

Use of Green Fluorescent Protein-Transgenic Strains to Study Pathogenic and Nonpathogenic Lifestyles in *Colletotrichum acutatum*

Sigal Horowitz, Stanley Freeman, and Amir Sharon

First and second authors: Department of Plant Pathology, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250; and third author: Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel.
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ABSTRACT

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Colletotrichum acutatum, which causes anthracnose disease on strawberry, can also persist on several other plant species without causing disease symptoms. The genetic and molecular bases that determine pathogenic and nonpathogenic lifestyles in *C. acutatum* are unclear. We developed a transformation system for *C. acutatum* by electroporation of germinating conidia, and transgenic isolates that express the green fluorescent protein (GFP) were produced. Details of the pathogenic and nonpathogenic lifestyles of *C. acutatum* were determined by using GFP-transgenic isolates. Major differences between colonization-mediating processes of strawberry and of other plants were observed. On the main host, strawberry, the germinating conidia formed branched, thick hyphae,

and large numbers of appressoria were produced that were essential for plant penetration. In strawberry, the fungus developed rapidly, filling the mesophyll with dense mycelium that invaded the cells and caused necrosis of the tissue. In nonpathogenic interactions on pepper, eggplant, and tomato, the conidia germinated, producing thin, straight germ tubes. Appressoria were produced but failed to germinate and penetrate leaf tissue, resulting in epiphytic growth without invasion of the plant. Penetration of the plant occurred only several days after inoculation and was restricted to the intercellular spaces of the first cell layers of infected tissue without causing any visible damage. Much of the new fungal biomass continued to develop on the surface of inoculated organs in the nonpathogenic interaction. The differences in fungal development on strawberry compared with the other plant species suggest that signal molecules, which may be present only in strawberry, trigger appressorial germination and penetration of the primary host.

Colletotrichum acutatum J.H. Simmonds is a major pathogen of strawberry, causing severe bud, crown, and fruit rots. Infected plants first develop typical anthracnose lesions that later coalesce, causing girdling of leaves and stems leading to plant death (9). *C. acutatum* is pathogenic on additional plant species including fruit trees, vegetables, and ornamental anemone (2,4,11). In cross-inoculation experiments of anemone and strawberry with *C. acutatum* isolates from the alternative hosts, plant death occurred in the infected anemone and strawberry plants regardless of isolate source, anemone or strawberry (12). In Israel, anemone is the only other known plant species attacked by *C. acutatum* strawberry isolates; however, the same isolates that cause strawberry anthracnose can persist on additional plant species that do not develop disease (8,13). In this study, we reisolated the pathogen from a variety of artificially inoculated plants, 2 to 3 months after inoculation, both from the leaf surface and from within the tissue, after surface sterilization. Moreover, *C. acutatum* was isolated from asymptomatic weeds growing in anthracnose-infected strawberry fields. Persistence of other *Colletotrichum* spp. on plants that do not develop disease has been reported in a number of cases (31), suggesting that nonpathogenic lifestyles may be common in this genus. These data raise the questions of how *Colletotrichum* host range is defined, and what are the differences between the symptomatic and asymptomatic interactions. It also is unclear how these fungi persist for long periods of time on plants without causing any visible damage to the tissues: where the fungus is

located, how it obtains nutrients, and the phytopathological significance of this mode of fungal-plant interaction.

Studies on infection processes in *Colletotrichum* spp. revealed a range of colonization strategies (3,27). Some species penetrate the host tissues through wounds (3) and stomatal pores (17) and cause immediate destruction of the host tissues, whereas other species breach the cuticle with the aid of appressoria (23,24,34). In these species, the fungus grows in the subcuticular spaces for several days without causing any symptoms, before the necrotrophic hyphae develop, invade the host cells and destroy them. A third mode of infection is characterized by an extended latent period that lacks any visible disease symptoms. These fungi breach the cuticle and the epidermal cell wall and establish biotrophic hyphae that interact with the host cells but do not kill them. At a certain stage, the fungus differentiates necrotrophic hyphae that cause disease (3,27). Very little is known about the interaction of *C. acutatum* and various hosts. The interactions between *C. acutatum* and citrus (35) and pine seedlings (25) have been previously studied, but only on a limited scale. A recent study has reported on the formation of secondary conidia of *C. acutatum* in strawberry, but only on the surface of detached leaves, which may have implications on disease epidemics (18). The interaction of *C. acutatum* with strawberry resembles the second group of fungi, i.e., the fungus uses appressoria to penetrate into the plant and symptoms appear 72 to 96 h later. Interaction of the *C. acutatum* strawberry isolates with other plant species resembles the third type of interactions, but without the transition to the necrotrophic phase. Most details of the pathogenic and nonpathogenic lifestyles in *C. acutatum* are poorly understood.

The study of fungus-plant interactions has been facilitated in recent years with the development of the green fluorescent pro-

Corresponding author: A. Sharon; E-mail address: amirsh@tauex.tau.ac.il

tein (GFP) as a reporter gene and vital marker in filamentous fungi (6,22,32). Transgenic fungi that express GFP are easily detected in planta without any external intervention, and therefore, such isolates provide valuable information on fungal development in planta and in situ. GFP has been used in a wide range of fungi to study temporal and spatial gene expression and protein localization, or as a viable marker to characterize fungal development (21).

In the current study, we used GFP-transgenic isolates to characterize pathogenic development of *C. acutatum* on strawberry, and compared it with the development of the fungus on plant species that do not develop disease symptoms. In order to obtain the transgenic isolates, a transformation system was developed for *C. acutatum* by electroporation of germinating conidia as previously described for *C. gloeosporioides* f. sp. *aeschyromene* (30).

MATERIALS AND METHODS

Fungi, plants, and growth conditions. *C. acutatum* isolate TUT-149 from strawberry was used for transformation (9). More than 50 transgenic strains were isolated and used throughout this study and all maintained pathogenicity on strawberry similar to the wild-type TUT-149 isolate. Plant inoculation studies were conducted with a single representative transgenic isolate M5 throughout this study. The fungi used in this study were maintained in the dark on modified Mathur's medium (MS) as previously described (33). Prior to plant inoculation, conidia were collected from plates and diluted to a final concentration of 5×10^6 conidia per ml. Plant species used in this study included strawberry (cv. Malach), tomato (*Lycopersicon esculentum* Mill. cv. 149), eggplant (*Solanum melongena* L. var. *esculentum* Nees cv. Classic), pepper (*Capsicum annum* L. cv. Maccabi), and garden bean (*Phaseolus vulgaris* L. cv. Hilda) provided by Hishtil Nursery, Nahsholim, Israel.

Plant inoculation. Plants were grown in 10-cm plastic pots (0.5-liter volume) in peat/vermiculite medium (vol/vol, 1:1), and were maintained in a greenhouse at 25°C. Plants were inoculated by spraying with conidial suspensions (5×10^6 conidia per ml) of either wild type or the transgenic isolates. All treatments contained 0.02% Tween 20 to increase dispersion of the conidia to the leaves. After inoculation, the plants were maintained at 100% relative humidity by covering with plastic bags for 72 h (8). Inoculation experiments consisted of 20 plants of each species, four replicates of five plants per species, which were organized in a randomized design. Six leaves were sampled at each time point. Five mock-inoculated plants (0.02% Tween 20) of each species were used as controls. Fungal germination, appressorial formation, and symptom appearance were recorded at different time points after inoculation. Fungal development was followed and documented on a plastic surface using petri dishes in order to compare development on different plant surfaces. In these experiments, conidial suspensions were germinated in 1 drop of MS

liquid medium and examined over a 14-day period. Each experiment was conducted twice with similar results being recorded.

Plasmids. A DNA fragment was amplified by polymerase chain reaction (PCR) from *C. acutatum* genomic DNA using the arbitrary microsatellite primer 5'-GACACGACACGACACGACACGACAC-3'. The PCR amplification product was subcloned into the plasmid pUC57 (MBI-Fermentas, Vilnius, Lithuania), and the sequence of the fragment was determined. The fragment size was 931 bp with no putative open reading frame identified. The fragment was excised by digesting with *Hind*III and *Xba*I or with *Hind*III, and the resulting fragments were cloned into corresponding sites in the gGFP (22) and pNOM102 (29) plasmids, respectively. The resulting plasmids were designated gGFP-CA and pNOM-CA.

Fungal transformation. Electroporation of germinating conidia was performed according to Robinson and Sharon (30) with the following modifications. The wild-type fungus (isolate TUT-149) was cultured on solid MS medium for 4 days. Conidia were collected in pea juice (30), diluted to a final concentration of 10^6 conidia per ml, and incubated at 28°C for 4.5 to 5 h to initiate germination. The germinating conidia were collected, washed with cold electroporation buffer (1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 50 mM mannitol [Sigma Chemical, St. Louis], pH 7.5), and concentrated to 10^8 conidia per ml, and 100- μ l aliquots were distributed to cold electroporation cuvettes (Bio-Rad, Hercules, CA). Five micrograms of circular or linear plasmid DNA was used in each cuvette. The linear plasmid was first digested at a single restriction site with *Hind*III. Electroporation was performed with a gene pulser (Bio-Rad) operating at 1.4 kV, 800 Ω , and 25 μ F. After application of the electric pulse, the conidia were plated on regeneration medium (145.7 g of mannitol [Sigma] per liter, 4 g of yeast extract [Difco Laboratories, Detroit] per liter, 1 g of soluble starch [Sigma] per liter, 16 g of agar [Difco] per liter, and 50 ml of pea juice per liter). After 10 h, a top overlay of water agar with 125 μ g of hygromycin B (Cal-Biochem, San Diego, CA) per ml was added. Transgenic colonies appeared 4 to 5 days after transformation.

Statistical analysis. The number of transformants obtained from 10 independent cuvettes per treatment (with linear and circular plasmids) was analyzed by least significant difference (LSD) according to the Tukey-Kramer multiple comparison test at a significance level of $P < 0.05$, using the JMP software package for PC version 3.2.6 (SAS Institute, Cary, NC).

DNA manipulation. Fungal DNA was extracted as previously described (10). Southern blotting and probe labeling were performed with enhanced chemiluminescence direct nuclear acid labeling and detection systems according to the manufacturer's instructions (Amersham Life Science, Little Chalfont, England). PCR conditions were as previously described (10).

Microscopy. Fluorescent microscopy was performed using an epifluorescent microscope (Leica DMRBE; Leica USA, Deerfield, IL). Examined tissues were placed on a microscope slide, submerged in a water droplet, and covered with a glass slip. The K3/L4 filter cube with a peak transmission for excitation at 470 to 490 nm and peak transmission for suppression at 515 to 560 nm was used for GFP detection. Light microscopy was performed with the same microscope without any filters. Confocal microscopy was performed with an inverted laser scanning confocal microscope (Zeiss 410; Carl Zeiss, Oberkochen, Germany) with excitation and emission at 488 and 510 nm, respectively.

RESULTS

Transformation. Conidia were harvested 4.5 to 5 h after submerging in pea juice. At this stage, a short germ tube had formed and the nucleus in the cell adjacent to the germ tube began to divide. Transformants were obtained only under these conditions, because exposure of conidia to the electric pulse after shorter or longer germination periods failed to produce any transgenic

TABLE 1. Effect of homologous sequence and plasmid form on transformation rates in *Colletotrichum acutatum* from strawberry

Plasmid	Plasmid form	No. of transformants ^a
gGFP ^b	Circular	0.8 \pm 0.25
gGFP	Linear ^c	1.8 \pm 0.51
gGFP-CA ^d	Circular	2.9 \pm 0.52
gGFP-CA	Linear	6.0 \pm 0.83

^a Values are standard error of the means of transformants obtained from 10 independent cuvettes per treatment that was analyzed by least significant difference (LSD) according to the Tukey-Kramer multiple comparison test at a significance level of $P < 0.05$. LSD = 2.16.

^b Transformants obtained with 5 μ g of the original gGFP plasmid (30).

^c Plasmids that were linearized by digestion with *Hind*III.

^d Transformants obtained with 5 μ g of plasmid gGFP-CA containing the 931-bp homologous GACAC fragment of *C. acutatum* genomic DNA.

colonies. Similarly, the exposure of transformed conidia to hygromycin selection requires precise timing. Selection was obtained only when the transformed conidia were exposed to the selective medium 8 to 10 h after transformation. Earlier or later exposure to the hygromycin resulted either in no colonies or in intense background, respectively. These results are consistent with those of Robinson and Sharon (30), who described the strict requirement for timing of the electric pulse and hygromycin exposure when transforming *C. gloeosporioides* f. sp. *aeschyromene*.

Less than a single transformant per cuvette (5 µg of DNA, 10⁷ conidia) was obtained, on average, when a circular form of the gGFP plasmid was used (Table 1). Almost two transformants per cuvette were obtained with a linear form of this plasmid, but

transformation rates were still very low. The inclusion of the *C. acutatum* homologous DNA fragment in the vector in its linearized form resulted in a threefold increase in transformation rates compared with that of the other treatments ($P < 0.05$) (Table 1). These numbers are still considerably lower than the transformation rates obtained with *C. gloeosporioides* f. sp. *aeschyromene*. However, the electrical conditions used in this study were the same as those used for *C. gloeosporioides* f. sp. *aeschyromene* that were not modified, and optimization of the electrical conditions may lead to higher transformation rates.

Southern blot analysis revealed integration into the chromosome of one or more copies of the gGFP-CA plasmid (data not shown). Integration of several copies usually occurred in tandem;

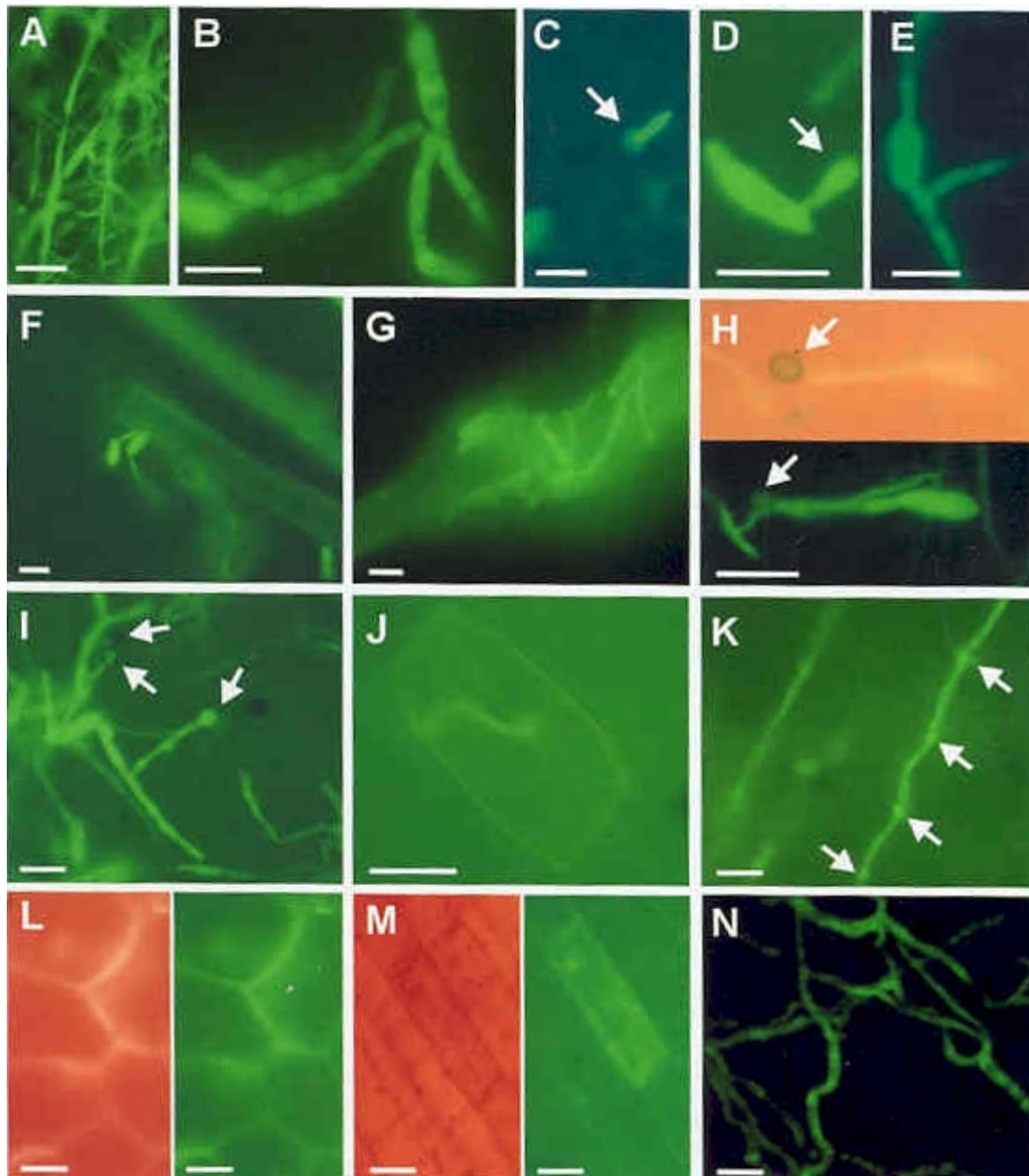


Fig. 1. Pathogenic development of *Colletotrichum acutatum* on strawberry. Strawberry plants were inoculated with *C. acutatum* transgenic isolate M5 and maintained for 72 h in a chamber with 100% humidity under greenhouse conditions. Visible and fluorescent light micrographs of the inoculated tissues were recorded at various times postinoculation (PI). **A**, Fluorescent image of inoculated leaf 3 days PI. **B**, Germination of conidia from both sides 15 h PI. **C**, Secretion of mucilage (denoted by an arrow) and adherence of a conidium to the leaf surface 15 h PI. **D**, Production of secondary conidia 15 h PI (denoted by an arrow). **E**, Formation of thick, highly branched, primary mycelium 15 h PI. **F and G**, Conidia adhere and invade trichomes filling them with mycelia. **H**, Light (top) and fluorescent (bottom) pictures of an appressorium (denoted by an arrow) that is formed at the tip of a germ tube 20 h PI (as denoted by an arrow). **I**, Different stages of appressorium formation; the immature appressoria fluoresce brightly (lower arrow) and gradually lose their fluorescence (middle and top arrows). **J**, Penetration of a hypha through stomatal pores on a petiole. **K**, Development of single hypha on a petiole; swollen internodes of the hypha are marked with arrows. **L**, A combination of visible and fluorescent light (left) and fluorescent light (right) images of mycelium growing between the epidermal cells 3 days PI. **M**, Colonization of a single petiole cell 4 days PI; note the development of necrosis in the light image (left). **N**, Development of mycelium inside the leaf 14 days PI. Image captured with a confocal microscope at a depth of 20 µm. Bars = 15 µm.

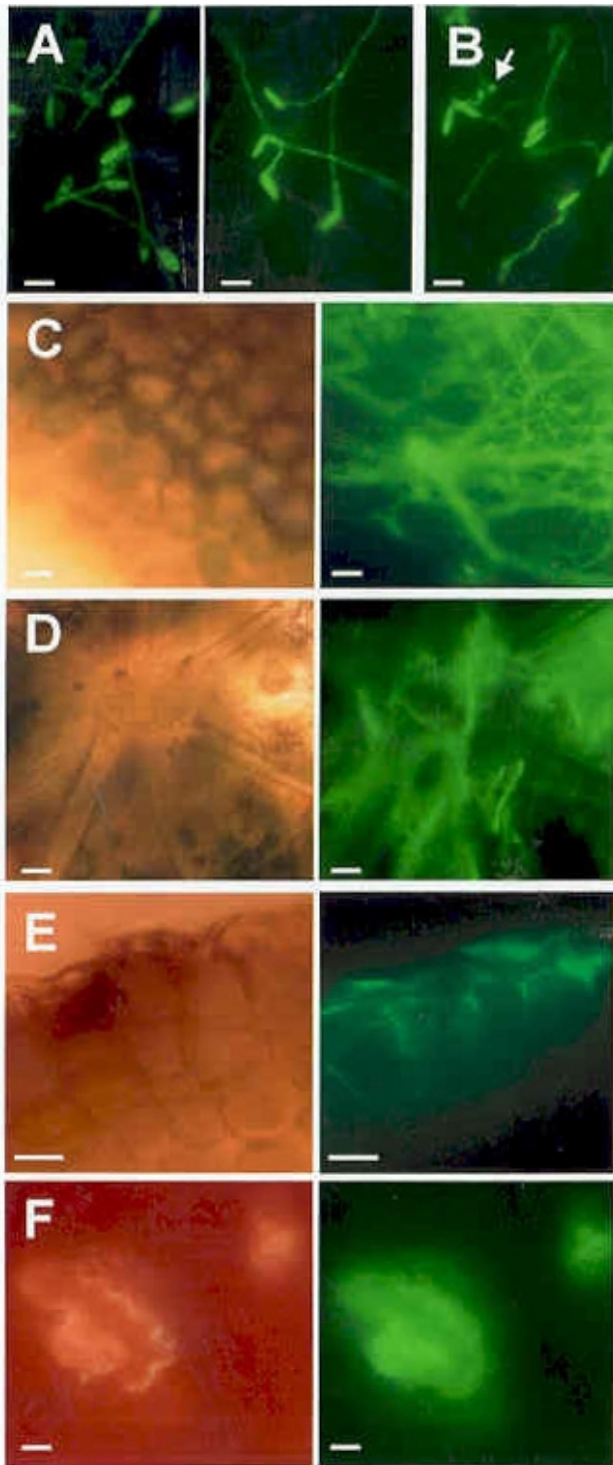


Fig. 2. Nonpathogenic development of *Colletotrichum acutatum* on asymptomatic plants. Eggplant and pepper plants were inoculated with *C. acutatum* transgenic isolate M5 and maintained for 72 h in a chamber with 100% humidity under greenhouse conditions. **A**, Germination of conidia on the surface of a pepper leaf 6 h (left) and 15 h (right) postinoculation (PI). Germination occurred only from one side of the conidium, and the hyphae are thin, unbranched, and linear. **B**, Formation of appressoria on a pepper leaf 20 h PI; note that only some of the germ tubes differentiate appressoria (denoted by an arrow). **C to F**, Light (left) and fluorescent (right) images of the same samples. **C**, Development of mycelium on the surface of a pepper leaf 5 days PI. **D**, Massive invasion of eggplant leaf-trichomes 5 days PI. **E**, A cross section of the stem of an eggplant leaf 14 days PI. Mycelium develops on the surface of the tissue and between (but not within) the epidermal cells. **F**, Infection of a pepper guard cell 7 days PI. Bars = 15 μ m.

however, some patterns were difficult to interpret, suggesting rearrangement of the plasmid. The transgenic isolates were stable without selection and maintained high level expression of the GFP transgene after several months during which they were cultured on MS medium and transferred periodically to fresh medium. GFP expressing isolates could be detected in plant tissues long after inoculation, whereas the wild type isolate was not visible on infected plants (Fig. 1A). All transgenic isolates (50 isolates all together) were tested on strawberry plants and produced typical anthracnose symptoms that were indistinguishable from those produced by the wild-type isolate (data not shown). On tomato, eggplant, pepper, and bean, no disease symptoms developed after inoculation with the wild type or transgenic isolates. These plants were therefore referred to as asymptomatic.

Development of *C. acutatum* on strawberry. The transgenic isolate was as infective as the wild type and was easily detected in infected tissues (Fig. 1A). Conidia attached and germinated on all plant organs several hours after inoculation. The germinating conidia developed multiple, thick germ tubes (Fig. 1B). Approximately 15 h after inoculation, a large amount of primary thick mycelium with many branches developed on the surface of the inoculated organs. The formation of secondary conidia also was observed at this stage (Fig. 1D). The fungus infected all parts of the leaf, but was especially pronounced on leaf trichomes. Large numbers of conidia germinated on the trichomes and infected them with mycelium (Fig. 1F and G). Appressoria were observed between 20 and 50 h after inoculation. The newly formed appressoria (between 20 and 40 h) had not yet developed a thick, melanized cell wall and therefore fluoresced brightly (Fig. 1H and I). During maturation, the appressoria gradually lost the green fluorescence until they became completely dark (Fig. 1I). Large numbers of brown, nonfluorescing appressoria were detected on inoculated leaves 50 to 60 h after conidial application. The massive production of appressoria and the collapse of the appressorium at a later stage indicated that appressorium-assisted penetration plays an important role in infection of strawberry by *C. acutatum*. Penetration through stomatal pores was observed occasionally (Fig. 1J), but only on stolons and petioles and not on leaves, even when the germinating conidia developed in the vicinity of a stomatal pore (data not shown). Large amounts of mycelium covered the inoculated tissues 3 days postinoculation. On leaves, the mycelium was dense without any special order (Fig. 1A), whereas on stolons and petioles, single hyphae grew in a straight line where swollen thick organelles were formed, which may serve for attachment or as penetration points (Fig. 1K). No disease symptoms were apparent on the plants 3 days postinoculation, but the fungus could be detected inside the leaves as bright mycelium that grew around the cells (Fig. 1L). Symptoms were first observed after 4 days in the form of necrotic lesions. The only organs that did not develop symptoms were senescing leaves, where despite massive colonization, no disease symptoms were evident. Necrosis began in single cells that were colonized by the fungus and gradually turned brown (Fig. 1M). Disease outbreak followed and was accompanied by heavy infestation of the cells, evidenced by intense fluorescence inside the cells of the necrotizing tissues (Fig. 1M). Concomitant with the transition to the necrotrophic phase, aerial acervuli were formed, giving rise to new conidia (data not shown). After transition to the necrotrophic phase and development of disease symptoms, the fungus was no longer observed on the surface of leaves, but it could be detected in the inner parts of the infected tissues with confocal microscopy (Fig. 1N).

Development of *C. acutatum* on asymptomatic plants. The development of *C. acutatum* was studied on all the plant species specified previously. These plants are not natural hosts of *C. acutatum* and do not typically develop anthracnose symptoms when inoculated with this fungus (8,13). There were no significant differences in the development of *C. acutatum* on all plant species

at all stages. The results presented describe the development of the fungus at different stages on specific plants (pepper and eggplant). Similar development of the fungus was observed on tomato, bean, and some weed species.

Conidia germinated on inoculated leaves within several hours after application as seen on strawberry. Germination occurred mainly from one end of the conidium, unlike on strawberry where germination occurred from both sides of the conidium (Figs. 1B and E and 2A). The developing hyphae were long, thin, and straight as opposed to the shorter, coiled and thick germ tubes and hyphae that were observed on strawberry. Secondary conidiation was observed occasionally, as seen on strawberry (data not shown). Immature, fluorescing appressoria began to differentiate at the end of some germ tubes after 20 h. Many hyphae did not give rise to appressoria but instead continued to grow without branching as straight, thin hyphae (Fig. 2B). Mature, melanized appressoria were observed between 20 to 50 h after inoculation, but unlike the appressoria that developed on strawberry, they did not germinate, even after several days (Fig. 3C to E). Moreover, many appressoria continued to fluoresce on the asymptomatic plants 2 to 3 weeks after formation. Direct penetration of asymptomatic leaves by the appressoria was not detected by leaf sections or by examination of leaves with a fluorescence stereoscope, although occasional penetration of a single hypha through stomatal pores was observed at this stage (data not shown). The fungus grew vigorously on the surface of inoculated tissues, forming dense mycelium on all inoculated parts several days after inoculation (Fig. 2C). Similar to strawberry inoculation, the fungus covered the leaf trichomes and penetrated them, forming dense

mycelium on and inside the trichomes (Fig. 2D). Disease symptoms were not detected on any of the inoculated plants at this stage, or several days later, unlike strawberry plants, which were heavily infected and showed severe disease symptoms 5 to 7 days after inoculation. Fluorescent hyphae were detected inside the leaf tissues approximately 1 week after inoculation, but growth was restricted to the subcuticular cell layer and did not progress into the leaf mesophyll space (Fig. 2E). In most cases, the mycelium grew between the epidermal cells without penetrating into the cells, exhibiting quiescent development. Necrosis was observed only in a few cases, in which the fungus invaded and colonized substomatal cells. These cells later became necrotic, but the necrosis was restricted to the specific cell in which the fungus had penetrated, while the surrounding cells remained unaffected (Fig. 2F).

Appressoria development. Appressoria formed on strawberry and other plant species 20 to 50 h after inoculation. On strawberry, the appressoria served to establish infection via direct penetration through the leaf cuticle, whereas on asymptomatic plants, there was no evidence of appressorium-associated leaf penetration. Melanin began to accumulate in the appressoria wall 20 to 40 h after conidial germination, similar to that detected in appressoria formed on the surface of a plastic petri dish (Fig. 3F). At this stage, the appressoria on strawberry and on pepper were indistinguishable from each other, symmetrical and rounded, with thick-pigmented walls, as also was seen on the petri dish. After approximately 50 h, appressoria on strawberry started to lose their symmetric shape, developed lobes and sockets, and eventually collapsed (Fig. 3A). These changes probably resulted from the formation of a penetration peg and the development of a pene-

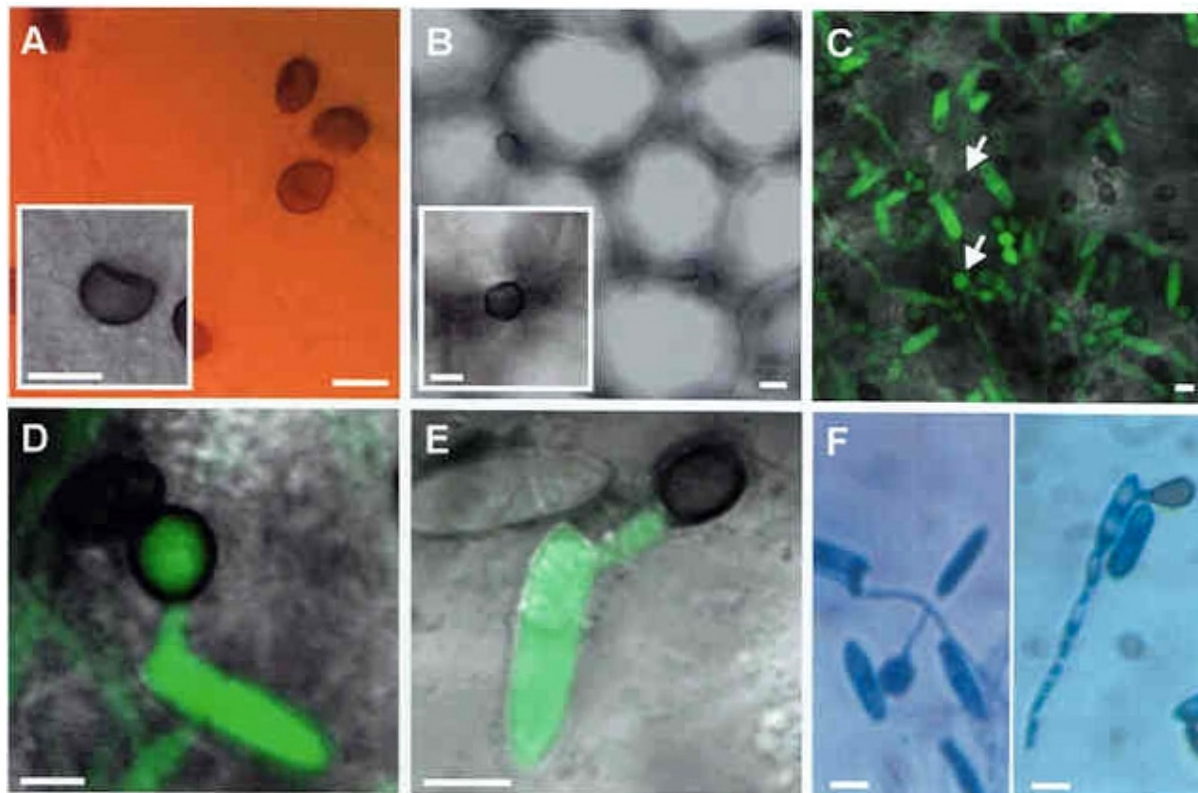


Fig. 3. Formation of appressoria on strawberry and pepper leaves and on a plastic surface. Conidia of *Colletotrichum acutatum* transgenic isolate M5 were inoculated on strawberry and pepper leaves or placed in an MS liquid medium droplet on the surface of a plastic petri dish. The appressoria that were formed on the plastic surface were stained with lactophenol blue solution (Fluka, Rehovot, Israel). Images were taken with **A and F**, light, and inset panels **A and B and C to E**, confocal microscopes. **A**, Appressoria on a strawberry leaf 3 days postinoculation (PI). Inset shows an appressorium after 7 days (note the development of sockets and deformations in the appressoria). **B**, Appressoria on a strawberry leaf 7 days PI (also inset). Appressoria are dark and tend to form between adjacent cells. **C**, Appressoria (denoted by an arrow) on a pepper leaf 10 days PI. **D and E**, A large proportion of the appressoria are fluorescing and **D**, nonfluorescing and fluorescing for up to 10 days. **E**, Melanized appressoria on pepper that maintain their regular shape, are not deformed, and remain symmetrical 10 days PI. **F**, Appressoria on a plastic surface 3 days PI. Appressoria were formed either at the end of a long germ tube (left) or on a very short germ tube adjacent to conidium (right). Sockets or deformations were not observed. Bars = 10 μ m.

trating hypha that occurred concomitant with losses of turgor pressure in the appressorium body (23,24,28). A large number of appressoria were formed above junctions between adjacent epidermal cells (Fig. 3B), probably reflecting a thigmotropic response. These junctions have been suggested to be weaker points in the cuticle, and therefore, formation of appressoria near these areas may facilitate host penetration (7,25,26). Unlike on strawberry, the appressoria on pepper retained their symmetrical shape and did not undergo any structural changes even after 2 weeks (Fig. 3C to E). These appressoria probably did not form a penetration peg, at least not at this stage, fluoresced for a longer period of time than in strawberry, and therefore did not seem to assist in fungal penetration into the tissues of these plants. Appressoria that developed on a plastic surface had similar morphology to those formed on asymptomatic plants: they did not develop a penetration peg, and they retained their symmetrical shape for up to 2 weeks (Fig. 3F).

DISCUSSION

Transgenic isolates of *C. acutatum* were obtained by electroporation of germinating conidia according to the method reported by Robinson and Sharon (30). In the original manuscript, it was suggested that additional fungal species with similar conidial parameters, i.e., small dimensions and a single nucleus, may be transformed by this method after appropriate adjustments. The authors proposed that the crucial factors that may vary between different species are the selection conditions and the time at which the conidia are exposed to the electric pulse. Indeed, the development of the current transformation protocol focused on adjustment of the selection and germination conditions. To our knowledge, transformation of *C. acutatum* has not been previously reported, and our attempts to transform *C. acutatum* protoplasts were unsuccessful due to high levels of tolerance to hygromycin. The development of an electroporation transformation protocol for *C. acutatum* solved these problems and provided an easy and efficient way to obtain stable transformants of this fungus. Although a relatively low number of transformants were produced, optimization of the electric conditions and DNA concentration can probably help to increase transformation rates. It should be noted that unlike *C. gloeosporioides* f. sp. *aeschynomene*, in which homologous and nonhomologous vectors yielded similar numbers of transformants, in *C. acutatum*, transformation efficiency was enhanced by inclusion of homologous sequences in the transformation vectors. This may reflect differences between the recombination processes in species of this fungal genus.

The *C. acutatum* wild-type isolate TUT-149 that was used in this study has been previously isolated from diseased strawberry (9). GFP transgenic strains of isolate TUT-149 were easily detected on and inside the plant and were equally pathogenic on strawberry, causing severe necroses on all parts of the plant. Therefore, these isolates provided a valuable tool for analysis and comparison of the colonization processes of strawberry and other plants by *C. acutatum*.

Conidia that germinated on strawberry developed thick, branched mycelium with short nodes. Germination occurred from both sides of the conidium and appressoria developed at the end of the short germ tubes within 20 to 40 h after the conidia were inoculated onto plants (Fig. 1). The melanized appressoria had apparently breached the cuticle within 48 h after conidial application. Thus, it appears that on strawberry, penetration of the fungus into the plant is assisted by appressoria and occurs within 2 days after establishment of the first contact between the fungus and the plant. Germination of conidia on asymptomatic plants occurred mainly from one side of the conidium and was characterized by formation of thin, unbranched hyphae (Fig. 2). This kind of development resembles the effects of mutations in signal transduction components observed in other fungal species. In *Cochliobolus*

heterostrophus, conidia germinate and develop short, coiled germ tubes that differentiate appressoria, whereas in a mutant defected in a G α i subunit, the germ tube is thin and straight and appressoria rarely develop (14). Mutations in other signal transduction components, such as components of the MAPK cascade, or elements of cAMP-dependant signal transduction are often associated with loss of pathogenicity on the natural hosts (1,5,15, 16,19). In *Magnaporthe grisea*, specific molecules on the leaves of rice plants are necessary for triggering appressoria differentiation and for pathogenicity (20). Lack of these molecules, or mutations in signal transduction elements that prevented activation of the corresponding genes in the fungus, resulted in epiphytic growth and reduced pathogenicity. The similarities between the developmental patterns of *C. acutatum* on asymptomatic plants with that of signal transduction deficient mutants suggest that strawberry plants may contain specific compounds that are important for activation of pathogenic development in *C. acutatum*. These compounds may be absent in other plant species. Therefore, on these plants, the pathogenic development of the fungus is incomplete.

Appressoria started to differentiate on asymptomatic plants approximately 15 h after plant inoculation, and mature, melanized appressoria were observed after 40 to 50 h, similar to that observed on strawberry. However, on strawberry, the appressoria developed lobes and sockets and eventually collapsed, whereas the appressoria that were formed on asymptomatic plants retained their symmetric, smooth structure even after several weeks, similar to those formed on plastic surfaces (Fig. 3). These differences may account for the lack of appressorial peg formation, and as a result, lack of plant penetration. This possibility is further supported by lack of mycelium inside the tissues of asymptomatic plants several days after inoculation. It is therefore suggested that a major difference in the early events associated with colonization of strawberry compared with asymptomatic plants is in the development of special structures such as thick, branched mycelium and anchoring bodies on the surface of the plant. The differences in the fungal organelles that develop on strawberry and on the asymptomatic plants include the formation of thick and highly branched mycelium and the role of appressoria in plant penetration and in the establishment of internal infection that occur only on strawberry. The heavy infection of strawberry is related to the massive penetration by appressoria that enables fast and profound colonization of the intracellular spaces with large quantities of mycelium. The fast-developing mycelium penetrates the cells, causing necrosis, and the fungus proliferates in all of the plant organs. This is in contrast to the asymptomatic plants in which direct penetration was not observed. Invasion of these plants was restricted to the intercellular spaces of the subcuticular cell layer without cell penetration. The only time that cell penetration was detected occurred during invasion of stomatal cells (Fig. 2F). Such cells became necrotic, and the fungus was restricted to these cells. It is thus concluded that on asymptomatic plants, the pathogen can proliferate on the surface of the inoculated organ, and after several days, it enters into the epidermal layers where it remains quiescent without causing damage to the surrounding cells or tissues. The fungus seems to reach a balance with the plant such that it does not develop massive hyphae and its growth is restricted to the upper cellular layer beneath the cuticle without causing cell damage. At this stage, the fungus obtains nutrients from the plant apoplast spaces in sufficient amounts to keep it viable. By this mode of infection *C. acutatum* can survive on healthy looking plants for long periods of time as reported in a related study (8,13).

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LITERATURE CITED

- Adachi, K., and Hamer, J. E. 1998. Divergent cAMP signaling pathways regulate growth and pathogenesis in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 10:1361-1373.
- Alahakoon, P. W., Brown, A. E., and Sreenivasaprasad, S. 1994. Cross infection potential of genetic groups of *Colletotrichum gloeosporioides* on tropical fruits. *Physiol. Mol. Plant Pathol.* 44:93-103.
- Bailey, J. A., O'Connell, R. J., Pring, R. J., and Nash, C. 1992. Infection strategies of *Colletotrichum* species. Pages 88-120 in: *Colletotrichum: Biology, Pathology and Control*. J. A. Bailey and M. J. Jeger, eds. CAB International, Wallingford, U.K.
- Bernstein, B., Zehr, E. I., Dean, R. A., and Shabi, E. 1995. Characterization of *Colletotrichum* from peach, apple, pecan, and other hosts. *Plant Dis.* 79:478-482.
- Dean, R. A. 1997. Signal pathways and appressorium morphogenesis. *Annu. Rev. Phytopathol.* 35:211-234.
- Dumas, B., Centis, S., Sarrazin, N., and Tugay, M. 1999. Use of green fluorescent protein to detect expression of an endopolygalacturonase gene of *Colletotrichum lindemuthianum* during bean infection. *Appl. Environ. Microbiol.* 65:1769-1771.
- Emmett, R. W., and Parbery, D. G. 1975. Appressoria. *Annu. Rev. Phytopathol.* 13:147-167.
- Freeman, S., Horowitz, S., and Sharon, A. 2001. Pathogenic and non-pathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. *Phytopathology* 91:986-992.
- Freeman, S., and Katan, T. 1997. Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. *Phytopathology* 87:516-521.
- Freeman, S., Pham, M., and Rodriguez, R. J. 1993. Molecular genotyping of *Colletotrichum* species based on arbitrarily primed PCR, A+T-rich DNA, and nuclear DNA analyses. *Exp. Mycol.* 17:309-322.
- Freeman, S., and Shabi, E. 1996. Cross infection of subtropical and temperate fruits by *Colletotrichum* species from various hosts. *Physiol. Mol. Plant Pathol.* 49:395-404.
- Freeman, S., Shabi, E., and Katan, T. 2000. Characterization of *Colletotrichum acutatum* causing anthracnose of anemone (*Anemone coronaria* L.). *Appl. Environ. Microbiol.* 66:5267-5272.
- Horowitz, S., Freeman, S., and Sharon, A. 2000. Host specificity and survival of *Colletotrichum acutatum* from strawberry. *Phytoparasitica* 28:280.
- Horwitz, B. A., Sharon, A., Lu, S., Ritter, V., Sandrock, T. M., Yoder, O. C., and Turgeon, G. B. 1999. A G protein alpha subunit form *Cochliobolus heterostrophus* involved in mating and appressorium formation. *Fung. Genet. Biol.* 26:19-32.
- Kahmann, R., Basse, C., and Feldbrugge, M. 1999. Fungal-plant signaling in the *Ustilago maydis*-maize pathosystem. *Curr. Opin. Microbiol.* 2:647-650.
- Kronstad, J. W. 1997. Virulence and cAMP in smuts, blights and blights. *Trends Plant Sci.* 2:161-202.
- Latunde-Dada, A., O'Connell, O., Nash, R. J. C., and Lucas, J. A. 1999. Stomatal penetration of cowpea (*Vigna unguiculata*) leaves by a *Colletotrichum* species causing latent anthracnose. *Plant Pathol.* 48:777-785.
- Leandro, L. F. S., Gleason, M. L., Nutter, F. W., Jr., Wegulo, S. N., and Dixon, P. M. 2001. Germination and sporulation of *Colletotrichum acutatum* on symptomless strawberry leaves. *Phytopathology* 91:659-664.
- Lev, S., Sharon, A., Hadar, R., Ma, H., and Horwitz, B. A. 1999. A mitogen-activated protein kinase of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: Diverse role for mitogen-activated protein kinase homologues in foliar pathogens. *Proc. Natl. Acad. Sci. USA* 96:13542-13547.
- Liu, S., and Dean, R. A. 1997. G protein α subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 10:1075-1086.
- Lorang, J. M., Tuori, R. P., Martinez, J. P., Sawyer, T. L., Redman, R. S., Rollins, J. A., Wolpert, T. J., Johnson, K. B., Rodriguez, R. J., Dickman, M. B., and Ciuffetti, L. M. 2001. Green Fluorescent protein is lighting up fungal biology. *Appl. Environ. Microbiol.* 67:1987-1994.
- Maor, R., Puyesky, M., Horwitz, B. A., and Sharon, A. 1998. Use of green fluorescent protein (GFP) for studying development and fungal plant interaction in *Cochliobolus heterostrophus*. *Mycol. Res.* 102:491-496.
- Mould, M. J. R., Boland, G. J., and Robb, J. 1991. Ultrastructure of the *Colletotrichum trifolii*-*Medicago sativa* pathosystem. I. Pre-penetration events. *Physiol. Mol. Plant Pathol.* 38:179-194.
- Mould, M. J. R., Boland, G. J., and Robb, J. 1991. Ultrastructure of the *Colletotrichum trifolii*-*Medicago sativa* pathosystem. II. Post-penetration events. *Physiol. Mol. Plant Pathol.* 38:195-210.
- Nair, J., and Corbin, J. B. 1981. Histopathology of *Pinus radiata* seedlings infected by *Colletotrichum acutatum* f. sp. *pinica*. *Phytopathology* 71:777-783.
- O'Connell, R. J., Bailey, J. A., and Richmond, D. V. 1985. Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. *Physiol. Plant Pathol.* 27:75-98.
- O'Connell, R. J., Perfect, S., Hughes, B., Carazaniga, R., Bailey, J. A., and Green, J. 2000. Dissecting the cell biology of *Colletotrichum* infection processes. Pages 57-76 in: *Colletotrichum: Host Specificity, Pathology, and Host-Pathogen Interaction*. D. Prusky, S. Freeman, and M. B. Dickman, eds. The American Phytopathological Society, St. Paul, MN.
- Porto, M. D. M., Grau, C. R., de Zoeten, G. A., and Gaard, G. 1988. Histopathology of *Colletotrichum trifolii* on alfalfa. *Phytopathology* 78:345-349.
- Robert, I. N., Oliver, R. P., Punt, P. J., and van den Hondel, C. A. M. J. J. 1989. Expression of the *Escherichia coli* β -glucuronidase gene in industrial and phytopathogenic filamentous fungi. *Curr. Genet.* 15:177-180.
- Robinson, M., and Sharon, A. 1999. Transformation of the bioherbicide *Colletotrichum gloeosporioides* f. sp. *aeschyromene* by electroporation of germinated conidia. *Curr. Genet.* 36:98-104.
- Rodriguez, R. J., and Redman, R. S. 2000. *Colletotrichum* as a model system for defining the genetic basis of fungal symbiotic lifestyles. Pages 114-130 in: *Colletotrichum: Host Specificity, Pathology and Host-Pathogen Interaction*. D. Prusky, S. Freeman, and M. B. Dickman, eds. The American Phytopathological Society, St. Paul, MN.
- Spellig, T., Bottin, A., and Kahmann, R. 1996. Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. *Mol. Gen. Genet.* 252:503-509.
- Tu, J. C. 1985. An improved Mathur's medium for growth, sporulation and germination of *Colletotrichum lindemuthianum*. *Microbiosis* 44:87-93.
- Wharton, P. S., Julian, A. M., and O'Connell, R. J. 2001. Ultrastructure of the infection of *Sorghum bicolor* by *Colletotrichum sublineolum*. *Phytopathology* 91:149-158.
- Zulfigar, M., Brlansky, R. H., and Timmer, L. W. 1996. Infection of flower and vegetative tissues of citrus by *Colletotrichum acutatum* and *C. gloeosporioides*. *Mycologia* 88:121-128.