APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1997, p. 2378–2383 Vol. 63, No. 6
0099-2240/97/$04.00+0
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Gene Expression Analysis during Conidial Germ Tube and Appressorium Development in *Colletotrichum trifolii*†

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Received 7 October 1996/Accepted 2 February 1997

Preinfection development in *Colletotrichum* spp. exhibits three morphologies (conidia, germ tubes, and appressoria) and is directed by a complex interplay of environmental signals. Germ tube morphogenesis for *Colletotrichum trifolii* and the related fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene* was shown to be partially dependent on a balance between self-germination inhibitors and environmental nutrients or cutin. The degree of responsiveness to these environmental signals was strikingly different between the two fungal species. A solid contact surface stimulated germ tube morphogenesis and was the only apparent requirement for appressorium morphogenesis in both fungi. A population of *C. trifolii* conidia was incubated on a solid surface in the presence of cutin to stimulate nearly synchronous preinfection morphogenesis for gene expression analysis. RNA analysis of signal-transducing genes from *C. trifolii*, including genes for a serine-threonine kinase (TB3), calmodulin, and protein kinase C, showed that maximum transcription of all three genes occurred in conidia prior to or during germ tube morphogenesis. Transcription of melanin biosynthetic genes *THR1* and *SCD1* (Y. Kubo, Y. Takano, and I. Furusawa, *Colletotrichum* Newsl. II:5–10, 1996; N. S. Perpetua, Y. Kubo, N. Yasuda, Y. Takano, and I. Furusawa, Mol. Plant-Microbe Interact. 9:323–329, 1996) was highest prior to and during appressorium morphogenesis.

Spores of many pathogenic fungi germinate under appropriate conditions and subsequently differentiate into appressoria (28, 29). Appressorium development, because of its importance in the early stages of host infection, is a primary focus of study and a potential pathogen-specific target for plant disease control strategies (28). An appressorium is an infection structure (usually melanized) that adheres to host surfaces, is typically formed prior to host penetration, and is a prerequisite to infection for many pathogenic fungi (28, 29). Appressorium differentiation is triggered by different signals in different species of phytopathogenic fungi, although substrate attachment appears to be a prerequisite for appressoriation in all fungi (14, 15, 18, 22, 28). Host-specific waxes or ethylene triggers appressorium formation in *Colletotrichum gloeosporioides* which infects avocado fruits (11, 25), while cutin monomers are important in the rice blast fungus *Magnaporthe grisea* (13). *Colletotrichum acutatum* germ tubes reportedly form appressoria as a result of nutrient stress (2).

Germination and germ tube morphogenesis precede appressorium differentiation and also are critical during preinfection fungal development. A germ tube is a young hyphal tip and is morphologically similar to an invading hyphal tip. Thus, hyphal development is required prior to infection as well as during host invasion and colonization. Furthermore, the pathogenicity of all pathogenic filamentous fungi relies on hyphal morphogenesis, although not all pathogenic filamentous fungi require appressorium formation.

The environmental requirements that stimulate germ tube morphogenesis vary among fungal species. Water or synthetic nutrient media stimulate germination in some *Colletotrichum* species (23). Cutin, the predominant surface polymer of the plant phyllosphere, may influence germination of many plant-pathogenic fungi in nature (21, 23). In addition, fungus-synthesized germination inhibitors that prevent untimely germination prior to spore dissemination have been characterized in several fungi (19), including *Colletotrichum* (31). Thus, neutralizing the activity of germination inhibitors is a requirement for germ tube morphogenesis in *Colletotrichum*.

The goal of our research is to identify and characterize genes expressed in *Colletotrichum* during preinfection development. Here, we identify environmental factors that influence the developmental preinfection sequence from conidia to germ tubes to appressoria in *Colletotrichum trifolii*, the causal agent of alfalfa anthracnose (8), and, for comparison, in *C. gloeosporioides* f. sp. *aeschynomene*, the causal agent of northern joint vetch anthracnose (7, 30). We identified environmental criteria that stimulate nearly synchronous morphogenesis of dense conidial populations (10^6 conidia/ml), isolated RNA from a synchronized population of *C. trifolii*, and demonstrated transcription of six fungal genes during preinfection development.

MATERIALS AND METHODS

Strains and plasmids. *C. trifolii* race 1 was isolated from the alfalfa cultivar Saranac. *C. gloeosporioides* f. sp. *aeschynomene* strain 3.1.3 was isolated from northern joint vetch and provided by David TeBeest, University of Arkansas.

Media and culture conditions. YpSs (32) agar plates were inoculated with glycerol spore suspensions of *Colletotrichum* strains stored at −70°C. Cultures were grown at 20°C with a 12-h photoperiod under white fluorescent light. Agar plugs of approximately 0.5 cm^2 containing fungal tissue were used as the inoculum for 0.5 to 1.2 liters of SOB liquid medium (27) containing 30 μg of streptomycin/ml and 30 μg of kanamycin/ml in nonbeveled Fernbach flasks. Liquid cultures were grown at room temperature (22 to 25°C) under continuous white fluorescent light at 175 rpm. *C. trifolii* was grown for 5 to 7 days, and *C. gloeosporioides* f. sp. *aeschynomene* was grown for 2 to 3 days to induce conidiation. Conidia were not more than 36 h old at the time of harvesting. Conidia were filtered through two layers of sterile Miracloth (Calbiochem) and collected by centrifugation at 10,000 × *g* at 20°C for 10 min. Conidia (1.5×10^9) were then suspended in 1.5 liters of sterile distilled water with stirring for 8 min prior to pelleting at 10,000 × *g* at 20°C for 10 min. Conidia were washed a total of three

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† Journal series no. 11712, Agricultural Research Division, University of Nebraska.

times with sterile distilled water and then suspended in sterile distilled water (>10^6 conidia/ml).

Conidia were then mixed with distilled water, nutrient media, cutin (16), or purified chemicals in Eppendorf tubes at 10^5 or 10^6 conidia/ml. Purified chemicals included 8,16-dihydroxyhexadecanoic acid and 9,10-dihydroxyhexadecanoic acid, gifts from Richard Bostock (U.C.-Davis, Davis, Calif.), 9,10,16-trihydroxyhexadecanoic (aleuritic) acid (Fluka Chemical), and 16-hydroxyhexadecanoic acid (Sigma). Media included 0.4% (wt/vol) yeast extract, 4.0% yeast extract, SOB medium, 0.05% (wt/vol) cutin, or 0.5% cutin. One hundred microliters of each sample was then aliquoted onto each of five glass slides (LabTek). Each 100-μl aliquot was subsequently used to count rates of germ tube and appressorium development for one time point. Samples on glass slides were set on wet sponges in closed plastic containers to provide and maintain a high-humidity environment. Samples were incubated at room temperature without agitation prior to microscopic examination. Microscopic examination of a minimum of 100 conidia per sample required less than 3 min per sample and was completed within 1.5 min of the designated time point. Conidia with emerging germ tubes were counted as germinated conidia. Conidia with bulbous germ tube ends (protoappressoria) eventually matured into fully melanized appressoria by 10 h and were counted as appressoria. Glass slides were brushed vigorously with a 1% sodium dodecyl sulfate (SDS) solution, exhaustively rinsed with distilled water, and air dried prior to each experiment. Alternatively, conidia were suspended in 100 ml of distilled water or SOB medium at 10^5 or 10^6 conidia/ml and shaken at 100 rpm at room temperature prior to microscopic examination. Samples were examined in a minimum of three independent experiments.

Fungal RNA preparation. Conidia of *C. trifolii* were isolated and washed as described above and then suspended in sterile distilled water with 0.05% cutin at 10^6 conidia/ml. Ten-milliliter conidial aliquots were pipetted onto Pyrex glass plates (88-mm diameter) and incubated at room temperature without agitation for 2, 3, 4, 5, 6, or 10 h before harvesting. The 0.05% cutin suspension in water was decanted, and conidia attached to the glass plate surface were then harvested for RNA extraction. RNA extraction buffer (5) was added directly to the plate prior to scraping with a rubber policeman. Conidia were harvested over a 10-min interval, within 5 min of the designated incubation period. Ten plates (10^8 conidia total) were harvested per time point. RNA was isolated by the method of Chomczynski and Sacchi (5) with 15 ml of RNA extraction buffer used per time point. Purified RNA was dissolved in 100% formamide.

Northern (RNA) analysis. Approximately 5 μg of total RNA was suspended with 0.96 μg of ethidium bromide, 2 μl of 10× MOPS-formaldehyde buffer [a 10× solution contains 0.2 M 3-(*N*-morpholino)propanesulfonic acid (pH 7), 80 mM sodium acetate, 10 mM EDTA, and 3 M formaldehyde] and formamide in an 18-μl volume. Samples were heated at 75°C for 5 min and placed briefly on ice before addition of 2 μl of 10× loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll, 10 mM EDTA). The samples were loaded onto a 1% agarose–1× MOPS-formaldehyde gel and run at 2 to 3 V/cm in 1× MOPS-formaldehyde buffer for 3.5 to 5 h. Ethidium bromide staining confirmed nearly equivalent RNA quantities loaded for each sample. Gels were soaked twice in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4]) at room temperature for 15 min. RNA was blotted onto nylon membranes (Micron Separations, Inc.) with 20× SSPE and subsequently fixed by UV irradiation. Filters were soaked briefly in 2× SSPE before incubation in hybridization solution (0.25 M Na_2HPO_4 [pH 7.4], 7% SDS, 2% blocking reagent [Boehringer Mannheim], 1 mM EDTA) at 65°C for 2 to 4 h. The hybridization solution was replaced with fresh solution containing the appropriate probe, and the filters were incubated 16 to 20 h at 65°C.

Hybridizations included 25 ng of a probe in 10 to 12 ml of solution. Probes were labeled with digoxigenin (Boehringer Mannheim) by the random priming method of Feinberg and Vogelstein (10) or by PCR. Probes included the following: a 420-bp fragment containing *C. gloeosporioides* f. sp. *aeschynomene TUB2*, a constitutive β-tubulin gene (3); an 870-bp fragment containing *C. trifolii TB3* cDNA (4); a 1,400-bp *Bam*HI-*Eco*RI fragment containing *Neurospora crassa* 17S ribosomal DNA (12); a 2,400-bp *Eco*RI fragment containing *C. trifolii* protein kinase C cDNA (9); a 1,000-bp *Eco*RI-*Xba*I fragment containing *Colletotrichum lagenarium* 1,3,8-trihydroxynaphthalene reductase (*THR1*) cDNA (24); a 720-bp *Eco*RI-*Xba*I fragment containing *C. lagenarium* scytalone dehydratase (*SCD1*) cDNA (17); and a 300-bp fragment containing *Aspergillus nidulans* calmodulin cDNA (26). Filters were washed under high-stringency conditions, including three or four washes with a mixture containing 40 mM Na_2HPO_4 (pH 7.2), 1 mM EDTA, and 1% SDS at 65°C for 20 min each. Chemiluminescent detection of probes was done as described in the manufacturer's (Boehringer Mannheim) instructions. Filters were exposed to Kodak X-Omat film. Filters were stripped in 50% formamide–6× SSPE at 68°C for 30 to 60 min and then in 0.2× SSPE–0.1% SDS for 10 min at 68°C. Northern autoradiograms were scanned with a densitometer, and RNA signals were normalized relative to 17S rRNA signals by use of the software package Image 1.49 (written by Wayne Rasband and available free from the National Institutes of Health).

RESULTS

Conidial germ tube and appressorium morphogenesis in *C. trifolii*. Germ tube and appressorium morphogenesis were initially examined in water since *C. trifolii* conidia are naturally dispersed by water (33). Table 1 shows morphogenesis over time of *C. trifolii* conidia prepared identically from six separate cultures (samples A to F). Each of the six *C. trifolii* cultures yielded a different number of conidia. After harvesting, conidia were washed three times with 200 ml of water and pelleted at 20°C. Conidia then were suspended in water at 10^6 conidia/ml, and 100-μl aliquots were pipetted onto glass slides. Samples were monitored for 6 h, and the rate of conidial germ tube formation was calculated as a percentage of total conidia. Interestingly, variations in the number of conidia harvested from individual *C. trifolii* cultures affected the rate of conidial morphogenesis. Germination of samples A and B was completed by 3 h and was nearly synchronous, while germination of samples E and F was delayed, continued through 6 h, and was not synchronous. Thus, the synchronicity of morphogenesis decreased as the initial population size of washed conidia increased. Furthermore, less than 5% of unwashed conidia germinated by 6 h (data not shown). These observations were likely the result of germination inhibitors (31) and indicated that the washes did not adequately remove germination inhibitors from cultures yielding large numbers of conidia. A specified number of conidia (1.5×10^9) were subsequently washed in a defined quantity of water (three washes each with 1,500 ml of water) prior to incubation of conidia on glass slides. This standardized washing procedure greatly reduced variations in the synchronicity of conidial morphogenesis that resulted from variable conidial yields among separate cultures (compare the statistical variability and timing of germination in water at 10^6 conidia/ml in Tables 1 and 2).

TABLE 1. Percentage of germ tubes produced by *C. trifolii* conidia from six individual cultures

Sample	No. of conidia[a]	Germ tubes produced (%) at[b]:					
		1 h	2 h	3 h	4 h	5 h	6 h
A	2.26×10^7	0	51	87	87	87	87
B	5.50×10^7	0	44	95	95	95	96
C	1.45×10^8	0	33	86	90	91	91
D	2.90×10^8	0	7	36	81	88	88
E	4.40×10^8	0	9	30	45	71	77
F	9.40×10^8	0	2	15	55	78	81

[a] Number of conidia harvested from an individual culture.
[b] The percentages of germ tubes produced were 0%, 24% ± 19%, 58% ± 32%, 75% ± 18%, 86% ± 8%, and 86% ± 6% at 1, 2, 3, 4, 5, and 6 h, respectively (means ± standard deviations).

The impact of complex nutrient media and cutin on germ tube and appressorium morphogenesis was examined (Table 2) by using conidia prepared by the standardized washing protocol. Germ tube morphogenesis was first observed by 1.5 h and was completed by 3 h in conidial populations incubated in water. Germ tube morphogenesis was slightly delayed in 0.4% yeast extract and 0.05% cutin and was completed by 4 h. Appressorium morphogenesis followed the appearance of germ tubes by 1 h in these samples. The synchronicity of appressorium differentiation mimicked germ tube development since the appearance of both structures was completed over similar time spans. Therefore, approximately 1- to 2-h time intervals represent windows for nearly synchronous germination and then appressoriation of *C. trifolii* conidia in these three different environments. Higher concentrations of nutrients (4% yeast extract or SOB medium) or cutin (0.5%) more effectively delayed germ tube and/or appressorium morphogenesis (Table 2), indicating a pronounced physiological impact on *C. trifolii* spores. Appressoria developed after germi-

TABLE 2. Conidial germ tube and appressorium morphogenesis in *C. trifolii* expressed as a percentage of total conidia

Medium[a]	Conidia/ml	% of total conidia											
		Germ tubes						Appressoria					
		1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h
Water	10^5	0	75 ± 5	93 ± 2	96 ± 2	95 ± 2	98 ± 1	0	0	76 ± 12	90 ± 5	92 ± 4	96 ± 1
Water	10^6	0	82 ± 10	92 ± 5	93 ± 2	96 ± 2	96 ± 1	0	0	68 ± 12	87 ± 8	95 ± 4	95 ± 2
0.4% YE	10^5	0	20 ± 3	86 ± 6	96 ± 1	97 ± 2	97 ± 1	0	0	48 ± 7	80 ± 17	93 ± 3	95 ± 1
0.4% YE	10^6	0	19 ± 6	73 ± 32	95 ± 2	97 ± 2	97 ± 2	0	0	20 ± 15	83 ± 8	96 ± 2	97 ± 3
4% YE	10^5	0	0	14 ± 6	42 ± 5	66 ± 11	81 ± 4	0	0	0	0	2 ± 2	3 ± 3
4% YE	10^6	0	0	12 ± 5	69 ± 8	82 ± 12	96 ± 3	0	0	0	0	0	5 ± 6
SOB	10^5	0	24 ± 9	86 ± 9	91 ± 4	95 ± 5	98 ± 1	0	0	0	34 ± 22	55 ± 40	74 ± 27
SOB	10^6	0	30 ± 16	90 ± 10	96 ± 2	97 ± 2	97 ± 3	0	0	0	32 ± 20	75 ± 34	82 ± 21
0.05% Cutin	10^5	0	20 ± 9	77 ± 3	90 ± 3	94 ± 2	94 ± 3	0	0	18 ± 3	38 ± 19	71 ± 11	75 ± 12
0.05% Cutin	10^6	0	34 ± 18	81 ± 3	90 ± 5	92 ± 2	94 ± 3	0	0	14 ± 5	47 ± 10	78 ± 4	88 ± 3
0.05% Cutin	10^6	0	0	2 ± 3	21 ± 26	59 ± 29	82 ± 14	0	0	0	0	0	13 ± 16

[a] YE, yeast extract.

nation on glass slides regardless of the environment (nearly all germ tubes in 4% yeast extract and cutin formed appressoria with longer incubation times), although the timing of appressoriation was influenced by nutrients or cutin (Table 2).

Implementation of the standardized washing procedure yielded consistent preinfection morphogenesis of conidia. However, an interesting difference between extensively washed conidia (i.e., conidia washed with the standardized protocol) (Table 2) and less extensively washed conidia (e.g., sample F in Table 1) was observed. Preinfection morphogenesis of extensively washed conidia incubated in water on glass slides was nearly synchronous but slightly delayed after water was replaced with 0.4% yeast extract or 0.05% cutin (Table 2). In contrast, morphogenesis of less extensively washed conidia (e.g., sample F in Table 1) was not synchronous in water but was greatly stimulated and nearly synchronous in the presence of 0.4% yeast extract or 0.05% cutin (data not shown).

C. trifolii conidia also were incubated in the presence of known cutin and cutin-like monomers on glass slides. Germination in the presence of these chemicals at 500 ng/ml to 5 μg/ml was identical to germination in water, but germination was inhibited at 50 μg/ml (data not shown).

Synchronous conidial germination is also stimulated by a contact surface. To more thoroughly compare germ tube development to appressorium morphogenesis, an attempt was made to induce synchronous conidial germination in the absence of a solid surface (glass) and to avoid appressorium morphogenesis. *C. trifolii* conidia were washed according to the standardized protocol and then shaken in water or SOB medium at 10^5 or 10^6 conidia/ml. Germination of conidia suspended in water at 10^5 or 10^6 conidia/ml or in nutrient medium at 10^6 conidia/ml never exceeded 38% by 6 h, was highly variable, and was not synchronous over a time course (data not shown). Furthermore, the majority of conidia with germ tubes were in contact with other conidia in the suspension. These observations, compared to germination efficiency on glass slides (Table 2), showed that a contact surface strongly stimulated conidial germination and was required for nearly synchronous morphogenesis. However, germination of conidia suspended in liquid nutrient media at a lower conidial density (10^5 conidia/ml) approached 90% by 4 h and was nearly synchronous, and the majority of germinating conidia were not in contact with other conidia. In addition, appressoria did not form in the shaken cultures (which were monitored for up to 10 h), demonstrating that a contact surface was required for appressorium morphogenesis, as has been shown for other fungi (28).

Germ tube and appressorium morphogenesis in *C. gloeosporioides* f. sp. *aeschynomene*. Preinfection development of *C. gloeosporioides* f. sp. *aeschynomene* was examined for intragenus comparison (Table 3). The extent and synchronicity of germination varied greatly among different environments and

TABLE 3. Conidial germ tube and appressorium morphogenesis in *C. gloeosporioides* f. sp. *aeschynomene* expressed as a percentage of total conidia

Medium[a]	Conidia/ml	% of total conidia											
		Germ tubes						Appressoria					
		1 h	2 h	3 h	4 h	5 h	6 h	1 h	2 h	3 h	4 h	5 h	6 h
Water	10^5	0	12 ± 9	26 ± 19	28 ± 14	29 ± 5	35 ± 11	0	0	13 ± 12	19 ± 16	28 ± 5	31 ± 6
Water	10^6	0	1 ± 1	7 ± 7	5 ± 2	3 ± 2	3 ± 3	0	0	4 ± 4	4 ± 2	3 ± 2	3 ± 3
0.4% YE	10^5	0	44 ± 13	79 ± 3	86 ± 5	87 ± 6	89 ± 3	0	0	42 ± 25	81 ± 6	84 ± 4	89 ± 3
0.4% YE	10^6	0	7 ± 6	17 ± 9	26 ± 16	26 ± 16	24 ± 12	0	0	5 ± 3	21 ± 16	23 ± 17	24 ± 12
4% YE	10^5	0	29 ± 17	85 ± 8	93 ± 2	93 ± 2	95 ± 3	0	0	2 ± 4	34 ± 13	82 ± 12	90 ± 7
4% YE	10^6	0	4 ± 3	22 ± 5	44 ± 14	54 ± 11	66 ± 14	0	0	0	4 ± 3	24 ± 9	60 ± 18
SOB	10^5	0	12 ± 7	76 ± 10	88 ± 8	90 ± 3	90 ± 4	0	0	3 ± 4	31 ± 17	78 ± 7	87 ± 3
SOB	10^6	0	0	6 ± 2	12 ± 7	9 ± 3	16 ± 6	0	0	0	1 ± 1	6 ± 2	13 ± 5
0.05% cutin	10^5	0	21 ± 12	70 ± 18	90 ± 7	92 ± 7	93 ± 8	0	0	42 ± 20	84 ± 5	91 ± 6	93 ± 7
0.05% cutin	10^6	0	1 ± 1	4 ± 3	12 ± 11	13 ± 7	10 ± 5	0	0	2 ± 2	12 ± 11	13 ± 7	10 ± 5
0.5% cutin	10^6	0	29 ± 12	79 ± 15	89 ± 9	89 ± 10	95 ± 2	0	0	61 ± 14	85 ± 8	87 ± 9	95 ± 3

[a] YE, yeast extract.

were highly dependent on spore concentration. Germination was more efficient at 10^5 conidia/ml than at 10^6 conidia/ml regardless of the nutritional environment (except for 0.5% cutin), which presumably reflects the effects of spore germination inhibitors (19, 31). These data show that nutrient media and especially cutin greatly stimulated germination. Cutin (0.5%) was the only inducer of nearly synchronous morphogenesis at 10^6 conidia/ml. Thus, germination in *C. gloeosporioides* f. sp. *aeschynomene* was markedly stimulated by cutin, which reflects a major difference compared to *C. trifolii* germination.

Appressorium morphogenesis followed germ tube morphogenesis by 1 h in all samples except 4% yeast extract (Table 3). Appressoria were first observed approximately 2 h after germ tubes in 4% yeast extract. Thereafter, the temporal pattern of appressorium differentiation followed the temporal pattern of germ tube morphogenesis in 4% yeast extract at 10^5 conidia/ml. Steady, continuous germ tube and appressorium morphogenesis was observed with 10^6 conidia/ml in 4% yeast extract. This nonsynchronous pattern of morphogenesis was different from that of all other samples. As in *C. trifolii*, a solid surface stimulated germination and was required for appressorium development (data not shown). Thus, appressoria developed in *C. gloeosporioides* f. sp. *aeschynomene* apparently by default in the presence of a solid surface since none of the nutritional regimens prevented appressoriation. Interestingly, 0.5% cutin did not delay appressorium morphogenesis in *C. gloeosporioides* f. sp. *aeschynomene* (Table 3) as it did in *C. trifolii* (Table 2).

C. gloeosporioides f. sp. *aeschynomene* conidia were incubated on glass slides in the presence of cutin and cutin-like monomers. Germination decreased in the presence of each cutin or cutin-like monomer at concentrations ranging from 500 ng/ml to 500 μg/ml when compared to germination in water (data not shown), contrasting with the germination-stimulating influence of the cutin polymer.

Northern analysis. *C. trifolii* was chosen to examine transcription during preinfection development because *C. trifolii* genes were available as probes. Analysis of developmental gene expression required a large conidial population (10^8 conidia per time point) to isolate an adequate quantity of RNA. A high population density (10^6 conidia/ml) was required to isolate sufficient amounts of RNA over a short time span and hence for accurate analysis of developmental gene expression. Preinfection morphogenesis was subsequently examined on glass plates as opposed to glass slides to fulfill these requirements.

Preinfection morphogenesis of *C. trifolii* conidia suspended in water and then incubated on Pyrex glass plates was highly variable (data not shown) and in stark contrast to the consistent morphogenesis observed on LabTek glass slides (Table 2). Differences in artificial substrates have been documented to dramatically influence preinfection morphogenesis in pathogenic fungi (1). Interestingly, preinfection morphogenesis of *C. trifolii* conidia on Pyrex glass plates in the presence of cutin or nutrient media was statistically identical to morphogenesis on LabTek glass slides (Table 2). Cutin is a component of the in vivo substrate and may influence gene expression in conidia on plants; hence, gene transcription was examined with conidia prepared by the standardized washing protocol and incubated with 0.05% cutin on Pyrex glass plates. Figure 1A graphically presents temporal morphogenesis of 10^6 conidia/ml in 0.05% cutin on Pyrex glass plates.

Northern blots of RNA isolated during *C. trifolii* preinfection development (Fig. 1A) were probed with six genes (Fig. 1B). Constitutive transcription of the β-tubulin housekeeping gene *TUB2* (3) was observed in all samples (Fig. 1B) and demonstrates the equivalency of RNA loading. *TB3* is a *C. trifolii* serine-threonine kinase gene required for normal hyphal elongation and branching (4). *TB3* transcripts were observed in conidia and throughout preinfection morphogenesis. Interestingly, smaller transcripts (that presumably are processed mRNA species) were prevalent after incubation of conidia in cutin on a morphogenesis-stimulating solid surface. *TB3* expression peaked during germ tube morphogenesis (Fig. 1). Similar to *TB3*, peak transcription of the calmodulin gene occurred during germ tube morphogenesis, 3 h after differentiation was induced (Fig. 1). However, peak expression of protein kinase C occurred after addition of cutin and prior to incubation on a solid surface. *THR1* and *SCD1* transcription was observed prior to and during appressorium development (Fig. 1).

DISCUSSION

Cumulatively, these data show that a complex interplay of multiple environmental factors influences preinfection development of *Colletotrichum*. Germ tube morphogenesis was affected by a balance between self-germination inhibitors and available nutrients or cutin in the environment. In addition, the necessity of a contact surface for appressorium morphogenesis is well documented (28, 29). Of note, our data indicate that a contact surface also stimulated germination and was required for a conidial population to undergo synchronous germination.

Germination inhibitors were apparently removed from *C. trifolii* and *C. gloeosporioides* f. sp. *aeschynomene* with water to stimulate germination. Interestingly, *C. trifolii* germinated more prolifically on a solid substrate in the presence of water than *C. gloeosporioides* f. sp. *aeschynomene*. Thus, germination inhibitors from *C. gloeosporioides* f. sp. *aeschynomene* were presumably leached slower than those from *C. trifolii* when conidia were washed and subsequently incubated in the presence of water on a solid surface. This could be attributed to possible differences in germination inhibitors produced by the two fungal species, including variations in water solubility, spore binding affinities, or levels of inhibitor production.

Cutin influenced germination in both species of *Colletotrichum* and, particularly, stimulated germination in *C. gloeosporioides* f. sp. *aeschynomene*, showing that a generic plant polymer that covers the phylloplane can influence preinfection morphogenesis of phytopathogenic fungi. Cutin constituents may serve as plant signal molecules that bypass or inactivate the effects of germination inhibitors in *Colletotrichum*. However, the cutin monomers which were tested did not stimulate germination. It is possible that the correct germination-stimulating monomer was not tested or that a complex mixture of cutin breakdown products (i.e., cutin oligomers) is required to stimulate germination. Alternatively, cutin may be a nontoxic polymer that effectively titrates a germination inhibitor(s). Furthermore, cutin may influence preinfection morphogenesis in *C. trifolii* and *C. gloeosporioides* f. sp. *aeschynomene* by different mechanisms, as suggested by the observation that appressorium development in 0.5% cutin was delayed in *C. trifolii* but not in *C. gloeosporioides* f. sp. *aeschynomene.*

Water dispersal or removal of self-germination inhibitors, cutin, and a contact surface are environmental factors encountered by naturally dispersed *Colletotrichum* conidia, and all were taken into account in developing a nearly synchronous morphogenesis system. This system was utilized to examine gene expression during preinfection development.

Large conidial populations of *C. trifolii* were stimulated to undergo nearly synchronous morphogenesis for subsequent

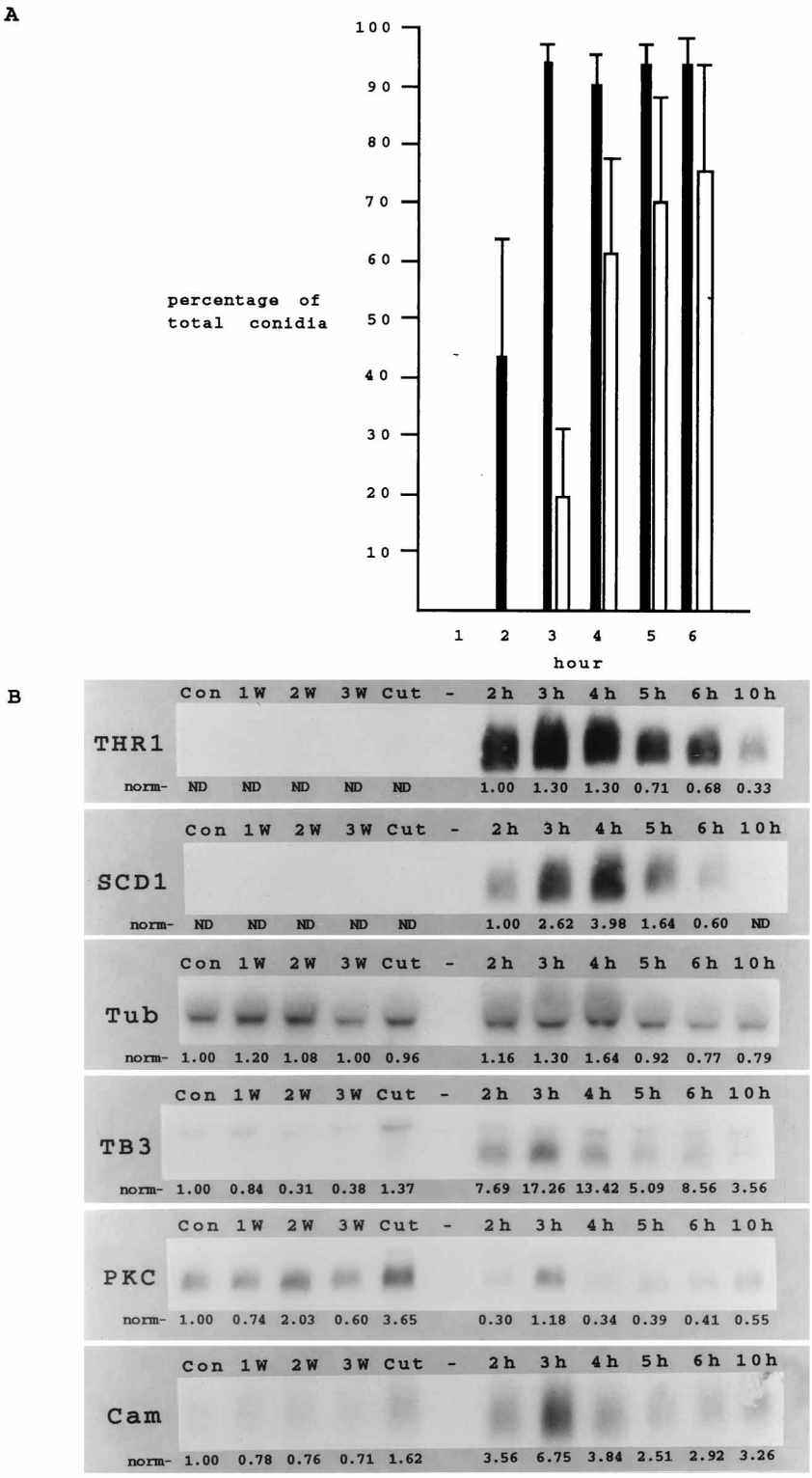

FIG. 1. (A) Developmental germ tube and appressorium morphogenesis of *C. trifolii* conidia suspended in 0.05% cutin at 10^6 conidia/ml and then incubated on Pyrex glass plates at room temperature. Symbols: closed bars, germ tube morphogenesis; open bars, appressorium morphogenesis. (B) Northern analysis of six fungal genes during preinfection *C. trifolii* development. Probes are described in Materials and Methods. Abbreviations: Con, RNA from conidia; 1W, 2W, and 3W, RNA samples from conidia washed with water once, twice and three times, respectively; cut, RNA from conidia washed three times and suspended in 0.05% cutin prior to incubation on glass plates; 2h, 3h, 4h, 5h, 6h, and 10h, RNA from conidia incubated on glass plates for 2, 3, 4, 5, 6, and 10 h, respectively; ND, no detectable hybridization signal. The numbers next to "norm" indicate normalized levels of RNA expression relative to a 17S ribosomal DNA probe. Tub, *TUB2*; Cam, calmodulin gene; PKC, protein kinase C gene.

transcription analysis. As expected, transcription of melanin biosynthetic genes *THR1* and *SCD1* occurred prior to and during appressorium development, while β-tubulin gene transcription was constitutive throughout preinfection morphogenesis. Transcription of the gene encoding protein kinase C peaked after addition of cutin and prior to incubation on a solid surface. Thus, peak transcription of the protein kinase C gene is an early event separate from and prior to the stimulus of a contact surface. Peak transcription of *TB3* and the calmodulin gene were observed on a solid surface during germ tube morphogenesis. These data show that individual environmental stimuli can affect transcription of specific genes. Protein kinase C, calmodulin, and the *TB3* product have been characterized as important components of signal-transducing pathways (4, 6, 20). These proteins typically serve as relays in preexisting systems that monitor environmental changes, and they are activated via posttranslational mechanisms such as second messenger (diacylglycerol or calcium) binding or phosphorylation. Elevated expression of these genes prior to and during germ tube morphogenesis suggests that these proteins are important for normal preinfection development and hence for initiation of infection. More definitive roles for these proteins during preinfection morphogenesis will require posttranslational characterization. Nearly synchronous developmental assays will assist in such explorations.

ACKNOWLEDGMENTS

We thank Cindy Stryker for technical assistance.

This work was supported by the United States-Israel Binational Agricultural Research and Development Fund (grant 2232-92) and DOE/NSF/USDA Program of Collaborative Research in Plant Biology (grant 92-37310-7821).

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