Nullification of Antagonism of Phoma betae by Bacillus subtilis var. niger in Soil and in a Simulated Rhizosphere

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ABSTRACT

Bacillus subtilis var. *niger*, a sugar beet rhizosphere colonist, was antagonistic to the seed-borne sugar beet pathogen *Phoma betae* in culture. The bacterium caused initial retardation of sugar beet seedling attack by the fungus, but gave no control either in steamed (autoclaved) or nonsteamed soils. Competitive parasitism of sugar beet seedlings by indigenous species of *Pythium* in raw soil was prevented by soil treatment with *p*-dimethylaminodiazobenzene sodium sulfonate. This chemical had no effect on *P. betae* or *B. subtilis* at the dosage used.

In a simulated rhizosphere, it was found that the antagonism of *B. subtilis niger* to *P. betae* was reduced by *Bacillus megaterium* or a mixed population of soil bacteria, and almost completely nullified by *Escherichia coli*, an atypical soil organism. The degree of antagonism of *E. coli* to *B. subtilis niger* was postively correlated with the population of *E. coli*. This antagonism was not eliminated by the addition of nutrients. *Bacillus subtilis niger* held in contact with *E. coli* for 24 hr became nonviable, and failed to recover even when transferred to new media. Phytopathology 61: 1447-1450.

Additional key words: Pyrenochaeta terrestris, Fusarium spp., *Rhizoctonia solani, Pythium ultimum.*

Forms of *Bacillus subtilis* were obtained in routine isolations from rhizospheres of maturing sugar beets (*Beta vulgaris* L.). This bacterium, a producer of antibiotics (7), is antagonistic to fungi (1, 2, 6, 9). Isolates of *B. subtilis*, therefore, were tested in culture against dominant parasitic and saprobic fungi colonizing sugar beet feeder-root systems. *Bacillus subtilis niger*, one of the most common forms encountered, was highly antagonistic in culture to the sugar beet root parasites, *Phoma betae* Frank and *Pyrenochaeta terrestris* (Hansen) Gorenz, Walker, & Larson. *Pyrenochaeta terrestris* is a common cortical parasite of sugar beet feeder roots, especially in old onion field soils in Colorado (5). In addition, *B. subtilis niger* was moderately antagonistic in the same tests to the parasite *Rhizoctonia solani* Kuehn, slightly inhibitory to saprobic and weakly parasitic forms of *Fusarium roseum* (Link) emend. Snyd. & Hans., *F. oxysporum* (Schlecht.) emend. Snyd. & Hans., and *F. solani* (Mart.) Appel & Wr. emend. Snyd. & Hans., and noninhibitory to the pathogen *Pythium ultimum* Trow (Kreutzer, *unpublished data*).

Phoma betae, a common seed-borne pathogen of sugar beet (3), was present in ca. 40% of our seed lots tested and was selected for further interaction studies with *B. subtilis niger*. Preliminary trials in which sugar beet seeds coated with spores of *B. subtilis niger* were planted in both steamed (autoclaved 15 lb./30 min) and nonsteamed soils showed no control of post-emergence attack of resultant seedlings by seed-borne *P. betae*. We believed that this result arose from interactions between *B. subtilis niger* and other microorganisms, thus nullifying the antagonistic effect of *B. subtilis niger* on *P. betae*. This hypothesis was investigated, using a simple model designed to simulate the nutrient-enriched rhizosphere of the sugar beet feeder root (8) in which interactions of *P. betae* and *B. subtilis niger* could be tested in the presence or absence of other common bacteria.

MATERIALS AND METHODS.—Sugar beet seeds were treated by immersion in suspensions of *P. betae* conidia (ca. 2×10^5/ml) or *B. subtilis niger* spores (ca. 10^7/ml), or in mixed spore suspensions of both organisms. Samples of treated seeds were transferred to nutrient agar in petri dishes to ascertain viabilities of the microorganisms. Other seed lots were planted in prepared soils.

The planting medium consisted of one part clay-loam field soil to three parts of a sand-loam-ground peat mixture, in approximately equal volumes. Portions of this medium were autoclaved (15 lb./30 min); other fractions were not. Prior to planting, soil lots were moistened to 50% of field capacity and placed in either plastic pots (116 mm diam) or in large test tubes (25 x 200 mm). To prevent attack of sugar beet seedlings in nonsteamed soil by indigenous species of *Pythium*, raw soil lots were treated with single applications of a 250-μg/ml aqueous solution of *p*-dimethylaminodiazobenzene sodium sulfonate (4).

A simulated rhizosphere model was prepared by pouring thin (2-mm) sheets of nutrient agars in petri dishes. Three media were used, standard 2% potato-dextrose agar (PDA), and yeast extract and petone agars. After the sheets solidified, the surface of each was seeded with a test organism. Sheets then were carefully removed and placed one on top of another, seeded side uppermost, each separated from an underlying layer by sterile cellophane film. In all tests, three layers of agar were employed, designated as

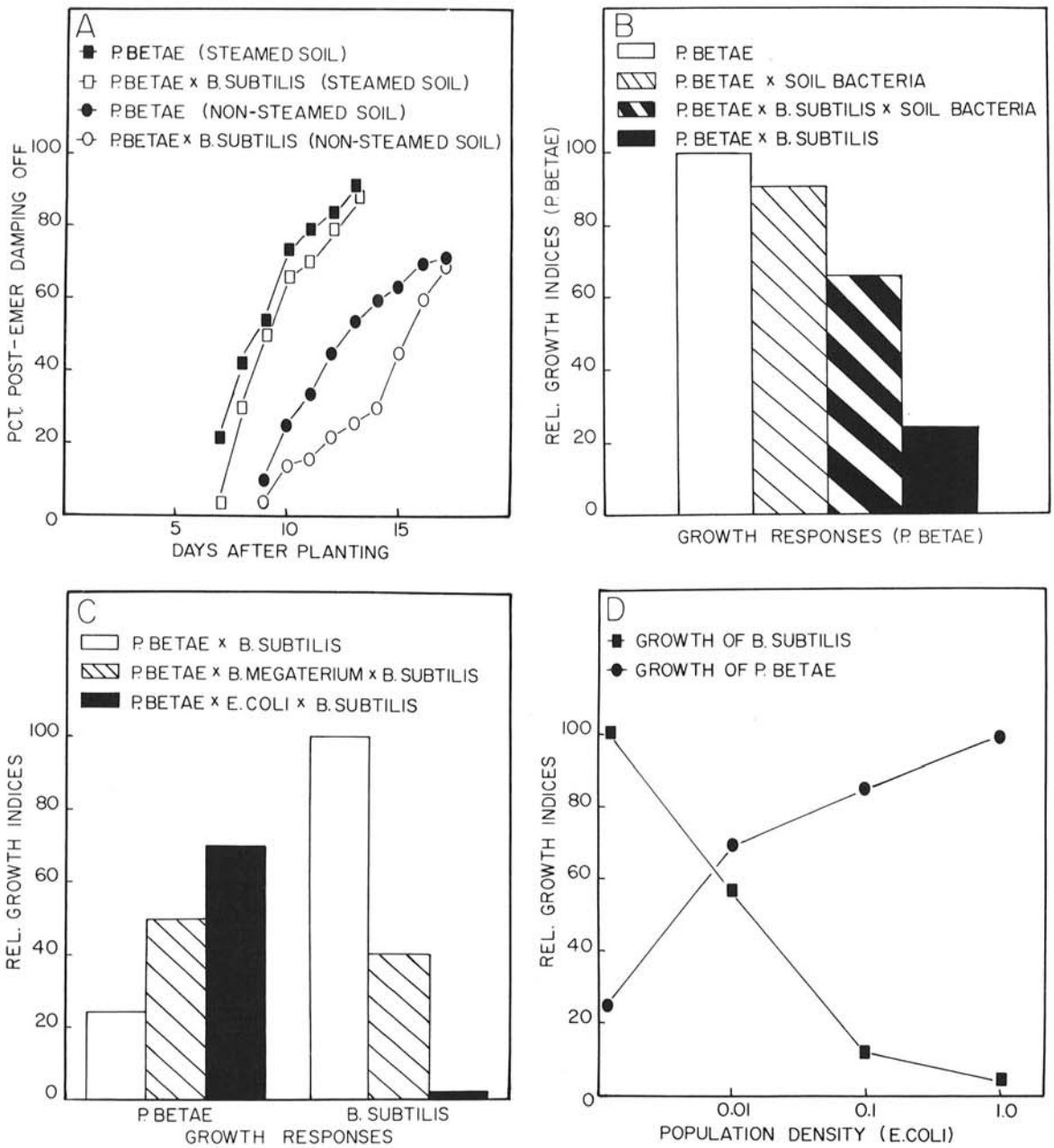

Fig. 1. Responses of *Phoma betae* and *Bacillus subtilis* var. *niger* to the presence or absence of other organisms. **A)** Effects of seed-borne *B. subtilis* on postemergence damping-off of sugar beets caused by seed-borne *P. betae* in steamed and nonsteamed soils. **B)** Growth of *P. betae* in the presence of soil bacteria and *B. subtilis*. **C)** Growth responses of *B. subtilis* and *P. betae* to interactions with *Bacillus megaterium* and *Escherichia coli*. **D)** Effects of population densities of *E. coli* on growth of *B. subtilis* and *P. betae*.

"top", "middle", and "bottom" layers. *Phoma betae, Bacillus subtilis niger, Bacillus megaterium*, and a mixed bacterial population from sugar beet rhizospheres, as well as *Escherichia coli*, an atypical soil organism, were used in these studies.

Growth of *B. subtilis niger* was estimated by comparing colony numbers and sizes in treatments with those of corresponding controls. Growth of *P. betae* was determined by measuring radial hyphal growth in millimeters microscopically. Growth indices for all test organisms were calculated in percentage of controls. All experiments were conducted at room temperature (22-24 C).

RESULTS.—*Interactions of Beta vulgaris, Bacillus subtilis var. niger, and Phoma betae in steamed and nonsteamed soils.*—Sugar beet seedlings from seeds naturally or artificially infested with *P. betae*, with or without *B. subtilis niger*, and grown in steamed and

nonsteamed soils, showed only minor differences in damping-off patterns. Summarized data from three trials involving a total of 3,600 seedlings, each consisting of four treatments and three replications, are presented in Fig. 1-A. The curves indicate limited initial retardation of postemergence attack by *P. betae* in the presence of *B. subtilis niger*. In no instance, however, was control obtained.

Treatment of raw soil with *p*-dimethylaminodiazobenzene sodium sulfonate controlled damping-off caused by indigenous species of *Pythium*, but did not interfere with postemergence attack by *P. betae*. The chemical also was nontoxic to *B. subtilis niger* at the dosage used (250 μg/ml), thereby permitting evaluation of the bacterium on pathogenicity of *P. betae*.

Results in soil differed from responses after placement of infested seeds on nutrient agars. Here, colonies of *P. betae* failed to develop in the presence of *B. subtilis niger*, and resultant seedlings were not attacked by *P. betae*. However, seedlings from seeds infested solely by *P. betae* were rapidly destroyed by expanding *P. betae* colonies.

Interactions of Phoma betae, Bacillus subtilis var. niger, and a mixed population of soil bacteria in a simulated rhizosphere.—The inability of *B. subtilis niger* to control *P. betae* in nonsteamed soil was not unexpected. The failure to obtain some disease control in steamed soil, however, suggested introduction via seeds of organisms capable of nullifying the antagonism of *B. subtilis niger* to *P. betae*.

The three-layered agar model designed to simulate an environment in which such postulated interactions might occur was used to determine the effect of *B. subtilis niger* on *P. betae* in the presence or absence of a mixed soil bacterial population. *Phoma betae* was always grown on a top layer of 2% PDA, using 4-mm-diam agar disc inoculum centers. Middle and bottom layers consisted of 2% peptone or yeast extract agars. Middle layers were either kept uninfested or were seeded with the bacterial mixture; bottom layers were either left sterile or were seeded with *B. subtilis niger* (10^6/ml). Radial measurements of *P. betae* colonies were made after 3 days' incubation.

Optimum growth of *P. betae* (mean diam 24.4 mm/72 hr = growth index 100) was obtained on PDA agar layers contiguous to sheets of the same medium, and in the absence of other organisms. Results from four experiments are shown (Fig. 1-B) giving mean growth indices of *P. betae* for each interaction. *Bacillus subtilis niger* markedly restricted the growth of *P. betae* (mean growth index 24.0). Soil bacteria alone only slightly retarded the growth of *P. betae* (index 91.0), but in the presence of *B. subtilis niger*, these bacteria apparently interfered with its antagonistic effect (mean growth index for *P. betae* 66.0).

Interactions of Phoma betae, Bacillus subtilis var. niger, Bacillus megaterium, and Escherichia coli in a simulated rhizosphere.—The effect of *B. subtilis niger* on *P. betae* was studied in the presence of *B. megaterium* or *E. coli* utilizing the simulated rhizosphere model. In these experiments, five selected interactions (including controls) x 10 replications were employed. *Phoma betae* was again placed on an uppermost layer of PDA, the middle layer of 2% peptone agar was kept sterile or was seeded with *B. megaterium* or *E. coli*, and the bottom layer of 2% yeast extract agar was either kept sterile or was inoculated with *B. subtilis niger*. Growth assessments were made after 3 days' incubation at 22-24 C. Data are summarized in Fig. 1-C.

Best growth of *P. betae* occurred in the absence of all bacteria (growth index 100). Where both *B. subtilis niger* and *E. coli* were present, the mean growth index for *P. betae* was 70. In the presence of both *B. megaterium* and *B. subtilis niger*, the index for *P. betae* was 50. In the presence of *B. subtilis niger* only, the index of *P. betae* was 25. Optimum growth of *B. subtilis niger* (index 100) occurred either in the absence of other organisms or in the presence of *P. betae* only. In the presence of *B. megaterium*, growth of *B. subtilis niger* was reduced 60% (index 40); in the presence of *E. coli*, *B. subtilis niger* showed little growth (index < 2).

Effects of population density of E. coli and preincubation of B. subtilis niger on antagonized microorganisms in a simulated rhizosphere.—The influence of population density of *E. coli* on the growth of *P. betae* and *B. subtilis niger* was investigated, using the in vitro model. In this experiment, *E. coli* was either absent or present in three concentrations (bottom layer, peptone agar) in interactions with *P. betae* (top layer, PDA) and *B. subtilis niger* (middle layer, yeast extract agar). A water suspension of *E. coli* cells (5×10^8/ml) was prepared and diluted 9:1 and 99:1 with sterile water. Six drops of sterile water or an equal quantity from each of the three prepared cell suspensions were added to 10 ml warm nutrient agar in each of 24 petri dishes (four treatments x six replications). This gave calculated *E. coli* populations of 10^7, 10^6, 10^5, and 0 cells/ml agar, equaling assigned density values of 1.0, 0.1, 0.01, and 0, respectively. Growth of *B. subtilis niger* was estimated by recording the numbers and sizes of colonies appearing after an incubation period of 72 hr at 22-24 C. Growth of *P. betae* was determined by measuring radial hyphal growth at the end of this time. Results summarized in Fig. 1-D show that as the density of *E. coli* cells increased, the growth of *P. betae* increased, with a corresponding drop in the growth of *B. subtilis niger*.

Preincubation effects of *B. subtilis niger* (bottom layer, yeast extract agar) were studied in three experiments in the presence or absence of soil bacteria (middle layer, PDA, peptone or yeast extract agars) and *P. betae* (top layer, PDA). In each experiment, *Bacillus subtilis niger* was seeded on the nutrient medium at the same time interacting organisms were introduced, or was preincubated by growing it for 18 hr in advance of test initiation (four treatments x six replications). Readings were made by measuring hyphal growth of *P. betae* microscopically at the end of 96 hr at 22-24 C.

Phoma betae showed 75% growth inhibition in the presence of nonpreincubated *B. subtilis niger*, 87% inhibition in the presence of preincubated *B. subtilis*

niger, 59% inhibition in the presence of soil bacteria x nonpreincubated *B. subtilis niger*, and 79% inhibition in the presence of soil bacteria x preincubated *B. subtilis niger*. This study indicated that preincubation of *B. subtilis niger* enhanced its antagonistic effect on *P. betae*. The mixed soil bacteria, although slightly antagonistic to *P. betae*, tended to nullify the antifungal properties of *B. subtilis niger*.

Finally, when *B. subtilis niger* cells on layers of nutrient agar were held in contact for 24 hr with sheets of media containing the growing antagonistic *E. coli*, they failed to grow when placed on sterile nutrient agars (peptone or PDA). Unexposed *B. subtilis niger* cells transferred to nutrient agar layers previously exposed to *E. coli* also failed to grow. Trials involving 16 preparations were conducted in an attempt to compensate for a possible nutrient deficiency as a cause of this antagonism by adding a layer of nutrient agar (2% peptone) between sheets on which *E. coli* and *B. subtilis* were seeded. In no instance was the antagonistic effect of *E. coli* eliminated.

DISCUSSION.—A common sugar beet rhizosphere isolate, *Bacillus subtilis* var. *niger*, was antagonistic in culture to certain fungal colonists of sugar beet feeder roots, especially *Phoma betae*. The seed-borne pathogen, however, was not prevented by *B. subtilis niger* from attacking sugar beet seedlings growing in either steamed or nonsteamed soils. This finding is at variance with several reports in the literature, indicating that *B. subtilis* coated on planted seeds or added to soils can effect a measurable control of disease caused by fungi sensitive to the antagonist (1, 2, 6).

The failure of *B. subtilis niger* to control damping-off of sugar beet seedlings by *P. betae* in our tests could be the result of inhibition of growth of the bacterium or inactivation of its metabolites in soil by other bacteria. The in vitro model used to simulate microbial interactions in the sugar beet rhizosphere showed that although *B. subtilis niger* antagonized *P. betae*, this bacterium was in turn antagonized by *E. coli*, *B. megaterium* and, to a lesser extent, by a mixture of soil bacteria.

We believe the methods used would rule out oxygen or nutrient deficiencies as growth retardants. Furthermore, since several microorganisms studied inhibited the growth of *B. subtilis niger*, it seems unlikely that the effect was due to a metabolite specific for any one organism. Rather, the inhibiting factor may be a metabolite common to many bacteria, and *B. subtilis niger* is quite sensitive to this substance. Thus, the capability of *B. subtilis* to exert an antagonistic effect upon causal agents of soil-borne plant diseases in natural rhizospheres may be quite limited.

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