Influence of Temperature, pH, Osmotic Potential, and Fungicide Sensitivity on Germination of Conidia and Growth from Sclerotia of *Colletotrichum coccodes* in Vitro

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ABSTRACT

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Germination of conidia of *Colletotrichum coccodes* was greatest at 22 C after 24 hr. Conidia did not germinate at 7 C after 24 hr and <70% of the conidia germinated at 10 or 31 C. Growth rates of cultures started with air-dried sclerotia were greatest at temperatures from 25 to 31 C. Germination of conidia and growth from sclerotia were optimum at pH 6. Water agar was osmotically adjusted using either KCl, NaCl, $CaCl_2$, or sucrose. Maximum germination of conidia and growth from sclerotia occurred at the highest osmotic potentials (−5 to −10 bars). Little or no germination of conidia occurred at −45 bars except when $CaCl_2$ was used to adjust the osmotic potential of the medium. Radial growth from sclerotia was less when KCl or NaCl amendments were used than when $CaCl_2$ or sucrose were used. Sensitivity of conidia and sclerotia to captafol, chlorothalonil, anilazine, mancozeb, and copper hydroxide were determined in vitro. Conidia were most sensitive to captafol, which significantly reduced their germination and germ tube elongation at concentrations of 0.01, 0.1, and 1 μg a.i./ml. Sclerotia were also most sensitive to captafol, which significantly reduced growth at concentrations of 1, 10, and 100 μg a.i./ml. Conidia and sclerotia were sensitive to chlorothalonil at the two highest concentrations tested (0.1, 1 μg a.i./ml and 10, 100 μg a.i./ml, respectively).

Additional keywords: *Lycopersicon esculentum*, tomato anthracnose.

Colletotrichum coccodes (Wallr.) Hughes has a wide host range primarily in the Cucurbitaceae, Leguminosae, and Solanaceae among agricultural crops and weeds (3,11,16,18). The fungus causes tomato anthracnose, which is a major threat to the processing tomato industry in New York and other northeastern states (4,5,8,15,21). A comprehensive study of factors affecting spore germination and growth of *C. coccodes* was initiated in part because of serious outbreaks of tomato anthracnose in New York State.

C. coccodes overwinters as sclerotia free in the soil or as sclerotia and mycelium in infested debris (2,7,10,15). The sclerotia may survive for several years in soil (2,7). Unlike most sclerotium-forming fungi, the sclerotia of *C. coccodes* originate as an acervulus differentiated from a stroma and germinate mycelogenically (22). Conidia produced in the acervulus serve as the primary inoculum for infection of tomatoes (3,8,15).

Failure to control tomato anthracnose is often attributed to poor timing of fungicide applications (4,5,11) and weather conditions conducive for disease development (8,11). However, little information is available on the biology of *C. coccodes* and on conditions that promote tomato anthracnose development. Preliminary studies examined the effect of temperature (14) and carbon sources (11) on growth of the fungus. Due to changes in the taxonomy of the causal agent and reports of isolations of other anthracnose fungi from tomato fruit (1), it is not clear whether early research was consistently conducted using *C. coccodes*. The objective of this study was to collect basic information on the biology of *C. coccodes*. The influence of temperature, pH, and osmotic potential on germination of conidia and growth from sclerotia were determined. The sensitivity of conidia and sclerotia of *C. coccodes* to fungicides commonly used for control of tomato anthracnose was characterized.

MATERIALS AND METHODS

Inoculum production and preparation. Isolate 210 of *C. coccodes*, which originated from tomatoes (*Lycopersicon esculentum* Mill.) grown in Lockport, NY, was used in this study. To produce sclerotia, cultures were grown on V-8 juice agar (30% V-8 juice, 3% Difco Bacto agar in distilled water, pH 4.6–5.0) plates for 2 mo. Cultures were incubated in the dark at 22–25 C. Sclerotia were gently scraped from the agar surface and washed twice in sterile distilled water by centrifugation to remove excess agar from the surface of the sclerotia. Sclerotia were air dried for 24 hr and stored at 5 C until needed. Conidia were obtained from 10–14-day-old cultures grown on V-8 juice agar by adding sterile distilled water to the plates and gently scraping the agar surface. The suspension was centrifuged and the supernatant discarded. Conidia were resuspended in sterile distilled water.

Effect of temperature. Petri plates containing autoclaved water agar (Difco Bacto agar, 2%) were preconditioned at 7, 10, 16, 19, 22, 25, 28, and 31 C before use. A conidial suspension was atomized onto the water agar plates, which were kept in the dark at the experimental temperatures. After 2, 4, 6, 8, 12, 22, and 24 hr, conidia were fixed by placing a drop of cotton blue in lactophenol in the center of each agar plate. Germination was determined by observing 40 conidia on each plate. A conidium was considered germinated if the germ tube length was >50% of the length of the conidium. Four plates were used at each temperature, and the experiment was repeated. Radial growth from sclerotia was examined on V-8 juice agar plates preconditioned at 4, 7, 10, 16, 19, 22, 25, 28, 31, and 36 C. The center of each plate was inoculated with a clump (1–3) of air-dried sclerotia and kept in the dark. Radial growth was measured after 5, 7, and 9 days. Five plates were used at each temperature, and the experiment was repeated.

Effect of pH. The effect of pH on germination of conidia and growth from sclerotia was determined using 2% Difco Noble water agar adjusted to the desired pH (range of 2–9) with combinations of HCl, $KHC_8H_4O_4$, H_3BO_3, KH_2PO_4, and NaOH. The pH of cooled agar (45–49 C) was readjusted to the final desired pH value when necessary, and the agar was poured into 9-cm-diameter plastic petri plates. The conidial suspension was atomized onto the agar plates and incubated in the dark at 24 C for 20–24 hr. Germination was determined by observing 20 conidia on each plate. Radial growth from sclerotia was determined from agar plates inoculated with a clump (1–3) of air-dried sclerotia. Plates were kept in the dark at 24 C, and radial growth was recorded after 5 days. For experiments with conidia or sclerotia, five plates were used at each temperature and the experiment was repeated.

Effect of osmotic potential. Difco Noble water agar (1%, pH 5.5) was adjusted to various osmotic potentials (−5 to −50 bars) by the addition of either NaCl, KCl, $CaCl_2$, or sucrose (13,19). The pH of the amended media at the low and high concentrations of each osmoticum was pH 5.9–6.0 for NaCl, pH 5.9–5.7 for KCl, pH 5.1–5.2 for $CaCl_2$, and pH 6.5 for sucrose. The conidial suspension was atomized onto the agar plates and incubated in the dark at 24 C for 20–24 hr. Germination was determined by observing 100 conidia on each plate. Radial growth from sclerotia was determined from agar plates inoculated with a clump (1–3) of air-dried sclerotia. The plates were sealed with Parafilm to prevent moisture loss and kept in the dark at 24 C. Radial growth was recorded after 7 days. Five replicate plates were used for each treatment, and the entire experiment using conidia and sclerotia was repeated.

Fungicide sensitivity. Inhibition of germination of conidia was determined using fungicide-amended agar. Chlorothalonil, anilazine, mancozeb, captafol, and copper hydroxide were used to amend autoclaved Difco Bacto agar (2%) to achieve final concentrations of 0.01, 0.1, and 1 μg a.i./ml. Media were prepared 1 day before use. The conidial suspension was atomized onto the fungicide-amended media and incubated at 23–25 C for 17 hr. After the incubation period, spores on the agar surface were fixed and stained with cotton blue in lactophenol. Spore germination was determined by counting the germinated spores out of 40 on each replicate plate. A spore was considered germinated if its germ tube length was >50% of the length of the spore. Twenty germ tube lengths were recorded for each treatment. Five replicate plates were used for each treatment. The experiment was conducted three times, and the data were analyzed by analysis of variance. The mean percent germination and germ tube length for each treatment in the three experiments was expressed as a percentage of the results obtained from the control plates.

The same fungicides were used to study inhibition of growth from sclerotia. Autoclaved V-8 juice agar was amended to achieve final concentrations of 1, 10, and 100 μg a.i./ml. Media were prepared 1 day before use. The fungicide-amended agar plates were inoculated with a clump of 1–3 air-dried sclerotia and kept in the dark at 23–25 C. Radial growth was measured after 9 and 12 days. Five replicate plates were used for each treatment. The experiment was conducted three times, and the data were analyzed by analysis of variance. The mean colony diameter for each treatment in the three experiments was expressed as a percentage of the results obtained from the control plates.

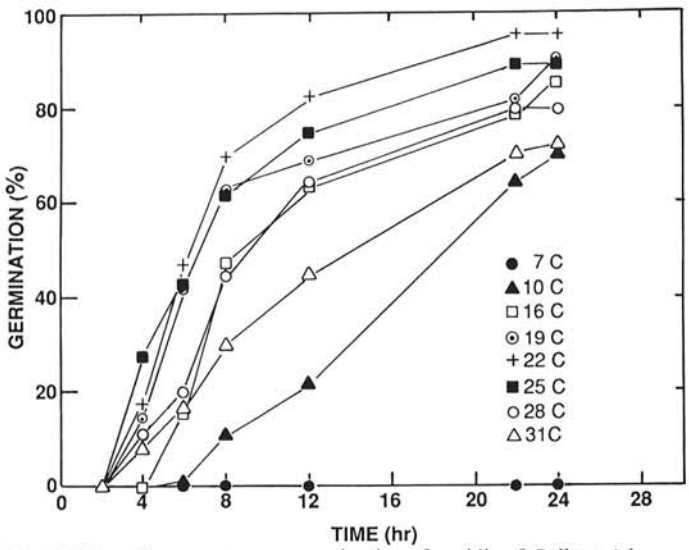

Fig. 1. Effect of temperature on germination of conidia of *Colletotrichum coccodes* on Difco Bacto water agar.

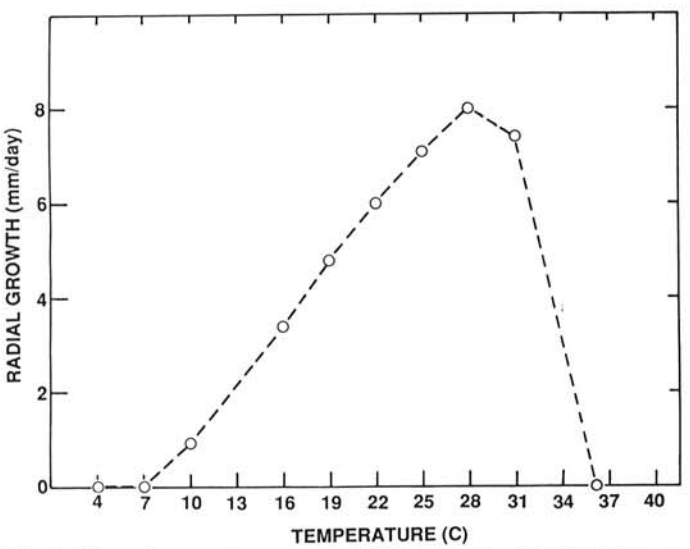

Fig. 2. Effect of temperature on growth from sclerotia of *Colletotrichum coccodes* on V-8 juice agar after 9 days.

RESULTS

Effect of temperature. Conidia germinated on water agar over a range of temperatures from 10 to 31 C (Fig. 1). Conidia did not germinate after 24 hr at 7 C, and <70% of the conidia germinated at 10 and 31 C. Germination of conidia was optimal at 22 C. Radial growth from sclerotia after 9 days occurred from 10 to 31 C (Fig. 2). No growth was detected at 4, 7, and 36 C. Growth was optimal at 28 C.

Effect of pH. Conidia germinated best on water agar adjusted from pH 5 to 7 (Fig. 3). Germination was 78.2, 84.8, and 65.6% at pH 5, 6, and 7, respectively. Germination was optimum at pH 6. Sclerotia responded similarly, with optimum radial growth at pH 6 (Fig. 3).

Effect of osmotic potential. The response of conidia to osmotic potential was influenced by the osmotica (Fig. 4). When $CaCl_2$ was used to adjust the osmotic potential from −6 to −50 bars, germination ranged from 82 to 52%, respectively. Germination was 64, 73, and 68% at −5 bars and was reduced to 0.2, 6, and 24% at −45 bars when KCl, NaCl, and sucrose were the osmotica, respectively. Conidia responded similarly to NaCl and KCl amendments. Radial growth from sclerotia was greatest at high osmotic potentials (−5 to −10 bars), and decreased as the osmotic

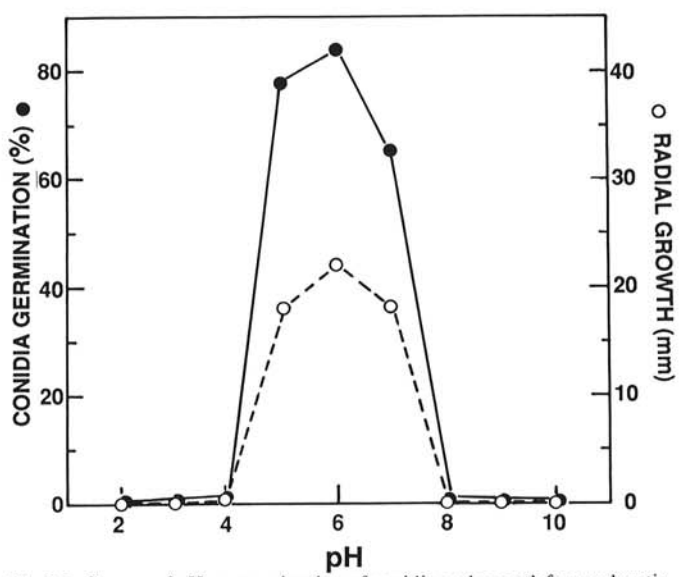

Fig. 3. Influence of pH on germination of conidia and growth from sclerotia of *Colletotrichum coccodes* on pH-adjusted Difco Noble water agar incubated at 24 C in the dark. Conidium germination was recorded after 20–24 hr and radial growth after 5 days.

potential decreased (Fig. 5). Growth was less when KCl or NaCl amendments were used to adjust the osmotic potential than when $CaCl_2$ or sucrose amendments were used.

Fungicide sensitivity. Captafol-amended water agar gave the greatest reduction in conidium germination and germ tube elongation at all concentrations tested (Table 1). Chlorothalonil also significantly reduced conidium germination and germ tube length. Anilazine and mancozeb provided modest reductions in germination and germ tube elongation at the 0.01 and 0.1 μg a.i./ml concentrations. Addition of copper hydroxide to the mancozeb-amended media significantly enhanced the reduction in conidium germination at 1 μg a.i./ml but had no effect on the other treatments. Copper hydroxide alone had little inhibitory effect on conidium germination and germ tube elongation.

Mean colony diameter was smallest on captafol-amended V-8 juice agar at all concentrations tested (Table 2). Chlorothalonil at 1 μg a.i./ml, caused some reduction in colony diameter, and greater colony diameter reduction at 10 and 100 μg a.i./ml. Anilazine at 100 μg a.i./ml, prevented radial growth but caused only modest reductions in radial growth at lower concentrations. Mancozeb at 100 μg a.i./ml moderately reduced radial growth and did not reduce radial growth at lower concentrations. Addition of copper hydroxide to the amended media did not enhance the reduction in radial growth by the test fungicides except when used with chlorothalonil at 100 μg a.i./ml or captafol at 1 μg a.i./ml. No fungicide tested prevented sclerotium formation. Different formulations of the commercial fungicides did not influence their efficacy.

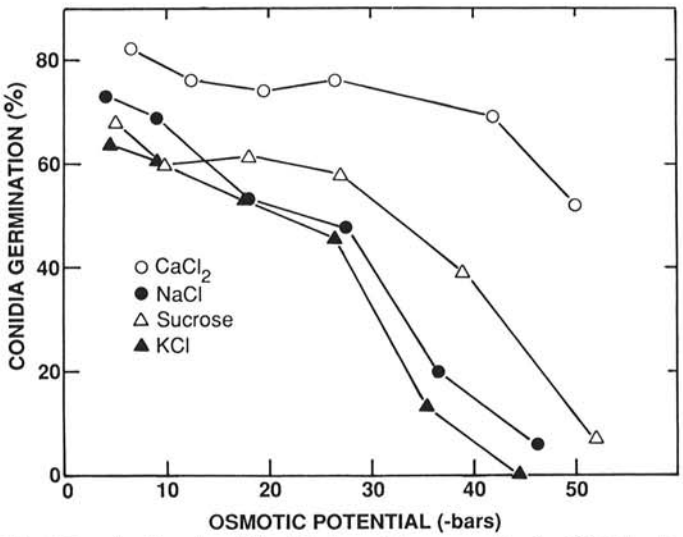

Fig. 4. Germination of conidia of *Colletotrichum coccodes* after 20–24 hr of incubation at 24 C on Difco Noble water agar osmotically adjusted with $CaCl_2$, NaCl, sucrose, or KCl.

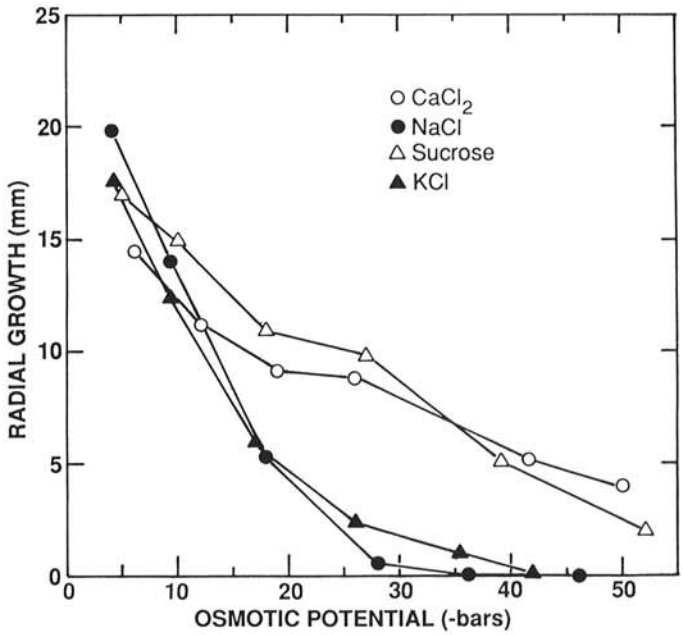

Fig. 5. Growth from sclerotia of *Colletotrichum coccodes* after 7 days of incubation at 24 C on Difco Noble water agar osmotically adjusted with $CaCl_2$, NaCl, sucrose, or KCl.

TABLE 1. Influence of fungicides on conidium germination and germ tube elongation of *Colletotrichum coccodes* in vitro

Treatment	Fungicide[a] concentration (μg a.i./ml)	Conidium germination[b] (% of control)		Germ tube length[c] (% of control)	
		− copper[d]	+ copper	− copper	+ copper
Chlorothalonil (Bravo 500)	0.01	93	93	80	73
	0.1	13	10	...[e]	...
	1.0	0	0	...	...
Chlorothalonil (Bravo 720)	0.01	94	91	71	66
	0.1	10	7	...	...
	1.0	0	0	...	...
Anilazine (Dyrene 4F)	0.01	93	93	75	73
	0.1	41	41	42	41
	1.0	0	0	...	...
Anilazine (Dyrene 50 WP)	0.01	91	92	67	76
	0.1	65	64	52	60
	1.0	2	2	...	...
Mancozeb (Dithane M-45)	0.01	92	90	62	74
	0.1	89	90	75	65
	1.0	67	0	38	...
Captafol (Difol. 80 Sprills)	0.01	51	45	20	22
	0.1	0	0	...	...
	1.0	0	0	...	...
Copper hydroxide (Kocide 101)	0.01	N/A	96	N/A	78
	0.1	N/A	92	N/A	74
	1.0	N/A	87	N/A	64

[a] Concentration of treatment fungicide and Kocide 101 when added.
[b] Data represent the mean from 15 plates (five per experiment) recorded after 17 hr, with 40 spores counted per plate. LSD for mean separation ($P = 0.05$) is 3.1.
[c] Data represent the mean from 15 plates (five per experiment), four germ tubes measured per plate. LSD for mean separation ($P = 0.05$) is 4.6.
[d] Copper hydroxide (Kocide 101) added (+) or not added (−) to test medium.
[e] Germ tubes not present or present in insufficient quantities to measure.

TABLE 2. Influence of fungicides on radial growth of *Colletotrichum coccodes* in vitro

Treatment	Fungicide[a] concentration (μg a.i./ml)	Colony diameter[b] (% of control)	
		− copper[c]	+ copper
Chlorothalonil (Bravo 500)	1	75	79
	10	51	48
	100	29	2
Chlorothalonil (Bravo 720)	1	82	79
	10	46	56
	100	39	2
Anilazine (Dyrene 4F)	1	95	94
	10	64	59
	100	0	0
Anilazine (Dyrene 50 WP)	1	93	94
	10	69	73
	100	0	0
Mancozeb (Dithane M-45)	1	93	93
	10	90	94
	100	45	55
Captafol (Difolatan 80 Sprills)	1	38	28
	10	1	0
	100	0	0
Copper hydroxide (Kocide 101)	1	N/A	98
	10	N/A	97
	100	N/A	95

[a] Concentration of treatment fungicide and copper hydroxide (Kocide 101) when added to V-8 juice agar.
[b] Data represent the mean from 15 plates (five per experiment) after 12 days. LSD for mean separation ($P = 0.05$) is 3.5.
[c] Copper hydroxide (Kocide 101) added (+) or not added (−) to test medium.

DISCUSSION

This study has shown that conidia and sclerotia of *C. coccodes* have very different temperature optima. Nightingale (14) reported the optimum temperature for growth as 26.6 C using mycelium as inoculum in vitro. Results from the current study indicated that optimum growth from sclerotia was 28 C and is within Nightingale's reported range. However, optimum germination of conidia occurred at 22 C. It is important to differentiate temperature optima between the two inoculum types because sclerotia serve as the overwintering and survival structures (2,7), and conidia serve as the primary inoculum for infection (3,8,15). The higher temperature optimum for growth from sclerotia may reflect adaptation of the sclerotia to germination during warm summer months when susceptible hosts are more readily available (tomato, potato, etc.). The lower temperature optimum for germination of conidia may reflect adaptation to germination during periods of free moisture availability. It has been observed that tomato anthracnose is most prevalent late in the growing season on ripe fruit during periods of high rainfall and frequent dew (8,11).

The optimum pH for germination of conidia and growth from sclerotia of *C. coccodes* was pH 6. The desired pH of processing tomatoes is <4.6 to avoid contamination by *Clostridium botulinum*, which has the ability to grow and produce toxins in tomato products at pH 4.8–5.0 (20). Tomatoes do not provide substrate with an optimum pH for germination of conidia of *C. coccodes*, which may account in part for its reputation as a weak parasite on tomato (3,8,11). However, deleterious effects of low pH appear to be overcome by the fungus once an infection is established; Sapers et al (20) reported that tomatoes inoculated with *C. coccodes* showed an increase in pH from 4.35 to 5.28 over a 13-day period.

Conidia and sclerotia of *C. coccodes* showed an increased tolerance to osmotic adjustments made with $CaCl_2$ and sucrose. This response was not related to the pH of the amended agar, which ranged from 5.1 to 6.5 when $CaCl_2$ or sucrose were used, and is within the optimal growth range. A similar increased tolerance to sucrose adjustment has been demonstrated with teliospores of *Tilletia indica* (6) and to $CaCl_2$ adjustment by basidiospores of *Athelia rolfsii* (17). The nature of these fungal responses is not known.

Fungicide applications are currently recommended for control of tomato anthracnose in New York State. The results of this study indicate that fungicide sensitivity of *C. coccodes* should be considered in disease control strategies. In vitro, conidia and sclerotia were most sensitive to captafol and chlorothalonil. The in vitro performance of these fungicides is consistent with field observations (4,5). Enhanced control of bacterial diseases through the use of premixed applications of copper-mancozeb sprays has been reported (12). Tomato growers in New York State have questioned whether these copper-mancozeb mixtures, which increase the available copper in solution, would enhance fungal disease control. In this study, addition of copper hydroxide to chlorothalonil (100 μg a.i./ml) or captafol (1 μg a.i./ml) significantly reduced radial growth from sclerotia. Further, addition of copper hydroxide to the mancozeb media enhanced reduction in conidia germination at the 1 μg a.i./ml concentration. It remains questionable whether enhanced control of *C. coccodes* in the field is possible with additions of copper compounds to fungicide sprays since conidia were only affected at the highest concentration in one treatment in this study.

Commercial tomato processors impose strict limits on the level of anthracnose that is acceptable because of the higher pH of infected fruit (20) and the increased spore and hyphal fragment counts in the processed tomato product (9). This study provides further information on factors affecting germination and growth of the propagules of *C. coccodes*, the causal agent of tomato anthracnose. Studies on factors that influence infection and lesion development on tomato fruit are in progress.

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