

Lignite and Stillage: Carrier and Substrate for Application of Fungal Biocontrol Agents to Soil

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ABSTRACT

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A lignite-stillage carrier system was tested for applying biocontrol agents to the soil. Lignite was ground into granules (425–2,000 μm) and twice amended with 50% (v/v) thin liquid stillage (TLS), a by-product of ethanol production. Two isolates of *Gliocladium virens* and one isolate of *Trichoderma harzianum* were used as test organisms. Seven days after inoculation, the colonized granules were air-dried and stored at 20 C. After 4 mo of storage, fungal viability remained >90% as determined by plating of granules. Colonized lignite-stillage carrier granules were applied in-furrow at a rate of 9.15 g/m to soil in growth boxes artificially infested with

Rhizoctonia solani. Root rot ratings and root and shoot dry weights revealed positive effects of the biocontrol agents and carrier. One isolate of *G. virens* significantly lowered the incidence of damping-off and root rot caused in peanuts by *R. solani*; however, the incidence of damping-off and root rot were still significantly higher than in the noninfested control. Thin liquid stillage supported significant production of gliotoxin by *G. virens* in broth culture. Gliotoxin was not an important factor in suppression of *R. solani*; a gliotoxin-producing isolate of *G. virens* did not lessen damping-off or root rot as effectively as a non-gliotoxin-producing isolate.

Field use of biological control agents in modern agriculture is hampered by the lack of suitable carriers and application methods (2,14). Seed-coating methods have been relatively successful when applied to small volumes of soil under greenhouse conditions (7,21), but these are limited by failure of the biocontrol agents to colonize the seed coat, inadequate spermatophane area for inoculation, or a restricted food base (4,24). In addition, antibiotic-producing biological control agents may have deleterious effects upon the seed if applied directly to the seed coat (22).

Fluid drilling with gels provides a promising new approach to the application of biological control agents to soil (5,6). However, costs and laborious methods of application may preclude the use of gels for large-scale application to field crops. A more successful and practical means of applying biocontrol agents, at any stage of a plant's life, has involved using a granular carrier system that contains an adequate food base. Backman and Rodriguez-Kabana (3) reported on such a two-component carrier system consisting of diatomaceous earth impregnated with an amended molasses food base. They listed several criteria for a successful carrier system: stability during handling, readily available low-cost components, and a granular formulation for easy application by existing farm machinery.

The widely accepted use of lignite as a carrier for applying inoculum of *Rhizobium* to seeds of legumes (10,18) prompted our investigation of the applicability of lignite as a carrier for fungal biocontrol agents. Thin liquid stillage, a malt extract-like by-product of grain alcohol production (17), was chosen as an inexpensive nutrient base for impregnation of the lignite.

The efficacy of the lignite-stillage carrier system for the application and study of fungal biocontrol agents is reported in this paper. An introductory report has been published (9).

MATERIALS AND METHODS

Test organisms. Two isolates of *Gliocladium virens* Miller, Giddens & Foster, designated GVI-3 and GV2-3, and an isolate of *Trichoderma harzianum* Rifai, designated TH2-3, were chosen as potential biocontrol agents. The choice of these fungi was based on their parasitism of *Rhizoctonia solani* Kuhn (peanut isolate, AG-4) on malt extract agar. The biocontrol agents were isolated from fallow pasture land.

Carrier system preparation. Thin liquid stillage (TLS), a by-product of sorghum fermentation, was obtained from the ethyl alcohol production plant of the Agricultural Engineering Department at Texas A&M University. Chemical analysis of TLS by the soil testing laboratory indicated that it contained 5.8% dry matter partitioned as 3% nitrogen, 17.5% starch, and 44.7% sugars. The pH of the TLS ranged from 3.9 to 4.2.

Lignite was obtained from mine spoils near Rockdale, TX. Moisture content ranged from 19 to 21% and pH 5.6. The lignite was ground into small granules with a Straub 4E mill. Granules 425–2,000 μm were retained on sieves (10–40 mesh) for amendment with TLS.

Preparation of the carrier system proceeded by the addition of 150 ml of TLS to 300 ml of granular lignite. The mixture was thoroughly stirred until the lignite absorbed the TLS. The amended granules were then spread on foil sheets and dried overnight at 30 C. After drying, the mixture was amended with an additional 50% v/v of TLS. The doubly amended granules were spread evenly in 14-cm-diameter petri dishes to a depth of 2–3 cm, covered, and autoclaved. Following cooling, individual batches of the mixture were inoculated with 0.5 ml of a conidial suspension (10^6 conidia per milliliter) of one of the selected biocontrol agents. The inoculated carrier system was stirred after 4 days of incubation to evenly distribute the sporulating fungus. After 7 days, the petri dish covers were removed and the fungus-covered granules were air-dried. The colonized granules were then placed in covered jars and stored at 20 C. Maintenance of fungal viability on the carrier was tested periodically over a 4-mo period by placing colonized granules on water agar (15).

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Application to the soil. The control of *Rhizoctonia* damping-off of peanuts by using the lignite-stillage carrier system was tested in an outdoor lath house. Twenty-four wooden boxes (60 × 40 × 20 cm) were each filled with 10 L of a sandy loam soil with the following analysis: pH 6.8; and (in µg/g) 7 P, 84 K, 560 Ca, 95 Mg,

0.64 Zn, 12.2 Fe, and 9.6 Mn. Two-week-old inoculum of *R. solani* grown in a sand:cornmeal mix (200 ml of sand, 10 ml of cornmeal, 30 ml of water), was incorporated at 100 ml per box in all but the noninfested controls. The carrier system, inoculated or uninoculated with the biocontrol agents, was added to the appropriate boxes at the rate of 9.15 g/m applied in the furrows. Eight peanut seeds (cultivar Starr) were planted in each of two furrows and covered with 2 cm of soil. Four replicates of each treatment were prepared. Treatments consisted of: noninfested controls, untreated controls in soil infested with *R. solani* only, soil infested with *R. solani* and treated with *G. virens* GV1-3, *G. virens* GV2-3, or *T. harzianum* TH2-3 applied on the lignite-stillage carrier, and soil infested with *R. solani* and treated with uncolonized lignite-stillage carrier. The soil was routinely watered with distilled water. After 2 wk, preemergence damping-off was recorded, and the remaining seedlings were removed, dried at 85 C, and the dry weights of the shoots and roots were recorded. Seedlings from each treatment were rated visually with a disease index of 0 to 10 in which 0 = no root rotting induced by *Rhizoctonia*, 5 = 50% root rotting, and 10 = total rotting of the main root system.

TABLE 1. Influence of various fungal biocontrol agents applied in-furrow on colonized thin-liquid-stillage-amended lignite granules on damping-off and root rot caused in peanuts by *Rhizoctonia solani*^a

Treatment	Damping-off (%)	Disease index ^b	Seedling dry weight (g)	
			Roots	Shoots
Noninfested control	3 a	0.17 a	2.52 a	8.16 a
<i>R. solani</i> + <i>G. virens</i> GV2-3	22 b	1.10 ab	1.38 b	4.20 b
<i>R. solani</i> + <i>T. harzianum</i> TH2-3	37 c	2.68 bc	0.79 c	3.35 c
<i>R. solani</i> + <i>G. virens</i> GV1-3	33 bc	2.95 c	0.85 c	3.15 cd
<i>R. solani</i> control	34 c	3.67 c	0.89 c	2.48 de
<i>R. solani</i> + uninoculated lignite-stillage control	56 d	3.25 c	0.72 c	1.83 e

^a *R. solani* was applied to soil in growth boxes as an infested cornmeal:sand mixture; biocontrol fungi were applied on carrier granules at 9.15 g/m of furrow. All data are means of four replicates, repeated twice. Means in columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

^b Visual rating of seedling root vigor from 0 to 10 in which 0 = no root rotting induced by *Rhizoctonia*, 5 = 50% root rotting, and 10 = total rotting of the main root system.

Production of gliotoxin. TLS was tested as a substrate for gliotoxin production by isolates GV1-3 and GV2-3 of *G. virens*. *G. virens* (NRRL 1828), a gliotoxin producer, was also used.

A total of 150 ml of TLS was added to each of nine 500-ml flasks. After autoclaving, a 1-ml conidial suspension (10^5 conidia per milliliter) of each isolate was pipetted into each of three replicate flasks. Each flask was shaken on a reciprocal shaker (150 rpm) for 60 hr. Contents of each flask were then vacuum filtered and the filtrates of each isolate were pooled. A total of 375 ml of filtrate was recovered from each isolate. The total filtrates of each isolate were

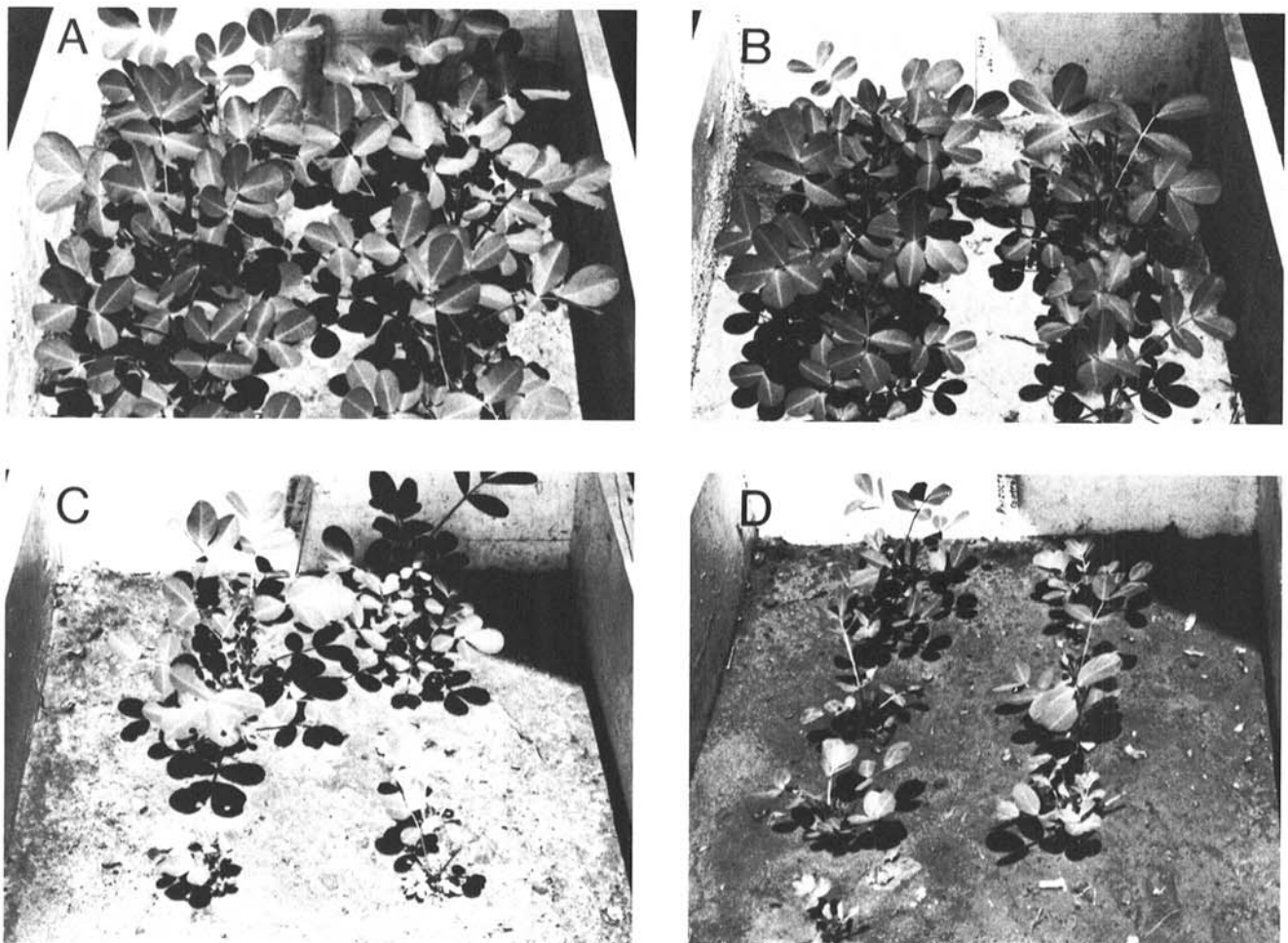


Fig. 1. Representative effects of various treatments on peanut seedling vigor. A, Noninfested soil; B, soil infested with *Rhizoctonia solani* and treated with lignite-stillage carrier colonized by *Gliocladium virens* GV2-3; C, infested soil treated with uninoculated lignite-stillage carrier; D, infested soil.

extracted with two one-tenth-volume additions of chloroform.

Chloroform extracts of each isolate were dried in vacuo, then resuspended in 4 ml of absolute ethanol. Thin-layer silica gel plates were prepared and spotted with 20 μ l of each isolate extract and a gliotoxin standard (Squibb, Princeton, NJ). Plates were developed in a chloroform:acetone (95:5) solvent. After drying, the gliotoxin was visualized by spraying the plates with 0.1M silver nitrate.

RESULTS

Gliocladium virens isolate GV1-3 produced 4.0–4.5 mg of gliotoxin on TLS substrate, *G. virens* NRRL 1828 produced one-third as much gliotoxin, and *G. virens* GV2-3 did not produce detectable levels of gliotoxin.

Sporulation of the isolates of *Gliocladium* and *Trichoderma* was profuse on the lignite-stillage carrier system. The isolate of *T. harzianum* produced conidia in abundance, some of which readily dispersed upon transfer of the dried carrier system to jars. The isolates of *G. virens* produced wet conidial heads which adhered to the lignite granules upon drying. Colonized granules maintained a fungal viability >90% during 4 mo of storage at 20 C. Chlamydospores were abundant in preparations of each fungus on the nutrient-amended lignite.

The effect of various treatments on damping-off and overall disease severity is summarized in Table 1. Seed and seedling damping-off in those treatments with the gliotoxin-producing isolate *G. virens* GV1-3 and *T. harzianum* TH2-3 was not significantly different from the *R. solani* infested control. Only one antagonist, *G. virens* GV2-3, significantly reduced the percentage of damping-off compared to the control infested with *R. solani*; however, damping-off was still significantly higher in the GV2-3 treatment compared to the noninfested control. The uninoculated lignite-stillage carrier treatment had significantly more damping-off of seeds and seedlings grown in soil infested with *R. solani*.

The disease index indicated no significant difference in root rotting between the noninfested control soil and the soil infested with *G. virens* GV2-3. Roots from the GV2-3 treatment and the noninfested control weighed significantly more than the infested control. Root weights from treatment GV2-3 were significantly lower than the noninfested control. Plants from these treatments and those treated with TH2-3 had shoot weights significantly greater than those in the infested control. The influence of the different treatments on peanut growth is illustrated in Fig. 1.

DISCUSSION

The components of the lignite-stillage carrier system are low-cost, readily-available, and easily standardized. Both lignite and TLS are quite inexpensive, costing only a few cents per pound and liter, respectively. Twice-amended, uninoculated lignite may be dried for storage and rehydrated as needed by the addition of water. The granules maintain their structural integrity during repeated handling.

Many carriers such as vermiculite, peat, or wheat bran are either too light for mechanical application or of unsuitable particle size. Lignite may be ground to various sizes and provides sufficiently dense granules to penetrate a foliar canopy. The lignite-stillage carrier system is suitable for growth and delivery of many different fungi including *Chaetomium globosum*, *Stilbum* sp., and *Laetisaria arvalis* (R. W. Jones, unpublished). Technology similar to that presently used in producing inoculant of *Rhizobium* might be applied to the production of bacterial biocontrol carriers utilizing a lignite substratum. Problems associated with the dispersal of nonadhering dry-spored fungal conidia during handling of the dry, colonized carrier system could limit the carrier's practical use. Limitations would arise not from loss of propagules (10^6 propagules per gram remained after dispersion of excess conidia of *Trichoderma*), but from the safety aspect of possible spore inhalation. To limit the dispersion of nonadhering conidia, a sticker, such as a starch solution or foliar pesticide adherent, might be applied to the dry, colonized carrier granules to promote conidial adhesion.

Significant amounts of gliotoxin were produced by *G. virens* GV1-3 when metabolizing the TLS. This may be important when attempting to control fungi such as *Pythium* spp., which are sensitive to gliotoxin (8). Gliotoxin, if produced in the soil by the gliotoxin-producing *G. virens* isolate GV1-3, did not suppress *R. solani* in comparison to the nonproducing strain. These results are consistent with recent studies by Howell (8) but contrary to earlier studies (1,20). In possible explanation, gliotoxin sensitivity could differ among isolates of *R. solani* or among anastomosing groups. TLS was quite similar to corn or grain-steep liquor, substrates which have been used to promote fungal antibiotic production (19) and provide additional nutrients to carbohydrate substrates (12). Corn-based TLS declined in pH after fungal utilization in broth culture while the pH of sorghum-based TLS increased (R. W. Jones, unpublished). Corn TLS may prove more effective in maintaining the stability of gliotoxin on the carrier system (20,23).

The increased damping-off in the soil infested with *R. solani* after treatment with uninoculated lignite-stillage suggests that the pathogen can utilize the carrier nutrients, thus increasing its inoculum potential. Previously, researchers have observed increased disease severity when they combined uninoculated carrier with inoculated carrier to provide an additional food base for the biocontrol agent (11,13). Concentrating the nutrients on the lignite granule by twice amending the lignite with TLS eliminates the need for an additional fresh food base when applying colonized granules to the soil.

While differences in the degree of damping-off were observed, the subsequent root decay was equally important. In some crops where seedling stand is reduced by damping-off, the surviving plants may compensate through increased growth and yield due to the reduced competition. However, if some of the remaining seedlings have damaged root systems they may develop into unthrifty plants. These unthrifty plants compete with thrifty, undiseased plants, thus lowering the ability of the latter to compensate. For example, yields of cotton have been reported to be reduced 5–75% when seedling roots were damaged by *R. solani* (16). The root disease index may provide a better view of the effectiveness of the treatment on subsequent yields, especially when evaluating treatments in the field.

Suppression of *R. solani* by competition alone seems unlikely since the ineffective *T. harzianum* isolate TH2-3 was observed sporulating on damped-off peanut seeds. The non-gliotoxin-producing *G. virens* isolate GV2-3 was effective against *R. solani*. Other toxins or enzymes may have been produced which aided in suppression of *R. solani*.

This paper reports on an in-furrow application of a carrier system for delivery of biological control agents at low rates (~100 kg/ha). Modifications of the carrier system through use of more acidic lignites, more effective biocontrol agents, or higher application rates, may provide a more effective method of controlling soilborne plant pathogens.

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