Evaluation of New Biotypes of *Trichoderma harzianum* for Tolerance to Benomyl and Enhanced Biocontrol Capabilities

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ABSTRACT

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Aqueous suspensions of conidia of *Trichoderma harzianum* wild strain WT-6 were placed on V-8 juice agar and exposed to ultraviolet (UV) radiation for 100 min. Conidia of WT-6 from surviving colonies of the first irradiation were allowed to germinate on the agar before a second exposure to UV for 100 min. The irradiated plates were incubated at 25 C under fluorescent light, and the resulting colonies of *T. harzianum* were isolated and grown on the medium. Conidia from the colonies that survived the second irradiation were placed on agar and UV-irradiated for 100 min. Of 36 colonies that survived the three irradiations, 19 colonies from the third series tolerated high concentration of benomyl (100–500 mg/ml) as indicated by growth in solid and liquid media and conidial germination tests on benomyl-amended agar. The UV-induced biotypes differed considerably from WT-6 in appearance, growth habit, fungitoxic metabolite production against a given assay pathogen (*Sclerotium cepivorum*), and ability to survive in soil. Certain UV-induced biotypes that were also tolerant to benomyl suppressed the saprophytic activity of *Rhizoctonia solani* in soil more effectively than did the wild strain. Several UV-induced biotypes were consistently more effective than the wild strain WT-6 in suppressing damping-off (*Pythium ultimum*) of peas, damping-off (*R. solani*) of cotton and radish, and white rot (*S. cepivorum*) of onion.

Twelve years ago, Rifai (12) stated in his monographic study of the genus *Trichoderma* that, "It is not yet known whether one can easily induce mutations in species of *Trichoderma* or whether they occur readily in nature. It is also not yet known whether polyploidy can be found among species of *Hypocrea* or among the different isolates of the same species or species aggregate of *Trichoderma* ...." Subsequent evidence from industrial microbiology showed that it is possible to improve the yield and quality of enzymes synthesized by *Trichoderma* spp. by genetic manipulation (5,6). Improved strains of *T. reesei* were isolated by mutation and selection, and these produced more cellulase than did the wild strains (6). Mandels et al (4) irradiated *T. viride* with a linear accelerator and produced a new strain that secreted twice as much cellulase as its wild parent.

Similar studies to induce new biotypes of *Trichoderma* strains that may be used for biological control of soilborne plant pathogens have not as yet been reported. Troutman and Matejka (13) induced tolerance of *T. viride* to benomyl by gamma irradiation, but did not report on the biological control capabilities of their benomyl-tolerant biotypes. The present study was initiated to develop new biotypes of *T. harzianum* Rifai, to select those that are resistant to certain fungicides, and that also possess enhanced biocontrol capabilities and superior survival in soil. A preliminary report of this work was presented (10).

MATERIALS AND METHODS

Strains T-1, Th-1, and WT-6 of *T. harzianum* were used. Strain Th-1 was isolated by the senior author from a Beltsville soil. Strains T-1 and WT-6 were provided by H. D. Wells, Tifton, GA. Strain WT-6 is easy to distinguish from other strains because the mycelium and the conidial masses are white. H. D. Wells (*personal communication*) characterized WT-6 as an excellent antagonist against *Rhizoctonia solani* Kühn in vitro, and it was effective as a biocontrol agent in the field against fruit rot of cucumber caused by *R. solani* (3). For comparison, benomyl-tolerant biotypes T-1(ben 100-5) and Th-1(ben 200-1) from another study (G. C. Papavizas, *unpublished*) also were used.

Conidia of *T. harzianum* were produced by growing the isolate on V-8 juice agar (200 ml V-8 juice, 800 ml water, 1 g glucose, 20 g agar, 6.0 ml 1.0 N NaOH) for 7 days under continuous fluorescent light (~700 μEin/m^2/sec). Conidia were removed from the agar surface by pipetting 3–5 ml of sterile distilled water on the surface and gently rubbing the surface with a sterile cotton-tipped applicator. Conidia were counted in a hemacytometer, and the suspensions were adjusted with water to provide the desired number of conidia in each experiment.

In all experiments, there were five replications, and each experiment was done twice. A randomized complete block design was used throughout.

Ultraviolet irradiation. Aliquots (1-ml) of a conidial suspension (10^5 conidia per milliliter) of isolate WT-6 were placed on 100 V-8 juice agar plates and immediately exposed to ultraviolet (UV) irradiation for 100 min. Irradiation was provided by two adjacent (7 cm apart) G-E Germicidal Lamps (G36T6, General Electric Co., Nela Park, Cleveland, OH 44112). The plates (with the lids removed) were irradiated at a distance of 29 cm from the agar surface to the lamps. The irradiated plates were covered and incubated at 25 C under fluorescent light. Seven colonies developed from 10^7 conidia irradiated. The colonies were isolated and grown on V-8 juice agar.

Conidia from the seven colonies of the first irradiation series were placed on the medium (50 plates), allowed to germinate for 18 hr, and exposed to UV irradiation for 100 min. Five colonies developed from 5×10^6 germinating conidia irradiated. The colonies (second irradiation series) again were isolated and grown

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on the same medium.

Conidia produced from survivors of the second series were placed on 100 plates and immediately exposed to UV irradiation for 100 min. Twenty-four colonies developed from 10^7 conidia (third irradiation series). Colonies of the third series were isolated on V-8 juice agar, and the 24 new colonies were tested for tolerance to benomyl together with the colonies of the first and second series. Since no other recognizable markers or characteristics were available, tolerance to benomyl was used as a criterion to distinguish mutants from wild strains. The wild strains in this paper are designated by one or two capital letters followed by a one-digit number (eg, WT-6, T-1, Th-1). The UV-induced biotypes are indicated by a second number or by a designation showing the highest benomyl concentration which each biotype tolerated (eg, WT-6-1, Th-1 [ben 200-1]).

Tolerance of the UV-induced biotypes to benomyl and other fungicides. In vitro fungistatic activity of 1-(butylcarbamoyl)-2-benzimidazole carbamate (benomyl, 50% wettable powder, E. I. du Pont de Nemours & Company, Wilmington, DE 19898) was studied on V-8 juice agar (20 ml per 9-cm-diameter petri plate), or in potato-dextrose broth (PDB) (40 ml per 250-ml Erlenmeyer flask).

Benomyl was suspended in sterile distilled water and added in appropriate quantities (w/v) to the media after they were autoclaved and before they were dispensed to flasks or petri plates. The fungicide was tested in the solid medium at 0.0 (controls), 0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50, 100, 250, and 500 μg active ingredient (a.i.) per milliliter of medium. In PDB, benomyl was tested at 0.0, 25, and 50 μg a.i./ml. Disks (5-mm-diameter) of 7-day-old colonies of WT-6 and of biotypes in the three irradiation series grown on benomyl-free V-8 juice agar were transferred to the center of petri plates or to flasks. Colony radii on solid media were measured at 4, 7, and 14 days, and dry weights of mycelial mats of flask cultures were determined after 7, 14, and 21 days.

Selected biotypes of the third irradiation series also were transferred to V-8 juice agar containing singly the following fungicides at the indicated concentrations (mg a.i./ml): tetrachloroisophthalonitrile (chlorothalonil, 75% wettable powder, Diamond Shamrock Corp., Painesville, OH 44077), 3; *cis*-*N*-[(1,1,2,2-tetrachloroethyl)thio]-4-cyclohexene-1,2-dicarboximide (captafol, Chevron Chemical Co., Richmond, CA 94804), 4; *N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide (captan, Chevron Chemical Co., Richmond, CA 94804), 2; 3-(3,5-dichlorophenyl)-1-isopropylcarbamoylhydantoin (iprodione; Rhone-Poulene Agrochemicals, Lyon, France), 2; and 3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione (vinclozolin, BASF Wyandotte Corp., Parsippany, NJ 07054), 2. Colony radii on solid media containing these fungicides were measured at 7 and 13 days.

Effect of benomyl on conidial germination. We studied comparative toxicity of benomyl (used at 0.0, 25, 50, 100, and 200 μg a.i./ml) on spore germination of strain WT-6 and a selected biotype (WT-6-1) from the third series of irradiations. We also used for comparison *T. harzianum* isolate Th-1 and a biotype derived from it that is resistant to benomyl (Th-1[ben 200-1]). Aliquots (1-ml) of conidial suspensions (10^4 conidia per milliliter) were pipetted on the surface of V-8 juice agar containing the fungicide and the plates were incubated at 25 C under fluorescent light. After 16, 24, and 36 hr, we stained selected areas in the plates with lacto-fuchsin and counted germinated and ungerminated spores. The germinability readings as they were affected by benomyl were based on 100 conidia per replication, four replications per treatment. The data were expressed as percent inhibition of germination.

In a second test, aqueous 1-ml suspensions of conidia (10^4/ml) of isolates WT-6, T-1, and Th-1, and of biotypes WT-6-1, T-1(ben 100-5), and Th-1(ben 200-1), were added separately to 250-ml Erlenmeyer flasks containing 30 ml of 0.2% (a.i.) suspensions of benomyl (spore suspension:benomyl suspension, 1:1, v/v). The conidia, therefore, were directly exposed to 0.1% benomyl (1,000 μg a.i./ml). The flasks were placed on a platform shaker and shaken continuously for 4 wk in the dark at 5 C to prevent germination and growth. At 1, 2, 3, and 4 wk, drops containing conidia were placed on V-8 juice agar in petri plates and the plates were incubated at 25 C under continuous fluorescent light. Percentage germination was recorded as before after 24, 36, and 48 hr.

Effect of *T. harzianum* on the saprophytic colonization of a substrate by *Rhizoctonia* in soil. Isolates R-5 and R-35 of *R. solani* Kühn (anastomosis group 4) were grown separately on sterilized sand-cornmeal (96% quartz sand, 4% cornmeal, water to 20% moisture, v/w) for 3 wk, and inoculum of both isolates combined was added to moist Beltsville soil (pH 6.0) at the rate of 1% (w/w). The infested soil batches were kept in the laboratory at approximately 24 C for 1 wk and then subdivided into 0.4-kg portions. Isolate WT-6 and 10 of its UV-induced biotypes were grown on sterile oat seeds (oats:water, 1:1, w/v) for 20 days. The inocula were air-dried at room temperature, ground in a hammer mill, and stored at 5 C. Dilutions ($1{:}10^9$) were prepared on the TME medium (8) to determine the number of colony-forming units (cfu) in the dry preparations. These were then added to soil infested with *R. solani* to provide a mixture containing 2.5×10^4 cfu per gram of soil. Autoclaved dry preparations were added to soil to equalize the amounts of organic matter added with the antagonists. The infested soil batches were placed in 600-ml beakers and covered with polyethylene film to prevent loss of moisture. The infested soils were kept at −9 bars moisture (approximately 50% of moisture holding capacity). After 2 and 4 wk, 100 g of soil was withdrawn, and the surviving *R. solani* population was determined by the table beet (*Beta vulgaris* L.) seed-colonization method described previously (9).

Survival of conidia of *T. harzianum* in soil. Aqueous suspensions of conidia of WT-6 and 19 UV-induced biotypes from V-8 juice agar were added to 200-g batches of natural soil at 10^4 conidia per gram of soil. The soils, kept at −9 bars moisture, were placed in 400-ml beakers, covered with polyethylene film, and kept at 24 ± 2 C. After 1, 3, 6, 16, and 22 wk, soil dilutions were made by suspending the equivalent of 5.0 g of oven-dry soil in 495 ml of sterile tap water and shaking the suspension vigorously by hand for 1 min. Aliquots (1-ml) from the final dilutions were removed from the beakers while the liquid was subjected to continuous agitation with a magnetic stirrer and spread over the surface of the TME medium (8). For isolate WT-6, TME, which is semiselective for *Trichoderma*, was used without any additives. For the UV-induced biotypes, TME was supplemented with benomyl (10 μg a.i./ml). Assay plates were incubated at 25 C under continuous fluorescent light and *T. harzianum* colonies were counted on the sixth or seventh day of incubation.

In a separate experiment, benomyl was added to soil first at 0 (control), 10, and 25 μg a.i./g of soil with half the total amount of water needed, which brought the moisture tension to −9 bars. Conidia of isolates WT-6 and Th-1, and of the respective UV-induced biotypes WT-6-24 and Th-1(ben 200-1), were added to benomyl-treated soils at 1×10^3 conidia per gram of soil with enough water to adjust the soil moisture content to −18 bars. After 1, 3, 6, and 9 wk, dilutions were prepared as before.

Production of fungitoxic metabolites. Isolate WT-6 and 19 UV-induced biotypes were grown for 10 days in the dark in 50 ml of the gliotoxin fermentation medium of Brian and Hemming (1). The culture filtrates were divided into two portions. One portion was sterilized by membrane filtration and the second by autoclaving at 121 C for 15 min. Culture filtrates were added at 10% (v/v) to Difco potato-dextrose agar (PDA) after it was autoclaved and cooled to about 55 C, and the medium was dispensed to petri plates (15 ml per plate). Disks (5-mm-diameter) of 4-day-old colonies of *Sclerotium cepivorum* Berk. were transferred to the center of plates. Colony radii of the assay fungus were measured at 4 days, and the effect of toxic metabolites was expressed as percent inhibition of radial growth of *S. cepivorum*. The term "fungitoxic metabolite" is used in this paper only in a general context; no attempts were made to determine the identity of the inhibitory substances produced by *T. harzianum*.

Disease suppression in the greenhouse. Conidia of WT-6 and of 10 UV-induced biotypes were harvested from 10-day-old cultures

growing on V-8 juice agar by rubbing (with a rubber policeman) sporulating surfaces to which 2 ml of 4% methyl cellulose (MC) solution had been added. Suspensions of conidia of all isolates were counted in a hemacytometer and adjusted to contain 2.5×10^8 conidia per milliliter, of which 97% germinated. One milliliter of the spore-MC suspensions was applied to 20 g of Perfected Freezer and Alaska pea seed and the seeds were allowed to dry. Treated and untreated seeds were planted in 11-cm-diameter plastic pots (10 seeds per pot) containing a Beltsville soil (sandy loam) naturally infested with *Pythium ultimum* Trow. Plant stands were determined 2 wk after planting.

In a second test, *R. solani* isolate R-35 was grown on chopped-potato-soil (CPS) substrate (14) for 3 wk. The soil inoculum mixture was air-dried and screened to yield large (>589 μg) propagules. The inoculum was added to soil at 1% (dry inoculum) 1 wk before antagonist addition and mixed thoroughly in a Hobart Mixer (Gill Marketing Co., Beltsville, MD 20705). One week after soil infestation, the soil was divided into 6-kg portions to receive treatments. Strain WT-6 and the UV-induced biotypes WT-6-1, WT-6-5, WT-6-6, WT-6-17, WT-6-19, and WT-6-24 were grown on V-8 juice agar for 10 days and on the SCB-GFM medium (J. A. Lewis and G. C. Papavizas, *unpublished*) containing the ingredients at the following proportions: quartz sand, 1,200 g; cornmeal, 40 g; wheat bran, 40 g; and modified gliotoxin fermentation medium (GFM), 150 ml. The modified GFM differed from the original medium of Brian and Hemming (1) in having double its concentration of ammonium tartrate and one-half its concentration of glucose. Aqueous suspensions of conidia from the V-8 juice agar plates were added to the soil infested with *R. solani* at 4.4×10^5 conidia per gram of soil. The inocula of WT-6 and of the UV-induced biotypes on the SCB-GFM medium were air-dried and mixed thoroughly to become a granular preparation. The numbers of colony-forming units in the dry preparations were determined on the TME medium as before and the preparations were added to soil at a rate to provide 4.4×10^5 cfu per gram of soil. Autoclaved dry preparations of the isolates on the SCB-GFM medium were added to soil to equalize the amounts of organic matter added with the antagonists.

One week after the treatments were applied, the soil was apportioned into 11-cm-diameter plastic pots (10 pots per treatment). Acala SJ-2 cottonseed (*Gossypium hirsutum* L.) and Scarlet Globe radish seed (*Raphanus sativus* L.) were planted at 10 seeds per pot and these were incubated at 25 ± 3 C. Plant stand was determined 3 wk after planting.

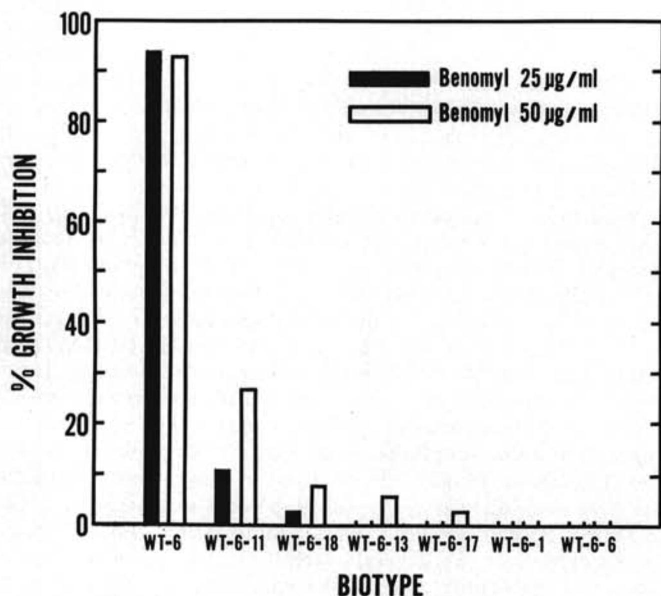

Fig. 1. Inhibition of mycelium dry weight gain of *Trichoderma harzianum* strain WT-6 and six UV-induced biotypes after 14 days of incubation in potato-dextrose broth containing benomyl.

In a third test, soil naturally infested with *S. cepivorum* was divided into 5-kg portions. Aqueous suspensions of conidia from V-8 juice agar plates and dry inocula (grown on the SCB-GFM medium as before) of WT-6 and of six UV-induced biotypes were added to the *S. cepivorum*-infested soil at 4.4×10^5 cfu per gram of soil. One week after treatments, the soil portions were subdivided into 1-kg batches and these were placed in 11-cm-diameter plastic pots. Six-week-old onion seedlings (*Allium cepa* L. 'Yellow Globe Danvers') were transplanted into infested soil (10 seedlings per pot) and grown in a growth chamber at 16 C with a 12-hr day length (Sylvania VHO cool-white, ~800 $\mu Ein/m^2/sec$) for 8 wk. Onion seedlings were harvested and disease severity data were recorded as the percentage of plants infected with *S. cepivorum* in each pot.

RESULTS

Induction of new biotypes resistant to benomyl. The wild isolate, WT-6, and the seven, five, and 24 colonies from the first, second, and third irradiation series, respectively, were transferred to V-8 juice agar to allow for normal growth and sporulation and then tested for tolerance to benomyl by transferring them to V-8 juice agar in petri plates containing the fungicide at concentrations ranging from 0 (control) to 500 μg a.i./ml of medium. The 12 isolates from the first two series of UV irradiation tolerated relatively low concentrations of benomyl (2.5 and 5.0 μg a.i./ml). These isolates were not tested further. The wild isolate WT-6 was sensitive to benomyl. The ED_{50} for inhibition of radial growth on V-8 juice agar by benomyl for this isolate was 0.5 μg of benomyl per milliliter; at 1.0 μg/ml, there was only a trace of growth. This ED_{50} value was as low as that observed for *T. harzianum* isolates T-1, T-3, T-5, T-14, H-54, and Th-1 (G. C. Papavizas, *unpublished*). In contrast, 19 of the 24 UV-induced biotypes of the third irradiation series grew as well on V-8 juice agar containing benomyl at 500 μg/ml as in the control. The ED_{50} for two biotypes (WT-6-17, WT-6-24) was about 50 μg/ml; and that for three biotypes (WT-6-11, WT-6-12, WT-6-13) was between 2.5 and 5 μg/ml.

In the PDB-benomyl experiment, growth of isolate WT-6 was completely inhibited at 25 and 50 μg of benomyl per milliliter (Fig. 1). Five of the nine biotypes (WT-6-1, -4, -6, -8, and -24) tested in this experiment grew as well at 50 μg of benomyl per milliliter of PDB as in the untreated control, and the growth of four (WT-6-11, -13, -17, and -18) was only slightly reduced at those concentrations of benomyl. Only representative isolates are shown in Fig. 1.

In the test performed to detect multiple tolerance to fungicides other than benomyl, isolate WT-6 grew as well in the presence of captan and captafol at 2 mg a.i./ml and 4 mg a.i./g, respectively, as in the untreated controls. Linear growth of WT-6 was restricted by about 50% at the seventh day of incubation on media containing iprodione, vinclozolin, and chlorothalonil at 2, 2, and 3 mg a.i./ml, respectively. After 14 days, linear growth of WT-6 was restricted in all cases by about 25%. None of the 19 UV-induced biotypes tested exceeded WT-6 in tolerance to the five fungicides.

Effect of benomyl on conidial germination. Benomyl prevented from 75 to almost 100% of conidia of the two wild isolates Th-1 and WT-6 from germinating at 25–200 μg a.i./ml (Fig. 2). In contrast, benomyl prevented only 10% of conidia of WT-6-1 from germinating, even at 200 μg/ml. With the new biotype Th-1(ben 200-1), only 18 and 37% of conidia did not germinate at 100 and 200 μg a.i./ml, respectively. All conidia of Th-1(ben 200-1) germinated at 25 and 50 μg/ml.

Fifty-seven percent of the conidia of isolate T-1 did not germinate on benomyl-free V-8 juice agar during a 48-hr incubation when exposed previously to a benomyl suspension of 1,000 μg/ml for 1–4 wk at 5 C (Fig. 3). Germination of conidia of the other two wild isolates, Th-1 and WT-6, was prevented by not more than 20%, when the conidia were exposed to the benomyl suspension for 4 wk. In contrast, benomyl prevented only 5% or less of conidia of the UV-induced biotypes from germinating, even after an exposure of 4 wk.

Effect of *T. harzianum* on the saprophytic colonization of table beet seed by *R. solani*. Five UV-induced biotypes at the 2-wk, and four at the 4-wk assay period were more effective in suppressing the

colonization of table beet seed by *R. solani* in soil than was WT-6 (Table 1). Two biotypes (WT-6-19 and WT-6-24) were more effective than WT-6 at both assay periods. Biotype WT-6-8 was more effective than WT-6 only at 4 wk. Two biotypes (WT-6-9 and WT-6-11) were less effective than WT-6, and four were as effective as WT-6 at the 4-wk assay period. Isolate WT-6 reduced colonization of table beet seed by *R. solani* by 46 and 48% at 2 and 4 wk, respectively.

Survival of conidia in soil. One week after the addition of conidia of WT-6 and of 19 UV-induced biotypes to soil, survival of a few biotypes (WT-6-1, WT-6-6, and WT-6-15) was relatively high (colony-forming unit recovery was 50% or more of original numbers of conidia added) (Fig. 4). Survival of WT-6 and some other isolates was moderate (colony-forming unit recovery ranged 25–50%); that of others (WT-6-11, WT-6-18) was poor (colony-forming unit recovery was <25%). Only isolate WT-6 and five biotypes are shown in Fig. 4. After 22 wk in soil, six biotypes had significantly higher population densities than WT-6; three were equal in density to WT-6; and 10 had significantly lower population densities than WT-6. Four of the biotypes were not detectable after 16 wk. At least 40% of the colony-forming units of biotypes WT-6-1, WT-6-15, WT-6-19, and WT-6-24 survived the 22-wk incubation period.

An experiment also was performed to determine whether biotypes tolerant to benomyl in vitro also are tolerant to the fungicide in soil. The tolerant biotypes WT-6-24 and Th-1(ben 200-1) were selected for this test because they survived as well as WT-6 and Th-1, respectively, in soil. Nine weeks after addition of conidia to soil that contained benomyl at 0.0 (control), 10, and 25 μg/g of soil, there were no differences in survival between WT-6 and WT-6-24; and between Th-1 and Th-1(ben 200-1). Approximately 6×10^3 and 7×10^3 colonies were counted for the two wild strains and the two biotypes after 9 wk of incubation in soil.

Fungitoxic metabolite production. The wild strains WT-6 and

TABLE 1. Effect of *Trichoderma harzianum* strain WT-6 and 10 UV-induced biotypes on the saprophytic colonization of table beet seed by *Rhizoctonia solani* in soil

Strain or biotype	Seed colonization (%)[a]	
	2 wk	4 wk
None (control)	70 a	68 a
Autoclaved oat seed (control)	73 a	70 a
WT-6-9	68 a	66 a
WT-6-11	64 a	60 a
WT-6-18	46 b	40 b
WT-6[b]	38 bc	40 b
WT-6-1	40 bc	20 de
WT-6-8	30 cd	22 de
WT-6-5	26 de	32 bcd
WT-6-6	18 ef	24 cde
WT-6-19	17 ef	18 de
WT-6-17	12 e	24 cde
WT-6-24	10 e	8 e

[a]In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[b]WT-6 is the wild strain; all other designations represent UV-induced biotypes from WT-6.

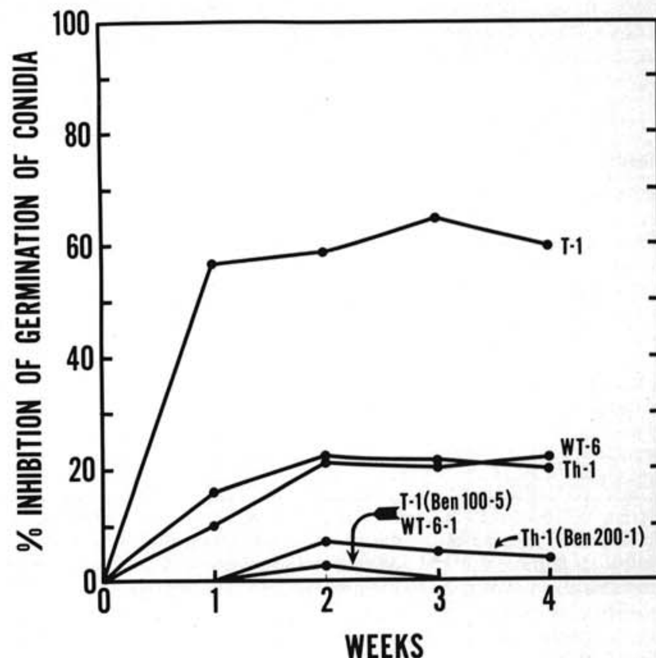

Fig. 3. Effect of exposure of conidia of *Trichoderma harzianum* strains WT-6, T-1, and Th-1 and the UV-induced biotypes WT-6-1, T-1(ben 100-1), and Th-1(ben 200-1) to benomyl suspension (1,000 μg/ml active ingredient) at 5 C for 4 wk on their subsequent germinability on benomyl-free V-8 juice agar.

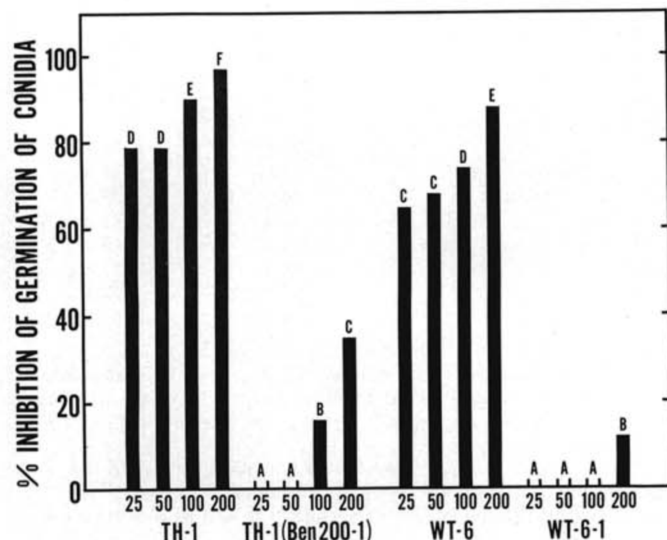

Fig. 2. Inhibition of conidial germination of *Trichoderma harzianum* strains WT-6 and Th-1 and of the UV-induced biotypes WT-6-1 and Th-1 (ben 200-1) after 36 hr of incubation in V-8 juice agar containing benomyl at 0, 25, 50, 100, and 200 μg a.i./ml.

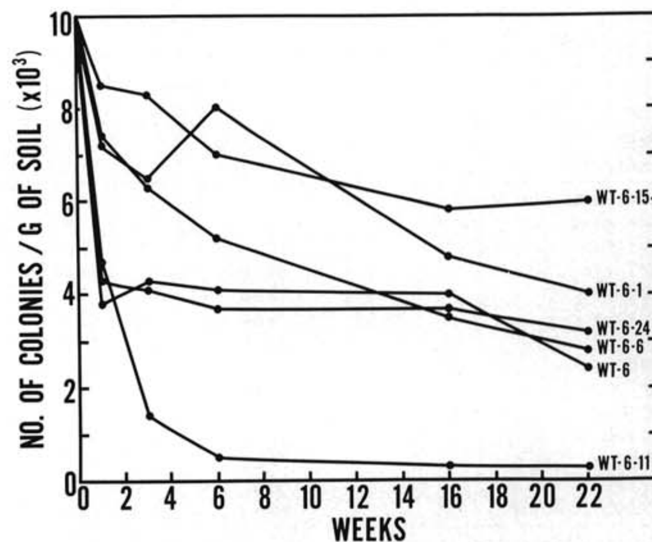

Fig. 4. Survival in soil of conidia of *Trichoderma harzianum* strain WT-6 and of five UV-induced biotypes after 22 wk of incubation as indicated by the number of colonies recovered on a special Trichoderma-selective medium.

the 19 biotypes were separated into three groups according to their ability to produce toxic metabolites against *S. cepivorum* after 10 days of growth in the dark at 25 C (Table 2). Group I strains (biotypes WT-6-10, WT-6-13, WT-6-18, and WT-6-20) were not inhibitory to *S. cepivorum*. Group II strains (WT-6 and 10 biotypes) produced a heat-labile toxic metabolite, but the amounts differed from one biotype to another. Seven of the biotypes in this group produced more toxic metabolite than did WT-6. Group III produced a heat-stable toxic metabolite or, possibly, two metabolites, one heat-labile, the other heat-stable. Biotype WT-6-11 produced the highest amount of toxic metabolite.

Disease suppression. Conidia of the UV-induced biotypes WT-6-6, WT-6-9, WT-6-15, WT-6-17, and WT-6-19, added to Perfected Freezer pea seed at equal population densities, resulted in a significantly better stand than that obtained in the untreated control or the methyl cellulose control (Table 3). Seed treatment with WT-6-6, WT-6-15, WT-6-17, and WT-6-19 resulted in significantly better stand than that obtained with the wild strain WT-6. With the exception of biotype WT-6-24, the wild strain and all biotypes tested significantly suppressed seed rot caused in Alaska peas by *P. ultimum*, but the differences between isolates were not as pronounced as those observed with cultivar Perfected Freezer, which is highly susceptible to *P. ultimum*. Alaska pea plants have considerable resistance to this pathogen.

In the *R. solani* test, aqueous suspensions of conidia of WT-6-5, WT-6-6, WT-6-17, and WT-6-24, or dry preparations of the same biotypes that had grown on the SCB-GFM medium, all added at 4.4×10^5 cfu per gram of soil, significantly suppressed seedling disease of cotton (Table 4). Biotypes WT-6-5, WT-6-6, WT-6-17, and WT-6-24 added as bare conidia; and WT-6-5, WT-6-6, and WT-6-24 added as dry inoculum-substrate preparations were more effective than WT-6. With radishes, bare conidia of WT-6-6, WT-6-17, and WT-6-24 resulted in stand counts significantly higher than that of the infested control and WT-6-5, WT-6-6, WT-6-17, and WT-6-24 were more effective than the wild strain. When dry preparations were used to protect radish from *R. solani*, WT-6-5, WT-6-19, and WT-6-24 were better than the infested control and WT-6-5 and WT-6-24 were better than WT-6.

In the onion experiment, biotypes WT-6-15 and WT-6-11 reduced incidence and severity of white rot significantly compared with that obtained with WT-6 (Table 5). The amount of suppression of white rot obtained with WT-6, and with biotypes WT-6-1, WT-6-6, WT-6-18, and WT-6-19 was not significantly different from the control.

TABLE 2. The effect of fungitoxic metabolite produced in culture by *Trichoderma harzianum* strain WT-6 and by UV-induced, benomyl-resistant biotypes on the radial growth of *Sclerotium cepivorum*

Strain or biotype	Group[b]	Percentage of radial growth inhibition[a] with indicated sterilization	
		Filtration[c]	Heat[d]
WT-6-10, -13, -18, -20	I	0 a[e]	0 a
WT-6 (wild strain)	II	13 b	0 a
WT-6-9	II	25 b	0 a
WT-6-7, -12	II	41 bc	0 a
WT-6-5, -6, -8, -15, -16	II	50 c	0 a
WT-6-1, -4	II	63 c	0 a
WT-6-21	III	73 cd	38 bc
WT-6-17	III	75 cd	25 b
WT-6-19	III	75 cd	38 bc
WT-6-24	III	88 d	50 c
WT-6-11	III	100 e	75 d

[a]Strain WT-6 and -19 biotypes were grown in the gliotoxin fermentation medium for 10 days in the dark; sterile culture filtrates were added to potato-dextrose agar and plates were inoculated with *S. cepivorum*; percentage of radial growth inhibition was determined by measuring radial growth after 4 days of incubation.
[b]Three arbitrary groups on the basis of fungitoxic metabolite production.
[c]Millipore filtration.
[d]Autoclaving at 121 C for 30 min.
[e]Values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

TABLE 3. Biological control of seed rot of Perfected Freezer and Alaska peas caused by *Pythium ultimum* with conidia of *Trichoderma harzianum* strain WT-6 and of selected ultraviolet light-induced biotypes produced on V-8 juice agar[a]

Seed treatment	Plant stand (%)[b] (2-wk postplant)	
	Perfected Freezer	Alaska
Untreated (control)	16 a	68 a
Methyl cellulose (control)	18 a	66 a
WT-6-24	18 a	78 ab
WT-6 (wild strain)	24 ab	84 bc
WT-6-1	26 ab	84 bc
WT-6-8	28 ab	92 cd
WT-6-5	30 ab	90 cd
WT-6-11	36 abc	88 bcd
WT-6-9	40 bcd	94 cd
WT-6-6	52 cd	90 cd
WT-6-19	52 cd	88 bcd
WT-6-17	54 cd	94 cd
WT-6-15	60 d	96 d
Thiram (control)	86 e	96 d

[a]One milliliter of conidial suspension of each isolate in 4% methyl cellulose, containing 2.5×10^8 conidia, was applied to 20 g of seed.
[b]Plant stand was rated 2 wk after planting. In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

TABLE 4. Biological control of seedling disease of cotton and damping-off of radish caused by *Rhizoctonia solani*[a] with *Trichoderma harzianum* strain WT-6 and with selected UV-induced biotypes

Biotype and kind of inoculum	Plant stand at 3 weeks (%)[b]	
	Cotton	Radish
Conidia in water[c]		
None (infested control)	16 ab	31 bc
WT-6 (wild strain)	20 abc	26 ab
WT-6-1	24 abc	37 bcde
WT-6-19	37 bcd	39 bcdef
WT-6-17	50 de	52 efg
WT-6-5	50 de	47 cdefg
WT-6-24	53 de	55 fg
WT-6-6	56 de	50 efg
None (uninfested control)	67 e	61 g
Dry antagonist preparation[d]		
WT-6-1	11 a	14 a
None (infested control)	16 ab	31 bc
WT-6 (wild strain)	20 abc	33 bcd
WT-6-19	20 abc	49 defg
WT-6-17	40 cd	46 cdefg
WT-6-24	48 de	53 efg
WT-6-5	50 de	52 efg
WT-6-6	54 de	37 bcde
None (uninfested control)	67 e	61 g

[a]Inoculum of *R. solani* isolate R-35 was produced on chopped-potato-soil (CPS) medium; large granular propagules (>589 μm) were separated as described by Wijetunga and Baker (14) and added to soil at 1% 1 wk before the antagonist, *T. harzianum*.
[b]In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.
[c]Conidia from V-8 juice agar plates added to soil to provide 4.4×10^5 conidia per gram of soil.
[d]Antagonists were grown on sand-cornmeal-bran-modified gliotoxin fermentation medium (SCB-GFM) for 3 wk at 25 C; preparations were dried, crumbled, and added to soil in amounts to provide 4.4×10^5 cfu per gram of soil.

DISCUSSION

From the evidence presented in this paper, there can be no doubt that the answer to the question raised by Rifai (12) as to whether one can easily induce mutations in the genus *Trichoderma* is positive. We presented evidence that it is feasible to induce new biotypes of *T. harzianum* with long and repeated exposures to UV irradiation. We do not know for certain, however, whether the new biotypes were mere selections of spores resistant to irradiation, or chromosomal mutations. From recent results with *Trichoderma* spp. in the field of industrial microbiology (4–6), however, it is most likely that the new biotypes obtained from the wild isolate WT-6 by irradiation were mutants rather than selections. There also can be no doubt that the field of research to improve strains of biocontrol agents for plant disease control by selection, genetic manipulation, or irradiation is now open for expansion in several areas.

Our assumption that the new biotypes were produced as a result of mutation rather than selection is supported by the fact that the wild strain WT-6 is sensitive to benomyl. The new biotypes possess considerable tolerance to the fungicide as indicated by their considerable growth or conidial germination in media containing benomyl. The demonstrated tolerance to benomyl by the biotypes is a characteristic that did not develop by prolonged exposure to increasing concentrations of benomyl, but it became evident immediately after irradiation in the third series. Prolonged and repeated exposures of WT-6 to increasing concentrations of benomyl did not yield any tolerant strains. Had there been variants within the wild strain with natural tolerance to benomyl, they would have appeared when conidia of WT-6 were placed on media containing the fungicide.

The experiment on fungitoxic metabolite production (Table 2) also strengthens our hypothesis that the new biotypes resulted from mutations. Isolate WT-6 produced only a heat-labile inhibitory factor. Of the 19 biotypes tested, five evidently produced two different factors, one heat-stable and the other heat-labile. Autoclaved filtrates of WT-6 and of 10 biotypes were not toxic to *S. cepivorum*, whereas autoclaved filtrates of the five biotypes were toxic to the assay organism. The activity of the filtrates sterilized by Millipore filters was almost twice as high as that of the autoclaved culture filtrates. Also, WT-6-11, the biotype that produced the highest amount of inhibitory factor against *S. cepivorum*, was also the most effective biotype against white rot of onion, and more effective than WT-6 (Table 5). However, that correlation was not observed when *R. solani* was used as the assay organism (T. H. Abd-El Moity and G. C. Papavizas, *unpublished*).

The ability of biotypes to produce an inhibitory factor against a given assay fungus, *S. cepivorum* in this case, could not be correlated with the ability to suppress saprophytic activity of *R. solani*. Neither could it be correlated with the ability of these biotypes to suppress disease caused by *R. solani* or to survive in soil. Biotype WT-6-11, the highest inhibitory factor producer, possessed low ability to suppress saprophytic colonization of a substrate by *R. solani* (Table 1) and to survive in the conidial state in soil (not included in Fig. 4). This observation suggests that *Trichoderma* spp. have diverse characteristics that may or may not be correlated with their efficiency as biocontrol agents.

Our results on cross-tolerance shown by biotypes that came from WT-6 to fungicides other than benomyl are inconclusive. It is difficult to show tolerance to fungicides such as captan, captafol, and even vinclozolin and iprodione. *T. harzianum* isolates, including WT-6, could tolerate high amounts (up to 2 mg a.i./ml) of these fungicides (7,8,11). Benomyl-resistant strains of *T. harzianum*, however, were resistant to thiabendazole, another benzimidazole fungicide (G. C. Papavizas, *unpublished*). With such natural tolerances, it is difficult to demonstrate additional tolerance. Development of multiple tolerant strains of biological control agents would be even more desirable than development of single fungicide tolerance for integrated pest management systems.

The tests on the effect of benomyl on conidial germination not only showed that conidia of biotypes tolerated relatively high amounts of benomyl in contrast to WT-6 conidia, but also revealed that it is possible to expose conidia to high concentrations of benomyl (suspensions containing 1,000 μg a.i./ml) for at least 4 wk without appreciable loss of viability. Conidia of *T. harzianum* possessing such tolerance can be used in spray programs in which the conidia and benomyl can be applied to the foliage or to soil in a single operation. It is interesting, however, that survival of conidia of WT-6 in the dormant state in soil was not affected by benomyl. This was expected since benomyl acts on the active phases of fungal development (the germination and growth processes), not on dormant spores (2).

We also showed that seed treatments and soil augmentation with conidia of WT-6 and of several UV-induced biotypes have the potential to significantly reduce white rot of onion, Pythium seed rot of peas, and *R. solani* damping-off of cotton and radish (Tables 3 to 5). Several UV-induced biotypes were consistently more effective in suppressing disease than WT-6. For instance, WT-6-5, WT-6-6, WT-6-17, and WT-6-24 were more effective in suppressing *R. solani* on cotton and radish than was WT-6. Two of these biotypes, WT-6-15 and WT-6-17, were also more effective than WT-6 in suppressing *P. ultimum* on Perfected Freezer peas. Also, WT-6-11 and WT-6-15 were more effective in suppressing white rot of onion than the wild strain WT-6. Therefore, the data on disease suppression in this paper, and data presented previously (7,8,11), suggest that considerable progress has been made in developing new effective strains of *T. harzianum*. Even more important may be the fact that it is possible to obtain new biotypes of biocontrol agents that differ from wild strains in many respects, including their compatibility with fungicides for integrated pest management programs.

TABLE 5. Biological control of white rot of onion caused by *Sclerotium cepivorum* with *Trichoderma harzianum* strain WT-6 and with selected UV-induced biotypes

Biotype	Infected seedlings (%)[a]	
	Conidia in water[b]	Dry antagonist preparation[c]
None (infested control)	46 a	46 a
WT-6-18	52 a	54 a
WT-6-1	50 a	52 a
WT-6-19	48 a	58 a
WT-6 (wild strain)	46 a	48 a
WT-6-6	34 ab	36 ab
WT-6-15	22 bc	26 bc
WT-6-11	8 c	12 c
None (uninfested control)	0 d	0 d

[a] Disease (percentage of plants in each pot infected with *S. cepivorum*) determined 8 wk after onion seedlings were transplanted in soil naturally infested with *S. cepivorum*.

[b] Conidia from V-8 juice agar plates added to soil to provide 4.4×10^5 conidia per gram of soil.

[c] Antagonists were grown on sand-cornmeal-bran-modified gliotoxin fermentation medium (SCB-GFM) for 3 wk at 25 C; preparations were dried and added to soil in amounts to provide 4.4×10^5 colony-forming units per gram of soil.

[d] In each column, values followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

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