

Evaluation of *Trichoderma* Spp. for Biological Control of Phytophthora Crown and Root Rot of Apple Seedlings

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ABSTRACT

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In a greenhouse bioassay, 223 isolates of five species of *Trichoderma* were evaluated as biological control agents of Phytophthora crown and root rot of apple seedlings. Effects of inoculum rate and ambient temperature on seedling mortality caused by *Phytophthora cactorum* were determined. Isolates of *Trichoderma* spp. were evaluated with two delivery methods. A viscous suspension of conidia in an aqueous gel was applied to seedling roots, or a colonized mixture of peat and wheat bran (peat-bran) was added to soil. After all isolates were evaluated twice with both delivery methods, six isolates delivered in peat-bran and five isolates delivered as conidium suspensions had increased survival time of seedlings compared with treatments without *Trichoderma* spp. These 11 treatments then were compared in a separate experiment. Isolate TW.055 of *T. virens* (= *Gliocladium virens*) in peat-bran consistently was superior to all other

treatments; seedlings survived an average of 30 (out of 44) days whereas those in the control survived only 19 days. Isolates TW.105 and TW.189 of *T. koningii* in peat-bran (both with an average seedling survival time of 24 days) and a conidium suspension of isolate TW.138 of *T. harzianum* (with an average seedling survival time of 22 days) were effective in most trials. *Trichoderma* spp. were promising as biological control agents of Phytophthora crown and root rot of apple seedlings under experimental conditions conducive to disease development and, therefore, should be pursued as a potential means of biological control of the disease on apple trees in the orchard. The bioassay developed to evaluate isolates of *Trichoderma* spp. was effective and probably could be adapted to evaluate other microorganisms for potential to control Phytophthora crown and root rot of apple trees.

Additional keywords: biocontrol, collar rot, *Malus × domestica*, *Trichoderma hamatum*, *Trichoderma viride*.

Phytophthora crown and root rot has been reported from virtually all apple (*Malus × domestica* Burkh.) producing regions of the world and is considered to be the most serious disease affecting apple rootstocks (13,15). Although the disease is caused by various species of *Phytophthora*, *P. cactorum* (Lebert & Cohn) J. Schröt. most frequently is associated with the disease worldwide. An integrated approach to manage Phytophthora crown and root rot in the orchard is recommended, and biological control is a management option that may offer potential (15).

Interest in biological control of *Phytophthora* spp. that attack apple trees has developed only recently (10,25,26), and the potential of this method of disease control has not been fully explored. *Trichoderma* spp. are well documented as effective biological control agents of plant diseases caused by soilborne fungi (reviewed in 4,19,28), including some pythiaceae fungi (7,8,23). However, research on *Trichoderma* spp. as biological control agents of root diseases of woody plants caused by *Phytophthora* spp. is very limited (16,17). When this research was initiated in 1986, *Trichoderma* spp. had not been evaluated as potential biological control agents of Phytophthora crown and root rot of apple trees.

In preliminary experiments, two isolates of *Trichoderma* spp. effective against *Pythium* spp. that attack seeds (7)—isolate T8m (ATCC 20736) of *T. koningii* Oudem. and isolate T12m (ATCC 20737) of *T. harzianum* Rifai—did not control Phytophthora crown and root rot of apple rootstocks (21). Consequently, isolates of *Trichoderma* spp. native to Wisconsin soils and adapted to persist in an apple root-crown environment were collected (22). Our goal in this research was to determine whether isolates of *Trichoderma* spp. could control Phytophthora crown and root rot of apple seedlings caused by *P. cactorum* under controlled

environmental conditions, which would indicate the potential of these fungi to manage the disease in the orchard.

MATERIALS AND METHODS

Sources of isolates of *Trichoderma* spp. Isolates of *Trichoderma* spp. (223 total) originally were recovered from soil (180 isolates) (22) or from underground runners of cranberry (*Vaccinium macrocarpon* Aiton) presumably killed by *Phytophthora* spp. (11) (24 isolates) or were obtained from the American Type Culture Collection (ATCC) (19 isolates). The 204 isolates of *Trichoderma* spp. from soil and cranberry runners were: *T. hamatum* (Bonord.) Bainier (65 isolates), *T. harzianum* (52 isolates), *T. koningii* (58 isolates), *T. virens* (Miller et al) Arx (= *Gliocladium virens* Miller et al) (27) (19 isolates), and *T. viride* Pers.:Fr. (10 isolates). The 19 isolates of *Trichoderma* spp. from ATCC were: *T. hamatum*, 26798, 28042, and 52198; *T. harzianum*, 24274, 42834, 48134, 52424, 52443, and 60850; *T. koningii*, 58953; *T. polysporum* (Link ex Pers.) Rifai, 34197; *T. pseudokoningii* Rifai, 28025, 58226, and 60641; *T. reesei* E. Simmons, 56767; and *T. viride*, 20476, 32098, 52438, and 52440. Several of these isolates have been effective biological control agents of other plant diseases.

Single-spore cultures were produced for each isolate, and all isolates were stored on both silica gel and potato-dextrose agar (PDA; Difco Laboratories, Detroit) in glass vials at 3 C. Isolates always were recovered from silica gel for inoculum production to promote uniformity over time among inoculum preparations for a given isolate.

Initial evaluation of isolates of *Trichoderma* spp. Isolates of *Trichoderma* spp. were evaluated in a greenhouse experiment for control of crown and root rot of apple seedlings caused by *P. cactorum*. Each isolate was evaluated with two delivery methods; an isolate of *Trichoderma* spp. and the delivery method were considered to be a treatment (1). In the first delivery method,

conidia from an isolate were suspended in an aqueous gel preparation of Viterra Agri-gel (Nepera, Inc., Harriman, NY), and roots were coated with the suspension. Viterra is a dry, synthetic organic polymer that becomes viscous when mixed with water and tends to be recalcitrant in soil. Isolates of *Trichoderma* spp. were grown on PDA in petri dishes for 13 days near a north-facing window at room temperature (20–25 C) to produce conidia. Conidia were collected by rinsing colonies with 15–25 ml of distilled water and pouring resultant suspensions through two layers of cheesecloth to remove fragments of hyphae. Conidia in suspension were quantified with the aid of a hemacytometer, and then an appropriate volume of suspension was added to 100 ml of aqueous Viterra (dry Viterra at 6.0 g/L of distilled water) so that a final concentration of 10^7 conidia per milliliter was achieved. The pH of three randomly selected Viterra treatments with *Trichoderma* spp. and Viterra without *Trichoderma* spp. were measured for each trial; pH ranged from 7.3 to 6.3 for all trials but always varied less than 0.5 pH units within a trial. Roots of apple seedlings were dipped into the conidium suspension just before transplanting into infested soil (discussed subsequently).

In the second delivery method, a 1:1 (v/v) mixture of peat and wheat bran (peat-bran) (23) colonized by an isolate of *Trichoderma* spp. was added to soil infested with *P. cactorum*. Isolates were grown individually in 250-ml Erlenmeyer flasks containing 50 ml of peat, 50 ml of wheat bran, and 40 ml of distilled water; flasks were autoclaved twice, once daily for 2 consecutive days. Three agar blocks with hyphae were cut from the edge of an actively growing culture on PDA and were added to the peat-bran in each flask. Flasks were placed at 24 C with a 12-h photoperiod for 6 days and were shaken daily to promote thorough colonization and sporulation. Just before transplanting seedlings, 6-day-old peat-bran inoculum was added at a rate of 5% (v/v) to soil infested with *P. cactorum*.

A soil sample was saved from each peat-bran treatment to determine the initial inoculum density of *Trichoderma* spp. Three 1- to 3-g subsamples of each soil were weighed, oven-dried, and reweighed to determine percent moisture so that inoculum densities could be expressed on a dry weight basis. In addition, approximately 1 g of each soil was weighed and added to 9 ml of sterile distilled water, and soil dilutions of 10^{-1} to 10^{-6} were prepared. From each dilution, 0.1 ml was spread onto each of three dishes of a medium selective for *Trichoderma* spp. (22), which was similar to a modification (24) of the original formulation (5). Dishes were placed at 24 C with a 12-h photoperiod, and cfu were counted 5 and 7 days later. The lower limit of detection was 100 cfu/g of dry soil.

The number of isolates of *Trichoderma* spp. that were tested in any one trial varied from 4 to 16. In all, 35 trials were conducted to evaluate each isolate twice; in 26 of 35 trials, 12 or more isolates were evaluated. Isolates were selected randomly for evaluation in a trial, and each isolate was evaluated once before any isolate was evaluated again. A few isolates were not evaluated with the Viterra delivery method, because they did not produce conidia under the incubation conditions used. In general, all trials were conducted similarly and always in the same greenhouse.

In each trial there were four additional treatments without *Trichoderma* spp. Apple seedlings were transplanted into uninfested soil or soil infested with only *P. cactorum* for two of the treatments. These two controls were not included in statistical analyses but were included in the experiment to demonstrate that apple seedlings would remain healthy in uninfested soil or die in infested soil under the experimental conditions used. In another treatment, Viterra without *Trichoderma* spp., roots of apple seedlings were dipped into unamended aqueous Viterra before transplanting into soil infested with *P. cactorum*; this was the treatment to which Viterra treatments with *Trichoderma* spp. would be compared. Likewise, a peat-bran treatment without *Trichoderma* spp. consisted of apple seedlings transplanted into soil infested with *P. cactorum* and amended with 5% (v/v) sterile peat-bran; this was the treatment to which peat-bran treatments with *Trichoderma* spp. would be compared.

Soil used throughout this study was a silt loam with the fol-

lowing physical characteristics: pH 6.2, 78 t/ha organic matter, 0.17% N, and 83 and 476 kg/ha of available P and K, respectively. Soil was pasteurized at 65–70 C for 30 min and mixed 2:1 (v/v) with medium-textured vermiculite before use.

To obtain apple seedlings, after-ripened apple seeds (open-pollinated cv. McIntosh) were planted into coarse-textured vermiculite and placed in a greenhouse for 10–14 days. Each seedling then was transplanted into a 115-ml planting cone (3.8 × 14.0 cm) (Ray Leach "Cone-tainer" Nursery, Canby, OR) containing approximately 100 ml of the appropriate soil. Transplanted seedlings were arranged in a randomized complete block design with 10 replicates per treatment. Cones were placed in alternate holes in planting-cone racks, and each rack served as a block. Seedlings were grown in a greenhouse for 44 days; average daily temperatures usually ranged from 22 to 25 C. Within a 24-h period, however, temperatures occasionally reached as low as 18 C or as high as 28 C, depending on the time of year. Plants were watered individually as needed; care was taken to prevent splashing among plants. Fourteen and 28 days after transplanting, seedlings were flooded for a period of 48 h to enhance disease development (12,13). Each plant was flooded individually by applying a latex rubber finger cot (VWR Scientific, Chicago) around the base of a cone and then adding tap water until approximately 1 cm of water remained on the soil surface.

Treatments in each trial were assessed by two criteria. Mortality of individual seedlings was recorded at 2- to 3-day intervals, beginning 4–7 days after transplanting, and is reported as survival time, i.e., the mean number of days after transplanting that seedlings in a treatment survived. In addition, shoots were collected at the end of each trial, and oven-dried weights were measured. In the initial test of each isolate of *Trichoderma* spp., roots of dead plants were placed on PAR medium, selective for *Phytophthora* spp. (14), to isolate *P. cactorum*. The frequency of pathogen recovery was 99.4% (3,264 successes out of 3,285 attempted isolations). Therefore, in the second evaluation of each isolate, isolations were attempted from roots of only five arbitrarily selected plants per trial; the frequency of recovery was 100% (75/75).

Inoculum of *P. cactorum*. Five isolates of *P. cactorum* were pooled to produce inoculum for each trial so isolates of *Trichoderma* spp. that were effective biological control agents would not be selected for specificity to only one isolate of the pathogen. Isolates, originally recovered from diseased apple trees, were NY.097, NY.181, and NY.188 from New York; NY.195 from Quebec, Canada; and W.024 from Wisconsin. Each isolate of *P. cactorum* was grown in three 250-ml Erlenmeyer flasks, each containing 100 ml of medium-textured vermiculite and 50 ml of V8 juice broth (800 ml of distilled water, 200 ml of V8 juice, and 2 g of CaCO₃) that previously had been autoclaved on each of 2 consecutive days. All flasks were placed on a laboratory bench (20–25 C) for 20 days and were shaken periodically to promote thorough colonization. Before use, particles from each flask were assayed on cornmeal agar (CMA; Difco Laboratories) to ensure that vermiculite was thoroughly colonized and not contaminated. Equal volumes of each isolate then were combined and mixed thoroughly before adding to pasteurized soil.

To determine an appropriate amount of inoculum of *P. cactorum* for maximum mortality in the absence of biological control treatments, the relationship between inoculum rate and seedling mortality was investigated. Fourteen seedlings were transplanted into infested soil in planting cones for each of six inoculum rates: 0, 1, 2, 4, 6, or 8% (v/v). Seedlings were arranged in a randomized complete block design with seven replicates per treatment in each of two blocks. Seedlings were grown as described above for 42 days, and mortality was recorded every 2 days. The experiment was conducted twice.

During the course of this research, a finer-textured vermiculite replaced that originally used to prepare inoculum of *P. cactorum* because of commercial availability. For the same volume of inoculum, more "propagules" of the pathogen (i.e., colonized vermiculite particles) would be available with the finer vermiculite. Therefore, the inoculum rate-disease incidence experiment was repeated

with inoculum prepared from this finer-textured vermiculite. The experiment was identical to that described above, except that the 8% inoculum rate was replaced with 1.36% (i.e., 15 ml of inoculum added to 1,085 ml of soil). The experiment was conducted twice.

Effects of temperature on seedling mortality. Mortality of apple seedlings in the greenhouse varied among trials and appeared to be related to ambient temperature in the greenhouse, which was thermostatically controlled but still varied with the season. Therefore, an experiment was conducted in growth chambers to determine the effect of temperature on mortality of apple seedlings transplanted into soil infested with *P. cactorum*. Inoculum of *P. cactorum* was prepared as described above. Two trials were conducted. In one trial, inoculum of *P. cactorum* was prepared with the original vermiculite and added to soil at 2% (v/v); in the other trial, inoculum was prepared with the finer-textured vermiculite and added to soil at 1.36% (v/v). For each trial, 20 seedlings in infested soil were placed at each of four temperatures: 16, 20, 24, and 28 C. Seedlings transplanted into uninfested soil also were placed at each temperature—two to four per temperature in the first trial and 20 per temperature in the other trial. Seedlings were grown for 44 days as described above, and mortality was recorded every 2 days beginning 4 days after transplanting.

Evaluation of selected treatments. Six peat-bran and five Viterra treatments with *Trichoderma* spp. that showed promising biological control in initial evaluation trials were compared in another experiment. Isolates were tested only with the delivery method that was effective in initial trials. Ten replicate seedlings per treatment in each of four blocks (40 seedlings for each treatment) were arranged in a randomized complete block design. This experimental design allowed for measurement of two dependent variables: mean survival time of individual plants and area under the disease progress curve (AUDPC). AUDPC was calculated from four replicates per treatment, with each block of 10 seedlings used as one replicate.

Inocula of *P. cactorum* and *Trichoderma* spp. were prepared as described above. A 2% rate of inoculum of *P. cactorum*, prepared with finer-textured vermiculite, was used to ensure adequate mortality of apple seedlings. Three additional treatments were planted. One treatment consisted of seedlings transplanted into soil infested with only *P. cactorum*, a situation analogous to that encountered in an orchard. This was the control treatment to which all treatments with *Trichoderma* spp. were compared (1). The other two were the peat-bran and Viterra treatments without *Trichoderma* spp. described above. Other than experimental design, the experiment was conducted identically to that used to initially evaluate isolates of *Trichoderma* spp. and was conducted three times.

Data analysis. Data were analyzed with MINITAB (release 7.2) statistical software (Minitab, Inc., State College, PA). Results from all analyses were judged significant at $P < 0.05$.

Data on seedling mortality (i.e., the number of seedlings dead) at any given time were binomial and, therefore, compared by chi-square analyses. These data are reported as percent mortality. In the experiments examining effects of inoculum rates or ambient temperature on mortality of seedlings growing in soil infested with *P. cactorum*, treatments were compared at 13–14, 28, and 42 days after transplanting—which corresponded to just before the first flooding, 2 wk after the first flooding, and 2 wk after the second flooding, respectively.

The relationship between initial inoculum density of *Trichoderma* spp. added to soil in peat-bran and biological control activity was examined by linear regression. For each trial, survival time of seedlings (the dependent variable) was regressed on \log_{10} inoculum density (the independent variable). Slopes of regression lines were analyzed, and coefficients of determination (R^2) were examined.

Data from trials to evaluate isolates of *Trichoderma* spp. for biological control activity were analyzed independently from each other, because environmental and experimental conditions were not identical among trials. For data from initial evaluation trials, two-way analyses of variance (ANOVAs) of survival time often

resulted in a significant interaction between isolates and delivery method; therefore, treatments were separated by delivery method, and the simple effects among Viterra and peat-bran treatments were examined by one-way ANOVAs. Treatment means for each delivery method within a trial were separated by Fisher's protected least significant difference (LSD; $P = 0.05$) because no pre-conceived comparisons were planned. Data from trials to further evaluate selected treatments were analyzed by one-way ANOVAs, and, again, means were separated by LSD ($P = 0.05$).

RESULTS

Inoculum rate of *P. cactorum* and seedling mortality. In trials with both particle sizes of vermiculite, plants growing in soil not infested with *P. cactorum* remained healthy throughout the 42-day duration of the experiment (Fig. 1). With the original, coarser-textured vermiculite (Fig. 1A), seedlings growing in 1% infested soil had only a low level of disease that occurred after the first flooding. Disease progress was similar for all other inoculum rates. The second flooding at 28 days after transplanting increased mortality at all inoculum rates except 1% (Fig. 1A). Significant differences among treatments occurred after the second flooding but not after the first. P values of chi-square statistics (5 df) comparing the number of seedlings surviving for all treatments at 13, 28, and 42 days were 0.097, 0.056, and < 0.001 , respectively. A significant difference also occurred at 42 days among seedlings

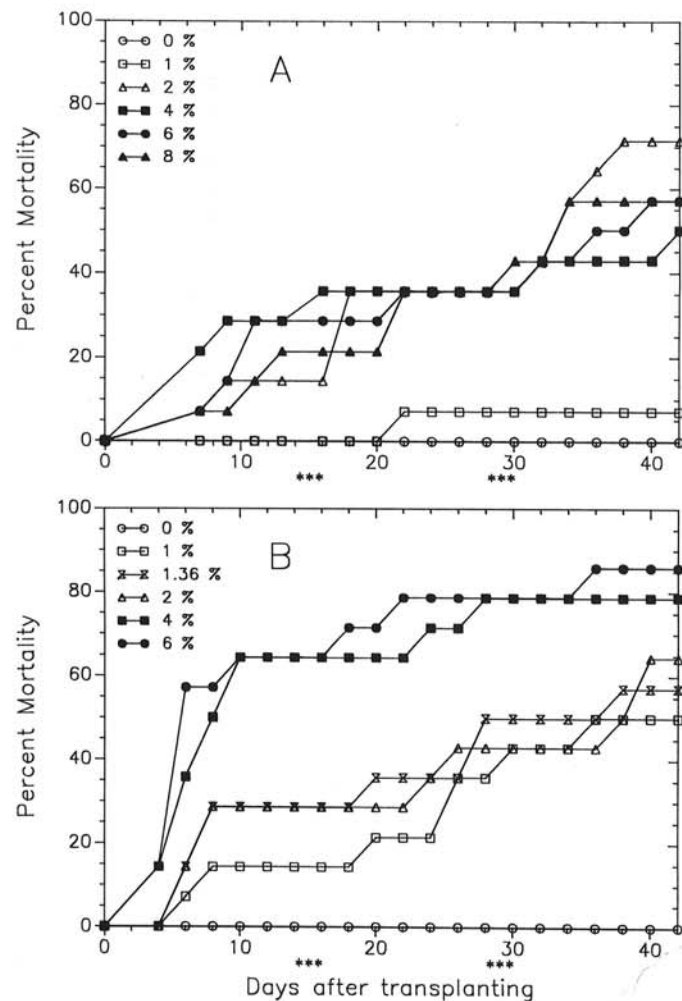


Fig. 1. Disease progress for the relationship between inoculum rate (v/v) of *Phytophthora cactorum* and mortality of apple seedlings (open-pollinated cv. McIntosh) in pasteurized soil. Inocula were prepared with, **A**, relatively coarse or, **B**, relatively fine medium-textured vermiculite. Percent mortality was calculated from 14 seedlings per inoculum rate. To enhance disease development, seedlings were flooded for 48 h (***) at 14 and 28 days after transplanting.

surviving in all infested treatments ($\chi^2 = 14.05$, $P = 0.007$, 4 df) but did not occur among seedlings growing in soil infested with 2–8% inoculum ($\chi^2 = 1.40$, $P = 0.706$, 3 df) (Fig. 1A). Results were similar when the experiment was repeated. Therefore, 2% was selected as the standard inoculum rate for *P. cactorum*, and two flooding periods were used for the initial evaluation of isolates of *Trichoderma* spp.

When the experiment was repeated with finer-textured vermiculite (Fig. 1B), significant differences among all treatments were established at 14 days; P values of chi-square statistics (5 df) at 14, 28, and 42 days were all < 0.001 . At the end of the experiment, a significant difference also occurred among treatments in infested soils ($\chi^2 = 10.01$, $P = 0.040$, 4 df). There was greater seedling mortality, particularly before the first flooding, at inoculum rates of 4 and 6% compared with 1, 1.36, and 2%. The second flooding period increased mortality at all inoculum rates except 4% (Fig. 1B). Results were similar when the experiment was repeated. With the finer-textured vermiculite as inoculum for *P. cactorum*, a rate of 1.36% was selected to replace the 2% rate used with the coarser-textured vermiculite as the standard for the remaining trials, because disease progress curves for these two rates were similar (Fig. 1).

Temperature and seedling mortality. As suspected, temperature had an effect on mortality of apple seedlings growing in soil infested with *P. cactorum*. Results were similar for both trials; therefore, data were combined to present a more representative picture (Fig. 2). At 44 days, 45% of seedlings at 16 C had died, 70% at 20 C had died, 92.5% at 24 C had died, and 55% at 28 C had died. Seedlings at the two higher temperatures died sooner than seedlings at the two lower temperatures. By day 6 of the experiment, 86.5% (32 of 37 seedlings) and 90.0% (20 of 22 seedlings) of the final mortality at 24 and 28 C, respectively, had occurred; in contrast, only 57.1% (16 of 28 seedlings) and 33.3% (6 of 18 seedlings) of the final mortality at 20 and 16 C, respectively, had occurred (Fig. 2). Significant differences among treatments occurred at 14, 28, and 42 days after transplanting; P values of chi-square statistics (3 df) were 0.044, 0.005, and 0.003, respectively. Seedlings in both trials that were transplanted into uninfested soil remained healthy throughout the experiment.

Initial evaluation of isolates of *Trichoderma* spp. The two variables, survival time and dry shoot weight, usually yielded similar results for overall treatment effects and relative performance of individual treatments in a given trial. Occasionally, however, a treatment had shoot weight significantly greater than

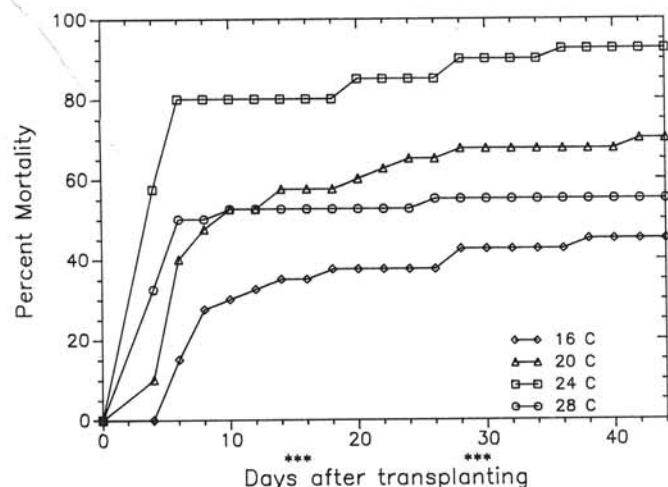


Fig. 2. Disease progress for the relationship between four ambient temperatures and mortality of apple seedlings (open-pollinated cv. McIntosh) transplanted into pasteurized soil artificially infested with *Phytophthora cactorum*. Percent mortality was calculated from 40 seedlings per temperature, 20 in each of two trials. To enhance disease development, seedlings were flooded for 48 h (***) at 14 and 28 days after transplanting.

but survival time similar to the treatment without *Trichoderma* spp. Isolates of *Trichoderma* spp. in such treatments may have been causing a growth promotion effect (2,29) that masked relatively poor biological control activity. Consequently, survival time was judged more reliable than dry shoot weight, and only data for survival time were used to evaluate treatments.

In all 35 trials combined, final mortality of seedlings differed significantly among the three treatments without *Trichoderma* spp. that were transplanted into infested soil ($\chi^2 = 21.66$, $P < 0.001$, 2 df). Mortality was 58.0% (203/350) for seedlings transplanted in soil infested with only *P. cactorum*, 56.6% (198/350) for those dipped in Viterra before transplanting, and 72.0% (252/350) for those transplanted into infested soil amended with peat-bran. Similarly, mean survival times among these treatments also were significantly different (one-way ANOVA, $P < 0.001$). Mean survival time for seedlings in the peat-bran treatment (20.8 days) was significantly less (LSD, $P = 0.05$) than that for seedlings in either of the other two treatments (26.8 days for the Viterra treatment and 26.0 days for the treatment with only *P. cactorum*). Consequently, not only did more seedlings die in soil infested with *P. cactorum* and amended with peat-bran, but they also died sooner than those in the other two treatments without *Trichoderma* spp.

This trend also occurred for treatments with *Trichoderma* spp.; seedlings in the peat-bran treatments with *Trichoderma* typically survived fewer days than seedlings in Viterra treatments with *Trichoderma*. In all 35 trials combined, mortality of seedlings in peat-bran treatments was 67% (3,205/4,807) and that in Viterra treatments was 58% (2,751/4,778); the difference between these two values was significant ($\chi^2 = 84.3$, $P < 0.001$, 1 df). In any one trial, however, there tended to be more variability among Viterra treatments compared with peat-bran treatments. In 26 of 35 trials, mean square errors from one-way ANOVAs of Viterra treatments were greater than those of peat-bran treatments, and this difference was significant in seven of these trials. In the other nine trials, mean square errors were greater in peat-bran than in Viterra treatments, but the difference was not significant.

Initial inoculum densities in soil of isolates of *Trichoderma* spp. delivered in peat-bran varied tremendously, even though all isolates were added at a uniform rate (5%, v/v). Inoculum densities of six isolates were below the threshold of detection in one of the two trials in which they were evaluated, but those of other isolates exceeded 10^8 cfu. On average, the maximum inoculum density in a trial was 10^5 – 10^6 times greater than the minimum

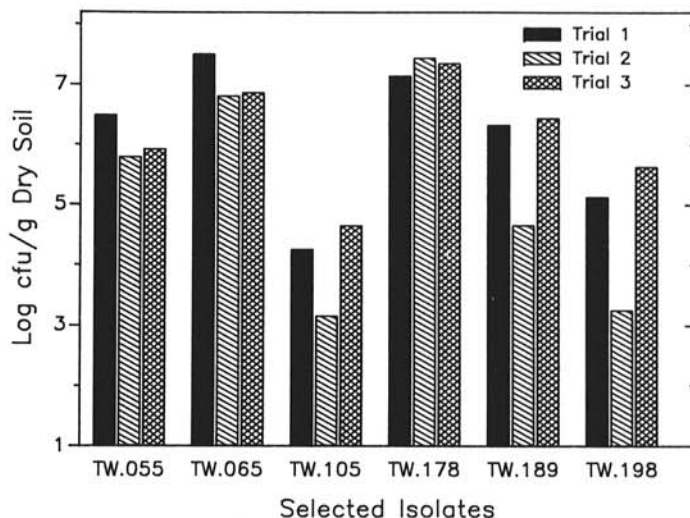


Fig. 3. A comparison of inoculum densities of six isolates of *Trichoderma* spp. initially present in pasteurized soil in three trials in which isolates were evaluated for biological control of *Phytophthora* crown and root rot of apple seedlings. Each isolate initially was added to soil as colonized peat-wheat bran (5%, v/v). Colony-forming units of *Trichoderma* spp. were determined from serial dilutions of each soil on a selective medium and are in \log_{10} units.

inoculum density. Initial inoculum density was not a good predictor of biological control activity. Slopes of regression lines between survival time and inoculum density were significantly different from zero in only two of 35 trials ($P = 0.049$ and $P = 0.031$); one of these slopes was positive and the other was negative. In 30 of the remaining 33 trials, t statistics to test significance of regression line slopes had $P > 0.200$. Values of R^2 for regression lines ranged from < 0.1 to 44.2% (in 27 trials, $R^2 < 10\%$).

Our goal in the initial evaluation was to identify isolates of *Trichoderma* spp. with the most potential for biological control. Compared with the peat-bran treatment without *Trichoderma* spp., six isolates in peat-bran—TW.055, TW.065, TW.105, TW.178, TW.189, and TW.198—significantly increased survival time of seedlings in each of the two trials in which they were evaluated. Other isolates of *Trichoderma* spp. delivered in peat-bran significantly increased survival time of seedlings in one of the two trials. However, only the six isolates effective in both trials were selected for further evaluation.

None of the isolates of *Trichoderma* spp. delivered as a suspension of conidia in Viterra significantly improved survival of seedlings over the Viterra treatment without *Trichoderma* spp. in both trials in which it was evaluated. Therefore, Viterra treatments in each trial were ranked by survival time, and the two relative ranks for each isolate were compared. Five isolates—TW.012, TW.123, TW.138, TW.190, and TW.212—that ranked either first or second in both trials were selected for further evaluation. Based on these selection criteria, no isolate was effective with both delivery methods. Of the 11 isolates selected for further evaluation, four were *T. virens* (TW.055, TW.065, TW.123, TW.190), three were *T. koningii* (TW.105, TW.189, TW.198), two were *T. harzianum* (TW.138 and TW.178), one was *T. hamatum* (TW.212), and one was *T. viride* (TW.012).

Evaluation of selected treatments. The six isolates of *Trichoderma* spp. delivered as colonized peat-bran at a constant rate of 5% resulted in different inoculum densities at the time of transplanting (Fig. 3). The interaction between isolates and trials was highly significant (two-way ANOVA, $P < 0.001$), indicating that variation in inoculum density for individual isolates was inconsistent among trials. In one-way ANOVAs, significant differences occurred among isolates for each trial ($P < 0.001$)

and among trials for each isolate ($P = 0.028$ for TW.178 and $P < 0.001$ for all other isolates). A similar trend occurred in the trials to initially evaluate isolates. For all 223 isolates of *Trichoderma* spp., inoculum densities at transplanting in the two trials in which an isolate was evaluated differed by less than a factor of 10 for 70% of the isolates, by more than a factor of 10 but less than a factor of 100 for 17% of the isolates, and by more than a factor of 100 for 13% of the isolates. As in initial evaluation trials, inoculum density of selected isolates in peat-bran was not a good predictor of seedling survival time. When survival time was regressed on \log_{10} of inoculum density for each of the three trials, slopes of regression lines were not significantly different from zero ($P = 0.646$, trial 1; $P = 0.748$, trial 2; $P = 0.461$, trial 3), and R^2 values were very low (5.8, 2.9, and 14.2%, respectively).

Three independent trials, each planted at a different time in the greenhouse, were conducted to test the 11 selected treatments. The two dependent variables, survival time and AUDPC, ranked treatments identically within each trial; therefore, only data for survival time are presented (Table 1). Results from the three trials varied, and relative ranks of many treatments were not consistent in all three trials. However, overall variability within a trial, indicated by LSD value (Table 1), was similar among trials.

In the first trial, five treatments significantly increased survival time compared with the control treatment, which consisted of apple seedlings growing in soil infested with only *P. cactorum*. Of these five treatments, three isolates—TW.055, TW.189, and TW.178—were delivered by the peat-bran method and two isolates—TW.138 and TW.190—were delivered by the Viterra method.

In the second trial, TW.055 in peat-bran again was the best treatment, and TW.105 in peat-bran, TW.189 in peat-bran, and TW.212 in Viterra were not significantly different from it. However, none of these differed significantly from the control. Seedlings in the control treatment survived longer in this trial than in the other two trials (Table 1) or in previous trials conducted under similar conditions. In contrast, mortality among the other treatments was greater, and, therefore, mean survival time overall was less compared with the other two trials (Table 1). During trial 2, early warm spring weather occurred before the air conditioning system was operational, and higher greenhouse tempera-

TABLE 1. Effect of 11 isolates of *Trichoderma* spp., each delivered by one of two different methods, on survival time of apple seedlings transplanted into pasteurized soil that was artificially infested with *Phytophthora cactorum*^a

Isolate number	Species	Delivery method ^v	Survival time (days) ^w			
			Trial 1	Trial 2	Trial 3	Average
TW.055	<i>T. virens</i>	P-B	32.40 a	26.75 a	30.55 a	29.90
TW.189	<i>T. koningii</i>	P-B	28.80 ab	20.17 a-d	23.75 a-d	24.24
TW.138	<i>T. harzianum</i>	Vit	27.75 ab	15.30 cd	24.30 a-c	22.45
TW.178	<i>T. harzianum</i>	P-B	26.45 a-c	17.35 b-d	18.75 cd	20.85
TW.190	<i>T. virens</i>	Vit	24.70 b-d	15.45 cd	18.10 cd	19.42
TW.212 ^x	<i>T. hamatum</i>	Vit	24.10 b-e	20.00 a-d	18.95 cd	21.02
TW.198	<i>T. koningii</i>	P-B	23.65 b-e	19.30 b-d	19.25 cd	20.73
TW.105	<i>T. koningii</i>	P-B	23.65 b-e	21.60 a-c	27.95 a	24.40
TW.065	<i>T. virens</i>	P-B	22.75 b-f	19.33 b-d	27.50 ab	23.19
(No <i>Trichoderma</i> spp.)		Vit	20.00 c-g	21.02 a-c	20.30 b-d	20.44
TW.123	<i>T. virens</i>	Vit	17.75 d-g	19.17 b-d	23.10 a-d	20.01
Control ^y			16.80 e-g	24.35 ab	16.20 d	19.12
TW.012	<i>T. viride</i>	Vit	16.00 fg	17.20 b-d	23.25 a-d	18.82
(No <i>Trichoderma</i> spp.)		P-B	14.10 g	13.27 d	20.10 b-d	15.82
Mean			22.78	19.30	22.29	21.46
P			< 0.001	0.042	0.011	
LSD ^z			7.45	7.25	7.59	

^a Forty seedlings (open-pollinated cv. McIntosh) were planted for each treatment with 10 replicates in each of four blocks. Seedlings were grown for 44 days and flooded for 48 h at 14 and 28 days after transplanting.

^v P-B: Soil infested with *P. cactorum* was amended with a 1:1 (v/v) mixture of peat and wheat bran colonized by an isolate of *Trichoderma* spp. or not colonized (no *Trichoderma* spp.). Vit: Roots of seedlings were dipped into a viscous suspension of Viterra Agri-gel containing 10^7 conidia/ml of an isolate of *Trichoderma* spp. or no conidia (no *Trichoderma* spp.) before transplanting into soil infested with *P. cactorum*.

^w Survival time = the mean number of days after transplanting that seedlings survived.

^x *T. hamatum* isolate TW.212 = ATCC 28042.

^y No *Trichoderma* spp. added, and no delivery method used.

^z Fisher's protected least significant difference with $P = 0.05$; means within columns followed by a common letter are not significantly different.

tures probably were responsible for the increased mortality. However, this does not explain the increased survival of seedlings in the control.

In trial 3, four treatments significantly increased survival time compared with the control treatment (Table 1). Again, TW.055 in peat-bran was superior to all other treatments; two other isolates in peat-bran, TW.105 and TW.065, also were effective. TW.138 was the only isolate effective with the Viterra delivery method.

DISCUSSION

Our goal was to evaluate a diverse array of isolates of *Trichoderma* spp. for efficacy in controlling crown and root rot of apple seedlings caused by *P. cactorum*. These results were to indicate whether *Trichoderma* spp. had potential to manage Phytophthora crown and root rot of apple trees in the orchard. To this end, a system was developed to evaluate these biological control treatments under controlled conditions conducive to disease development. Even under such conditions, some isolates of *Trichoderma* spp. consistently suppressed crown and root rot. Although there must be caution in extrapolating results from seedlings in a greenhouse to trees in the orchard, the potential for *Trichoderma* spp. to biologically manage Phytophthora crown and root rot of apple trees clearly exists and should be pursued. The same conclusion recently was reached by Smith et al (24) based on results from a study similar to this one. Limited success with biological control of crown and root rot has been demonstrated previously with other microorganisms (10,25,26).

Of the 11 isolates of *Trichoderma* spp. selected for further study, one isolate originally had been recovered from a cranberry runner, nine isolates were from soils collected in Wisconsin (22), and one isolate was obtained from ATCC. This ratio among sources of effective isolates, 1:9:1, is similar to that for sources of all isolates evaluated (i.e., 24 from cranberry runners, 180 from soil, and 19 from ATCC). Consequently, the source of an isolate apparently had no effect on its potential efficacy. In all, approximately one isolate out of 20 (i.e., 11 out of 223) was effective in the initial evaluation. The isolates obtained from soil were recovered at three temperatures: 8, 16, and 24 C (22). Of the nine isolates from soil that were effective initially, six were recovered at 8 C, whereas only two and one were recovered at 16 and 24 C, respectively, which suggests that isolates recovered at cooler temperatures may be more antagonistic to *P. cactorum*.

Each of the five species of *Trichoderma* evaluated was represented by at least one effective isolate. However, unlike the sources of isolates, the ratios of isolates effective to isolates tested for the five species were very different. Four of 19 isolates (21%) of *T. virens* that were tested were effective, but only 1 of 68 (1%), 2 of 52 (4%), 3 of 59 (5%), and 1 of 10 (10%) isolates for *T. hamatum*, *T. harzianum*, *T. koningii*, and *T. viride*, respectively, were effective. *T. virens* appears to be more antagonistic to *P. cactorum* than other species of *Trichoderma*; perhaps this species should be selectively evaluated for biological control of crown and root rot in future research.

Isolate TW.055 of *T. virens* delivered in peat-bran consistently was the most effective of all treatments and offers the most potential to biologically manage Phytophthora crown and root rot of apple trees. An isolate of *Gliocladium virens* (= *T. virens* [27]) also was the most effective one evaluated by Smith et al (24). Other promising isolates in our evaluation of selected treatments were TW.138 of *T. harzianum* in Viterra and TW.105 and TW.189 of *T. koningii* in peat-bran.

To further evaluate *Trichoderma* spp. as biological controls of Phytophthora crown and root rot, several factors must be considered. First, *Trichoderma* spp. must be shown to be effective in natural, unpasteurized soil; the apple seedling bioassay developed here would be useful for this. Then control of the disease on woody plants, including both nursery stock and orchard trees (12), should be assessed. Additionally, *Trichoderma* spp. must be proven to be effective against other species of *Phytophthora* that attack apple trees (15). Although *Trichoderma* spp. are known to exist naturally in apple orchard soils (22), ultimately

it must be demonstrated that introduced isolates will be effective as biological control agents in an orchard setting.

The use of *Trichoderma* spp. in an orchard not only must be effective but should be compatible with other disease management practices, including the use of fungicides to control Phytophthora crown and root rot as well as those used to control other diseases (19). Previously, researchers have shown *Trichoderma* spp. to withstand concentrations of chemicals lethal to other species of fungi and then to reestablish an antagonistic population (6,18). Similarly, the use of metalaxyl to control *Phytophthora* spp. in orchard soils may give *Trichoderma* spp., which usually are not inhibited by metalaxyl (24), a competitive advantage that will allow these fungi to control crown and root rot more effectively than metalaxyl alone.

Another potential benefit from introducing *Trichoderma* spp. into the rhizosphere is enhanced plant growth in the absence of root pathogens (2,29). Growth promotion of apple seedlings inoculated with *Trichoderma* spp. only recently has been explored (24) and should be studied further. We did not evaluate isolates in this study for growth promotion, nor did we observe any positive growth response from apple seedlings inoculated with isolate T8m of *T. koningii* or T12m of *T. harzianum* in preliminary experiments (20). Ideally, an isolate of *Trichoderma* spp. used for biological control also would promote plant growth.

The efficacy of individual isolates of *Trichoderma* spp. as biological control agents often was not consistent among trials. Several factors may have contributed to inconsistent performance, including variability associated with the apple seedlings, the amount of inoculum of *Trichoderma* spp. applied, the method of watering plants, and seasonal fluctuations in greenhouse temperature. A separate lot of apple seedlings was grown for each trial, and seedlings for each treatment were selected for similarities in size and vigor. However, inevitably there were differences among seedlings used for both treatments and trials. The inherent genetic variability of seedlings grown from open-pollinated seeds also may have contributed to the variable responses observed. We tried to account for at least some of this variability by using large numbers of replicates for each treatment.

Differences among trials in the amount of inoculum applied for any one isolate may have been an important source of variability that contributed to inconsistent performance. Viterra treatments usually had greater variability among replicates than peat-bran treatments in a given trial, which made it more difficult to identify significant effects. One reason for more variability could have been due to less consistent numbers of propagules applied to seedling roots by the Viterra method. Although concentrations of conidia in Viterra suspensions were standardized, the absolute amount of inoculum applied to roots depended on the root mass of the seedling, which varied considerably.

There also was variability among trials in the quantities of propagules of individual isolates added in peat-bran inocula. It is likely that many factors influenced the number of propagules produced in peat-bran, including light, temperature, and mixing to ensure thorough colonization. *Trichoderma* spp. grow so rapidly that a slight difference from one trial to the next in one of these factors may have resulted in a relatively large difference in final propagule numbers. Although inoculum density of *Trichoderma* spp. was not related to effective biological control when all isolates were compared, it may be associated positively with control by individual isolates. Therefore, incubation conditions that maximize propagule numbers in peat-bran should be identified.

Seedlings routinely were top-watered only as needed, based on a visual assessment of soil moisture. By this watering method, propagules of *Trichoderma* spp. may have been dispersed differentially along the root surface of individual plants (3). Therefore, seedlings that grew more vigorously, i.e., that remained healthy, were watered more often, which may have aided preferentially the movement of *Trichoderma* spp. on these plants. Any effect of watering on the dispersal of inoculum probably was greater in Viterra treatments, in which inoculum was applied only to root systems, than in peat-bran treatments, in which inoculum

was incorporated uniformly in the soil.

One of the most important factors causing inconsistent biological control performance of individual isolates among trials was temperature in the greenhouse. Survival time of apple seedlings decreased as temperatures increased between 16 and 24 C. Overall mortality of apple seedlings in a trial fluctuated with the season; greater mortality occurred in spring and summer when average daily greenhouse temperatures were higher, compared with autumn and winter when average daily temperatures were lower. The effect of temperature on the biological control efficacy of isolates of *Trichoderma* spp. was not investigated but also may have been a factor.

Two delivery methods to apply *Trichoderma* spp. were utilized in this experiment. Dry delivery methods, like the peat-bran used here, have been used commonly (4,19,23). The Viterra delivery method was devised as a more practical method for potential commercial use. An orchard manager easily could adapt current tree-planting practices to accommodate the Viterra method for delivering *Trichoderma* spp. In this research, however, the peat-bran delivery method was more effective. Peat-bran provided a food source and, perhaps, physical protection that may have given the introduced antagonist a competitive edge; the inert Viterra could not provide such advantages. In addition, conidia were the only type of propagule applied by the Viterra method, whereas peat-bran presumably contained conidia as well as hyphae and, possibly, other propagules. Because of uniformity of propagule type, conidia in Viterra suspensions may have been more sensitive to varying conditions in the soil environment.

Alternatively, the difference in activity between peat-bran and Viterra treatments may have been one of pH. The pH of Viterra treatments was consistently near neutral, whereas that of peat-bran treatments was acidic due to the peat (23). Previously, Harman and Taylor (9) found that lowering the pH of Methocel, the viscous carrier they used to deliver conidia of *Trichoderma* spp., to between 3 and 4 greatly improved biological control activity. Our attempts to lower the pH of Viterra were unsuccessful because the viscous, gelled nature of the solution was disrupted by the addition of acid. Interestingly, no isolate of *Trichoderma* spp. was effective in both delivery methods. Instead, there often was a significant interaction between isolates and delivery method, indicating that isolates performed differentially when delivered by the two methods.

The bioassay developed in this research to evaluate isolates of *Trichoderma* spp. as biological control agents of Phytophthora crown and root rot of apple seedlings had several features that contributed to its success. The use of planting cones to grow plants allowed for numerous replicates to be used for each treatment and multiple treatments to be evaluated simultaneously under controlled greenhouse conditions. Therefore, significant differences among treatments were more likely to be identified. Because of the relatively small volume in each cone, large volumes of inocula of *P. cactorum* and *Trichoderma* spp. were not needed. Multiple isolates of *P. cactorum* were used to produce a pooled inoculum to encourage the selection of isolates of *Trichoderma* spp. that had broad biological control activity. An inoculum rate for *P. cactorum* was selected that consistently produced adequate mortality in control treatments, and plants were inoculated at a young, susceptible age. The use of finger cots to flood each plant individually prevented contamination among plants that could occur if plants were flooded together. In addition, seedlings were flooded twice to enhance disease development. The system reported here should be useful for initially evaluating other potential biological control treatments for Phytophthora crown and root rot of apple trees and may be useful for evaluating biological controls for Phytophthora root rots of other woody plants.

Recently, Smith et al (24) also developed a system for evaluating the biological control potential of isolates of *Trichoderma* spp.; however, their experimental design and criteria for evaluation contrasted with ours. Smith et al used multiple plants in a pot as an experimental unit and fewer experimental units per treatment. In a given area of greenhouse space, fewer treatments could

be examined simultaneously, or the ability to identify significant differences among treatments would be reduced with fewer replicates in their system compared with ours. Use of multiple plants per pot could allow competition among plants, which ultimately may affect plant growth. In their study, only one isolate of *P. cactorum* and only a single flooding period were used. We used multiple isolates of *P. cactorum* for the reason mentioned above, and found that two floodings increased disease pressure. Finally, Smith et al used plant weight as the primary determinant to evaluate isolates of *Trichoderma* spp. We found that shoot weight and survival time as parameters to identify successful biological control agents occasionally conflicted, presumably due to growth promotion by *Trichoderma* spp. (2,24,29). Therefore, survival time appeared to be a more objective measure for evaluating biological control potential.

Regardless of the system used, *Trichoderma* spp. effectively managed Phytophthora crown and root rot of apple seedlings. However, the question remains whether an antagonistic microorganism effective on apple seedlings in the greenhouse will be effective on apple trees in an orchard. This question must be answered before biological control can become part of an integrated approach to Phytophthora crown and root rot management.

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