cuticle needed clarification. Other *Colletotrichum* species that cause anthracnose on plants gain entry by direct penetration of the epidermis with a primary infection mycelium produced from an appressorium, or through natural openings (Muirhead and Deverall, 1981; Russo and Pappelis, 1981; Chau and Alvarez, 1983; Porto et al., 1988). Since Gardner's (1918) report there has been little information developed on events occurring at the site of infection of *C. orbiculare* on watermelon. An increased knowledge of this process could lead to better control of *C. orbiculare*. The objective of this study was to employ microscopy and cytochemistry to clarify the process of infection and colonization of watermelon foliage by a race of *C. orbiculare* which causes typical symptoms on watermelon (Russo et al., 1997).

MATERIALS AND METHODS

Preparation of live tissue for inoculation. Watermelon, cv. 'Sugar Baby' (2N), seeds were planted in potting mix (Redi-Earth 3CF, Grace Sierra, Milpitas, CA) in 3.5 L pots in a greenhouse. Fully expanded second true leaves were removed from plants and placed adaxial side up on moistened filter paper in 100 X 15 mm petri dishes.

Preparation of dead tissue for inoculation. Watermelon and white onion (*Allium cepa* L. cv. 'Sterling') (non-host), were used. Second true watermelon leaves were harvested as before, and disks were cut from the leaves with a 12 mm dia metal cork borer. To disable chemical defense mechanisms some of the disks were placed in boiling water for 1 min. To visualize fungal development on host tissue, other disks were cleared by boiling in 70% ETOH, and then rinsed in water. All disks were placed, adaxial side up, on two moistened layers of filter paper in 100 X 15 mm petri dishes. Onion pieces were prepared so that dead tissue, comprised of intact adaxial walls and cuticles, could be inoculated as described by Russo and Pappelis (1981).

Preparation of inoculum and inoculation. Cultures of *C. orbiculare*, race 6 (Jenkins et al., 1964), ATCC #15471, were maintained for 7 days on potato dextrose agar (Becton Dickinson and Company, Cockeysville, MD). Cultures were flooded with 10 ml of sterile distilled water followed by gentle agitation with a camel hair brush to harvest spores. The suspension was diluted to 10^6 conidia/ml.

For live tissue, 5 µL droplets of the suspension were placed on the adaxial surface to the left or right of the main vein servicing lobes on the leaf. There were 6 to 8 sites per leaf. Droplets of the same volume were placed on the adaxial surface in the center of pieces of dead onion tissue, and on dead watermelon tissue not associated with major veins. One site was inoculated on dead onion and watermelon tissues. All inoculated tissues were incubated in closed petri dishes under constant cool-white fluorescent light (Duro-Test, Fairfield, NJ) at 23 ± 1°C. A minimum of 10 sites from sections from living

Table 1. Number, objective of test, stain of reagent, expected positive response, and citation.

Number	Target of test:	Stain or reagent:	Expected positive response:	Citation
1a	Flavins	2-aminoethyldiphenyl[z]	Yellow	Dai et al., 1995
1b		citric acid-boric acid[z]	Yellow	Hariri et al., 1991
2a	Lignin	Mirandé reagent	Red	Dai et al., 1995
2b		Phloroglucin	Red	Gahan, 1984
3a	Phenols	Modified Hoepfner	Orange-brown	Reeve, 1951
3b		Phenosafranin[y]	Red[x]	Gahan, 1984
4	Polyphenol	Ferric chloride	Green	Gahan, 1984
5a	Cellulose	ZnCl-KI	Dark blue to black	Johansen, 1940
5b		Iodide - sulfuric acid	Dark blue to black	Johansen, 1940
6	Halo	lactophenol - cotton blue	Area around mycelia blue	Kunoh and Akai, 1969
7	Tannins (catechins)	Vanillin	Red	Dai et al., 1995
8	Peroxidase	tetramethylbenzidine	Blue	Imberty et al., 1984
9a	Suberin	Sudan IV	Blue-black	Johansen, 1940
9b		dibasic sodium phosphate[z]	Yellow	Gahan, 1984
10	Callose	Aniline blue - sucrose[z]	Light green	Currier and Strugger, 1955
11	Cellulose brightener	Calcofluor MZR New[z,y]	Pale blue green	Galbraith, 1981
12	Nuclear material	Acridine orange[z]	DNA-green, RNA-red	Yamamoto and Uchida, 1982
13a	General	Methylene blue	Blue	Johansen, 1940
13b		Safranin red	Red	Johansen, 1940
14	Pectin	Ruthenium red	Pink to red	Johansen, 1940
15	Reaction beneath attempted infection	autofluorescence	Light-green	Harris and Hartley, 1976, Mayama and Shishiyama, 1978

[z]Fluorescence microscopy required for test.
[y]Vital dye.
[x]If cell is dead

Table 2. Positive or negative reactions in host or *Colletotrichum orbiculare for* selected tests[z] on living watermelon tissue at 40-48, 72-96, and 120-168 hours after inoculation.

	Host observed at:			Fungus observed at:		
Test	40-48 h	72-96 h	120-168 h	40-48 h	72-96 h	120-168 h
2a	-	-	-	+	+	+
3a	-	-	+	-	-	-
3b	-	-	+	-	+	-
4	-	-	+	-	-	-
5a	-	-	-	+	+	+
7	-	-	+	-	-	-
9a	-	-	-	+	+	+
10	-	-	-	+[y]	+[y]	+[y]
11	-	-	-	+[y]	+[y]	+[y]
12	-	-	-	+[y]	+[y]	+[y]
13a	-	-	-	+	+	+
13b	-	-	+	+	+	+
14	-	-	+	+	-	-
15	+	-	-	-	-	-

[z]If test number is not shown, the result was negative.
[y]Mycelia fluoresced.

Table 3. Positive or negative reactions[z] on dead onion and watermelon tissue observed at 20-24 hours after innoculation.

	Onion		Watermelon	
Test	Plant	Fungus	Plant	Fungus
6	-	+	-	+
10	-	+	-	+
11	+	+	-	+
12	DNA-	DNA+	DNA-	DNA+
	RNA-	RNA+	RNA-	RNA+
13A	-	+	-	+

[z]If test number is not shown, the result was negative.

and dead tissue samples were viewed at each observation time.

Microscopy and cytochemistry. Observations were made with an Olympus BX60 system (20X UPlanFl objective, 1X to 2X variable magnifinder, WH10X/22 oculars, 200X to 800X total magnification) for bright-field, phase-contrast, and reflected-light, 100W halogen, fluorescence microscopy. For fluorescence, excitation was at 330-385, 400-440 or 460-490 nm.

The target of the test, stain or reagent, expected positive response, and citation providing further information on methodology is provided (Table 1). All tissues were treated with stains or reagents on slides or in small petri dishes. Prior to observation tissues were placed on slides and covered with a cover glass.

All tissues were observed beginning 1 h after inoculation. For the first 6 hr, and at 12 and 18 hr, observations were made with bright-field or reflected-fluorescence microscopy with the latter being used to visualize autofluorescence responses (Harris and Hartley, 1976; Mayama and Shishiyama, 1978). Other cytochemical and microscopic tests were observed on dead tissues at 18 and 24 h after inoculation, and on live tissue at 20 to 24, 40 to 48, 72 to 96, and 120 to 168 h after inoculation.

RESULTS

Live tissue. Phase contrast microscopy was not effective on disks because of specimen thickness. However, observations could be made with bright field and reflected fluorescence microscopy. Germination of more than 90% of spores, principally by appressoria, was observed after 24 h, and small amounts of mycelia were observed by 40 to 48 h (Fig. 1A). Mycelial growth was superficial. Primary infection mycelia were not observed to be developing below appressoria, penetration into stomata, or subsequent ramification of mycelia through epidermal or sub-epidermal tissues. Extensive superficial mycelia and acervuli were formed by 120 to 168 h (Fig. 1B,C).

The hypersensitive response, as defined by Goodman and Novacky (1994), was observed in plant cells at 40 to 48 h (Fig. 1D), and cellular collapse leading to formation of lesions was observed by 96 h (Fig. 1E). For most cytochemical tests there were no positive responses in plants. Where responses in plants were observed they were most obvious at 120 to 168 h post-inoculation (Table 2). In *C. orbiculare*, prior to 120 to 168 h, positive responses were observed when spores and/or mycelia were stained with methylene blue or safranin (general

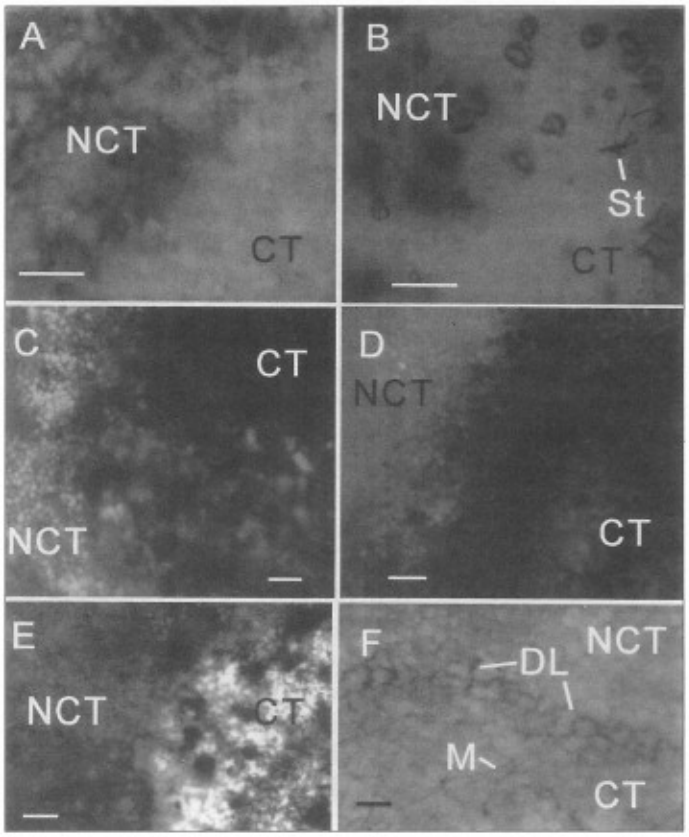

Fig. 2. Host responses to colonization by *Colletotrichum orbiculare* in living watermelon tissue at 120 to 168 h. A, Deposition of polyphenols, dark area, in non-colonized tissue (NCT) treated with ferric chloride. B, Deposition of tannis, dark area, in NCT on tissue treated with vanillin. Shown are setae (St) from developing acervuli. C, Graduation of dense to diffuse staining from colonized tissue (CT) to NCT on tussue stained with safranin. D, Graduation of dense to diffuse staining from CT to NCT on tissue stained with phenosafranin. E, Cells with pectin removed, light area, in CT on tissue stained with Ruthenium Red. F, Demarcation line (DL) between CT and NCT with mycelia (M) shown in CT on tissue stained with Ruthenium Red. Bar = 100μm.

stains), phenosafranin (vital stain), phloroglucin (lignin), ruthenium red (pectin), and ZnCl (cellulose) (Table 2). In addition, the mycelia fluoresced when treated with aniline blue (callose) and Calcofluor MZR New (cellulose brightener), and nuclei in mycelia could be distinguished when stained with acridine orange.

Positive responses for polyphenols and tannins were observed at 120 to 168 h after inoculation (Fig. 2A,B). Host cells were stained most densely with safranin and phenosafranin at the inoculation site, and the staining density decreased in host cells as distance from the inoculation site increased (Fig. 2C,D). Cellulose was not degraded, but pectin was removed from cell walls (Fig. 2E). Methylene blue and ruthenium red stains indicated a line of demarcation between tissues on which mycelia were growing and those which were free of mycelia (Fig. 2F).

Dead tissue. Phase contrast microscopy was not effective on dead watermelon tissue. Bright field microscopy was adequate for observations, especially on cleared tissue. On onion tissue phase contrast microscopy presented an improved image. More than 90% of spores were germinated by mycelia and/or appressoria by 18 h post-inoculation on dead onion and on dead cleared, or non-cleared watermelon tissue (Fig. 3A,C). There was no evidence of a primary infection mycelium developing below appressoria, penetration into stomata, or subsequent ramification of mycelia through epidermal or subepidermal tissues. By 40 to 48 h after inoculation large amounts of superficial mycelia were produced (Fig. 3B,D). After that time mycelial development was extensive and further observations of spores could not be made. The only difference between responses on dead onion and watermelon tissues was that onion cell walls fluoresced with Calcofluor White, while those of watermelon did not (Table 3).

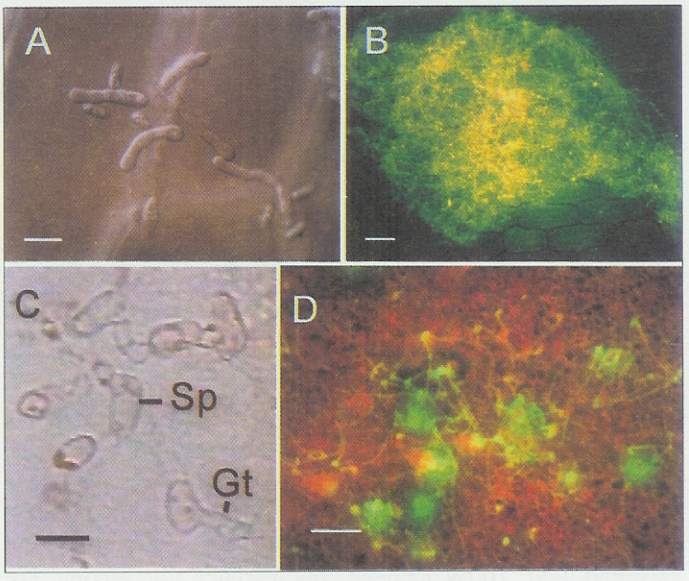

Fig. 3. Germination of *Colletotrichum orbiculare* and colonization of dead onion and watermelon tissue. A, Germinating spores on dead onion tissue at 18 h viewed with phase contrast microscopy. B, Mycelia on dead onion tissue at 40 to 48 h stained with aniline blue and viewed with reflected fluorescence microscopy. C, Germinating spores (Sp) and germ-tubes (Gt) on cleared, dead, watermelon tissue at 20 to 24 h viewed with bright field microscopy. D, Mycelial development at 40 to 48 h on dead, not cleared, watermelon tussue stained with acridine orange and viewed with reflected fluorescence microscopy. Bar=20μm, A and C, an 100 μm, B and D.

DISCUSSION

Ultimate susceptibility of a plant to a disease organism does not preclude an initial period of resistance, especially immediately after inoculation and prior to the formation of visible lesions. The interaction between a host and a fungus is a balance of chemical activities affecting identification of suitable sites (Hadwiger and Loschke, 1981; Sheng and Showalter, 1994), and neutralization of chemicals produced by the host or fungus (Albersheim and Anderson, 1971; Cervone et al., 1989). A successful pathogen must overcome plant defenses, which in this watermelon cultivar appears to be due to a combination of physical barriers and chemical responses.

The lack of primary infection mycelia produced below *C. orbiculare* appressoria on dead watermelon or onion tissues, in cellular responses to infection have presumably been disabled, suggests that physical barriers are involved. Papillae are chemical and physical barriers to penetration (Aist, 1976), often deposited in plant cells below appressoria which autofluoresce (Mayama and Shishiyama, 1978; Russo and Pappelis, 1983). These deposits were not observed in this study, and therefore did not impair penetration below appressoria. Why penetration of the cuticle or cell wall by an infection peg from *C. orbiculare* appressoria is inhibited needs more study.

Alternatively on living tissue, spore germination was delayed beyond the time observed on dead tissues. This suggests that chemical defense mechanisms exist in living tissues and must be overcome by *C. orbiculare*. These chemical defenses may be involved in the observed hypersensitive response (Schmelzer et al., 1995). Plant defenses did not appear to prevent eventual colonization of watermelon tissue which occurred within the time frame previously described (Sitterly and Keinath, 1996).

Glomerella cingulata (Ston.) Spauld. & Schrenk, often described as the teleomorph of *Colletotrichum* species, can transform plant-produced toxins to non-toxic substances (Miyazawa et al., 1995). The pattern of staining with phenosafranin suggests that a fungal toxin diffuses into host tissue in advance of the expanding colony and kills host cells. The area where toxic compounds produced by *C. orbiculare* interact with compounds which are components of the plant defense system may be illustrated by the observed demarcation zone. Fungal enzymes may also contribute to removal of pectin from host cell walls during colonization which could compromise cell integrity. Leaking material from cells may be utilized by *C. orbiculare*. Fungal enzymes are used by other

Colletotrichum species to degrade pectin in cell walls during colonization (Benhamou et al., 1991; Russo and Pappelis, 1981).

Some *Colletotrichum* species directly penetrate host cells through the cuticle and/or the adaxial surface of the epidermis (Nicholson and Hammerschmidt, 1992; Morin et al., 1993). Some of these produce primary infection mycelia or vesicles below appressoria (Muirhead and Deverall, 1981; Russo and Pappelis, 1981; Irwin et al., 1984; O'Connell and Bailey, 1988; Byrne et al., 1997;). This is like the penetration method described by Gardner (1918) for *C. lagenarium* (syn. *C. orbiculare*). A modification of this infection method is that described by Porto et al. (1988) for *C. trifolii* et Essary, where cells were penetrated directly, and extensive hyphae was produced on stem surfaces of alfalfa (*Medicago sativa* L.).

Morin et al. (1993) reported that primary infection hyphae occurred beneath appressoria of *C. orbiculare*. However, in that case *C. orbiculare* was parasitizing cells of Cocklebur (*Xanthium occidentale* Bertol.) that had previously been penetrated by a rust fungus. In this study the colonization method used by *C. orbiculare* is like that of *C. acutatum* Simmonds on Valencia orange (*Citrus sinensis* (L.) Osbeck) where abundant mycelia was found on tissue surfaces, and acervuli formed several days after inoculation (Zulfiqar, et al., 1996).

The objective of this research was to employ modern techniques to obtain additional details about events occurring at sites of infection by a *C. orbiculare* isolate that forms lesions on watermelon (Russo et al., 1997). It was determined that this fungal isolate does not use the same method to infect as previously reported (Gardner, 1918). This suggests that different races of the same fungus use different physiological processes during infection. There is insufficient information in the literature to make categorical statements about the methods used by *C. orbiculare* to penetrate and colonize watermelon tissues. It remains to be determined how other races of *C. orbiculare* colonize watermelon, and ascertain how those methods are related to resistance.

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