A Rapid, Reliable Bioassay for Pathogenicity of *Colletotrichum magna* on Cucurbits and Its Use in Screening for Nonpathogenic Mutants

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ABSTRACT

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A rapid, reliable bioassay for large-scale screening of pathogenicity of *Colletotrichum magna*, a causal agent of anthracnose in cucurbits, was developed. This method involved exposing whole seedlings and cuttings (plants with excised root systems) to conidial suspensions in scintillation vials. The results corresponded well to disease responses observed by the standard leaf inoculation procedures. This continuous dip method allowed for a rapid disease response in cuttings; mortality occurred within 48–72 hr after inoculation. The method was also reliable for determining susceptible and resistant cucurbit cultivars and enabled screening of more than 300 UV-irradiated potential pathogenicity mutants. Three nonpathogenic mutants, isolates HU 25, HU 36, and HU 43, were identified. Isolate HU 25 did not cause mortality in whole plants but did induce a hypersensitive response when conidia were assayed on cotyledons. The banana-specific pathogen, *C. musae*, did not cause disease in cuttings and cotyledon-inoculated seedlings. The proposed method enables rapid, reliable, and large-scale screening, and requires less time and greenhouse space than the standard leaf inoculation techniques. The continuous dip method may also be well-suited for screening pathogenicity in isolates of soilborne fungi and especially for evaluating resistance of cultivars to wilt pathogens.

Additional keywords: cucumber, *Fusarium* spp., mutagenesis, watermelon

An anthracnose disease of cucurbits, caused by *Colletotrichum magna* S. F. Jenkins and Winstead (teleomorph: *Glomerella magna* S. F. Jenkins and Winstead), was first detected in 1959 (6). Symptoms are similar to those caused by *C. lagenarium* (Pass.) Ellis & Halst. (4,6) and include reddish-brown lesions of angular shape with yellow-soaked borders on cotyledons and leaves; stem lesions are sunken and advance rapidly. Because of symptom similarity between *C. lagenarium* and *C. magna*, *C. magna* may cause disease more frequently in cucurbits than previously reported (6,10).

Fungi from the genus *Colletotrichum* collectively cause disease on the majority of significant agricultural crops by infecting either foliar parts of the plant or persisting as soilborne pathogens (1,3, 4,8). Therefore, there is a need to generate and screen for resistant cultivars or genotypes of many crops.

Some species of *Colletotrichum*, such as *C. acutatum* J. H. Simmonds, a soilborne fungal pathogen of strawberry, are bioassayed by spray-inoculating plants with a suspension of naturally infested soil (3). The standard leaf inoculation procedures for evaluating anthracnose resistance involve foliar application of fungal conidia by spraying, pipette drop techniques, or injection (1,2,8). These procedures are time- and labor-consuming and require considerable greenhouse and/or growth chamber space.

The objective of this study was to develop a rapid, reliable bioassay for large-scale screening of *C. magna* isolates, primarily for obtaining mutants that are nonpathogenic on cucurbits. The proposed method involves continuous exposure of whole plants or cuttings (plants with root system excised) to fungal conidia suspensions contained in scintillation vials. Our bioassay and standard leaf inoculation procedures were compared with regard to rate of disease development, reliability, consistency, and labor and greenhouse requirements. The utility of this method in screening soilborne pathogens is discussed.

MATERIALS AND METHODS

Pathogens. Pathogenic isolates of *C. magna* (L2.5 and DXD) were obtained from O. C. Yoder of Cornell University.

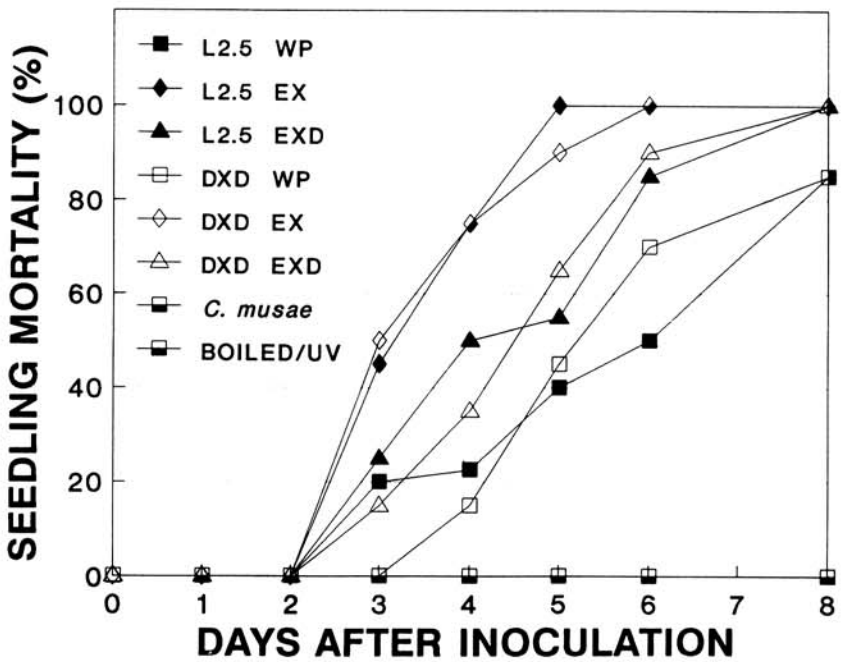

Fig. 1. Disease incidence in watermelon seedlings inoculated with conidia of isolates L2.5 and DXD of *Colletotrichum magna* and isolate 1116 of *C. musae*. Whole plants (WP) and cuttings with root system excised (EX) were immersed continuously in conidial suspensions ($2.5–3 \times 10^6$ conidia per milliliter), whereas cuttings dipped for 3 hr only (EXD) were transferred to tap water. Control treatments represent *C. musae*, boiled (100 C for 3 min) and UV-killed conidia of isolate L2.5, all inoculated in WP, and EX and EXD cuttings. After probit transformation of percentage of seedling mortality and log transformation of days after inoculation, the linear regression coefficients ($0.67 < R^2 < 0.72$) are significant ($P = 0.05$). The slope values of the different treatments are not significantly different ($P = 0.05$). The time required to reach 50% disease incidence is significantly different between the WP, EX, and EXD treatments of each isolate alone ($P = 0.05$).

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Nonpathogenic mutants (HU 25, HU 36, and HU 43) of *C. magna* were derived from isolate L2.5 by UV mutagenesis as described below. Pathogenic isolates of *C. musae* (Berk. & M. A. Curtis) Arx (927 and 1116) were isolated from diseased banana fruits and provided by A. Johanson of ODNRI, England.

Inoculum production. Conidial suspensions of *Colletotrichum* were plated on a modified Mathur's medium (MS) (9) (0.1% yeast extract, 0.1% Bacto Peptone, 1% sucrose, 0.25% $MgSO_4 \cdot 7H_2O$, 0.27% KH_2PO_4, 1.2% agar supplemented with 500 μl of ampicillin in 1 L of sterile distilled water) and incubated for 5 days at 23 C under cool fluorescent illumination. Thereafter, conidia were suspended in sterile water amended with 0.05% agar and filtered through four layers of sterilized surgical gauze. The conidial suspensions were adjusted to a concentration of $2.5–3 \times 10^6$ conidia per milliliter for all plant inoculation trials.

Plants. Anthracnose-susceptible (Sugar Baby) and partially resistant (Jubilee) cultivars of watermelon (*Citrullus lanatus*) (Atlee Burpee Co., Warminister, PA), and susceptible (Burpee Pickler) and resistant (Poinsett 76-S) cultivars of cucumber (*Cucumis sativas*) (Harris Moran Seed Co., Davis, CA) were used to evaluate disease incidence.

Mutagenesis. Conidia of L2.5 of *C. magna* from 5-day-old cultures, grown on solid MS medium, were harvested by washing the cultures with liquid MS medium and filtering through surgical gauze. Spore concentration was adjusted to 5×10^7 conidia per milliliter without washing, and aliquots of 5 ml were dispensed aseptically in glass petri plates. Plates containing the conidia were placed at a distance of 3 cm directly under UV light (254 nm, 1,200 μW cm^{-2}; Gates [MR-4] New York) and exposed for 30 min. Viability of conidia after UV radiation was determined by dilution-plating on MS medium, and plates were incubated under fluorescent and incandescent light at 22 C. Mortality was calculated by comparing UV-treated conidia with untreated controls. An exposure of 30 min resulted in the death of about 99.99% of the conidia. Surviving individual colonies were screened for pathogenicity as described below. In addition, UV-irradiated conidia (5×10^6 per milliliter) were plated on a minimal medium (0.17% yeast nitrogen base,

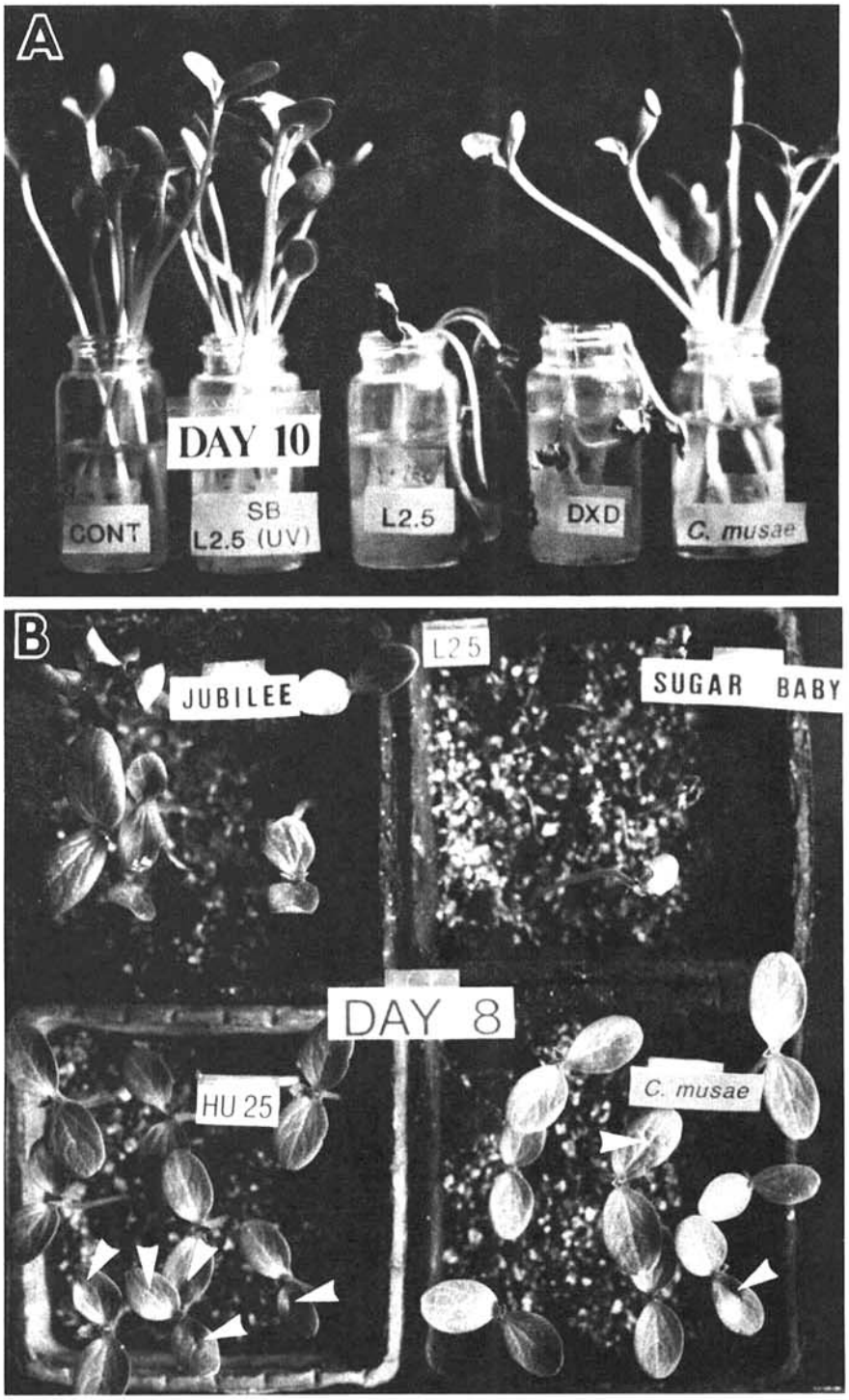

Fig. 2. Disease incidence in whole plants and cuttings (root system excised) inoculated by **(A)** the continuous dip method compared to that of seedlings inoculated by **(B)** the standard procedure. In **(A)**, whole plants and cuttings of a Sugar Baby watermelon (susceptible) were dipped continuously in conidial suspensions ($2.5–3 \times 10^6$ conidia per milliliter) of pathogenic isolates of *Colletotrichum magna* (isolates L2.5 and DXD), a nonpathogenic mutant HU 25 (L2.5 [UV]), and the host-specific pathogen of banana (*C. musae*), and were compared with water-inoculated controls. In L2.5 and DXD vials, all dead cuttings were removed at day 3, whereas cuttings remain in all the other vials. In **(B)**, the partially resistant Jubilee watermelon was leaf-inoculated with conidial suspensions of isolate L2.5 ($2–3 \times 10^6$ conidia per milliliter), whereas the susceptible cultivar (Sugar Baby) was leaf-inoculated with conidial suspensions of L2.5, HU 25, and *C. musae* (at the same concentration). Arrows indicate the hypersensitive reaction on infected cotyledons.

Table 1. Time required for 50% seedling mortality in susceptible cultivar Sugar Baby of watermelon plants dip-inoculated with *Colletotrichum magna*

Isolate	Inoculation treatment[x]	Time[y] (days)
L2.5	Whole plants	6.2 a[z]
L2.5	Excised plants, 3-hr dip	4.3 b
L2.5	Excised plants	3.2 c
DXD	Whole plants	5.8 a
DXD	Excised plants, 3-hr dip	4.5 b
DXD	Excised plants	3.1 c

[x] Whole plants or plants with root system excised were dipped continuously or for 3 hr in conidial suspensions of isolates L2.5 and DXD of *C. magna*.

[y] Values for 50% seedling mortality were estimated from the respective regression equations of disease progress of each treatment.

[z] Values with a common letter are not significantly different ($P = 0.05$).

0.07% $[NH_4]_2SO_4$, 1% sucrose, 1.2% agar) amended with 0.5% hydroxyurea for the selection of hydroxyurea-resistant mutants. No growth of wild-type conidia was observed on the hydroxyurea-amended medium. Hydroxyurea-resistant colonies were single-spored and also tested for pathogenicity by the method described below. From more than 250 UV-irradiated survivors and approximately 50 hydroxyurea-resistant colonies, three potential nonpathogenic mutants were obtained, isolates HU 25, HU 36, and HU 43. Isolate HU 25 was used extensively throughout this study.

Inoculation of whole seedlings and cuttings with isolates of *C. magna* and *C. musae*. Watermelon and cucumber seeds were planted in vermiculite rooting medium. After 5–6 days, seedlings were removed and washed in running tap water. Complete seedlings or cuttings (seedlings that had the root system excised at the crown with a razor blade) were exposed continuously or for 3 hr in 18 ml of conidial suspensions (2.5–3 $\times 10^6$ conidia per milliliter) in scintillation vials. The plants and cuttings were maintained in a controlled growth chamber (EGC, Chagrin Falls, OH) at 22 C under a 12-hr diurnal cycle. Artificial light intensity originated from fluorescent and incandescent lamps of 980 $\mu E \cdot m^{-2} \cdot s^{-1}$ (80,000 lx). Susceptible cuttings and seedlings showed wilt symptoms before seedling mortality, 2–3 days after the continuous dip inoculation. Resistant and control cuttings regenerated a new rooting system 10 days after inoculation. The causal agent was reisolated from infected tissue and used to reinfect healthy cuttings, thus conforming to Koch's postulates. Disease incidence was expressed as the percentage of seedling mortality; five replicates of six seedlings each per treatment were used. Initial screening for nonpathogenic mutants was performed with five cuttings and five whole plants per isolate. The experiments were done in a completely randomized design.

Inoculation of seedlings by the standard methods. The method of Dean and Kuć (2) essentially was used for cotyledon inoculations of seedlings. Seedlings of watermelon and cucumber were germinated in peat pots (10 × 10 × 15 cm in height) containing a vermiculite rooting medium. After 5–6 days, cotyledons were inoculated by pipetting 30 5-μl drops of conidial suspensions (2.5–3 $\times 10^6$ conidia per milliliter) from isolates of *C. magna* and *C. musae* (1,2). Cotyledon inoculations also were done by inverting the pots and dipping the seedlings in the same conidial suspensions and allowing excess water to drain off the leaves. Similar results were achieved by both methods. The plants were then sealed in clear polyethylene bags and placed in a growth chamber at 22 C under the same light intensity as described previously. Sterile water was used as a control for uninoculated plants. After 2 days, the bags were removed from the plants, and plants were then monitored on a daily basis for disease development. Susceptible watermelon seedlings exhibited extensive lesions on the cotyledons, and the plant died. The susceptible cucumber cultivar was not killed but exhibited extensive lesions on the cotyledons (1–2 cm in diameter), and plants were stunted and underdeveloped compared to the resistant cultivar and water-inoculated controls. Disease percentage was determined as described in the previous section. Experiments were done with four replicates of nine seedlings each per treatment in a completely randomized design.

Statistical analyses. All experiments in this study were repeated at least twice with similar results; the figures represent data from one such experiment. Statistical analyses of the data were done by linear regression after probit transformations of percentage of seedling mortality and log transformations of days after inoculation; significance level was $P = 0.05$. Values for 50% disease incidence were estimated from regression equations and compared by Duncan's multiple range test ($P = 0.05$).

RESULTS

Disease response of Sugar Baby after inoculation by the continuous dip method. A very rapid disease response was observed in Sugar Baby cuttings (EX) exposed to L2.5; 50% mortality was observed after 3 days and 100% mortality at 5 days (Fig. 1; Table 1). The disease response in cuttings, inoculated initially for 3 hr (EXD), was significantly slower than in cuttings of the continuous dip treatment (Table 1). A further significant delay in disease incidence was observed in the continuous dip treatment of whole plants (WP) (Figs. 1,2A; Table 1). The host-specific banana pathogen *C. musae* as well as boiled or UV-killed conidia of *C. magna* did not induce disease symptoms in cuttings (Fig. 2A). Furthermore, control cuttings regenerated new rooting systems 8–10 days after continuous exposure to the respective conidial suspensions, demonstrating full recovery from the initial wounding, and did not develop symptoms of disease.

Comparison between the standard and dip inoculation procedures with nonpathogenic mutants and nonspecific pathogens. Intact seedlings (WP) of Sugar Baby inoculated with the nonpathogenic mutant HU 25, by both the continuous dip and standard leaf methods, did not exhibit wilt symptoms after 8 and 16 days, respectively (Figs. 2A,3,4). However, minor lesions did occur on inoculated cotyledons (Fig. 2B). A low mortality rate in HU 25 inoculated cuttings (EX) was observed, although disease progress was significantly slower than in seedlings inoculated with the wild-type controls, L2.5 and DXD (Fig. 3). Disease progress also was delayed in cuttings inoculated with HU 25 (Fig. 3).

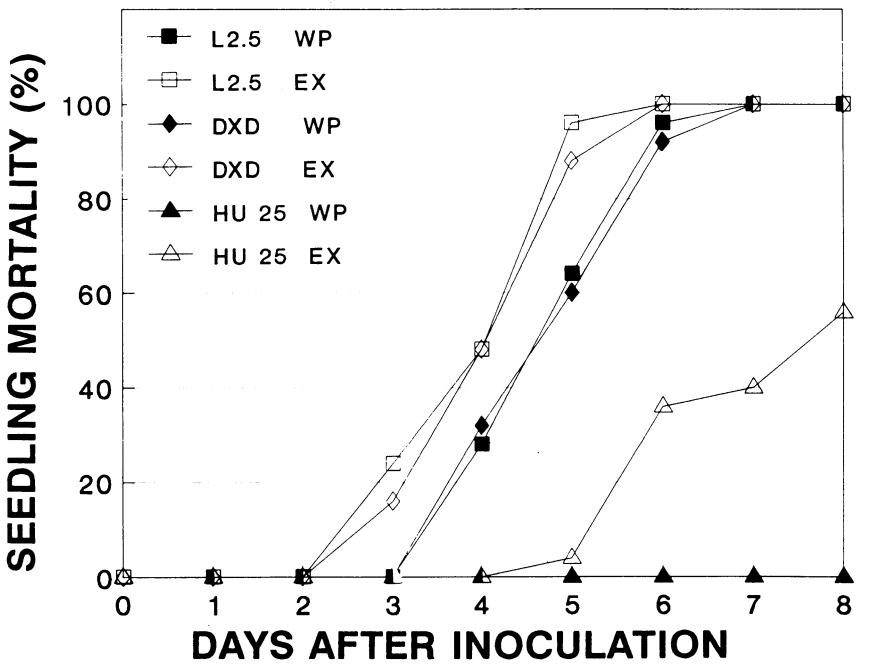

Fig. 3. Disease incidence in watermelon seedlings inoculated with conidia of isolates L2.5, DXD, and HU 25 (a nonpathogenic mutant of L2.5) of *Colletotrichum magna*. Whole plants (WP) and cuttings with root system excised (EX) were immersed continuously in a conidial suspension (2.5–3 $\times 10^6$ conidia per milliliter). After probit transformation of percentage of seedling mortality and log transformation of days after inoculation, the linear regression coefficients ($0.79 < R^2 < 0.94$) are significant ($P = 0.05$). The slope value of cuttings inoculated with the mutant (HU 25 EX) is significantly different from those of the cuttings (EX) inoculated with DXD and L2.5 ($P = 0.05$).

Similar results were also observed with two other nonpathogenic mutants, HU 36 and HU 43 (data not shown). Isolations were done from diseased Sugar Baby cuttings that had been inoculated with HU 25, and the fungi obtained were rescreened for pathogenicity. The reisolated fungi had phenotypes identical to HU 25, indicating that the occurrence of disease was not due to reversion or additional mutations.

The host-specific pathogen of banana, isolate 1116 of *C. musae*, did not induce disease symptoms in leaf-inoculated seedlings of Sugar Baby (Figs. 2B,4), although minor hypersensitive lesions were detected. Similar results were obtained with another banana-specific isolate, 927, of *C. musae* (data not shown).

Response of susceptible and resistant cucurbit cultivars after inoculation by both techniques. Disease responses of susceptible and resistant cultivars were examined by the continuous dip and standard leaf inoculation procedures. Cuttings of resistant cucurbit cultivars were resistant, and the susceptible cucurbit cultivars were susceptible after exposure to the standard leaf and continuous dip inoculation methods. Disease incidence with isolate L2.5 of *C. magna* in the partially resistant Jubilee cultivar of watermelon was 9.3% 10 days after the continuous dip inoculation of cuttings as compared to 17% 16 days after leaf inoculation (Fig. 4). Similarly, after leaf inoculations with isolate L2.5, the resistant Poinsett cultivar of cucumber showed no lesions on the cotyledons (Fig. 4), and all cuttings of this cultivar had rerooted 10 days after continuous dip inoculations and remained asymptomatic.

The susceptible Sugar Baby cultivar reacted similarly after leaf and continuous dip inoculations of cuttings with isolate L2.5; mortality was 92% and 100%, respectively. Disease progress was more rapid in the cutting method; 100% mortality was recorded 5–6 days after inoculation (Figs. 1,4). Seedlings of the susceptible cucumber cultivar Burpee Pickler showed lesions on all inoculated cotyledons (Fig. 4) as well as an 81.3% mortality in cuttings 10 days after continuous dip inoculations in a conidial suspension of isolate L2.5.

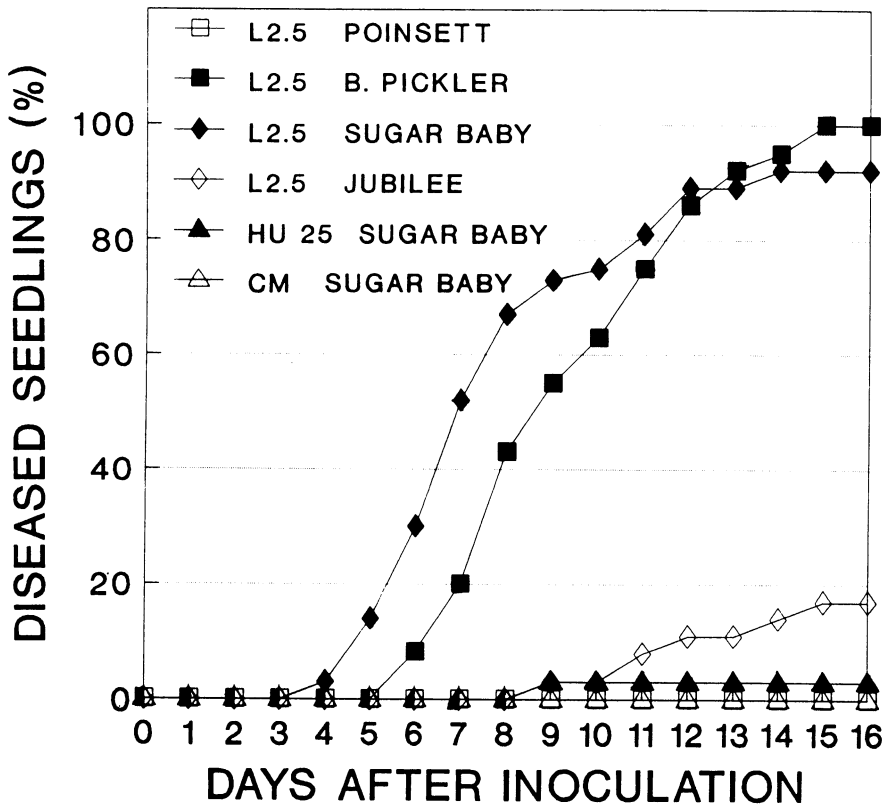

Fig. 4. Disease incidence in resistant and susceptible cultivars of cucumber and watermelon, leaf-inoculated ($2.5–3 \times 10^6$ conidia per milliliter) with isolates L2.5 and HU 25 (a nonpathogenic mutant of L2.5) of *Colletotrichum magna* and isolate 1116 of *C. musae* (CM). Sugar Baby and Burpee Pickler represent susceptible cultivars, whereas Jubilee and Poinsett represent resistant cultivars of watermelon and cucumber, respectively. After probit transformation of percentage of seedling mortality and log transformation of days after inoculation, the linear regression coefficients ($0.79 < R^2 < 0.89$) are significant ($P = 0.05$). Slope values for percentage of seedling mortality in the resistant cultivars are significantly different from those of the susceptible ones ($P = 0.05$). The slope value for percentage of seedling mortality, inoculated with the mutant HU 25 and the CM isolate, is significantly different from that of seedlings inoculated with the wild-type isolate L2.5. In cucumber cultivars, disease percentage was calculated according to the presence of lesions (1–2 cm in diameter) on cotyledons, whereas in watermelon cultivars disease percentage was calculated by seedling mortality.

DISCUSSION

The continuous dip method of whole plants and cuttings proved reliable as a bioassay for determining pathogenicity of *C. magna* isolates on watermelon and cucumber cultivars. The method was also reliable for determining susceptible and resistant cultivars of watermelon and cucumber as well as those of muskmelon, squash, zucchini, and pumpkin (data not shown). In all of these experiments, results from the continuous dip method corresponded to those of the standard leaf inoculation methods.

The continuous dip method may be used either on intact seedlings or cuttings. A very rapid disease response was observed in cuttings of susceptible cultivars; death occurred 48–72 hr after exposure. Wilting was observed in dip-inoculated seedlings before plant death, although this symptom was not observed in leaf inoculations and in naturally infected seedlings. The rate of disease progress was slower when whole plants were used, however, death occurred more rapidly than in plants inoculated by the standard leaf method (Figs. 3,4). The continuous dip method allows for discrimination of nonspecific host-pathogen interactions, whereby the specific banana pathogen, *C. musae*, was unable to cause disease in the susceptible Sugar Baby cultivar. Although minor hypersensitive lesions were observed on *C. musae*-inoculated cotyledons, leaf and dip inoculations did not cause death in either whole plants or cuttings. Furthermore, cuttings regenerated a new root system during exposure to conidial suspensions of *C. musae*. The regeneration of a new root system in cuttings of tolerant seedlings demonstrated their resistance to the pathogen and indicated a full recovery from the initial severe method of wounding.

Seedlings leaf-inoculated with the HU 25 mutant displayed a hypersensitive response, similar to those inoculated with *C. musae*. However, a low mortality rate was present in cuttings inoculated with the mutant. *C. musae*-inoculated cuttings remained healthy, suggesting that the genetic defect resulting in the nonpathogenic phenotype was different than the genetic basis of host-specific resistance.

The continuous dip method has enabled the isolation of nonpathogenic mutants after large-scale screening of more than 300 UV-irradiated survivors. The time required for this screening process, which involves two people, was approximately 6 hr. The proposed method requires considerably less space because of the use of scintillation vials as containers rather than plants in pots. The rapid screening of potential mutants could not have been performed with such ease with the standard leaf inoculation methods. Results were also routinely consistent with the continuous dip method, whereas inconsistent and irreproducible results were sometimes observed when spores were

injected into the host via the stem (data not shown). The continuous dip method is, therefore, well-suited for rapid screening of large numbers of potential mutants.

Preliminary studies have shown that this alternative inoculation method may be well-suited for screening resistant cultivars to soilborne pathogens, primarily the wilt-causing fungi, *Fusarium* and *Verticillium* spp., and damping-off diseases caused by *Pythium* and *Rhizoctonia* spp. Cross-protection of cucumber plants against anthracnose has been previously shown by similar exposure of cuttings to conidial suspensions of *F. oxysporum* f. sp. *cucumerinum* (5). The standard inoculation methods for screening resistance to *Fusarium* (7) have similar constraints as those described for the standard leaf inoculation methods of *Colletotrichum*. Therefore, adapting this rapid screening technique to encompass screening procedures especially for soilborne pathogens and, in particular, wilt-causing fungi would be beneficial.

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