Alginate Prill Formulations of *Talaromyces flavus* with Organic Carriers for Biocontrol of *Verticillium dahliae*

D. R. Fravel, J. A. Lewis, and J. L. Chittams

First and second authors: Biocontrol of Plant Diseases Laboratory, BARC-West, ARS, USDA, Beltsville, MD 20705; third author: University of Maryland/USDA Statistical Consulting Service, Beltsville, MD 20705.

ABSTRACT

Fravel, D. R., Lewis, J. A., and Chittams, J. L. 1995. Alginate prill formulations of *Talaromyces flavus* with organic carriers for biocontrol of *Verticillium dahliae*. Phytopathology 85:165-168.

Pyrophyllite clay (Pyrax), milled chitin, corn cobs, fish meal, neem cake, peanut hulls, soy fiber, and wheat bran were used to make alginate prill with or without ascospores of *Talaromyces flavus*. The formulations were compared for their ability to induce *T. flavus* to control Verticillium wilt of eggplant in the greenhouse in field soil and to increase populations of *T. flavus* in three field soils (two loamy sands, one silty clay). Survival of *T. flavus* in prill at 5 C or ambient temperature (22–24 C), as well as the carbon (C) and nitrogen (N) contents of the prill, were also determined. Two formulations (corn cobs and pyrophyllite) consistently enhanced biocontrol activity. In treatments without *T. flavus,* half of all plants were wilted 59 days after transplanting whereas more than half of the plants treated with *T. flavus* in either pyrophyllite or corn cob prill remained symptomless 90 days after transplanting. In some experiments, *T. flavus* in soy fiber prill delayed the median time for symp-

tom development by about 10 days. Experiments on survival and proliferation in soils indicated that there were highly significant interactions among carrier, soil, and sampling time. These interactions indicate that the formulations performed differently in different soils. Populations of *T. flavus* from some prill × soil × combinations increased during the 18-wk experiment while populations from other combinations remained constant or decreased. Populations of *T. flavus* in two soils (Hatboro loamy sand, silty clay) amended with wheat-bran prill were greater than those with other formulations in the first 2 wk of assay in these two soils. Populations of *T. flavus* in the loamy sand amended with peanut hull prill were greater than those from other prill at the 8- and 12-wk samplings in this soil. Prill with pyrophyllite and corn cobs had significantly greater C/N ratios than prill with other carriers. Carriers significantly affected survival of *T. flavus* at 5 C and at ambient temperature. Survival at both temperatures was best in prill formulated with corn cobs, soy fiber and peanut hulls. Temperature did not alter the survival pattern during the 18-wk sampling period.

Formulation of a biocontrol agent may greatly affect the success of biocontrol (4). Formulation can also influence the length of time the biocontrol agent can be stored (shelf life) and the survival and proliferation of the biocontrol agent in soil (17). The entrapment of propagules of biocontrol agents in alginate prill can be customized for a particular situation through addition of nutrients, fungicides, or other materials that may confer a selective advantage on the antagonist in soil. The basic formulation consists of an aqueous suspension with 1–20% sodium alginate, approximately 10–70 % of a carrier or combination of carriers and propagules of the biocontrol agent (5,13). This suspension is added dropwise to a gellant, usually a $CaCl_2$ solution. The resulting beads are collected, rinsed, and dried to produce uniform prill, usually 0.5 mm in diam.

Alginate prill have been used to deliver several biocontrol agents, including *Talaromyces flavus* (Klöcker) A. C. Stolk & R. A. Samson (3) as well as isolates of *Trichoderma* spp. (12) and *Gliocladium virens* (12,13). *T. flavus* is a potential biocontrol agent against *Verticillium dahliae* Kleb. (6,10,15), *Sclerotinia sclerotiorum* (Lib.) de Bary (16), and *Rhizoctonia solani* Kühn (1). Previous work on delivery of *T. flavus* with alginate prill was confined mainly to prill made with pyrophyllite (3). The present study was undertaken to determine the effects of various inexpensive organic carriers as the carrier in alginate prill on the efficacy of control of *V. dahliae* by *T. flavus*. The effects of these organic carriers on the shelf life of formulated *T. flavus* and on the survival and proliferation of *T. flavus* in soil were also determined.

MATERIALS AND METHODS

Alginate prill. The following organic carriers were milled to pass through a 0.425-mm pore-size screen: chitin (Protan Laboratories, Redmond, WA), corn cobs (Grit-o-Cobs, The Anderson's, Maumee, OH), fish meal (Menhanden, H.J. Baker Brothers, NY), neem cake (W.R. Grace and Co., Columbia, MD), peanut hulls (Birdsong, Franklin, VA), soy fiber (Nutrisoy, Archer Daniels Midland Corp., Decatur, IL), and wheat bran. Alginate prill were prepared using each of these carriers or pyrophyllite (hydrous aluminum silicate) (Pyrax, R. T. Vanderbuilt Co., Norwalk, CT) as the carrier. For each carrier, 63 g of the material was mixed with 450 ml of distilled water. An alginate suspension was prepared by blending (Waring, Winstead, CT) 9 g of alginate (IG-350, Corpco, NY) with 450 ml of distilled water for approximately 1 min. Following overnight refrigeration, 450 ml of each carrier suspension was mixed with 450 ml of the alginate suspension and with either 150 ml of sterile distilled water or 150 ml of a 10^6 ascospores per milliliter aqueous suspension of *T. flavus*. The mixture was stirred approximately 5 min using a magnetic stir bar. Samples of the resulting suspensions were diluted and plated on a medium semiselective for *T. flavus* (TF agar) (14) to monitor viability of *T. flavus*. The suspensions were added dropwise to 0.2 M $CaCl_2$. After approximately 20 min, prill were collected on 1-mm pore-size plastic mesh, rinsed with tap water, spread on paper, and dried overnight under ambient conditions. Dried prill contained 10^5 cfu/g. Additional prill not containing ascospores of *T. flavus* were prepared in the same manner. Prill were manufactured on five different dates (lots).

Biocontrol ability. One-half gram of each formulation, with or without ascospores of *T. flavus,* were mixed with 100 g of soilless potting mix (Pro-Mix BX, Premier Brands, New Rochelle,

NY) and placed in a 8.9-cm-diameter plastic pot. Additional pots contained only 100 g of Pro-Mix BX (control treatment). Two seeds of eggplant (cv. Black Beauty, Harris Seed, Rochester, NY) were planted in each pot. After emergence, pots were thinned to one plant per pot. There were 9, 16, and 16 pots per treatment for the first, second, and third repeats of the experiment (three lots of prill), respectively.

Six to nine weeks after seeding, plant height was recorded, and plants were examined for phytotoxicity. Plants, along with the soilless mix in which they were grown, were transplanted into a Rumford Loamy Sand (RLS) infested with 30 microsclerotia per gram of *V. dahliae*. Microsclerotia *V. dahliae* isolate V-1 (6) were produced by growing the pathogen at 21 C for 4–6 wk on Czapex-Dox agar (Difco, Detroit, MI). The soil was collected from the field the day before use and was screened through a 0.5-cm pore-size screen to remove rocks and plant debris. In addition to treatments of prill with the carriers but without *T. flavus*, a *V. dahliae*–alone treatment (disease check) and a treatment not infested with either the pathogen or the antagonist (healthy check) were included as additional controls. Plants were arranged in a randomized complete block design. Plants were examined every 2–3 days for symptoms of Verticillium wilt. The date of first symptom expression for each plant was recorded and petiole isolations were made on polygalacturonic acid–soil extract agar (9) to confirm infection by *V. dahliae*. The period from transplanting until first symptom expression was called the survival time.

Analysis of disease progress curves is usually based on populations of plants and percentage incidence in each replicate is recorded. In our experiments, data recorded were binary, that is, each plant was either diseased or not diseased. Thus, an alternative data analysis was needed. A survivor analysis was employed using SAS (proc lifetest, SAS, Cary, NC) to produce Kaplan-Meier product limit estimates (8). Kaplan-Meier product limit estimates of survival rates are used routinely in medical clinical survivorship studies. The most commonly used summary statistic in survival analysis is the median survival time (the date at which half of the individuals in the treatment are diseased).

Gehan's generalized Wilcoxon test was used to compare survival curves of the various treatment groups (7). This test gives more weight to early wilting than to late wilting. Thus, the generalized Wilcoxon test is more likely to detect early differences in survival distributions. Specifically, this test allowed us to determine which formulations with *T. flavus* significantly delayed onset of Verticillium wilt. In addition, the Wilcoxon test was used to determine if there were significant block effects and if the three runs of the experiment were similar.

Shelf life and C and N content. When prill were dry (24 h after prill manufacture), populations of *T. flavus* in the prill were determined by dissolving the prill in a mixture of 8.7×10^{-2} M KH_2PO_4 and 3.0×10^{-2} M Na_2HPO_4 for 1 h followed by dilution plating onto TF agar (5). The number of cfu were recorded 10 days later. Approximately 15 g of each formulation were stored at either ambient temperature or at 5 C. Every other week for 12 wk, populations of *T. flavus* in the prill were determined. The experiment was repeated with four different lots of prill.

The total C and N in each formulation containing ascospores was determined by analytical methods by the Soils Laboratory of the University of Maryland. Carbon/nitrogen (C/N) ratios were calculated for each type of prill (Table 1). Three lots of prill were used and the lots were considered replicates.

Survival and proliferation in soil. To study the effect of the formulated carriers on survival and proliferation of *T. flavus* in soils, 0.5 g of each type of prill, with or without ascospores of *T. flavus*, were mixed with 100 g of each of three soils: *(1)* the RLS used in the biocontrol experiment (74.3% sand, 17.1% clay, 8.6% silt, 0.3% humic matter, pH 5.4); *(2)* a Hatboro Loamy Sand (HLS) (59.4% sand, 28.8% clay, 11.8% silt, 3.2% humic matter, pH 4.5); and *(3)* a silty clay soil (SiC) (17.7% sand, 40.1% clay, 42.2% silt, 0.0% humic matter, pH 2.4). Soils were collected from the field the day before use and were screened as in the biocontrol experiment. Treatments were replicated four times. Soils were kept in 8.9-cm-diameter pots in the greenhouse at 20–23 C and watered as if they contained plants. At 2, 4, 6, 8, 10, and 12 wk, soil in each pot was transferred to a plastic bag and mixed thoroughly. Soils were mixed due to the extremely aggregated distribution of propagules resulting from the point-source inoculum nature of the prill. Approximately 5 g of soil was removed and the remaining soil was returned to the pot and used in subsequent samplings. The sample was dried overnight under ambient conditions, then 1 g of the dried soil was used for dilution plating onto TF agar. The number of cfu was determined after 10 days. Since there was an indigenous population of *T. flavus*, the number of cfu in soil amended with prill not containing *T. flavus* was subtracted from the cfu count for the prill containing ascospores of *T. flavus*. There were 10–14 pots per treatment and the experiment was repeated with four different lots of prill.

TABLE 1. Carbon/nitrogen (C/N) ratios of alginate prill formulations with organic carriers

Formulation	C/N
Pyrophyllite	158.8 a[z]
Corn cobs	96.6 b
Peanut hulls	39.2 c
Soy fiber	25.4 c
Wheat bran	21.1 c
Neem cake	14.8 c
Chitin	8.0 c
Fish meal	5.1 c

[z] Values followed by same letter are not significantly different from each other according to Duncan's multiple range test ($P < 0.05$).
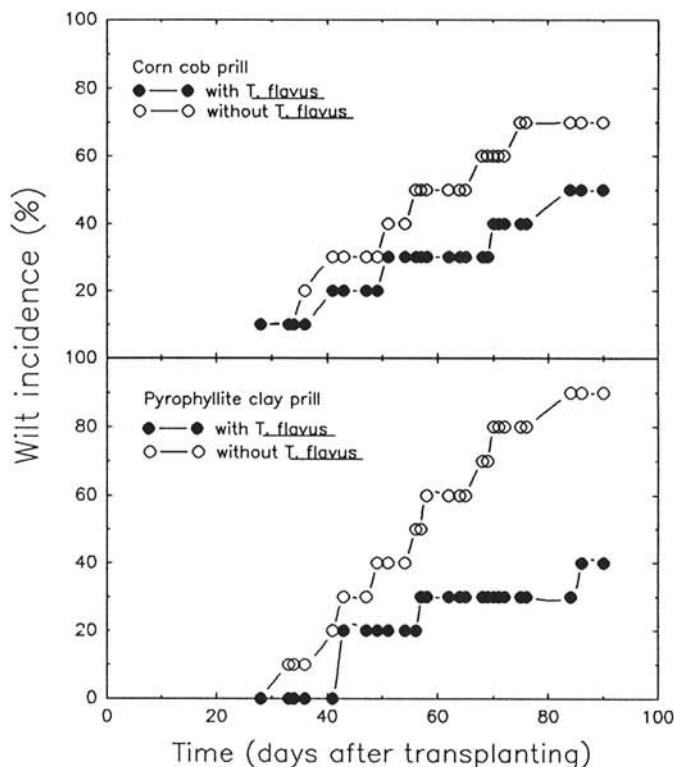

Fig. 1. Effect of alginate prill carrier and *Talaromyces flavus* on disease progress curves for Verticillium wilt of eggplant. Alginate prill with or without ascospores of *T. flavus* incorporated in transplant soil at seeding. After 9 wk, plants transplanted into soil infested with *Verticillium dahliae*. Corn cobs or pyrophyllite used as carrier in prill. Differences among curves determined using Gehan's generalized Wilcoxon test. Median survival times compared using Kaplan-Meier product limit estimates.

RESULTS

Biocontrol ability. Median wilting times for all treatments containing ascospores of *T. flavus* were not different from the median wilting time of the healthy control ($P \leq 0.05$). No treatments without *T. flavus* were similar in median wilting time to the healthy control ($P \leq 0.05$). Only the two formulations containing ascospores of *T. flavus* plus the carriers pyrophyllite or corn cobs significantly delayed ($P \leq 0.05$) the onset of Verticillium wilt in eggplants compared with the disease control and with the respective formulations without ascospores of *T. flavus* (Fig. 1). By 59 days after transplanting, half of all plants in treatments without *T. flavus* were wilted while more than half of the plants treated with *T. flavus* in either corn cob or pyrophyllite prill had no symptoms 90 days after transplanting. In two out of three experiments, *T. flavus* in soy fiber prill significantly delayed the median time for symptom development by about 10 days ($P \leq 0.05$). There were no differences between blocks and no differences, except for neem cake and soy fiber, among the three repeats of the experiment.

There were no differences in plant heights among treatments at the time of transplant. During the first experiment, possible phytotoxicity was observed in plants receiving neem cake prill. Plants with symptoms showed a stippling of nearly white lesions similar to ozone damage. Symptoms were primarily on the upper leaf surface,. No phytotoxicity was observed during the second experiment.

Shelf life and carbon and nitrogen content. Carriers significantly affected survival of *T. flavus* at 5 C and at ambient temperature. Survival at both temperatures was best in prill formulated with corn cobs, peanut hulls, and soy fiber ($P \leq 0.05$). While survival tended to be better at 5 C than at ambient temperature, temperature did not alter the survival pattern during the 18-wk sampling period. Although populations of *T. flavus* in all prill fluctuated, populations generally declined greatly during the first 4–6 wk, followed by a slight increase from 6–12 wk, then declined
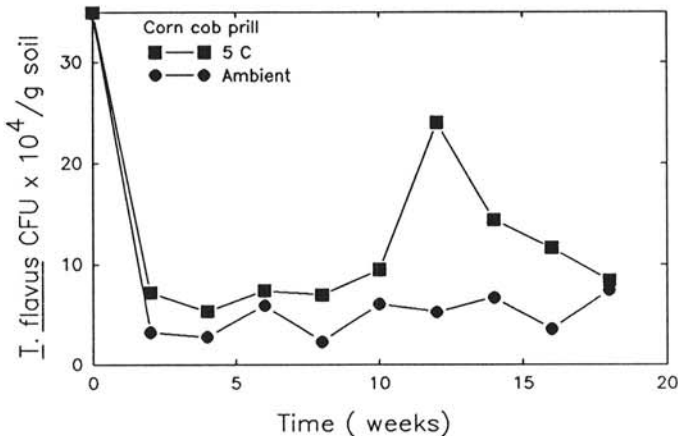

Fig. 2. Survival of *T. flavus* in alginate prill formulated with corn cobs as carrier. Prill stored at 5 C or ambient temperature. Data analyzed using a linear model (proc glm) with time as a continuous variable and other variables as classification variables.

TABLE 2. Analysis of variance on population sizes of *Talaromyces flavus* from prill with each of eight carriers at 2-wk intervals in three soils

Source	Degrees of freedom	Sum of squares	F value	$P > F$
Carrier	7	3,048,732,698	7.48	0.0001
Soil	2	2,533,908,807	21.75	0.0001
Time	7	4,949,871,710	12.14	0.0001
Replicate	2	297,788,526	2.56	0.0790
Experiment	3	529,588,216	3.03	0.0924
Carrier × Soil	14	3,167,977,069	3.88	0.0001
Carrier × Soil × Time	87	14,238,136,673	2.81	0.0001

again for the remainder of the experiment. Data for corn cob prill are shown in Fig. 2. There was a trend ($P \leq 0.10$) toward an interaction between carrier and temperature, indicating that *T. flavus* may survive better in some carriers when refrigerated while surviving better in other carriers under ambient conditions.

Pyrophyllite and corn cob prill had significantly greater C/N ratios than other prill ($P \leq 0.05$) (Table 1).

Survival and proliferation in soil. Considering data from the three soils and from all repeats of the experiment, there was a highly significant ($P \leq 0.0001$) interaction among carrier × soil × time (Table 2). *T. flavus* survived and proliferated differently from the various prill, depending on which soil the prill were in, and populations of *T. flavus* from some prill increased while others remained constant or declined over time. In the HLS, *T. flavus* increased significantly ($P \leq 0.05$) over the 12-wk experiment when introduced in prill from chitin, corn cobs, fish meal, or wheat bran. In the SiC, there were significant ($P \leq 0.05$) increases in population from prill made with neem cake, soy fiber, or wheat bran. Since the maximum population size from wheat bran was 6×10^3 cfu/g soil, the increase from wheat bran prill in the SiC may not be sufficient to result in disease control. A similar increase was observed for populations of *T. flavus* from fish meal prill in the RLS in which populations reached 6×10^3 cfu/g soil. Analyzing data from four repeats of the experiment together and for each soil × week separately, during the first 2 wk populations of *T. flavus* from bran prill were higher ($P \leq 0.05$) than populations from other prill in the RLS and the SiC. Data for wheat bran prill for the three soils are shown in Fig. 3. In the HLS, populations of *T. flavus* from peanut hull prill were greater than populations from other prill for the 8- and 12-wk sampling only.

DISCUSSION

The highly significant interactions among the carrier, soil, and time indicate that performance of formulated biocontrol agents needs to be tested in a variety of soils. These interactions may explain some of the variability often associated with biocontrol experiments and suggest that, in some cases, formulations of biocontrol agents may need to be customized for particular soils.

The ability of various carriers in prill to stimulate population proliferation of *T. flavus* in soil does not correlate with the ability of the prill to delay onset of Verticillium wilt of eggplant. In this study, peanut hulls and wheat bran with *T. flavus* were the only carriers tested that yielded soil populations of *T. flavus* significantly greater than other prill. However, peanut hull and wheat bran prill with *T. flavus* did not delay onset of wilt compared with the disease control. In contrast, prill containing ascospores of *T. flavus* formulated with corn cobs or pyrophyllite significantly delayed the onset of Verticillium wilt on eggplant in the green-
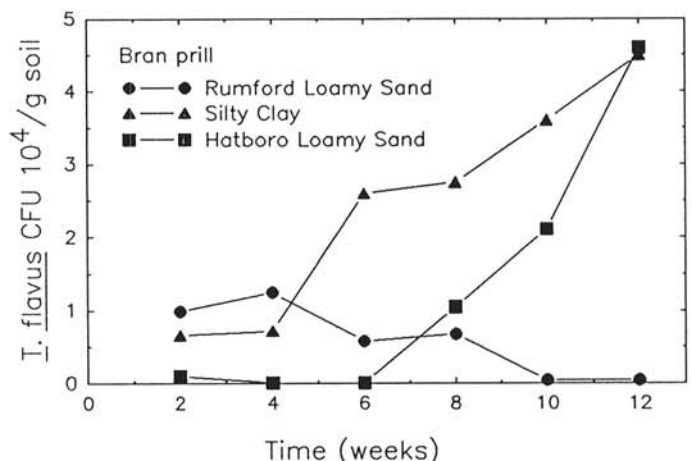

Fig. 3. Survival and proliferation of *T. flavus* in wheat bran prill in three soils. Data analyzed using a linear model (proc glm) with time as a continuous variable and other variables as classification variables.

house. Neither corn cob nor pyrophyllite prill yielded populations of *T. flavus* greater than other prill at any sampling. Further, pyrophyllite prill did not increase populations of *T. flavus* in the three soils studied while corn cob increased populations of *T. flavus* in only the HLS. No prill without *T. flavus* affected wilt incidence. Population sizes cannot be compared between soils since the efficiency of recovery of the TF agar varies from 68–130% (14).

Pyrophyllite, corn cob, and soy fiber prill had significantly higher C/N ratios than prill formulated with other carriers. Carbon/nitrogen ratios are known to affect sporulation and biomass production by *T. flavus* (2) and colonization of soil by other biocontrol fungi (18). In a study of the nutrition of *T. flavus* using maltose or lactose as a carbon source and hypoxanthine as a nitrogen source in *Neurospora* minimal medium, a 5:1 C/N ratio produced the highest hyphal dry weights of *T. flavus* while a 20:1 ratio resulted in the greatest ascospore production. Ascospore production was inhibited by C/N ratios of 25:1 and 30:1 (2). However, neither high ascospore nor high biomass production was related to biocontrol ability, and the best disease control was provided by ascospores of *T. flavus* grown on potato-dextrose agar. Prill made with pyrophyllite, corn cobs, and soy fiber have C/N ratios of 159, 97, and 25, respectively, which may inhibit ascospore formation (2). The role of ascospore production in biocontrol has not been established and further work is needed to determine if biocontrol by *T. flavus* can be enhanced by manipulation of the C/N ratio.

Formulation significantly affected survival of *T. flavus* at 5 C and ambient temperature. Survival at both temperatures was best in prill formulated with corn cobs, soy fiber, and peanut hulls. Temperature did not affect viability in storage (shelf life) during the 18-wk experiment. This is contrary to the work by Lewis and Papavizas (11) with 12 isolates of *Trichoderma* and *G. virens,* in which propagules in wheat bran prill retained more viability when stored at 5 C than at ambient temperature. Viability at ambient temperature remained high (>70%) after 1 wk of storage, but declined to less than 10% after 24 wk. If the time of this study were extended, we would expect longer shelf life with all formulations at 5 C than under ambient conditions (17). Following an initial decline in population, there was an apparent increase in populations of *T. flavus* at 6–12 wk for most prill. This apparent increase is consistent with previous observations of shelf life of *T. flavus* (D. R. Fravel, *unpublished*) and may be due to breaking ascospore dormancy. The factors responsible for this apparent increase merit further investigation to develop formulations with acceptable shelf life.

LITERATURE CITED

1. Boosalis, M. G. 1956. Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and *Trichoderma* sp. Phytopathology 46:473-478.
2. Engelkes, C. A., Nuclo, R. L., and Fravel, D. R. 1993. Effect of source and concentration of carbon and nitrogen on biomass and ascospore production of the biocontrol agent *Talaromyces flavus.* (Abstr.) Phytopathology 83:1365.
3. Fravel, D. R., Davis, J. R., and Sorenson, L. H. 1986. Effect of *Talaromyces flavus* and metham on Verticillium wilt incidence and potato yield 1984-1985. Biol. Cult. Tests 1:17.
4. Fravel, D. R. and Lewis, J. A. 1992. Production, formulation and delivery of beneficial microbes for biocontrol of plant pathogens. Pages 173-179 in: Pesticide Formulations and Application Systems: 11th Volume, ASTM 1112. L. E. Bode and D. G. Chasin eds., ASTM, Philadelphia.
5. Fravel, D. R., Marois, J. J., Lumsden, R. D., and Connick, W. J., Jr. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75:774-777.
6. Fravel, D. R. and Roberts, D. P. 1991. *In situ* evidence for the contribution of glucose oxidase to biocontrol of Verticillium wilt by *Talaromyces flavus.* Biocontrol Sci. Technol. 1:91-99.
7. Gehan, E. A. 1965. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. Biometrika 52:203-223.
8. Kaplan, E. L., and Meier, P. 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Assn. 53:457-481.
9. Keinath, A. P., Fravel, D. R., and Papavizas, G. C. 1991. Potential of *Gliocladium roseum* for biocontrol of *Verticillium dahliae.* Phytopathology 81:644-648.
10. Kim, K. K., Fravel, D. R., and Papavizas, G. C. 1990. Glucose oxidase as the antifungal principal of talaron from *Talaromyces flavus.* Can. J. Microbiol. 36:760-764.
11. Lewis, J. A., and Papavizas, G. C. 1985. Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. Plant Pathol. 34:571-577.
12. Lewis, J. A., and Papavizas, G. C. 1987. Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of Rhizoctonia damping-off. Plant Pathol. 36:438-446.
13. Lumsden, R. D., and Locke, J. C. 1989. Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. Phytopathology 79:361-366.
14. Marois, J. J., Fravel, D. R., and Papavizas, G. C. 1984. Ability of *Talaromyces flavus* to occupy the rhizosphere and its interaction with *Verticillium dahliae.* Soil Biol. Biochem. 16:387-390.
15. Marois, J. J., Johnston, S. A., Dunn, M. T., and Papavizas, G. C. 1982. Biological control of Verticillium wilt of eggplant in the field. Plant Dis. 66:1166-1168.
16. McLaren, D. L., Huang, H. C., and Rimmer, S. R. 1986. Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus.* Can. J. Plant Pathol. 8:43-48.
17. Papavizas, G. C., Fravel, D. R., and Lewis, J. A. 1987. Proliferation of *Talaromyces flavus* in soil and survival in alginate pellets. Phytopathology 77:131-136.
18. Stack, J. P., Kenerley, C. M., and Pettit, R. E. 1987. Influence of carbon and nitrogen sources, relative carbon and nitrogen concentrations, and soil moisture on the growth in nonsterile soil of soilborne fungal antagonists. Can. J. Microbiol. 33:626-631.