Liquid Fermentation Technology for Experimental Production of Biocontrol Fungi

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ABSTRACT

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Large batches of biomass of *Gliocladium virens, Trichoderma hamatum, T. harzianum, T. viride,* and *Talaromyces flavus* were produced in liquid fermentation in 20-L vessels simulating industrial conditions by utilizing commercially available, inexpensive ingredients (molasses and brewer's yeast). The maximum biomass yield of *T. harzianum, T. viride,* and *G. virens* was obtained after 15 days of agitated incubation and that of *T. hamatum* at 10 days. The biomass of *T. flavus* consisted of hyphae devoid of spores and that of *Trichoderma* and *Gliocladium* of mycelial fragments, conidia, and chlamydospores. At 6 and 10 days of incubation, about half of the spores were mature chlamydospores, and at 15 days 75% were mature chlamydospores. About 80–90% of the chlamydospores in the fresh biomass germinated on a medium selective for *Trichoderma.* Air-dried mats were ground and mixed (25 or 50%, w/w) with a commercially available pyrophyllite carrier. Conidia of *Trichoderma* in pyrophyllite survived better at −5, 5, 15, 25, and 30 C than biomass propagules (BP) alone. The best temperatures to prolong shelf life of conidia and BP in pyrophyllite were −5 and 5 C and the worst were 25 and 30 C. Biomass propagules of *T. flavus* had 44% survival at room temperature after 5 mo. Biomass propagules of *Gliocladium* and *Trichoderma*, added to soil at 5×10^3 colony-forming units per gram, fragmented or proliferated greatly and attained numbers from 2×10^6 to 6×10^6. In no case did conidia of *Trichoderma* or *Gliocladium*, added without a food base, proliferate in soil.

The rapid expansion of fermentation biotechnology over the past 30 yr has led to a greater awareness of the usefulness of filamentous fungi for the production of large amounts of acids, antibiotics, enzymes, and fuels from inexpensive and/or waste ingredients (10). Although fungal biotechnology has been oriented toward the food and energy industry, fungal and bacterial biotechnology has moved to new areas such as production of biological insecticides (2) and mycoherbicides (1,11). Fermentation technology for biocontrol agents of plant diseases is almost nonexistent, especially for small-scale experimentation.

If widespread biological control of soilborne plant pathogens is to be achieved by augmentation or seed treatment, it is necessary to mass-produce promising antagonists rapidly in the form of spores, mycelia, or mixtures. This can be achieved with liquid media in agitator-stirred fermentors. If infectious inocula from such systems are viable after being dried, milled, and formulated, the liquid fermentation approach is preferable to growing the antagonists in semisolid or solid fermentations.

In this paper we report on: a small-scale fermentation process for experimental production of several biocontrol agents of soilborne plant pathogens, optimum biomass production and the kinds of propagules produced, and behavior and survival of propagules in soil and in storage. A preliminary report has been presented (3).

MATERIALS AND METHODS

The biocontrol fungi used were: *Talaromyces flavus* (Klöcker) Stolk & Samson (teliomorph of *Penicillium vermiculatum* Dangeard) strain TF1 previously shown to have good biocontrol ability against Verticillium wilt of eggplant (6); *Gliocladium virens* Miller et al strains Gl-3, Gl-9, and Gl-17 isolated from sclerotia of *Sclerotium rolfsii*; *Trichoderma hamatum* (Bon.) Bain. strain Tm-1; *T. harzianum* Rifai strains Th-7 and Th-58; and *T. viride* Pers. ex Gray mutants T-1-R4 and T-1-R9. With the exception of Tm-1 obtained from Taiwan, all strains used were from the collection of the Soilborne Diseases Laboratory. The two mutants, both resistant to benomyl, were produced by UV irradiation (7,8). Stock cultures of *T. flavus* were maintained on potato-dextrose agar

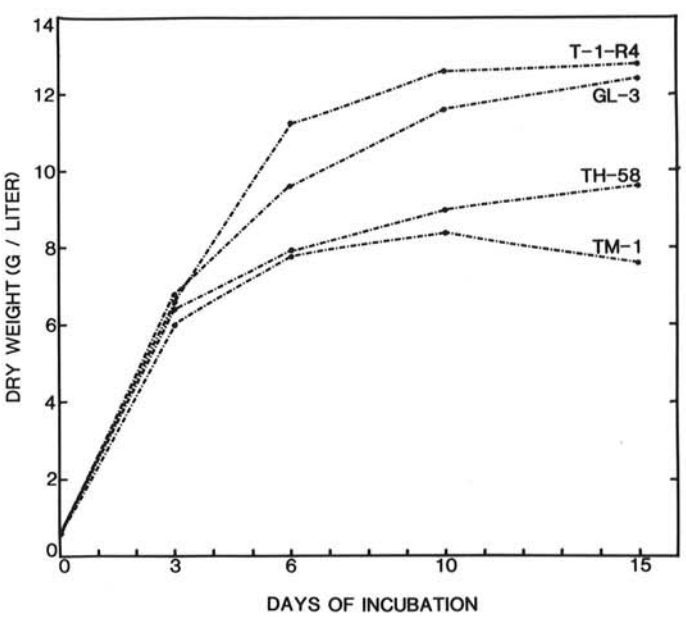

Fig. 1. Biomass (expressed as grams dry weight per liter) of *Trichoderma hamatum* (Tm-1), *T. harzianum* (Th-58), *T. viride* (T-1-R4), and *Gliocladium virens* (Gl-3) in agitated 17-L submerged cultures of molasses-brewer's yeast medium in 20-L carboys. Each point represents the mean of five replications.

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(PDA) slants and those of the other biocontrol fungi on V-8 juice agar (200 ml of V-8 juice, 800 ml of water, 15 g of agar, and 6.0 ml of 1.0 N NaOH).

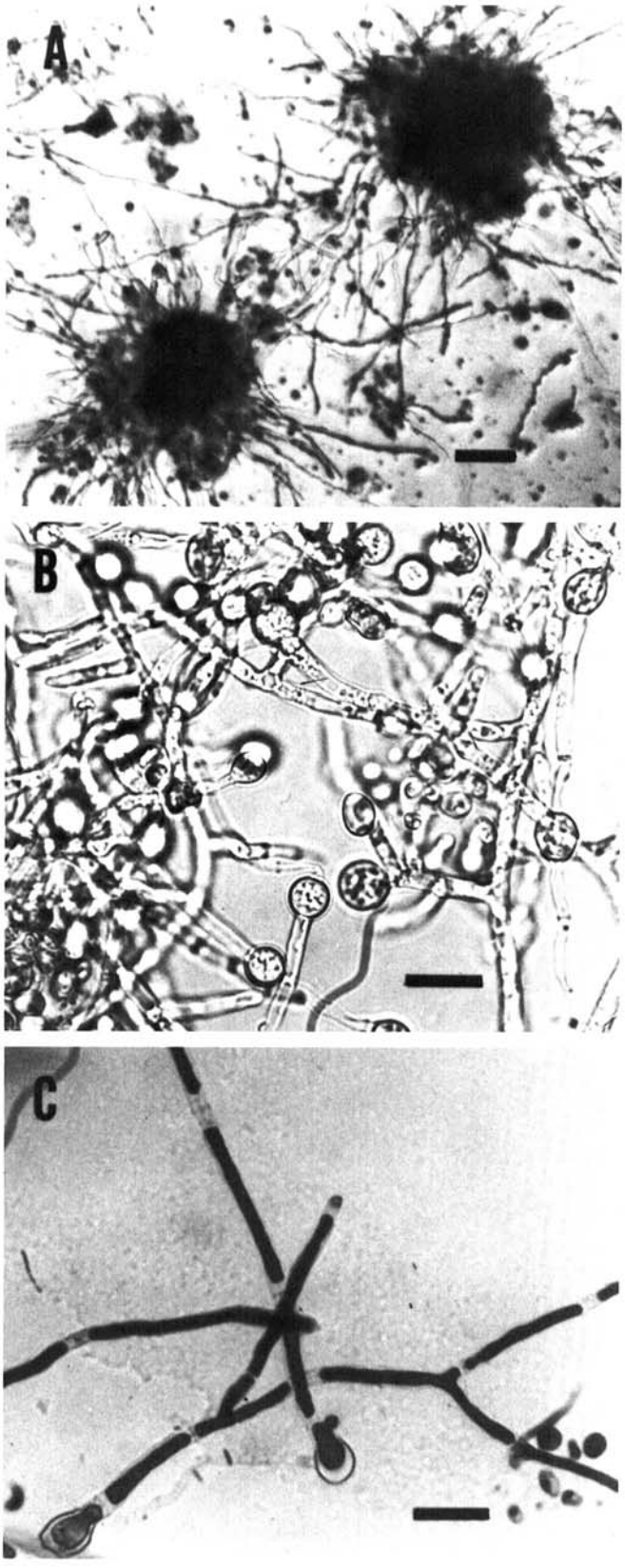

Fig. 2. Fermentor biomass preparations of *Trichoderma* spp. **A,** Aggregates of germinated chlamydospores of *T. harzianum* (Th-58) (bar = 60 μm). **B,** Mycelial fragments and intercalary chlamydospores of *T. viride* (T-1-R9) (bar = 30 μm). **C,** Germinated, aniline-blue stained chlamydospores of *T. viride* (T-1-R9) (bar = 30 μm).

Liquid fermentation. To simulate industrial production, 20-L autoclavable polypropylene carboys with 8-cm-diameter screw caps were used. A 3-cm-diameter hole was made close to the neck of each carboy and each hole was plugged with a rubber stopper perforated to allow air to flow into the medium through a glass tube that reached 5 cm from the vessel bottom. Compressed air from a centralized building system was passed through a 3.0-μm filter and allowed to enter the broth, simultaneously aerating and agitating it during the entire growth period. Foam control was obtained by adding an antifoam compound (Antifoam A; Sigma Chemical Co., St. Louis, MO 63178). The fermentation system is a modification of that used by McCabe (D. McCabe, *personal communication*). The system is similar to commercial systems in that it utilizes inexpensive growth materials under deep-tank fermentation conditions.

Both the fermentation medium and the starter medium contained (per liter) 30 g of molasses and 5 g of brewer's yeast. Fifteen liters of medium were placed in each carboy and these were autoclaved for 1 hr on each of two consecutive days. The fungi were first inoculated into 500-ml portions of the starter medium in 1-L flasks and the flasks were shaken on a rotary shaker for 5 days at room temperature. Two liters of inoculum suspension were used to inoculate the 15 L of medium in the carboys, thus raising the total volume in the carboys to 17 L. The fermentation broth was then agitated by bubbling filtered air through it for 5–15 days.

Separation of the fungal biomass from the aqueous broth was accomplished by filtration through a cotton muslin filter on an 18-cm-diameter Büchner funnel. The mats were air-dried for 3 days, weighed, and ground in a Wiley mill through a 425-μm (40-mesh) screen. The resulting powder was either used as such or mixed (10, 25, or 50% [w/w]) with Pyrax® ABB (pyrophyllite [anhydrous aluminum silicate, pH 7.0], R. T. Vanderbilt Company, Inc., Norwalk, CT 06855) as a diluent to increase the volume for distribution purposes.

Viability and survival of propagules of *Trichoderma*. A small amount (100 mg) of the powder that resulted from grinding the solids from the fermentors as described above (hereafter referred to as fermentation biomass) (FB), was suspended in water, comminuted in a homogenizer for 5 min, diluted with water (1:10), and 1-ml aliquots were pipetted and spread on the surface of the Trichoderma-selective agar medium TME (9) in petri plates. The preparations were examined microscopically after 0 and 18–24 hr to determine the kinds of propagules in the FB and the germinability of the chlamydospores. Propagule numbers were determined with a hemacytometer.

An FB batch of *T. viride* T-1-R9 in Pyrax® was subdivided into 10-g samples, placed in 50-ml vials (five replications), covered loosely, and stored at −5, 5, 15, 25, and 30 C. After 0, 3, 8, 15, and 20 wk, 100-mg samples were removed from the vials after the contents were mixed and suspended in sterile distilled water to make dilutions of 1 in 10^4, 1 in 10^5, 1 in 10^6, and 1 in 10^7. One-milliliter aliquots were removed from the containers while the liquid was agitated by a magnetic stirrer and spread on the TME medium (six plates per replication, four replications). The plates were incubated for 6 days under continuous fluorescent light at room temperature and colonies were counted to determine the number of viable colony-forming units (cfu) per gram of preparation.

Survival of propagules of *T. flavus* TF1 in the FB (in a 25% mixture with Pyrax®) was also monitored over time at room temperature (23 ± 3 C). Viability was tested with the dilution-plate method on a recently described selective medium (5).

Survival in soil and in a soilless mix. Conidia and FB of *T. hamatum* Tm-1 and *G. virens* Gl-3 in Pyrax® were suspended in water and added to 100-g (dry-weight equivalent) portions of natural Rumsford sandy loam (RSL, pH 6.0) and Sassafras loamy sand (SLS, pH 6.4) in 400-ml beakers at 5×10^3 cfu/g. Conidia, used for comparison, were obtained from 6-day-old cultures grown on V-8 juice agar under continuous fluorescent light by adding a few milliliters of sterile water to the cultures and gently rubbing the surface with a sterile cotton-tipped applicator. Conidia were counted in a hemacytometer. The propagule numbers of the FB were determined with the dilution-plate method on the TME

medium. The soils were brought to the laboratory from the field before use and the propagules were added with enough water to adjust the moisture tension in all samples to −0.3 bars and the containers were covered with polyethylene film to prevent water evaporation. Conidia and FB of Gl-3 were also added at 2×10^3/g of a soilless mix (Redi-earth®, W. R. Grace & Co., P.O. Box 238, Fogelsville, PA 18051) in 100-g portions in 600-ml beakers. Four replications were used and the soils and the soilless mix were incubated at 25 ± 2 C.

The two soils and the soilless mix were assayed for viable colony-forming units at 0, 10, 20, 40, 60, 80, and 130 days and at 0, 7, 21, 42, and 112 days, respectively, with the dilution-plate method (dilution, 1:1,000 or higher) on the TME medium. The plates were incubated at 25 ± 2 C under continuous fluorescent light and colonies were counted after 6 days.

All experiments were done twice with four or five replications.

RESULTS

In preliminary tests, large batches of biomass of *G. virens* (Gl-3, Gl-9, and Gl-17), *T. hamatum* (Tm-1), *T. harzianum* (Th-7 and Th-58), *T. viride* (T-1-R4 and T-1-R9), and *T. flavus* (TF1) were produced in liquid fermentation in 20-L vessels. These contained 15–17 L of molasses-brewer's yeast broth and thus simulated industrial fermentation conditions by utilizing commercially available, inexpensive ingredients. For instance, during 5 days of growth in the molasses-yeast medium with aeration, *T. flavus* produced 8 g of dry mat per liter. *T. hamatum* yielded 7–8 g of dry mat per liter which, when mixed with Pyrax® (10%, w/w), contained 10^7 propagules per gram. Substitution of cottonseed flour or corn steep liquor for brewer's yeast also gave good results.

Yield of fermentation biomass. A test was performed with *T. hamatum* Tm-1, *T. harzianum* Th-58, *T. viride* T-1-R4, and *G. virens* Gl-3 to determine the effect of length of incubation on dry weight of FB in 17 L of medium in the 20-L fermentors. Aliquots of 1 L were aseptically removed from the fermentors 0, 3, 6, 10, and 15 days after the medium in the fermentors was inoculated. The solids were dried overnight at 80 C and the dry weights were determined. With the exception of Tm-1, the maximum FB yield (expressed as grams per liter) was obtained after 15 days of incubation; the yield was only slightly higher than that obtained after 10 days (Fig. 1). The best FB yield with Tm-1 was obtained after 10 days of incubation. With no exceptions, the pH of the fermentation medium increased from 5.6 initially to between pH 7 and 8.

Fungal components and numbers in FB. Because of the method of filtering and grinding the mats, most of the biomass of the nine fungi that were studied existed as clumps of hyphae, spores, and yeast cells (Fig. 2A). The comminuted and diluted biomass of *T. flavus* TF1 consisted of hyphae devoid of ascospores, conidia, or chlamydospores. The diluted biomass of *Trichoderma* isolates Tm-1, Th-58, T-1-R4, and T-1-R9 and of *G. virens* Gl-3 consisted of mycelial fragments, immature chlamydospores, and mature chlamydospores (Fig. 2B). After 3 days of incubation, few, if any, chlamydospores were formed. At 6 and 10 days, about half of the spores of the isolates of *Trichoderma* and Gl-3 were mature chlamydospores. At 15 days, about 75% of the spores were mature chlamydospores, the remainder being conidia and immature chlamydospores. The benomyl-resistant biotype T-1-R9 produced more chlamydospores in the fermentor than any of the other isolates or biotypes of *Trichoderma* or *Gliocladium* that were studied. About 80–90% of the chlamydospores in fresh biomass germinated on the TME medium within 24 hr (Fig. 2C).

TABLE 1. Spore formation by *Trichoderma hamatum* (Tm-1), *T. harzianum* (Th-58), *T. viride* (T-1-R4), and *Gliocladium virens* (Gl-3) in molasses-brewer's yeast broth in 20-L carboys

	Spores[y] (× 10^9/g [dry weight] of biomass) at day:			
Isolate	3	6	10	15
Tm-1	2.4 a[z]	2.1 c	2.6 b	1.7 a
Th-58	3.6 a	4.0 ab	2.8 ab	3.2 a
T-1-R4	3.3 a	4.4 a	4.2 a	2.4 a
Gl-3	3.0 a	2.7 bc	3.3 ab	2.7 a

[y] Conidia, chlamydospores, and immature chlamydospores.

[z] Numbers in each column followed by the same letter do not differ significantly from each other according to Duncan's multiple range test, $P = 0.05$.

The number of spores produced per gram of dry FB varied with the isolate studied and, to some extent, with the length of incubation (Table 1). At 3 and 15 days of incubation, there were no significant differences in the number of spores per gram of FB among the four isolates (Tm-1, T-1-R4, Th-58, Gl-3) studied in the fermentor experiment. At 6 days, Tm-1 had produced fewer spores per gram of FB than Th-58 or T-1-R4, and at 10 days Tm-1 had produced fewer spores than T-1-R4. Statistical analysis based on Duncan's multiple range test performed to detect differences as affected by the length of incubation showed that the number of spores per gram of FB of Tm-1, Th-58, and Gl-3 did not differ with length of incubation up to 15 days. Isolate T-1-R4 had fewer spores per gram at 15 days than at 3, 6, and 10 days.

Shelf life of conidia and FB as affected by temperature. Viability of conidia and propagules in the FB of *T. viride* T-1-R9 stored at various temperatures was reduced with length of incubation. The reduction was more pronounced at 25 and 30 C than at lower temperatures (Fig. 3). In general, conidia in Pyrax® survived better than biomass propagules at most temperatures tested (Fig. 3A).

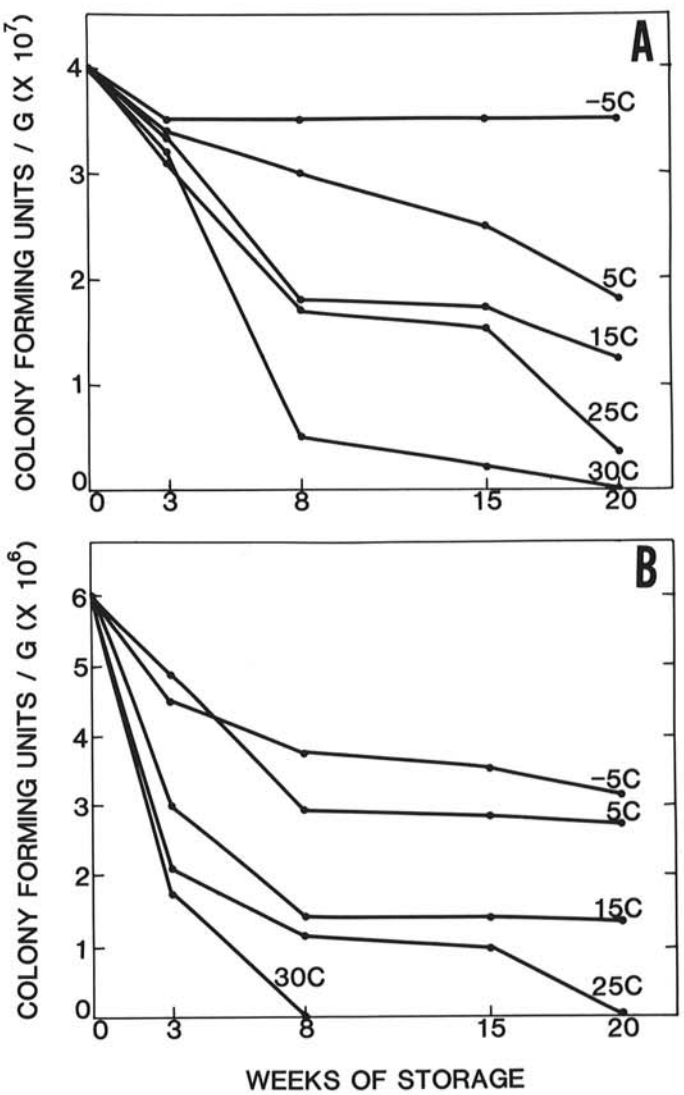

Fig. 3. Shelf life of **A,** conidia alone and **B,** colony-forming units (conidia, chlamydospores, and mycelial fragments) in fermentor biomass of *Trichoderma viride* (T-1-R9) as affected by length of storage at various temperatures. Each point is the mean of five replications.

Similar amounts (about 50%) of conidia and FB propagules survived at 5 C during 20 wk of storage. About 8% of the conidia lost viability at −5 C within 3 wk of storage with no further loss in viability after 20 wk of storage. Approximately 50% of the conidia were viable at 15 and 25 C after 8 wk of storage. In contrast, less than one-third of the propagules in the biomass were viable at 15 and 25 C after 8 wk; and after 20 wk, none of the propagules in the biomass survived at 25 or 30 C (Fig. 3B).

Although the *T. flavus* FB consisted of hyphae only, these propagules mixed with Pyrax® survived better than those of *Trichoderma*. For instance, after 5 mo, 44% of the mycelial propagules in the biomass of *T. flavus* survived.

Survival in soil and in a soilless mix. Ten days after addition of conidia to the two soils (SLS and RSL), survival of *T. hamatum* Tm-1 and *G. virens* Gl-3 was relatively high (recovery of colony-forming units was about 100% of the original numbers of conidia added) (Table 2). After 60 and 130 days, colony-forming units recoverable from the conidia-infested soils had dropped considerably. In no case did the conidia proliferate in soil. FB of both fungi, added at zero time at 5×10^3 cfu/g of soil, survived well in soil. Approximately 2.34×10^6 and 4.08×10^6 colonies of Tm-1 were counted from SLS and RSL, respectively, after 10 days of incubation; and Gl-3 had 1.9×10^6, and 5.56×10^6 in SLS and RSL, respectively. At 60 and 130 days the number of colony-forming units of both fungi dropped to about one-third or one-fourth of those obtained at 10 and 20 days.

Similar results were obtained when *G. virens* Gl-3 was added at 2×10^3 cfu/g as conidia or FB to a soilless mix (Table 3). After 21, 42, and 112 days numbers of colony-forming units recoverable from the conidia-infested soilless mix were lower than those added at zero time, whereas colony-forming units from FB-infested mix were 250- to 300-fold higher at 21, 42, and 112 days than those added at zero time.

DISCUSSION

From the evidence presented in this paper, there can be no doubt that it is possible to use inexpensive liquid media such as molasses and brewer's yeast to produce viable inocula of various organisms (*Gliocladium, Talaromyces*, and *Trichoderma*) with a liquid fermentation system simulating large-scale industrial production. Previous research (4) showed that several isolates of *Trichoderma* spp. can develop large amounts of biomass containing conidia and chlamydospores in both liquid and solid media containing inexpensive ingredients. The liquid fermentation system used is simple, the medium inexpensive, the biomass yield satisfactory, and contamination is not a serious problem if reasonable precautions are taken. The only problems encountered were related to filtering, drying, and grinding the biomass. This was due to lack of sophisticated equipment during experimentation. Availability of proper equipment would solve the problems under industrial production. Such problems would be more aggravating if our antagonists were grown in solid fermentations. No attempts were made in our tests to study the relationship between the rates of aeration and agitation to the yield and kinds of propagules produced in the system. Also, the importance of culture pH on the morphological form and the rheological properties of the antagonists grown in continuous culture was not studied. In another investigation (4), numbers of conidia and chlamydospores produced were not influenced by initial pH of the media, or by continuous maintenance of the media at pH 4 or 7.

The fermentation test with *T. harzianum* (Th-58), *T. viride* (T-1-R4), and *G. virens* (G1-3) showed that the highest biomass yield was obtained after 12 and 15 days of incubation (Fig. 1). The highest yield with *T. hamatum* (Tm-1) was obtained after 9–10 days of incubation. Although 10–15 days of incubation may be quite long under industrial conditions, this disadvantage is compensated by the increased concentration of mature chlamydospores in the biomass. High numbers of mature chlamydospores may prolong shelf life of preparations. This, however, is only an assumption since our survival studies (Fig. 3) with *T. viride* T-1-R9 at various temperatures were done with FB preparations obtained from 5-day-old agitated cultures. More studies are needed to determine the relationship between culture media, length of incubation, kinds of propagules obtained, diluents used, and shelf life.

Shelf life of conidia, and especially propagules in FB (both mixed with Pyrax®) of *Trichoderma* spp. and *G. virens*, was shorter than one might expect. In contrast, shelf life of mycelia of *T. flavus* in FB was considerably longer than that of *Trichoderma* and *Gliocladium*, despite the fact that the *T. flavus* FB was composed exclusively of mycelial fragments. This may be explained by assuming that the mycelium of *T. flavus* is more resistant to desiccation, grinding, and exposure to Pyrax® than the propagules of the other two antagonists. The effects of diluents used to formulate biocontrol agents have been studied with mycoinsecticidal fungi (11), but not with biocontrol fungi of plant pathogens.

Our studies on the comparative survival of conidia and FB in soil and in a soilless mix showed that FB of *Trichoderma* and *Gliocladium*, added in small numbers (2×10^3 or 5×10^3/g of mix and soil, respectively), appeared to proliferate to such an extent that populations as high as 5×10^6 and 6×10^6/g of soil were not uncommon 10 or 20 days after addition of the FB to soil. In contrast, numbers of conidia remained static and were even reduced after 20 days or more in soil. The apparent proliferation of FB, but not of conidia, could be accounted for by assuming that FB aggregates that passed the 425-μm screen broke into smaller, individual units, most likely chlamydospores, after exposure to various soil factors, including the lytic activity of soil microbiota. Microscopic examination of FB aggregates showed that these were composed of hundreds of chlamydospores held by mycelial mats. We have repeatedly observed, however, that soil fortified with *Gliocladium* or *Trichoderma* FB at as low as 10^3 cfu/g allowed the antagonists to grow and proliferate as evidenced by the masses of green conidia and mycelia that appeared on the soil surface after 5–7 days (G. C. Papavizas and J. A. Lewis, *unpublished*). No such proliferation was observed even when conidia were added to soil at

TABLE 2. Recovery of *Trichoderma hamatum* Tm-1 and *Gliocladium virens* Gl-3 from two soils as estimated by the dilution plate method on Trichoderma medium E

Isolate and sampling time (days)	Colony-forming units per gram of soil ($\times 10^3$)[z]			
	Conidia		Fermentor biomass	
	SLS	RSL	SLS	RSL
Tm-1				
10	4.2	4.6	2,340	4,080
20	4.0	2.6	1,500	5,600
60	3.4	1.8	880	1,500
130	1.6	1.6	720	1,020
Gl-3				
10	6.0	5.8	1,910	5,560
20	5.2	4.8	1,380	6,060
60	3.8	3.0	946	2,040
130	3.0	2.2	800	1,920

[z]Conidia and fermentor biomass were added to the soil at 5×10^3 propagules per gram at zero time. Acronyms: SLS = Sassafras loamy sand and RSL = Rumsford sandy loam.

TABLE 3. Recovery of *Gliocladium virens* Gl-3 from a soilless mix by using the dilution plate method on Trichoderma medium E

Days of sampling	Colony-forming units per gram of soilless mix ($\times 10^3$)[z]	
	Conidia	Fermentor biomass
7	2.4	723
21	1.5	681
42	1.2	511
112	0.8	520

[z]Conidia and fermentor biomass were added to the soil at 2×10^3 propagules per gram at zero time. Soilless mix was a commercial preparation containing ground vermiculite, sphagnum peat moss, and processed bark.

10^6/g. It was not determined whether the increase in colony-forming units resulting from FB, but not from conidia, is due to aggregate fragmentation, or to other factors such as carry-over of nutrients in the biomass, differential impact of fungistasis on the propagules, sporulation in soil, colonization of organic matter, or other factors.

Irrespective of whether we are dealing with fragmentation of FB or actual proliferation, the extremely high numbers resulting by augmenting the soil with FB, but not with conidia, may be sufficient to suppress soilborne plant pathogens, especially during the first 10–20 days after soil augmentation. In fact, tomato fruit rot caused by *Rhizoctonia solani* was considerably reduced by FB of various isolates of *Trichoderma* and *Gliocladium*, but not when equal or higher numbers of their conidia were added to soil (J. A. Lewis and G. C. Papavizas, *unpublished*). We may assume at this time that conidia of *Trichoderma* and *Gliocladium* may not be the most favorable spore forms for biological control, unless methods are developed to stimulate their survival and proliferation in soil. In addition, unless solid fermentations are used, conidia are not produced abundantly in submerged cultures, an approach most likely to be favored by industry. However, the large number of chlamydospores produced in relation to conidia in both liquid and solid fermentation in this study and in a previous one (4) suggests that the chlamydospore of *Trichoderma* or *Gliocladium* should be considered as the most important propagule produced during fermentation and the propagule that should be studied for its survival and proliferation in soil.

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