Semiselective Medium for *Colletotrichum gloeosporioides* and Occurrence of Three *Colletotrichum* spp. on Pepper Plants

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ABSTRACT

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Inhibition of mycelial growth of *Colletotrichum capsici* and *C. gloeosporioides* was significantly ($P = 0.01$) less than that of *Alternaria* sp. and *Fusarium* spp. when grown on a semiselective medium, *C. gloeosporioides* pepper isolate medium (CGPIM) containing one-quarter strength potato-dextrose agar amended with fenarimol and vinclozolin at 5 μg/ml each, chloramphenicol and erythromycin at 6.5 μg/ml each, iprodione at 15 μg/ml, neomycin sulfate at 20 μg/ml, and tetracycline hydrochloride at 25 μg/ml. Fenarimol enhanced the detection of *C. gloeosporioides* as cream-yellow sporulating colonies formed around infected and/or infested pepper (*Capsicum* spp.) seeds. When pepper seeds were placed on CGPIM and wet filter paper, *C. capsici* occurred at equal frequencies, but the frequency of *C. gloeosporioides* was significantly ($P = 0.01$) higher on CGPIM than on wet filter paper. *C. capsici* was detected on 14.5% of the seeds from var. LSU Sport, while *C. gloeosporioides* detection was less frequent. *C. gloeosporioides* was isolated from 30 and 1% of diseased fruits harvested and stored for 130 and 225 days, respectively. CGPIM and wet filter paper were equally effective in evaluating the occurrence of *C. capsici*, but the occurrence of *C. gloeosporioides* and *Glomerella cingulata* appressoria was significantly ($P = 0.01$) higher on CGPIM than on wet filter paper. *C. capsici* was recovered more frequently than either *C. gloeosporioides* or *G. cingulata* on inoculated leaves.

Colletotrichum capsici (Syd.) E.J. Butler & Bisby and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. are the most important *Colletotrichum* spp. reducing marketable fruit yields of pepper (*Capsicum annuum* L. and *Capsicum frutescens* L.) in the tropics and the subtropics (8,12). *Colletotrichum* spp. infect plants by germinating conidia deposited on plant parts by splashing rain (24). Conidia germinate to form appressoria, which facilitate penetration of host tissue or serve as survival units (15,16). Appressoria of *C. graminicola* (Ces.) G.W. Wils. were shown to remain dormant until higher temperatures occurred after their formation (21). The occurrence and viability of appressoria of *Colletotrichum* spp. has not been reported on pepper foliage.

Colletotrichum spp. are seedborne in crop plants. *C. capsici* (5–9,17,22) and *C. gloeosporioides* (syn. *C. piperatum* Ellis. & Everh. in Halst.) (5,8,22) occur either externally or internally in pepper seeds. Survival of mycelia and stromata in infected pepper seeds has been reported (22). Moist filter paper is commonly used to detect seedborne *Colletotrichum* spp. (9,12,18). In preliminary studies, *C. capsici* grew from pepper seeds and other plant parts when placed on wet filter paper, but slow-growing *C. gloeosporioides* was not detected (J. B. Manandhar, *unpublished*). Fast-growing organisms such as *Alternaria* and *Fusarium* spp. and bacteria often interfere with the detection of slow-growing organisms. Semiselective media, made by incorporating certain fungicides and antibiotics to inhibit growth of fungi and bacteria, were used to detect other *Colletotrichum* spp. (7,10). In addition, a selective medium differentiated two citrus strains of *C. gloeosporioides* (1,2). A semiselective medium to detect *C. gloeosporioides* and *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk in pepper seeds and other plant parts would be useful for monitoring fungal survival and studying host–pathogen interactions. The objectives of this study were to develop a semiselective medium to detect *C. capsici, C. gloeosporioides,* and *G. cingulata* on pepper leaf disks, petioles, and seeds, and to determine their occurrence on seeds and inoculated pepper leaves.

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MATERIALS AND METHODS

C. capsici (conidia 19.8–28.3 × 2.7–4.8 μm, mean 23.7 × 3.7 μm), a nonteleomorphic isolate of *C. gloeosporioides* (conidia 11.1–18.5 × 2.7–5.0 μm, mean 15.5 × 3.6 μm), and *G. cingulata* (formed glomerate perithecia on pepper plant parts, anamorph *Colletotrichum* sp., conidia 11.1–17.7 × 3.5–6.5 μm, mean 14.4 × 4.8 μm) were originally isolated from hot red pepper fruits of line PBC 595 grown during August 1992 at the Asian Vegetable Research and Development Center (AVRDC), Shanhua, Tainan, Taiwan. Single-conidial isolates of the *Colletotrichum* spp. were maintained on slants of acidified (pH 5) potato-dextrose agar (PDA) at 28 C. The semiselective medium consisted of a basal medium, one-quarter strength PDA (10 g of PDA and 15 g of agar in 1 L of water). To suppress bacterial growth, chloramphenicol and erythromycin at 6.5 μg/ml each, neomycin sulfate at 20 μg/ml, and tetracycline hydrochloride at 25 μg/ml were added when the basal medium cooled to 50 C after autoclaving. In addition, iprodione (Rovral 50W), 15 μg a.i./ml; fenarimol (Rubigan 11.76%E), 5 μg a.i./ml; and vinclozolin (Ronilan 50W), 5 μg a.i./ml, were added to suppress fungal growth other than *Colletotrichum* spp. Stock solutions of chloramphenicol, erythromycin, and tetracycline HCl were prepared in 10% methanol; iprodione, fenarimol, and vinclozoline in 20% methanol; and neomycin sulfate in distilled water. All were filter-sterilized through a 0.2-μm Nalgen filter. The basal media with combinations of fungicides and antibiotics were tested to develop the best semiselective medium, which was named *C. gloeosporioides* pepper isolate medium (CGPIM).

Inhibition of mycelial growth on fungicide-amended media. Three-millimeter-diameter disks from the margins of 3- to 5-day-old colonies on PDA of an isolate of an *Alternaria* sp., two isolates of *Fusarium* spp. (isolated from pepper seeds), and *C. capsici* and *C. gloeosporioides* (one isolate of each previously mentioned and five isolates of each from the AVRDC pepper isolate collection) were transferred to the middle of 9-cm-diameter plastic plates containing either basal medium and antibiotics (control) or basal medium amended with either iprodione, iprodione + fenarimol, or iprodione + fenarimol + vinclozolin. Inoculated plates were incubated under 12/12-h day–night cycles at 28 C. Three plates were used as replicates. The colony diameter was measured at 5 and 7 days. Percent inhibition of the mycelial growth was calculated: [(colony diameter of control − colony diameter of amended medium)/colony diameter of control] ×

100. Inhibition data for each isolate of *Colletotrichum* spp. were combined. The experiment was repeated once.

Detection of *Colletotrichum* spp. from foliage and seeds on fungicide-amended media. One hundred twenty leaves with petioles were randomly sampled at the eighth fruit harvest from field-grown pepper plants of var. Povaska that had been inoculated four times prior to fruit set with a mixed conidial suspension (10^6 conidia per milliliter) of *C. capsici* and *C. gloeosporioides*. Petiole sections (1 cm long, one per leaf) and leaf disks (6 mm diameter, one disk per leaf obtained by boring at random with a potato-screw borer no. 3) were excised and surface-sterilized in 0.5% sodium hypochlorite for 4 min. Twenty petioles per plate were placed on medium amended with either iprodione, iprodione + fenarimol, or iprodione + fenarimol + vinclozolin, or on nonamended medium. In addition, 400 nonsterilized seeds (20 seeds per plate) obtained from field-grown diseased pericarps of pepper line PBC 371 were plated on medium amended with either iprodione, iprodione + fenarimol, or iprodione + fenarimol + vinclozolin, or on nonamended medium. Leaf disks and petioles (two samples for each of three replicates) and seeds (20 subsamples of five samples in four replicates) were incubated under 12/12-h light–dark cycles for 7 days at 28 C.

Detection of *Colletotrichum* spp. on seeds. Four hundred seeds each of pepper var. Fyuco, KA-6-5, and LSU Sport, harvested from a spring-planted crop that had not been sprayed with fungicides or inoculated with cultured *Colletotrichum* spp., were plated on CGPIM or on wet filter paper (20 seeds per plate, five plates per replicate). Four shelves were used as four blocked replicates and incubated under continuous fluorescent light for 7 days at 28 C. Seeds plated on wet filter paper were incubated under continuous fluorescent light near 100% relative humidity for 14 days at 28 C. *C. capsici* and *C. gloeosporioides* were detected using a binocular microscope, and other *Colletotrichum* spp. (11) were discounted. Data from CGPIM and wet filter paper were bulked for analysis.

Detection of *C. gloeosporioides* on stored seeds. Ripe red fruits of varieties–lines KA-6-5, PBC 371, PBC 473, and Rotan, with anthracnose lesions caused by *C. gloeosporioides* covering 25–50% of the surface area, were harvested and air-dried. Seeds were extracted and cleaned manually and then stored in the laboratory at 25 ± 4 C. Four hundred seeds of each line–variety from two harvest times corresponding to 130 and 225 days after harvest (DAH) were plated on CGPIM (20 seeds per plate, 100 seeds per replicate) and incubated under fluorescent light for 7 days at 28 C. *C. gloeosporioides* was detected using a binocular microscope.

Detection of *Colletotrichum* spp. on inoculated pepper seedlings. Forty-eight seedlings at the five- to six-leaf stage of var. Long Fruit grown in 5-cm-diameter plastic pots (one plant per pot) containing greenhouse soil mix (soil, rice husk, sand, and compost in a 3:1:1:1 ratio) were spray-inoculated with a conidial suspension (10^6 conidia per milliliter) of either *C. capsici, C. gloeosporioides,* or *G. cingulata* until runoff. There were 16 pots for each *Colletotrichum* spp. Plants were incubated for 48 hr in a chamber programmed for 1 min of mist per 15 min at 28 C, and then transferred to 18-cm plastic pots and placed on screenhouse benches in a completely randomized design with three replicates for each isolate for an additional 83 days. The two lowest attached leaves and petioles were detached from each plant at 5, 30, 52, and 83 days after inoculation (DAI). Petioles were cut into two 1-cm lengths. Two 6-mm-diameter leaf disks from each leaf were cut out with a cork borer. Samples were washed 1 h in running tap water, surface-sterilized in 0.5% sodium hypochlorite for 4 min, and rinsed twice for 2 min each in sterilized distilled water. Sixteen petioles and leaf disks were plated either on double-layered wet Whatman No. 1 filter paper or on CGPIM. Samples on CGPIM were incubated under continuous fluorescent light for 7 days, and samples on wet filter paper were incubated for 14 days under 12/12 light–dark cycles and 100% relative humidity at 28 C. *Colletotrichum* spp. were detected using a binocular microscope.

Data analysis. Inhibition data were analyzed to determine if experiments could be combined (23) by using JMP (20). Data from this experiment and others were analyzed by ANOVA and means separation by Duncan's multiple range test ($P = 0.01$).

RESULTS

Inhibition of mycelial growth on fungicide-amended media. As a source of variation, experiments were not significantly ($P = 0.01$) different, and data from the two experiments were combined for analysis. *Alternaria* sp. and *Fusarium* spp. were inhibited significantly ($P = 0.01$) more than *C. capsici* and *C. gloeosporioides* when grown on iprodione-amended basal medium (Table 1). *Alternaria* sp. and *Fusarium* spp. did not grow when the basal medium was amended with either iprodione + fenarimol or iprodione + fenarimol + vinclozolin. Mycelial growth of *C. capsici* was inhibited significantly ($P = 0.01$) less than *C. gloeosporioides*. Colonies of *C. gloeosporioides* had floccose white mycelial growth on iprodione-amended medium and smaller cream-yellow sporulating colonies on media amended with iprodione + fenarimol and iprodione + fenarimol + vinclozoline.

Detection of *Colletotrichum* spp. from foliage and seeds on fungicide-amended media. *Colletotrichum* spp. were detected on leaf disks, petioles, and seeds significantly ($P = 0.01$) more on fungicide-amended media than on basal medium (Table 2). The frequency of *Colletotrichum* spp. was significantly ($P = 0.01$) more on leaf disks placed on medium amended with iprodione + fenarimol + vinclozolin than with iprodione or iprodione + fenarimol; but the frequency on petioles was not significantly ($P = 0.01$) different among iprodione, iprodione + fenarimol, or iprodione + fenarimol + vinclozolin. Seedborne occurrence of *Colletotrichum* spp. was detected significantly ($P = 0.01$) more on iprodione + fenarimol + vinclozolin medium than on either iprodione, iprodione + fenarimol, or on basal medium. Incorporation of fenarimol into the medium enhanced detection of *C. gloeosporioides*. Mycelial growth was restricted, and colonies were cream-yellow with abundant sporulation (Fig. 1).

Detection of *Colletotrichum* spp. on seeds. Recovery of *C. capsici* on seeds did not differ between CGPIM (4.8%) and wet filter paper (3.5%), but recovery of *C. gloeosporioides* differed significantly ($P = 0.05$, 2% on CGPIM and 0.1% on wet filter paper). Seeds of var. LSU Sport had the highest frequency of *C. capsici* (14.5%), but the fungus was not detected on the other two varieties. *C. gloeosporioides* was recovered at a significantly ($P = 0.05$) higher rate from var. Fyuco (3.8%) than from var. LSU Sport (0.5%), while recovery from var. KA-6-5 (1.8%) was not significantly different from the other two varieties.

Table 1. Percent inhibition of *Alternaria* sp., *Colletotrichum capsici*, *C. gloeosporioides*, and *Fusarium* spp. on basal medium amended with different fungicides

	Fungicide combination[y]		
Fungus	Iprodione	Iprodione + fenarimol	Iprodione + fenarimol + vinclozolin
Alternaria sp.	94 a[z]	100 a	100 a
C. capsici	15 d	12 c	13 c
C. gloeosporioides	44 c	62 b	65 b
Fusarium spp.	70 b	100 a	100 a

[y] Iprodione at 15 μg a.i./ml, fenarimol at 5 μg a.i./ml, and vinclozolin at 5 μg a.i./ml.
[z] Numbers followed by the same letter within columns are not significantly different using Duncan's multiple range test at $P = 0.01$.

Detection of *C. gloeosporioides* on stored seeds. The recovery of *C. gloeosporioides* was significantly ($P = 0.01$) higher at 130 DAH (30%) than at 225 DAH (1%) in seeds obtained from diseased fruits of four pepper varieties–lines when assayed on CGPIM.

Detection of *Colletotrichum* spp. on inoculated pepper seedlings. Recovery of *Colletotrichum* spp. was significantly ($P = 0.05$) higher on CGPIM than on wet filter paper at 30, 52, and 83 DAI, but not at 5 DAI. Recovery of *C. capsici, C. gloeosporioides,* and *G. cingulata* were significantly ($P = 0.05$) reduced over time from 5 to 83 DAI (Fig. 2A and B). Recovery of *C. capsici* was significantly ($P = 0.05$) higher at 30, 52, and 83 DAI than recovery of either *C. gloeosporioides* or *G. cingulata* on either CGPIM or on wet filter paper. Similarly, recovery of *G. cingulata* was significantly ($P = 0.01$) higher than *C. gloeosporioides* at 30, 52, and 83 DAI on both CGPIM and wet filter paper. *C. gloeosporioides* was detected on 64% of the samples on CGPIM at 30 DAI, which was significantly ($P = 0.05$) higher than detection on wet filter paper (6%); and 31% detection on CGPIM compared to almost none on wet filter paper at 52 DAI was significant ($P < 0.05$) (Fig. 2A and B).

DISCUSSION

Rose Bengal medium (7) or a modification of the blotter method (18) has been used to detect *C. capsici* from pepper seeds. In our study, there was no difference between wet filter paper and CGPIM in detecting *C. capsici*, but the CGPIM enhanced detection of *C. gloeosporioides* while allowing for detection of other species of *Colletotrichum*. The seedborne nature of *C. gloeosporioides* on pepper seeds has not been intensely studied, probably because of its obscure, slow-growing nature compared to other faster growing fungi that occur on seeds. Using CGPIM, the growth of *Alternaria* and *Fusarium* species was reduced; thus *C. gloeosporioides* was detected on seeds of some lines at a high frequency. Use of copper hydroxide in the medium stimulated sporulation of slow-growing orange colony types of *C. gloeosporioides* citrus isolates (1). Tolerance to benomyl in slow-growing orange colony types was used to differentiate these from ubiquitous fast-growing gray colony types of *C. gloeosporioides* in citrus foliage washings (12). From our studies, isolates of *C. gloeosporioides* from pepper were neither stimulated to sporulate on medium amended with copper hydroxide at 42.5 μg/ml nor tolerant to benomyl at 2 μg/ml (J. B. Manandhar, *unpublished*). Amending culture media with cytokinin or phenolic compounds stimulated sporulation of *Colletotrichum dematium* (Pers.) Grove (19). We found that, in media amended with fenarimol, *C. gloeosporioides* was stimulated to sporulate, producing distinct cream-yellow sporulating colonies with a restricted margin; the growth of other fungi was reduced.

Poor seed quality has been associated with fruits having anthracnose (14). Mycelia and stromata of *Colletotrichum* spp. have been reported as overseasoning structures in pepper seeds (5,22). Recovery of *C. gloeosporioides* from seeds of infected fruits from four pepper lines declined rapidly from 30 to 1% from seeds stored 130 to 225 DAH. One-year-old seeds of var. Fyuco, KA-6-5, and LSU Sport stored at room temperature and plated on CGPIM and wet filter paper had a high incidence of *C. capsici* and a low incidence of *C. gloeosporioides,* indicating that the viability of either overseasoning mycelia or stromata was greatly reduced in *C. gloeosporioides.*

Leaf and stem lesions that produce collar rot and dieback of pepper branches caused by *C. capsici* have been reported from India (5–7,17,18). Conidia and/or ascospores of *Colletotrichum* spp. and/or *G. cingulata* germinate quickly after deposition on pepper foliage to form dark appressoria that resist desiccation. Appressoria are known to form adhesive disks for adhering to plant surfaces and remain latent until physiological changes occur in the fruits (15,16). Marks et al (13) reported abortive penetration attempts of the infection hyphae similar to what we observed. During wet periods, appressoria have been reported to produce secondary conidia (15), which may be involved in secondary spread to pepper fruits. The amount of dark appressoria on older leaves of mango was used to estimate the level of anthracnose incidence in fruits and blossoms (4). Survival

Table 2. Percent frequency of *Colletotrichum* spp. on leaf disks, petioles, and seeds on basal medium and basal medium amended with fungicides

Fungicide combination[y]	*Colletotrichum* spp. (%)		
	Leaf disk	Petiole	Seed
Iprodione	55 b[z]	43 a	38 ab
Iprodione + fenarimol	61 b	46 a	35 b
Iprodione + fenarimol + vinclozolin	74 a	54 a	42 a
Basal medium	13 c	13 b	14 c

[y] Iprodione at 15 μg a.i./ml, fenarimol at 5 μg a.i./ml, and vinclozolin at 5 μg a.i./ml.
[z] Numbers followed by the same letter within columns are not significantly different using Duncan's multiple range test at $P = 0.01$.

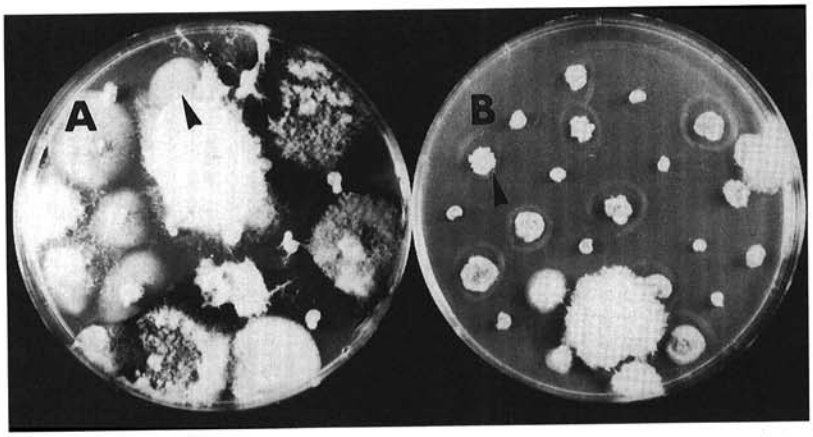

Fig. 1. Detection of *Colletotrichum gloeosporioides* on **(A)** basal medium and **(B)** on semiselective medium (*C. gloeosporioides* pepper isolate medium) with bright cream-yellow sporulating colonies of *C. gloeosporioides* around pepper seeds. Arrows and rings around the fungal colonies were *C. gloeosporioides* marked by a marking pen on the bottom of 9-cm-diameter dish during evaluation.

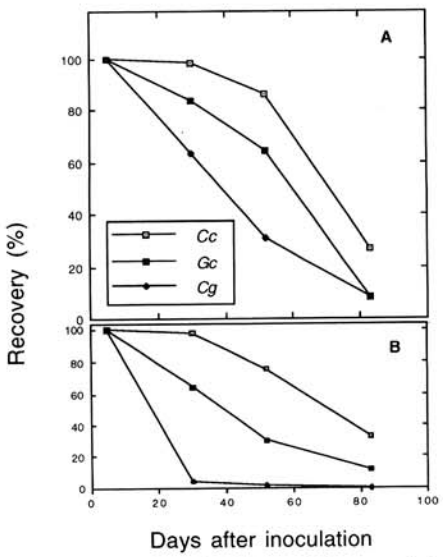

Fig. 2. Detection of *Colletotrichum capsici* (Cc), *C. gloeosporioides* (Cg), and *Glomerella cingulata* (Gc) (combined means of leaf disk and petiole) at different days after inoculation on **(A)** semiselective medium (*C. gloeosporioides* pepper isolate medium) and on **(B)** wet filter paper.

of the sclerotia of *C. graminicola* in sorghum stalks (3) and appressoria of *C. graminicola* on barley leaves (21) have been demonstrated. The detection of three species of *Colletotrichum* on pepper foliage in our study showed that *C. gloeosporioides* declined rapidly compared to *C. capsici* and *G. cingulata*. This could indicate a difference in infection frequency, survival capacity, and/or ability to produce appressoria.

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