

## Competitive Saprophytic Ability and Cellulolytic Activity of Rhizosphere-Competent Mutants of *Trichoderma harzianum*

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### ABSTRACT

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Competitive saprophytic ability (CSA) of strains of *Trichoderma* spp. was determined by the modified Cambridge method (sensu Garrett). Two rhizosphere-competent mutants of *T. harzianum* (T-95 and T-12B) had higher CSA indices than four rhizosphere-incompetent *Trichoderma* spp. and strains. CSA was directly correlated with rhizosphere competence (RC). When the strains were grown for 6 days on Czapek-Dox broth with

cellobiose, carboxymethyl cellulose, or cotton linters as sole sources of carbon, mutants produced more cellulase than the wild types. The amount of cellulase produced by these strains was directly correlated with CSA and RC. RC of the mutants, therefore, can be at least partially explained by their capacity to utilize cellulose substrates associated with the root.

Benomyl-tolerant mutants of *Trichoderma harzianum* Rifai applied to seeds colonized roots; however, the wild-type strains and species of the same fungus applied to seed did not colonize the rhizosphere (1,2). The reason(s) why such mutants were rhizosphere-competent, however, was not explored. Here we test one hypothesis to explain why the mutants were rhizosphere-competent—that is, mutation resulted in (besides benomyl tolerance) more efficient utilization of substrates found in the rhizoplane or root surface.

According to Garrett (8), “the share of a substrate obtained by any particular fungal species will be determined partly by its intrinsic competitive saprophytic ability and partly by the balance between its inoculum potential and that of competing species.” In tests for rhizosphere competence (RC), both the mutants and the wild types had the same population density when applied to seed (1,2). The ability to colonize the root, therefore, could be linked to the competitive saprophytic ability (CSA) of the mutants.

Garrett (8) lists production of and tolerance to antibiotics among attributes of successful root-surface and rhizosphere fungi. None of our strains have obvious antibiotic activity *in vitro* except for a routing factor seen in the hyphal cytoplasm at microscopic levels affecting *Pythium* spp. (9). Tolerance to antibiotics among benomyl-tolerant mutants currently is being explored.

Garrett (8) does not list “enzymes for cellulase degradation” as a necessary attribute of rhizosphere fungi, presumably, because amino acids and reducing sugars commonly are thought to be the predominant root exudates. Recently, however, Foster et al (7) demonstrated that the mucilage overlying the root surface in the region of elongation constitutes the remains of the outer primary wall of root epidermal cells. Microorganisms were seen embedded in this mucilage, and cellulose is a major component of primary cell walls. Conversely, mutants of *Trichoderma* spp. have been easily obtained with higher cellulase activity than their wild-type parents (11,12). The purpose of this study was to test the hypothesis that rhizosphere-competent mutants utilize cellulose substrates on or near roots more efficiently than wild types.

### MATERIALS AND METHODS

**Soil.** Nunn sandy loam was used in these investigations. Water content of 43.2-kg portions was adjusted to  $-0.03$  bar, and the soil was stored for 48 hr before use. Soil characteristics were reported (2).

***Trichoderma* spp. and strains.** Four strains of *T. harzianum* (T-95 [ATCC 60850], T-12B, WT, and T-12) and one strain each of *T. koningii* (T-8) and *T. viride* (T-S-1) used in these investigations were described previously (2). Only benomyl-tolerant mutants T-95 and T-12B were rhizosphere-competent (1).

**CSA assay.** To test CSA of *Trichoderma* spp., the Cambridge method (8) was modified. Strains of *Trichoderma* spp. were grown on potato-dextrose agar (PDA). Mutants tolerant to benomyl were grown on PDA containing  $10 \mu\text{g}$  a.i. benomyl per milliliter. Plates were incubated for 8 days at 25 C, flooded with sterile distilled water, and conidia were gently freed from the culture with a brush. The suspension was sieved through four layers of cheesecloth, centrifuged at 2,500 g for 15 min, and resuspended in sterile distilled water three times. Conidia were counted with a hemacytometer and then adjusted to the desired concentrations.

Freshly harvested conidia were added to 7.2 kg of previously moistened and incubated field soil at the rate of  $10^1$ ,  $10^2$ ,  $10^3$ , and  $10^4$  conidia per gram of soil. No conidia were added in controls. The soil was mixed thoroughly by hand and distributed in nine 11-cm-diameter plastic pots.

Clean, mature, polished winter wheat straw was cut in 1-cm segments; each segment included a node. Twenty pieces were buried randomly in each pot. The pots were arranged in a completely randomized design, covered with plastic to conserve moisture at  $-0.03$  bar, and incubated in the dark. No water was added to the pots. All 20 pieces, from each treatment including a noninfested control, were removed from the pots after 2, 4, or 6 days, washed in tap water to remove all adhering soil and debris, and surface-disinfested in a mixture of 1.1% sodium hypochlorite solution and 5% ethanol for 5 min. Segments were plated on a medium selective for *Trichoderma* (5) and incubated at 25 C for 5 days. Percent colonization of wheat pieces by *Trichoderma* for each treatment at a given time was determined. There were three replicates per treatment, and all experiments were repeated twice.

In experiments where cellophane disks were substituted for straw pieces, the disks were obtained by punching holes (6 mm diameter) in an untreated cellophane sheet. Disks were removed from the pots after incubation for 2, 4, or 6 days, washed in sterile

distilled water, and plated on a medium selective for *Trichoderma* (5).

**Growth of *Trichoderma* spp. in liquid culture.** Strains of *Trichoderma* spp. were grown in 250-ml Erlenmeyer flasks containing 50 ml of Czapek-Dox broth on a rotary shaker at 100 rpm at 26 C for 6 days. Finely ground cotton linters, carboxymethyl cellulose, or cellobiose (Sigma) were used as sole sources of carbon. Each flask was seeded with a 4-mm-diameter disk of PDA on which the strains had been grown for 2 days. After 6 days, the hyphal mat was removed aseptically and dried for 2 days at 60 C to obtain the weight of mycelium. There were six replicates per strain.

**Enzyme assay.** Cellulase (EC 3.2.1.4) was assayed spectrophotometrically ( $A_{340nm}$ ) by following the release of free glucose from the substrates listed above according to the manufacturer's directions (Sigma). Cellulase activity was expressed as units of cellulase produced per milliliter of culture filtrate of each strain when grown in the substrate for 6 days. There were six replicates per strain.

**CSA index.** A CSA index for each strain was developed as follows:

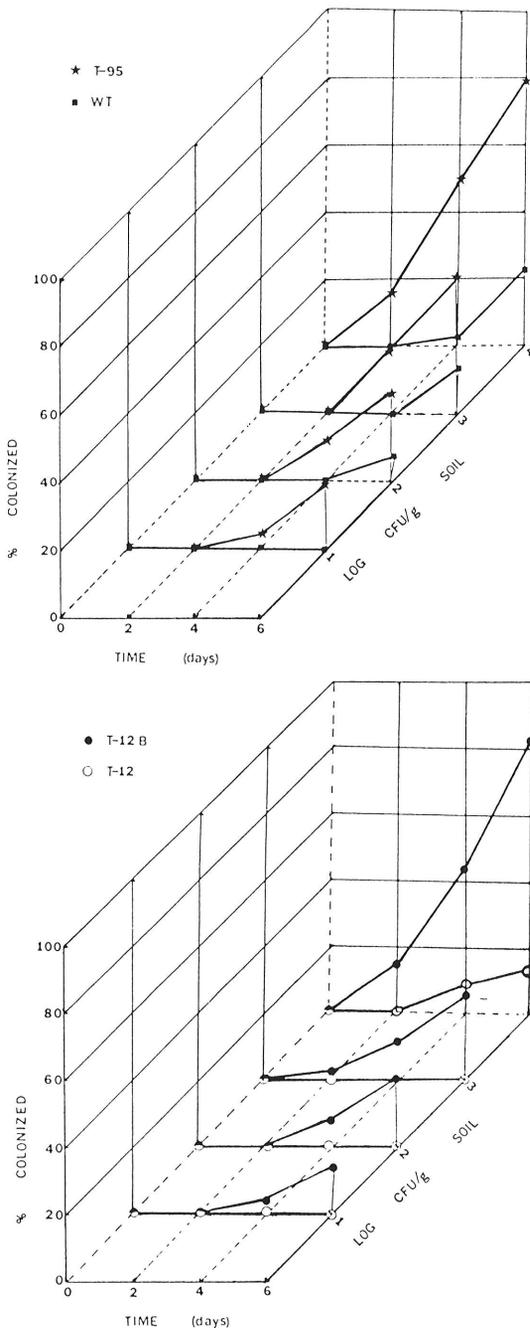
$$CSA \text{ index} = \frac{\sum_{i=1}^n [\ln(1/1-C_i)/(t_i)(\log p_i)]}{n} \quad (1)$$

where  $c$  = frequency of isolation of a specific strain of *Trichoderma* from the segments,  $t$  = time of incubation,  $p$  = population density of conidia added to the soil, and  $n$  = number of treatments.

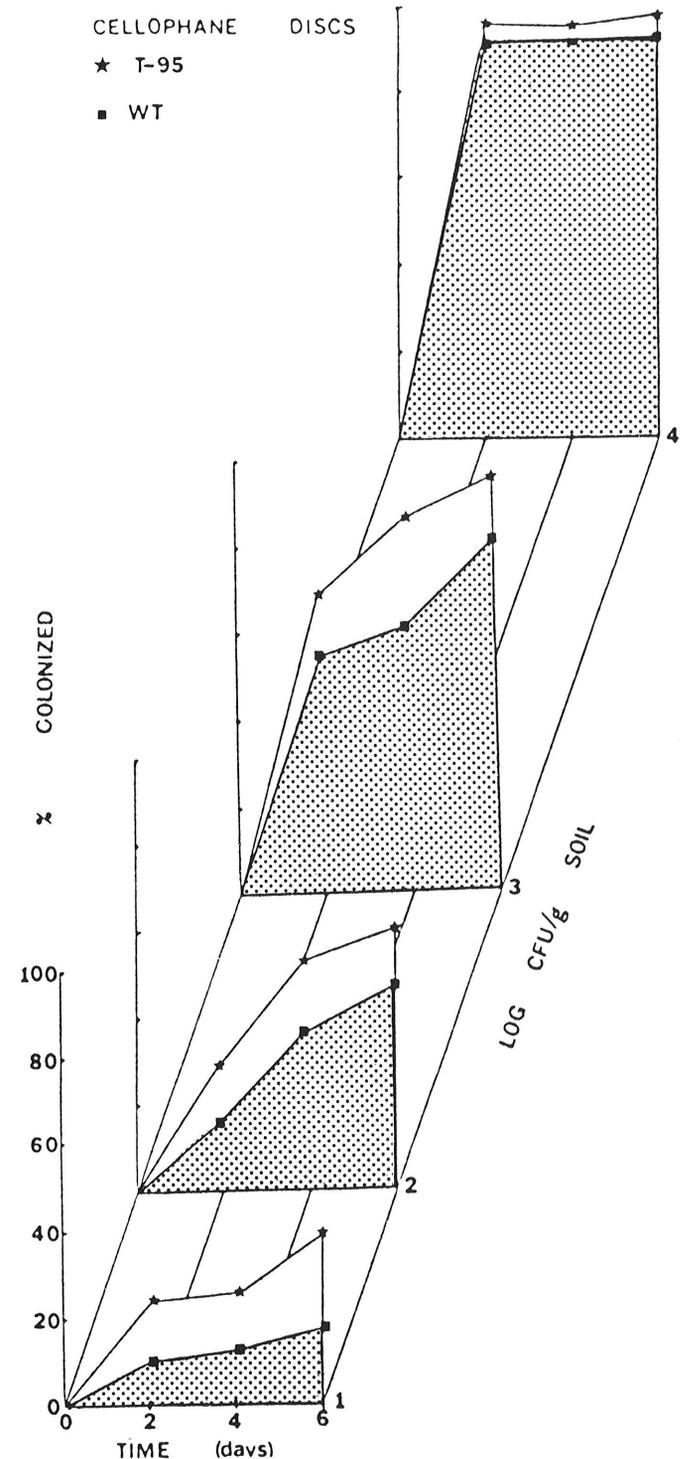
**RC index.** An RC index for each strain was developed from data reported previously (2), using the equation:

$$RC \text{ index} = \frac{\sum_{i=1}^n [\log(p_i + 1) \cdot \ln(d_i + 1)]}{n} \quad (2)$$

where  $p$  = population density per milligram of rhizosphere soil,  $d$  =



**Fig. 1.** Percent colonization of wheat straw segments by four strains of *Trichoderma harzianum*. (T-95 and T-12B are benomyl-tolerant mutants.)



**Fig. 2.** Percent colonization of cellophane disks by two strains of *Trichoderma harzianum*. (T-95 is the benomyl-tolerant mutant derived from the wild type [WT].)

root depth, and  $n$  = total root length.

**Statistical analysis.** The data for mycelial weight and cellulase units produced was subjected to one-way analysis of variance, and the means were separated with an FLSD ( $P = 0.05$ ). The data of CSA were subjected to multiple-regression analysis, and the slopes values were separated with an FLSD ( $P = 0.05$ ).

## RESULTS

**Colonization of straw by *Trichoderma* spp.** When polished wheat straw pieces were buried in soil infested with conidia of *Trichoderma* spp. and removed after 2, 4, and 6 days, *T. koningii* (T-8) and *T. viride* (T-S-1) were not isolated at any population

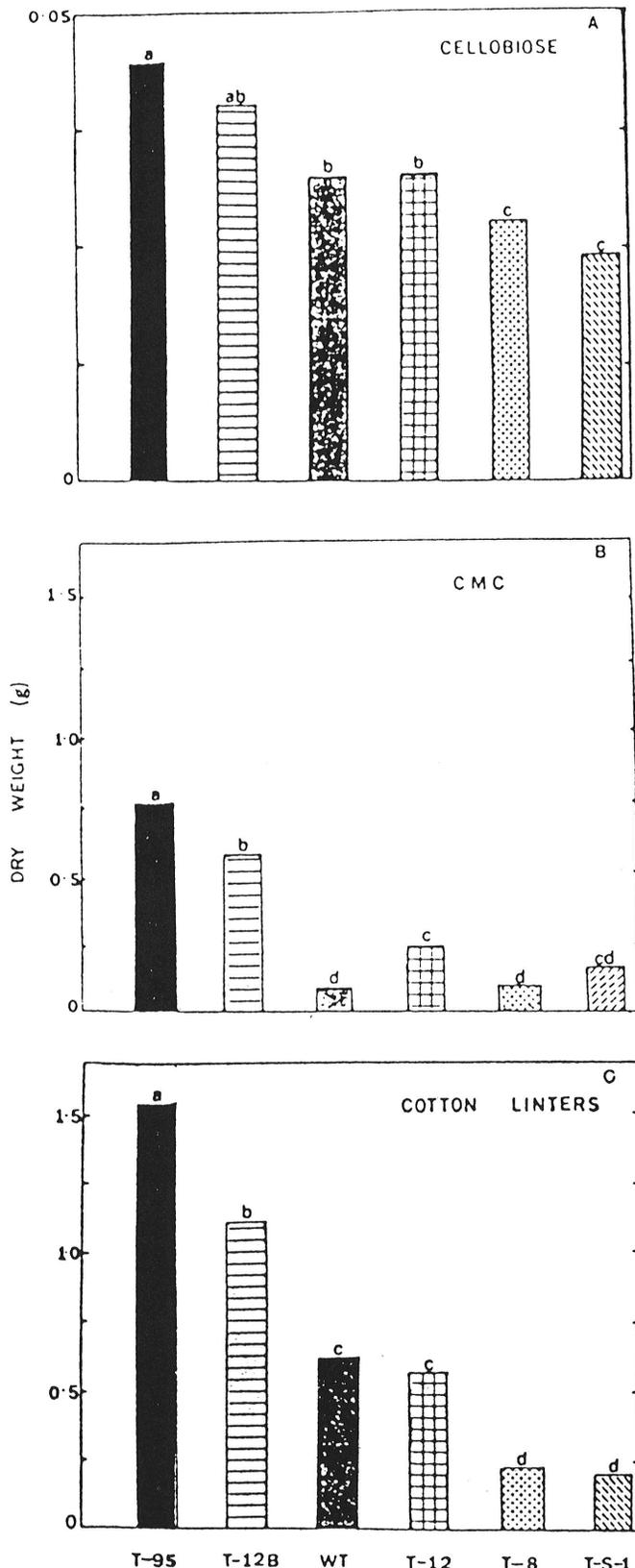


Fig. 3. Dry weight of mycelium of strains of *Trichoderma* spp. grown for 6 days in Czapek-Dox broth with A, cellobiose, B, carboxymethyl cellulose (CMC), and C, cotton linters as the sole carbon sources.

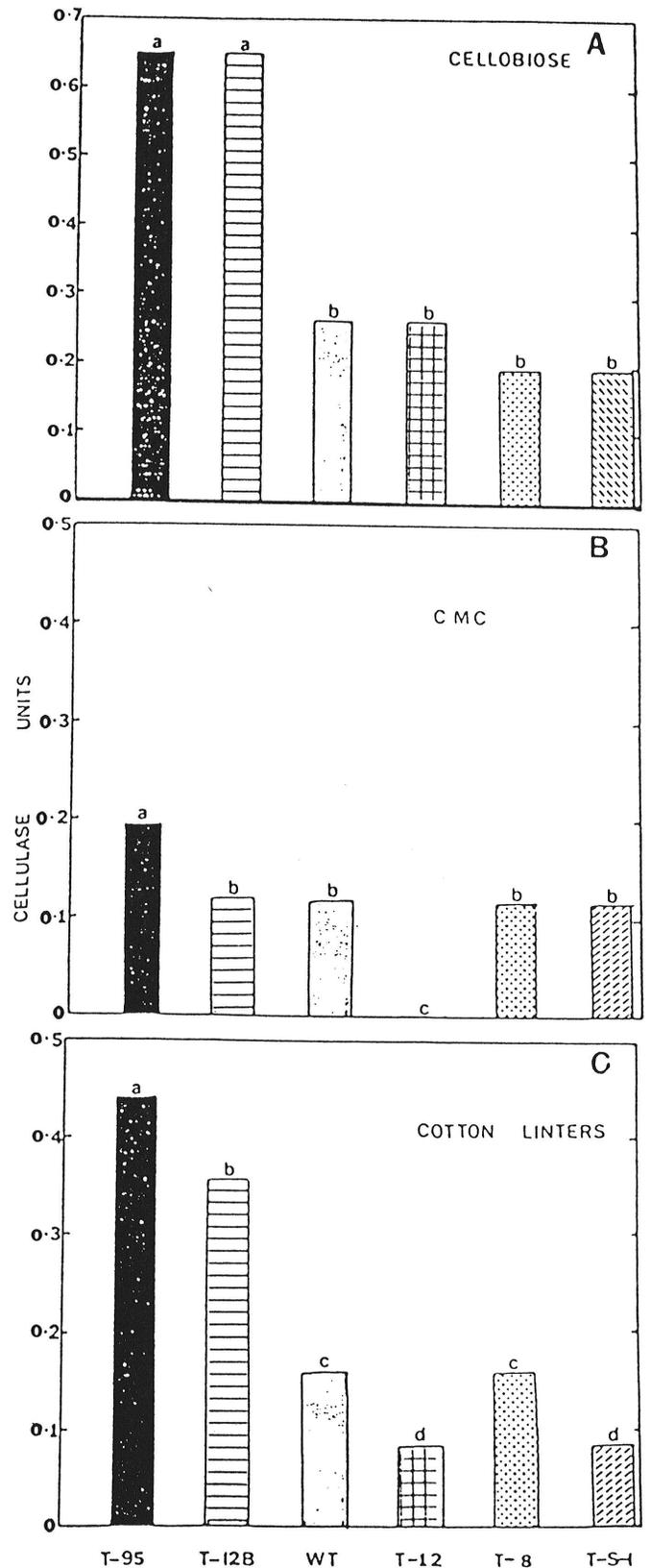


Fig. 4. Cellulase activity of strains of *Trichoderma* spp. A, Cellobiose, B, carboxymethyl cellulose (CMC), and C, cotton linters were the sole sources of carbon.

density. *T. harzianum* T-12 and WT were recovered from straw less frequently than the other strains and were slow to colonize the straw segments at higher population densities (Fig. 1). However, the mutants of these wild types, T-12B and T-95, respectively, were isolated from the straw segments at any population density (Fig. 1). Strains T-95 and T-12B showed significantly higher percent colonization than WT and T-12 at any population density on all days. Strain T-95 showed significantly higher percent colonization than T-12B at  $10^1$ ,  $10^2$ , and  $10^3$  cfu/g of soil on all days, but there were no significant differences between the two strains at  $10^4$  cfu/g. Strain WT showed significantly higher percent colonization than T-12 at  $10^2$  and  $10^3$  cfu/g of soil after 6 days, but there were no significant differences between the two strains at  $10^1$  and  $10^4$  cfu/g. When strains T-12 and WT were added at  $10^1$  cfu/g of soil, neither were isolated from wheat straw pieces after 2, 4, or 6 days of incubation. *Trichoderma* spp. were not isolated from controls.

When washed cellophane disks were buried in soil infested with conidia of T-95 or WT and removed after 2, 4, and 6 days, both strains could be isolated from the disks at any population density (Fig. 2). Strain T-95 showed significantly higher percent colonization than WT at  $10^1$ ,  $10^2$ , and  $10^3$  cfu/g of soil on all days, but there were no significant differences between the two strains at  $10^4$  cfu/g.

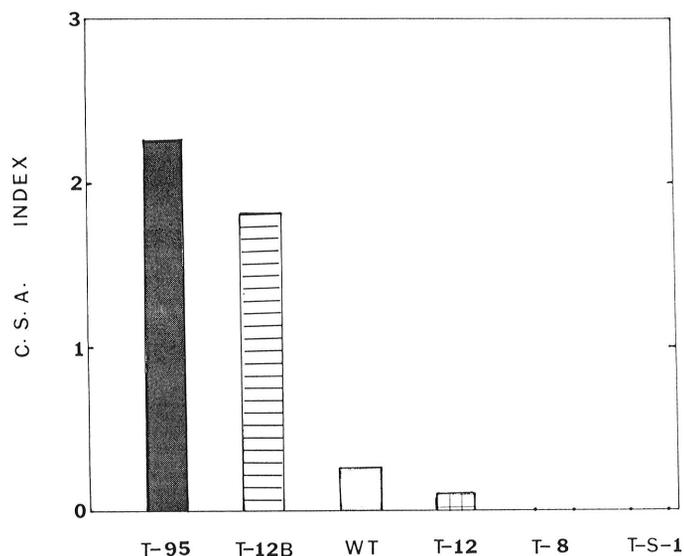


Fig. 5. Competitive saprophytic ability (CSA) index of *Trichoderma* spp. and strains.

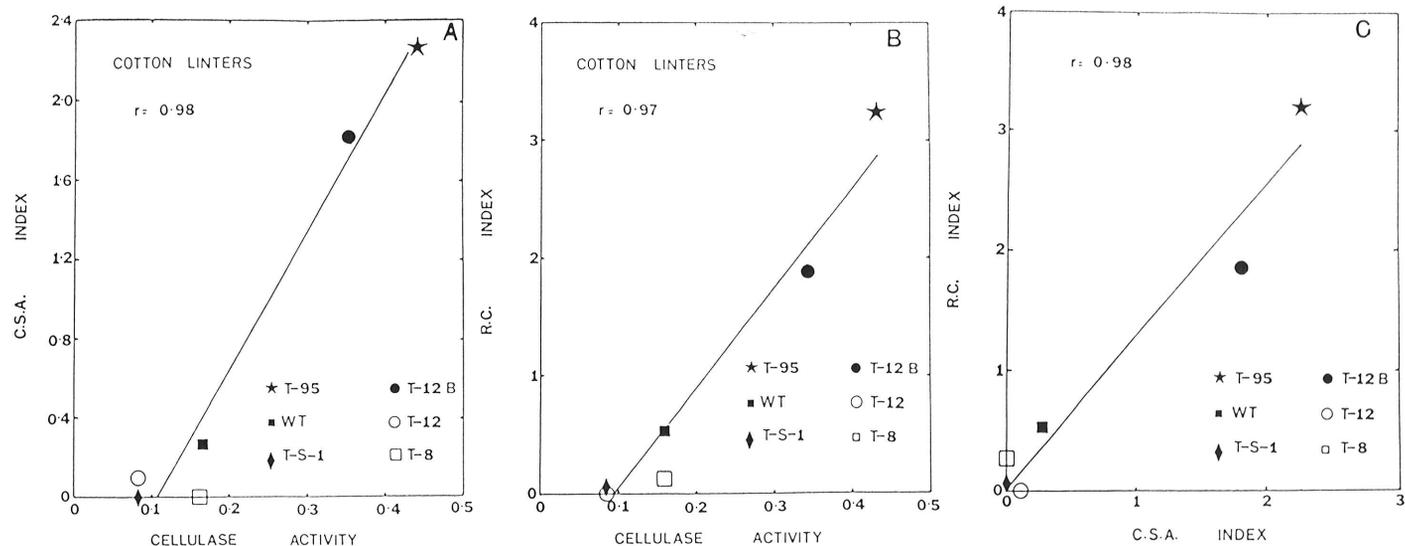


Fig. 6. Relation of A, competitive saprophytic ability (CSA) index of *Trichoderma* spp. and strains to the cellulase activity, B, rhizosphere competence (RC) index of *Trichoderma* spp. and strains to the cellulase activity when cotton linters were the sole source of carbon, and C, rhizosphere competence (RC) index to CSA index of *Trichoderma* spp. and strains.

**Growth of *Trichoderma* spp. in liquid culture.** When strains of *Trichoderma* spp. were grown in Czapek-Dox broth with cellobiose as the sole source of carbon, the mutant (T-95) mycelium attained significantly higher dry weight than all other wild-type strains (Fig. 3A). Strains T-12B, T-12, and WT had significantly higher dry weights than T-8 and T-S-1. When carboxymethyl cellulose or cotton linters were the sole source of carbon, the mutants T-95 and T-12B had significantly higher dry weights than the wild types (Fig. 3B,C). In both cases, strain T-95 had significantly higher dry weight than T-12B. With cotton linters, strains WT and T-12 had significantly higher dry weights than T-8 and T-S-1.

**Production of cellulase.** All strains produced cellulase when grown in Czapek-Dox broth with cellobiose as the sole source of carbon (Fig. 4A). Mutants T-95 and T-12B produced significantly higher amounts of cellulase than the wild-type or other strains. When carboxymethyl cellulose was the sole source of carbon, strain T-12 failed to produce any cellulase, strain T-95 produced significantly higher amounts of cellulase than all other strains, and mutant T-12B produced significantly more than T-12 (Fig. 4B). When cotton linters were the sole source of carbon, the mutants of *T. harzianum* produced significantly higher amounts of cellulase than the wild-type or other strains and strain T-95 produced significantly higher amounts than all other strains (Fig. 4C).

## DISCUSSION

Isolation of fungi from baits of dead plant material buried in field soil provides direct evidence that recovered fungi can colonize these substrates as competitive saprophytes (8). Therefore, many investigators have used wheat straw pieces rich in cellulose in the Cambridge method to determine the CSA of root-infecting fungi. The CSA index measured the capacity of different strains and species of *Trichoderma* to compete effectively in the colonization of wheat straw (Fig. 5). Benomyl-tolerant mutants had higher CSA indices than the wild types. Garrett (8) has included the ability to produce enzymes for utilization of specific substrates among the attributes of fungal species that contribute to their CSA.

Strains of *Trichoderma* spp. produce cellulase and other cell-wall-degrading enzymes (3,4,6). In our study, the CSA indices were correlated directly with production of cellulase (Fig. 6A). Also, mutants of *T. harzianum* produced significantly greater amounts of cellulase units than the wild types when natural cellulose (cotton linters) was the sole source of carbon (Fig. 4C). With the RC index, RC was directly correlated with amounts of cellulase units produced by the mutants (Fig. 6B) and the CSA of the mutants (Fig. 6C). Therefore, there are correlations suggesting that mutants

with higher cellulase activity than wild types can utilize cellulose substrates on or near the root more efficiently and thus are rhizosphere-competent. Utilization of cellulose substrates is not associated with parasitism because microscopic examination revealed no evidence of such a relationship (13). A more likely source of cellulose substrates is the remains of the primary cell walls in the mucegel (7).

The pattern of hydrolytic enzymes used by strains of *Trichoderma* spp. for the hydrolysis of cellulose has been well studied (10). Exo- and endo- $\beta$ -1,4-glucanases act on cellulose that is broken down to cellobiose and glucose. Cellobiose is further hydrolyzed by  $\beta$ -1,4-glucosidases to glucose. In an attempt to distinguish the amounts of these enzymes produced, different carbon sources were used as substrates. When cellobiose was used as the sole carbon source, the mutants produced significantly greater amounts of  $\beta$ -1,4-glucosidases than the wild types. The mutants not only produced greater amounts of  $\beta$ -1,4-glucanases but evidently also produced significantly greater amounts of  $\beta$ -1,4-glucosidases and utilized the substrate more efficiently. This is also evident from the dry weight of mycelium produced (Fig. 3).

This study suggests that certain strains of *T. harzianum* are rhizosphere-competent because of increased enzyme activity that results in higher CSA for possession of cellulose substrates on or near the root surface (7). This attribute of rhizosphere competence has not been recognized (8). If the extension rate of fungal thalli are sufficient to keep pace with root growth, the attribute of higher efficiency of cellulose degradation could be a key factor for inducing these microorganisms to become rhizosphere-competent.

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