A Rapid Inoculation Technique for Assessing Pathogenicity of *Fusarium oxysporum* f. sp. *niveum* and *F. o. melonis* on Cucurbits

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ABSTRACT

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A continuous-dip inoculation technique for rapid assessment of pathogenicity of *Fusarium oxysporum* f. sp. *niveum* and *F. o. melonis* was developed. The method, adapted from a similar procedure for determining pathogenicity of *Colletotrichum magna* (causal agent of anthracnose of cucurbits), involves constant exposure of seedlings and cuttings (seedlings with root systems excised) of watermelon and muskmelon to conidial suspensions contained in small scintillation vials. Disease development in intact seedlings corresponded well to disease responses observed with the standard root-dip inoculation/pot assay. The continuous-dip inoculation technique resulted in rapid disease development, with 50% of watermelon cuttings dying after 4–6 days of exposure to *F. o. niveum*. A mortality of 30% also was observed in watermelon cuttings exposed to conidia of *F. o. melonis*, as opposed to only a 0–2.5% mortality in seedlings with intact roots. Disease response was similar with muskmelon seedlings and cuttings continuously dip-inoculated with *F. o. melonis* isolates. However, no disease symptoms were observed in muskmelon seedlings or cuttings inoculated with *F. o. niveum*. Four nonpathogenic isolates of *F. oxysporum* did not cause disease symptoms in either watermelon or muskmelon cuttings and seedlings when assayed by this technique. The proposed method enables a rapid screening of pathogenicity and requires less time, labor, and greenhouse space than the standard root-dip inoculation/pot assay. The reliability of the continuous-dip inoculation technique is limited, however, to exposure of intact seedlings at a concentration of 1×10^6 conidia per milliliter; the method is not accurate at this range for excised seedlings.

The fungal genus *Fusarium* predominantly contains soilborne pathogens that collectively cause disease on a number of significant agricultural crops (2,5). In cucurbits, typical wilt symptoms resulting in mortality are manifested in young and mature plants and may be especially severe when infection occurs at the seedling stage (10). Therefore, there is a need to develop and screen for wilt-resistant plant cultivars or genotypes (3,10). Most pathogenic isolates of *F. oxysporum* Schlechtend.:Fr. have a remarkably high degree of host specificity. For example, *F. o. melonis* W.C. Snyder & H.N. Hans. is a specific pathogen of muskmelon (*Cucumis melo* L.) and is unable to cause disease in watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), whereas *F. o. niveum* (E.F. Sm.) W.C. Snyder & H.N. Hans. is specific to watermelon and does not cause disease in muskmelon (2,12). The standard inoculation method for determining pathogenicity usually involves transferring 7- to 10-day-old seedlings from a germination medium, washing their roots, dipping the roots in a conidial suspension, and transplanting the seedlings into a noninfested soil (2,6,10,12). However, this procedure is cumbersome and time-consuming and may require considerable greenhouse and/or growth chamber space if a large amount of germ plasm is tested.

A rapid and reliable technique was recently developed for screening for pathogenicity of *Colletotrichum magna* S.F. Jenkins & Winstead, a fungal pathogen causing anthracnose disease in cucurbits (7). This method involves continuously exposing intact seedlings and cuttings (plants with excised root systems) to fungal conidia contained in small glass scintillation vials. Disease development with this technique was consistent and correspended well with results obtained with the standard leaf inoculation procedures (4).

The objective of this study was to adapt the continuous-dip inoculation bioassay to determining pathogenicity of *F. o. niveum* and *F. o. melonis*, causal agents of Fusarium wilt of watermelon and muskmelon, respectively. The reliability, rate of disease development, and labor and greenhouse requirements of the dip-inoculation procedure were compared with those of the standard seedling root-dip inoculation/pot assay. The utility of this method for large-scale screening for nonpathogenic mutants is also discussed.

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MATERIALS AND METHODS

Fungal isolates. Pathogenic isolates of *F. o. niveum* (FON ATCC = ATCC 18467 race 1 and FON OK-270 race 2) and nonpathogenic *F. oxysporum* isolates (TX-RS = TX RS-IRGC [86] and TX-CG-1) were obtained from R. D. Martyn (Texas A&M University). Pathogenic isolates of *F. o. melonis* (FOMPZ = PZ, vcg 0131, race 2, and FOM660 = 660A, vcg 0134, race 1) and nonpathogenic *F. oxysporum* isolates (PB602 and MW1-1S) were provided by T. R. Gordon (University of California at Berkeley).

Inocula production. Conidial suspensions of each *Fusarium* isolate were spread on a medium containing yeast extract (5 g/L), peptone (5 g/L), glucose (20 g/L), and Difco agar (12 g/L) in petri plates and incubated for 5 days at 23 C under cool-light fluorescent illumination. After 5 days, the plates were rinsed with sterile water and filtered through eight layers of surgical gauze. The conidial suspensions then were washed with sterile distilled water, centrifuged at 5,000 *g* for 7 min at 25 C, and adjusted with sterile distilled water amended with 0.05% agar to a concentration of 1×10^6 conidia per milliliter. The ratio of microconidia to macroconidia in *F. o. niveum* isolates FON ATCC and FON OK-270 were 1:4 and 1:1, respectively, and the ratio in *F. o. melonis* isolates FOM660 and FOMPZ was 4:1.

Plants. A Fusarium-wilt susceptible cultivar (Sugar Baby) and a partially resistant cultivar (Crimson Sweet) of watermelon and a Fusarium-wilt susceptible cultivar (Hales Best Jumbo) of muskmelon were donated by Harris Moran Seed Co. (Davis, CA).

Continuous-dip inoculation of intact seedlings and cuttings. The procedure used in this study was similar to that previously described (7). Watermelon and muskmelon seedlings were germinated in a vermiculite rooting medium. After 5–6 days, seedlings were removed and the roots were washed under running water. Intact seedlings or cuttings (seedlings that had the root system excised at the crown with a razor blade) were exposed continuously in 18 ml of conidial suspensions of the *Fusarium* isolates (1×10^6 conidia per milliliter) in standard scintillation vials (20-ml volume), 10 plants per vial. Vials were replenished with sterile distilled water amended with 0.05% agar every 5 days. The plants and

cuttings were maintained in a controlled environment growth chamber at 24 C, 95% RH, and a 12-hr diurnal light cycle. Artificial light intensity (980 $\mu E \cdot m^{-2} \cdot s^{-1}$) originated from fluorescent and incandescent lamps. Disease symptoms in cuttings and seedlings, typified by cotyledon wilting and yellowing accompanied by lesions along stems, were recorded daily. Disease incidence was expressed as the percentage of seedling mortality compared with that of uninoculated controls. Each experiment consisted of four replicates of 10 seedlings per treatment arranged in a completely random design.

Seedling inoculation in pots by the standard root-dip method. The standard method used was essentially as previously described (2,6,10,12). Watermelon and muskmelon seedlings were germinated in vermiculite under the same conditions described for the continuous-dip inoculation technique. After 5–6 days, seedlings were removed and roots were washed with running water, then dipped in conidial suspensions (1×10^6 conidia per milliliter) of the respective *Fusarium* isolates for 30 sec. Seedlings were transplanted into peat pots ($10 \times 10 \times 15$ cm tall) in a steam-pasteurized U.C.-4 potting mix (1) composed of 0.382 m^3 of plaster sand, 0.382 m^3 of bark humus, 1,134.0 g of $CaCO_2$, 3,401.9 g of dolomite, 1,134.0 g of treble superphosphate, 56.7 g of KNO_3, 56.7 g of KCl, 62 g of $FeSO_4$, 22.6 g of $ZnSO_4$, 43 g of $CuSO_4$, 17 g of $MnSO_4$, and 34 g of $MgSO_4$. Disease incidence was observed after 6–7 days and expressed as the percentage of seedling mortality compared with that of uninoculated controls. Experiments consisted of four replicates with 10 seedlings per treatment arranged in a completely random design.

Statistical analyses. All experiments 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 transformation of seedling mortality and log transformation of days after inoculation at a significance level of $P = 0.05$, as previously demonstrated (6,7). These types of transformation systems were chosen because they were appropriate for conversion of rates of disease incidence over time to values best suited for linear regression analyses. Values for 50% mortality were estimated from regression equations and compared by Duncan's multiple range test ($P = 0.05$).

RESULTS

Disease incidence in watermelon cultivars after continuous-dip inoculation. A rapid disease response of 4 and 6 days was observed (50% mortality) in cuttings of Sugar Baby and Crimson Sweet, respectively, after continuous exposure to conidia of FON ATCC (Figs. 1 and 2). The time required to reach 50% mortality in excised plants and whole plants of cv. Sugar Baby inoculated by the continuous-dip treatments, 4.26 and 5.04 days, respectively, was significantly different from that with the root-dip/pot inoculation method (Fig. 1A). Values for identical treatments of Crimson Sweet seedlings were 5.87, 9.1, and 11.4 days for excised plants, whole plants, and root-dip/pot plants, which differed significantly from each other (Fig. 1B). Similar results were observed with another *F. o. niveum* isolate, FON OK-270 (Fig. 2). Disease progress in seedlings of both cultivars inoculated by the standard assay in pots was significantly slower than that of continuous-dip inoculated intact seedlings (Fig. 1). Mortality of approximately 30% was detected in cuttings of both Sugar Baby and Crimson Sweet dip-inoculated with the muskmelon-specific pathogen, isolate FOM660 of *F. o. melonis* (Fig. 1). In addition, lesions also developed on stems of most surviving cuttings; however, these plants subsequently recovered and continued to develop healthy rooting systems. No mortality occurred in intact seedlings of Crimson Sweet dip-inoculated with FOM660 (Fig. 1B), whereas a 2.5% mortality was observed in seedlings of Sugar Baby (Fig. 1A). Occasional lesions were observed on stems of intact seedlings similar to those on cuttings. Two nonpathogenic *F. oxysporum* isolates (TX-RS and TX-CG-1) did not cause any disease symptoms in cuttings or whole plants of either cultivar that were dip-inoculated (Fig. 2). Resistant cuttings rerooted in conidial suspensions after 7–10 days, demonstrating full recovery from wounding. No mortality

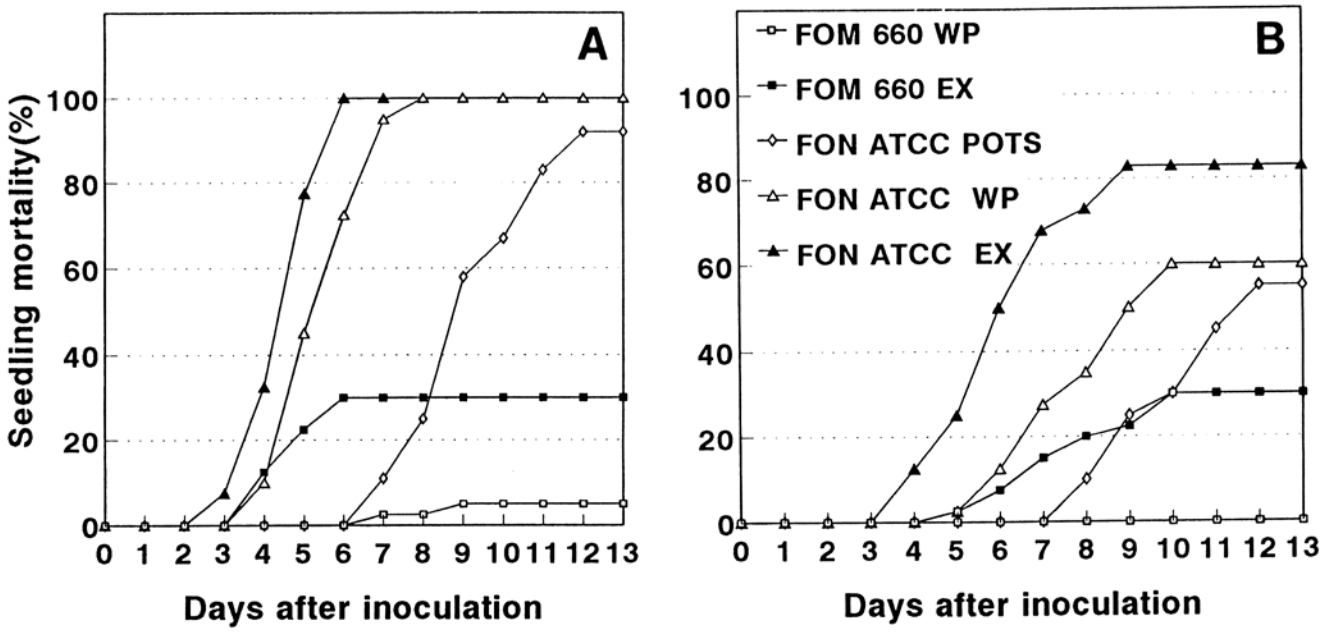

Fig. 1. Seedling mortality in watermelon cultivars of **(A)** Sugar Baby and **(B)** Crimson Sweet inoculated with isolate FON ATCC of *Fusarium oxysporum* f. sp. *niveum* and isolate FOM660 of *F. o. melonis*. Whole plants (WP) and cuttings with excised root systems (EX) were immersed continuously in conidial supensions (1×10^6 conidia per milliliter). Whole plants were also assayed by the standard root-dip procedure in pots (POTS) by immersing roots for 30 sec in conidial suspensions (1×10^6 conidia per milliliter) and then transplanting them into noninfested potting mixture. Probit transformation of seedling mortality and log transformation of days after inoculation indicated that the coefficients of determination associated with linear regression ($0.71 < R^2 < 0.91$) were significant ($P = 0.05$). The slope values of seedlings and cuttings inoculated with FOM660 in both Sugar Baby and Crimson Sweet were significantly different from all treatments inoculated with FON ATCC ($P = 0.05$). The time required to reach 50% seedling mortality was significantly different between POTS treatments and WP and EX treatments for both cultivars ($P = 0.05$).

was observed in Sugar Baby and Crimson Sweet seedlings root-dip-inoculated in pots with FOMPZ and FOM660 of *F. o. melonis* and the nonpathogenic isolates TX-RS and TX-CG-1 (*data not shown*).

Disease incidence in muskmelon after continuous-dip inoculation. Results were similar for cuttings and seedlings of cv. Hales Best Jumbo dip-inoculated with the muskmelon-specific isolates FOMPZ and FOM660 of *F. o. melonis*. Mortality occurred sooner in cuttings exposed to both FOMPZ and FOM660 than with the standard inoculation assay in pots (Fig. 3). The time required to reach 50% mortality in muskmelon inoculated with isolate FOMPZ in excised plants, whole plants, and root-dip plants in pots was 6.1, 6.85, and 9.45 days, respectively (Fig. 3A). Values for 50% mortality for the identical treatments inoculated with isolate FOM660 were 7.74, 8.89, and 10.12 days for excised plants, whole plants, and root-dip/pot plants, respectively (Fig. 3B). The disease progress in cuttings and whole plants was similar for isolate FOMPZ; however, a delay in progress of disease in intact plants was observed for isolate FOM660 (Fig. 3). Seedlings and cuttings that were continuously dip-inoculated and root-dip-inoculated and potted with the watermelon-specific isolate FON ATCC and two nonpathogenic isolates (*F. oxysporum* isolates PB602 and MW1-1S) of melon did not show any disease symptoms (*data not shown*). Cuttings of Hales Best Jumbo that were continuously dip-inoculated rerooted in these conidial suspensions, similar to the watermelon plants, indicating recovery from the severe wounding.

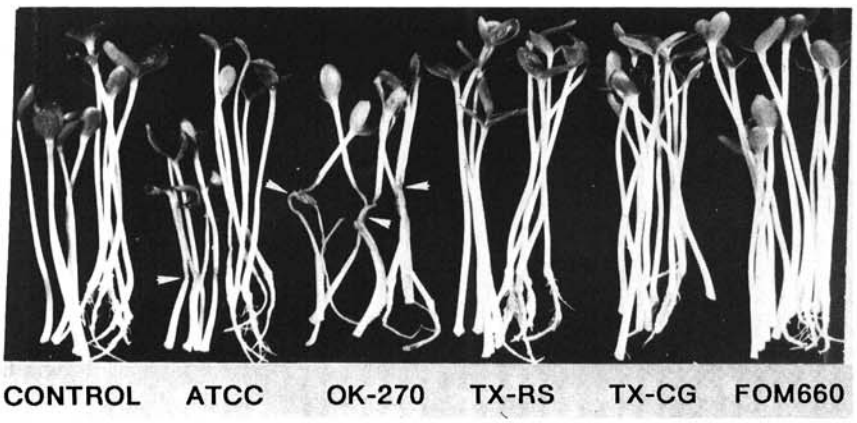

Fig. 2. Disease incidence in whole seedlings and cuttings (root system excised) of watermelon cultivar Sugar Baby after 4 days of continuous-dip inoculation. Seedlings and cuttings were immersed in conidial suspensions (1×10^6 conidia per milliliter) of isolates FON ATCC and FON OK-270 of *Fusarium oxysporum* f. sp. *niveum*, isolate FOM660 of *F. o. melonis*, nonpathogenic isolates TX-RS and TX-CG of *F. oxysporum* and uninoculated controls. Arrows indicate lesion development and seedling collapse.

DISCUSSION

The continuous-dip inoculation technique was reliable and consistent for pathogenicity determination of various *Fusarium* isolates in whole-plant assays of watermelon and muskmelon, and disease development corresponded well with that of the standard pot-inoculation procedure for whole plants. However, excised seedlings of watermelon reacted unfavorably to isolates of *F. o. melonis* during continuous-dip inoculation. Therefore, this technique should be limited to whole plants for an accurate assessment of pathogenicity at the tested concentration of 1×10^6 conidia per milliliter. No disease symptoms were observed in either cuttings or intact plants of both watermelon and muskmelon continuously exposed to four nonpathogenic isolates of *F. oxysporum*.

In watermelon cultivars, isolate FON ATCC caused significantly faster disease responses in both cuttings and seedlings with the continuous-dip inoculation method than with the standard pot bioassay. Although the continuous-dip method may be used on intact seedlings and cuttings, 30% mortality did occur in watermelon cuttings of both Sugar

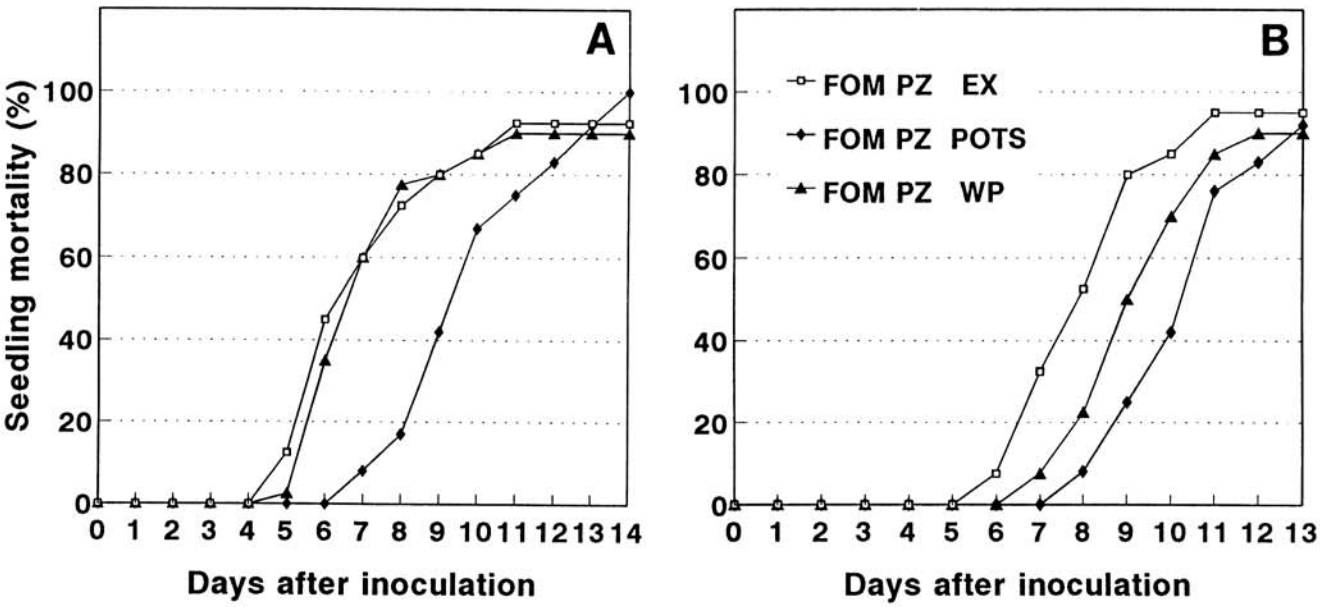

Fig. 3. Seedling mortality of muskmelon cultivar Hales Best Jumbo inoculated with isolates **(A)** FOMPZ and **(B)** FOM660 of *Fusarium oxysporum* f. sp. *melonis*. Whole plants (WP) and cuttings with excised root systems (EX) were immersed continuously in conidial suspensions (1×10^6 conidia per milliliter). Whole plants were assessed by the standard root-dip procedure in pots (POTS) by dip-inoculating roots for 30 sec in conidial suspensions (1×10^6 conidia per milliliter) and then transplanting them into noninfested potting mixture. Probit transformation of seedling mortality and log transformation of days after inoculation indicated that the coefficients of determination associated with linear regression ($0.74 < R < 0.95$) were significant ($P = 0.05$). Slope values of the different treatments were not significantly different ($P = 0.05$). The time required to reach 50% seedling mortality was significantly different between POTS treatments and WP and EX treatments for both isolates ($P = 0.05$).

Baby and Crimson Sweet cultivars inoculated with the muskmelon-specific pathogen, isolate FOM660 (Fig. 1). It is possible that removal of the rooting system in cuttings may have predisposed these plants to the muskmelon pathogen. In addition, the effect of conidial concentration and type of inocula, i.e., ratio of microconidia to macroconidia, may also be a considerable factor in inducing the disease response in watermelon. It should be noted that the initial concentration of inoculum (1×10^6 conidia per milliliter) may have changed over time during the continuous-dip procedure, and with lower inoculum concentrations the proposed method may be suitable for assessing pathogenicity on excised seedlings. Watermelon plants with an intact root system showed a very low disease incidence (2.5 and 0% in Sugar Baby and Crimson Sweet cultivars, respectively) when exposed to isolate FOM660 of *F. o. melonis*. We have previously shown that the continuous-dip method allowed for discrimination of a nonspecific host-pathogen interaction whereby *C. musae* (Berk. & M.A. Curtis) Arx, specific to banana, was unable to cause disease in the anthracnose-susceptible cultivar Sugar Baby (7).

The response of the susceptible muskmelon cultivar Hales Best Jumbo was similar to that of the watermelon cultivars, i.e., disease response was more rapid in continuously dip-inoculated plants than in those assayed with the standard method. In the case of Hales Best Jumbo, no detrimental effects were observed when intact seedlings or cuttings were dip-inoculated with the watermelon-specific FON ATCC.

The continuous-dip inoculation method requires considerably less greenhouse or growth chamber space because small scintillation vials are used instead of pots. Furthermore, less time and labor are required to set up the dip-inoculation experiments because 10 plants can be inserted directly into a single vial, whereas seedlings must be transplanted individually into pots for the standard assay.

Initially, the continuous-dip inoculation bioassay was devised for large-scale screening and isolation of nonpathogenic mutants of *C. magna* (7). We recently isolated a nonpathogenic mutant HU 25 (designated later as path-1), which has been characterized (8). This organism has been genetically converted from its wild-type pathogenic form to an endophytic mutualist that is capable of protecting cucurbit seedlings against wild-type *C. magna* and isolate FON OK-270 of *F. o. niveum* (8). Therefore, the potential exists to utilize the continuous-dip method for screening and identifying nonpathogenic mutants in broad host range *Fusarium* species that may be useful as biocontrol agents, as indicated with *C. magna* (8). These mutants may be used to improve biocontrol strategies that have previously utilized hypovirulent strains or nonpathogenic saprophytes by competing more efficiently for infection sites and nutrient sources (9,11,13,14).

Breeding programs could also possibly benefit by utilizing the continuous-dip method as a rapid screening process for resistant and susceptible genotypes and for determining causal agents of disease. Recently, we have successfully adapted this method (7) for rapid screening of tomato seedlings to the pathogens *F. o. lycopersici* (Sacc.) W.C. Snyder & H.N. Hans. and *F. o. radicis-lycopersici* W.R. Jarvis & Shoemaker, the respective causal agents of wilting and crown rot, and the potential exists for further application of the procedure to other wilt pathogens.

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LITERATURE CITED

1. Baker, K. F., ed. 1957. The U.C. system for producing healthy container grown plants. Univ. Calif. Div. Agric. Sci. Agric. Exp. Stn.
2. Beckman, C. H. 1987. The Nature of Wilt Diseases of Plants. American Phytopathological Society, St. Paul, MN.
3. Biles, C. L., and Martyn, R. D. 1989. Local and systemic resistance induced in watermelons by formae speciales of *Fusarium oxysporum*. Phytopathology 79:856-860.
4. Dean, R. A., and Kuć, J. 1986. Induced systemic protection in cucumber: Effects of inoculum density on symptom development caused by *Colletotrichum lagenarium* in previously infected and uninfected plants. Phytopathology 76:186-189.
5. Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. Fungi on Plants and Plant Products in the United States. American Phytopathological Society, St. Paul, MN.
6. Freeman, S., and Katan, J. 1988. Weakening effect on propagules of *Fusarium* by sublethal heating. Phytopathology 78:1656-1661.
7. Freeman, S., and Rodriguez, R. J. 1992. A rapid, reliable bioassay for pathogenicity of *Colletotrichum magna* on cucurbits and its use in screening for nonpathogenic mutants. Plant Dis. 76:901-905.
8. Freeman, S., and Rodriguez, R. J. 1993. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260:75-78.
9. Ishiba, C., Tani, T., and Murata, M. 1981. Protection of cucumber against anthracnose by a hypovirulent strain of *Fusarium oxysporum* f. sp. *cucumerinum*. Ann. Phytopathol. Soc. Jpn. 47:352-359.
10. Latin, R. X., and Snell, S. J. 1986. Comparison of methods for inoculation of muskmelon with *Fusarium oxysporum* f. sp. *melonis*. Plant Dis. 70:297-300.
11. Mandeel, Q., and Baker, R. 1991. Mechanisms involved in biological control of Fusarium wilt of cucumber with strains of nonpathogenic *Fusarium oxysporum*. Phytopathology 81:462-469.
12. Martyn, R. D., and McLaughlin, R. J. 1983. Effects of inoculation concentration on the apparent resistance of watermelons to *Fusarium oxysporum* f. sp. *niveum*. Plant Dis. 67:493-495.
13. Tjamos, E. C., Papavisas, G. C., and Cook, R. J. 1992. Biological Control of Plant Diseases: Progress and Challenges for the Future. Plenum Press, New York.
14. Yamaguchi, K., Sano, T., Arita, M., and Takahashi, M. 1992. Biocontrol of Fusarium wilt of tomato by non-pathogenic *Fusarium oxysporum* MT0062. Ann. Phytopathol. Soc. Jpn. 58:188-194.