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Effects of Annual Applications of Sodium Azide on Soil Fungal Populations with Emphasis on *Trichoderma* Species

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The effect of a granular formulation of sodium azide, applied annually to pine nursery beds at rates of 0, 67.2, and 134.5 kg a.i. ha^{-1} under water seal or plastic seal, on soil fungal populations was determined over a 3-year period. Populations of fungi in the soil decreased following application of the sodium azide each year; the greatest decrease occurred at the highest rate of application. Populations of fungi in soil treated with the azide generally remained lower than in the controls throughout each of the 3 years; however, the population disparity between treated and control plots decreased in magnitude with each succeeding year. Populations of *Trichoderma* spp., in plots treated with 134.5 kg sodium azide ha^{-1}, increased 2 weeks after treatment each year, and the population peaks increased in magnitude each year. In addition the effect of sodium azide (technical grade > 99%) at concentrations of 0, 2, 5, 10, 20 and 50 μg ml^{-1} in potato dextrose agar and blackstrap molasses agar media was determined *in vitro* for 14 isolates of *Trichoderma harzianum*. Growth and sporulation differed among the isolates and between the two media tested. Generally, the azide temporarily inhibited growth of the fungi, but the majority of the isolates were able to grow on either medium containing 50 μg sodium azide ml^{-1}, although sporulation was more profuse on the molasses than on the potato dextrose agar medium.

1. Introduction

The fact that sodium and potassium azides have a broad spectrum of biological activity has created interest in these compounds for possible agricultural use as soil treatment chemicals. Several previous studies have led to reports of activity against nematodes,[1–3] fungi,[4–11] bacteria,[5] and weeds.[3,7,12,13] However, most of these studies were short-term in nature and were directed toward effects on a single target organism. Thus, information on the long-term effects of these compounds on the soil microbiota has been lacking. Previous reports described the long-term effects of sodium azide on nematodes[14] and on soil bacterial populations, soil enzymic activities and certain other biological variables.[15] This paper is a part of the same study and is concerned with populations of soil fungi over a 3-year period in plots treated with sodium azide.

2. Materials and methods

2.1. Field plots

Field plots were established at the Stauffer State Nursery near Auburn, Alabama. Soil texture was 73.8% sand, 10.6% silt, and 15.6% clay; soil organic matter was 1.5% (by wt) and the soil pH was 5.4. A split-plot experimental design with four replications per treatment was used in the study. Plots 1.5 m × 9.1 m in size were maintained for a 3-year period. In April of each year (soil temperature > 10°C), a granular formulation of sodium azide containing 8% active ingredient ('Smite 8G') at rates of 0, 67.2, and 134.5 kg a.i. ha^{-1} was applied to the plots with a calibrated fertiliser spreader

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(Gandy) and incorporated into the top 15 cm of soil with a Roto-tiller; the 67.2 kg ha^{-1} rate was not included during the third year. One-half of each plot (1.5 m × 4.6 m) was covered with a polyethylene sheet and sealed around the edges with soil; the other half of each plot was water-sealed by wetting the soil surface with an overhead irrigation system. Untreated control plots were prepared in a similar manner. The plots were split and sealed the same way during the second and third years. Plastic sheets were removed after 10 days. All plots were planted each year with slash pine seed (*Pinus elliottii* Engelm.) 2 weeks after removal of the plastic sheets. Meteorological data, including wind speed, daily soil and air temperatures, and precipitation, are available.[16–18]

2.2. Soil sample collections

Each year, initial soil samples were collected, as described previously,[15] 10 days after treatment. Additional samples were taken periodically throughout the rest of the year. Soil samples consisted of 25 randomly-collected cores taken from the top 15 cm of each half-plot with a soil tube and composited. Each composite sample was thoroughly mixed and screened through a 4.75-mm mesh sieve prior to processing.

2.3. Laboratory analyses

2.3.1. Fungal populations

For determining fungal populations, soil (5 g fresh weight; approximately 8% moisture) was placed in a 500-ml Erlenmeyer flask containing sterile water (225 ml) and a magnetic stirring bar. After 2 min of stirring on a magnetic stirrer, a Pasteur pipette was used to transfer one drop of the soil–water suspension to a sterile Petri dish. The suspension was stirred continuously while the drops were being removed with the pipette. A total of ten Petri dishes was prepared for each soil sample. About 15 ml of peptone–dextrose–Rose Bengal–streptomycin–agar (PDRBSA) medium[19] was added to each of the dishes which were then incubated at room temperature (approximately 26°C) for 4 days, after which, the total numbers of fungal colonies and colonies of *Trichoderma* spp. were counted and recorded.

To determine the dilution factor for each soil–water suspension, 20 drops of the suspension were placed in a tarred aluminium weighing dish and oven-dried overnight at about 85°C; the oven-dry weight of the soil/drop was then calculated.

2.3.2. Azide-tolerant fungi

During the third year of the study, fungal populations were determined on PDRBSA medium, with and without the addition of 50 μg sodium azide (technical grade >99%) ml^{-1} and on Ohio State agar,[20] with and without 50 μg sodium azide ml^{-1}. The azide was added to the melted media just prior to dispensing into Petri dishes. The Ohio State agar was included in order to compare the results with those obtained using the PDRBSA medium. The two azide-containing media were included to test for fungi tolerant to sodium azide. Procedures for preparing and incubating the dishes and for counting the colonies were as described in section 2.3.1.

2.3.3. Tolerance of *Trichoderma harzianum* to sodium azide

Several isolates of *T. harzianum* were obtained from Homer Wells, ARS-USDA, Department of Plant Pathology, Tifton, Georgia, USA, and tested *in vitro* for tolerance to sodium azide. Potato–dextrose–agar (PDA)[20] and blackstrap molasses agar [blackstrap molasses (25 ml), agar (20 g) and water to 1 litre], to both of which sodium azide (technical grade >99%) at rates of 0, 2, 5, 10, 20, and 50 μg ml^{-1} had been added, were dispensed into Petri dishes. Each dish of agar was inoculated in the centre with a 7-mm disc of the test fungus cut from the periphery of an actively growing colony with a flame-sterilised cork borer. Each disc was placed mycelium-side down on the agar surface. Dishes were incubated at room temperature (about 26°C) for the duration of the study. The diameter of each colony was measured and the degree of sporulation of each colony was estimated after 3, 7, 13, and 20 days.

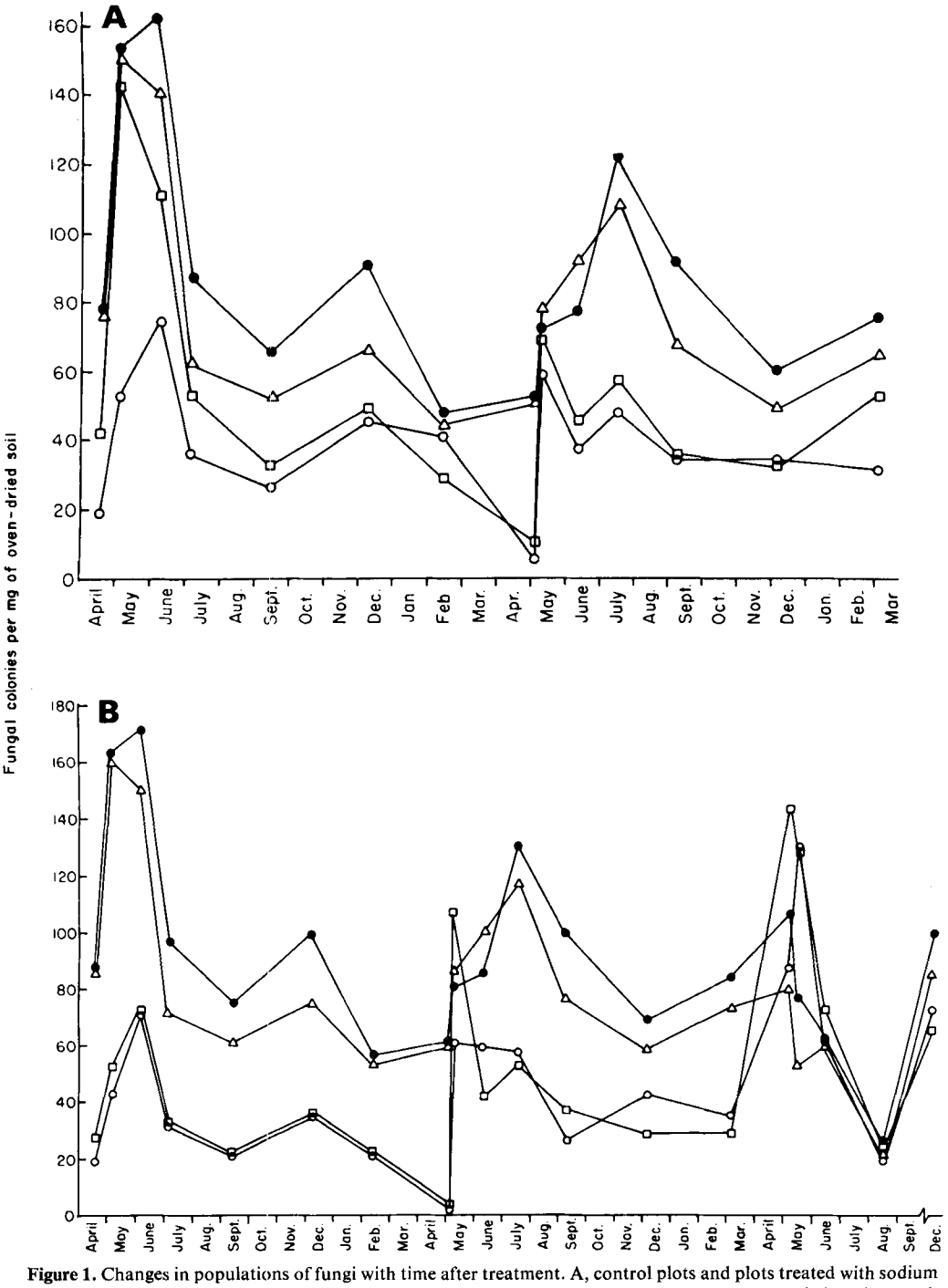

Figure 1. Changes in populations of fungi with time after treatment. A, control plots and plots treated with sodium azide at 67.2 kg ha^{-1}: (●) control, water-sealed; (△) control, plastic-sealed; (□) treated, water-sealed; (○) treated, plastic-sealed. B, control plots and plots treated with sodium azide at 134.5 kg ha^{-1}: (●) control, water-sealed; (△) control, plastic-sealed; (□) treated, water -sealed; (○) treated, plastic-sealed.

All data were subjected to analysis of variance, and means were compared for significance by Duncan's multiple range test. Differences referred to as significant in this paper were significant at the 1% level of probability.

3. Results

3.1. Fungal populations

Populations of fungi, in plots treated for each of the first 2 years with sodium azide at 67.2 kg ha^{-1}, were lower in numbers than the controls on all sampling dates, although the differences were not always significant (Figure 1A). In both years, the lowest populations were observed on the first sampling date following application of azide. Generally, populations of fungi were lower in the treated plots sealed with plastic than in plots sealed with water; this was especially obvious on the first four sampling dates of the first year.

Populations of fungi, in plots treated with sodium azide at 134.5 kg ha^{-1}, were significantly lower than the controls on all sampling dates throughout the first year (Figure 1B); no differences were observed between azide-treated plots sealed with plastic and those sealed with water. In the second year, populations of fungi on the first sampling date in the treated plots were again significantly lower than in the control plots; however, a significant recovery was observed on the second sampling date. Populations of fungi in the treated plots on the last five sampling dates of the second year were significantly lower than in the untreated controls. In the third year, populations of fungi on PDRBSA medium, at the first sampling date for the azide-treated plots that had been plastic-sealed, were not different from the controls but populations in the azide-treated plots that had been water-sealed were significantly greater than in the controls. On the second sampling date both the water-sealed and plastic-sealed plots treated with azide had populations of fungi significantly greater than the controls; however, on the last three sampling dates no differences were observed among the treatments. Populations of fungi as determined on Ohio State agar during the third year were generally lower in numbers than on PDRBSA medium (data not shown).

3.2. *Trichoderma* populations

The number of *Trichoderma* colonies from plots treated with sodium azide at 67.2 kg ha^{-1} was not different from the controls on most sampling dates during the 2-year study (Figure 2A). However, a significant effect was observed on the second sampling date of each year. In the first year, the azide-treated plots that had been water-sealed had a much higher population of *Trichoderma* spp. than the plastic sealed azide-treated plots, or the controls. In the second year, significantly higher populations of *Trichoderma* spp. were observed in the azide-treated plots, regardless of the method of sealing.

Populations of *Trichoderma* spp., in plots treated with sodium azide at 134.5 kg ha^{-1}, were not different from the controls during the first year of study (Figure 2B). However, on the second sampling date of the second year, *Trichoderma* spp. in the azide-treated plots were more abundant than in the controls; the highest populations were observed in the azide-treated water-sealed plots. No differences in *Trichoderma* populations were observed between the azide-treated plots and the controls during the remainder of the second year. On the first sampling date of the third year, *Trichoderma* populations in the water-sealed azide-treated plots, as determined on PDRBSA medium, were significantly greater than the controls; no difference was observed between the plastic-sealed plots and the controls. On the second sampling date of the third year, both the water-sealed and the plastic-sealed, azide-treated plots had populations of *Trichoderma* spp. in significantly higher numbers than the controls. The peaks in *Trichoderma* populations in the azide-treated plots were similar to those observed early in the second year of the study, but were much greater in magnitude. No differences in *Trichoderma* populations were observed among treatments on the last three sampling dates of the third year. Populations of *Trichoderma* spp., as determined on Ohio State agar during the third year, were generally lower in numbers than on PDRBSA medium; however, the percentage of colonies that were in the genus *Trichoderma* did not differ between the two media (data not shown).

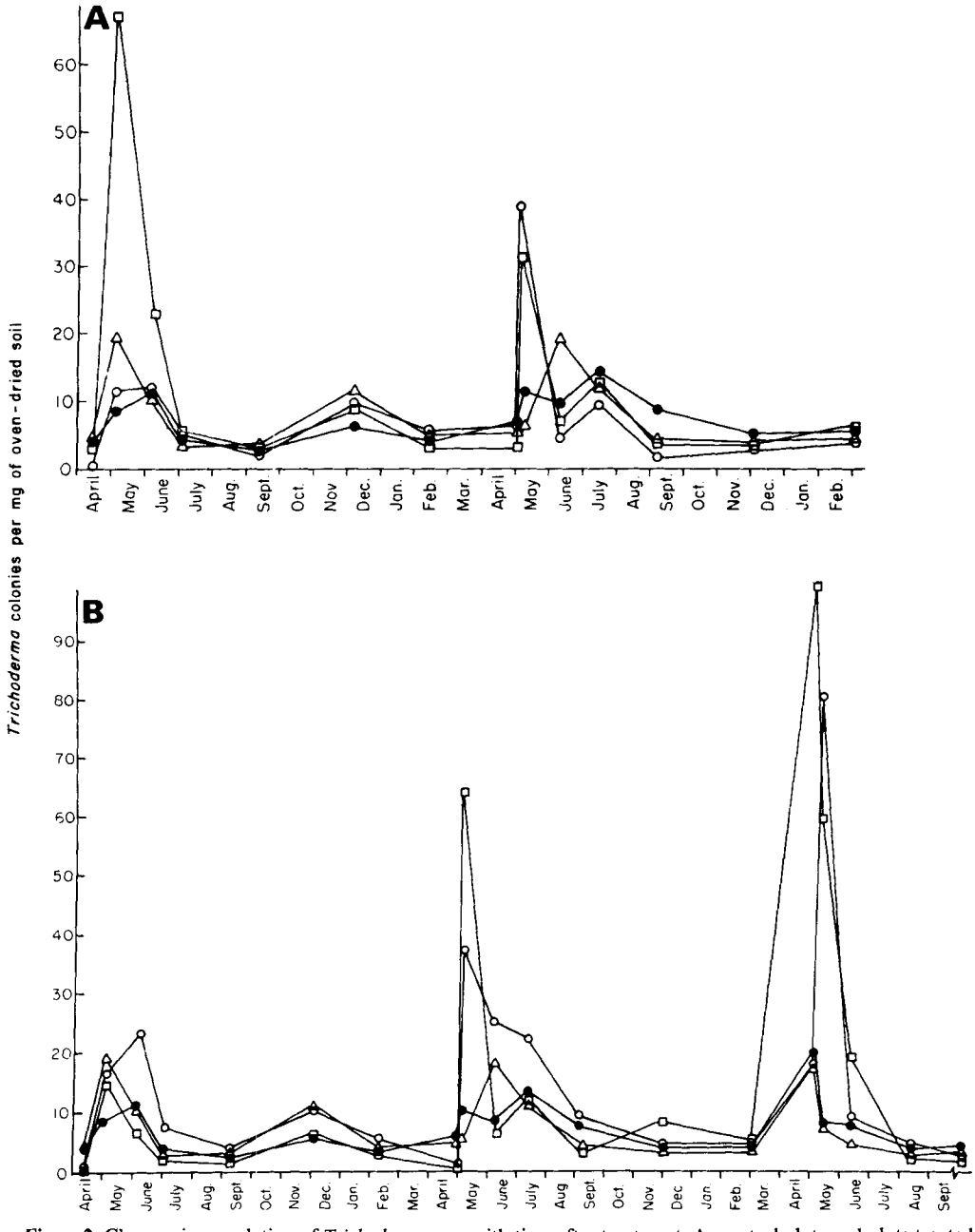

Figure 2. Changes in population of *Trichoderma* spp. with time after treatment. A, control plots and plots treated with sodium azide at 67.2 kg ha^{-1}: (●) control, water-sealed; (△) control, plastic-sealed; (□) treated, water-sealed; (○) treated, plastic-sealed. B, control plots and plots treated with sodium azide at 134.5 kg ha^{-1}: (●) control, water-sealed; (△) control, plastic-sealed; (□) treated, water-sealed, (○) treated, plastic-sealed.

3.3. Azide-tolerant fungi

Throughout the year, significant numbers of fungi, capable of growing on the two azide-treated media, were isolated from plots treated with sodium azide at 134.5 kg ha^{-1}; data are shown only for the Ohio State agar (Figure 3A). Water-sealed plots treated with azide favoured azide-tolerant fungi throughout the year, whereas the population increase in the plastic-sealed plots was significant only on the first, third and fourth sampling dates.

A significant increase in numbers of azide-tolerant *Trichoderma* spp. occurred only on the first and second sampling dates (Figure 3B); on these dates, populations in the azide-treated plots that were water-sealed were higher than in plots where plastic sealing was used.

3.4. Tolerance of *Trichoderma harzianum* to sodium azide

Growth and sporulation differed among the 14 isolates and between the media tested. After 20 days on media treated with 50 μg sodium azide ml^{-1}, six of the isolates had made no growth on the molasses medium; nor did four of the six isolates grow on the PDA medium (Figure 4A). Conversely, five of the isolates had reached the maximum diameter of 8.5 cm by the 20th day on molasses medium, while two isolates reached the maximum on the PDA medium.

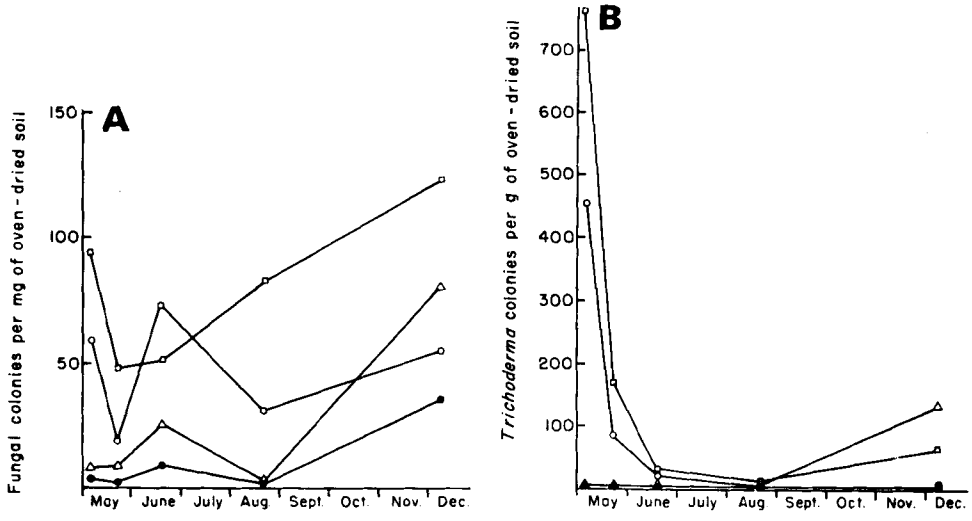

Figure 3. Effect of annual applications of sodium azide on: A, numbers of fungi capable of growing on Ohio State agar containing 50 μg sodium azide ml^{-1} Controls: (●) water-sealed; (△) plastic-sealed. Treated with 134.5 kg sodium azide ha^{-1}: (□) water-sealed; (○) plastic-sealed. B, numbers of *Trichoderma* spp. capable of growing on Ohio State agar containing 50 μg sodium azide ml^{-1}. Controls: (●) water-sealed; (△) plastic-sealed. Treated with 134.5 kg sodium azide ha^{-1}: (□) water-sealed; (○) plastic-sealed.

Sporulation was generally more profuse on the molasses medium than on the PDA medium. After 20 days on medium treated with 50 μg sodium azide ml^{-1}, eight of the isolates were rated at the highest sporulation index on the molasses medium (the sporulation index is determined subjectively as a range of indices from 0 to 5; 0= no sporulation, 5 = profuse sporulation); the other six isolates did not sporulate (Figure 4B). On the PDA medium, five of the isolates failed to sporulate, while the sporulation index for the other nine ranged from 1.0 to 4.0. After 3 days, growth of the fungi generally was inversely proportional to the azide concentration in the medium; with time this inverse relationship became less evident. Growth curves for isolate SOB No. 2 on molasses medium are presented in Figure 5A.

Sporulation was also affected by the concentration of azide in the medium (Figure 5B). No sporulation was observed for isolate SOB No. 2 on molasses medium after 3 days; however, after

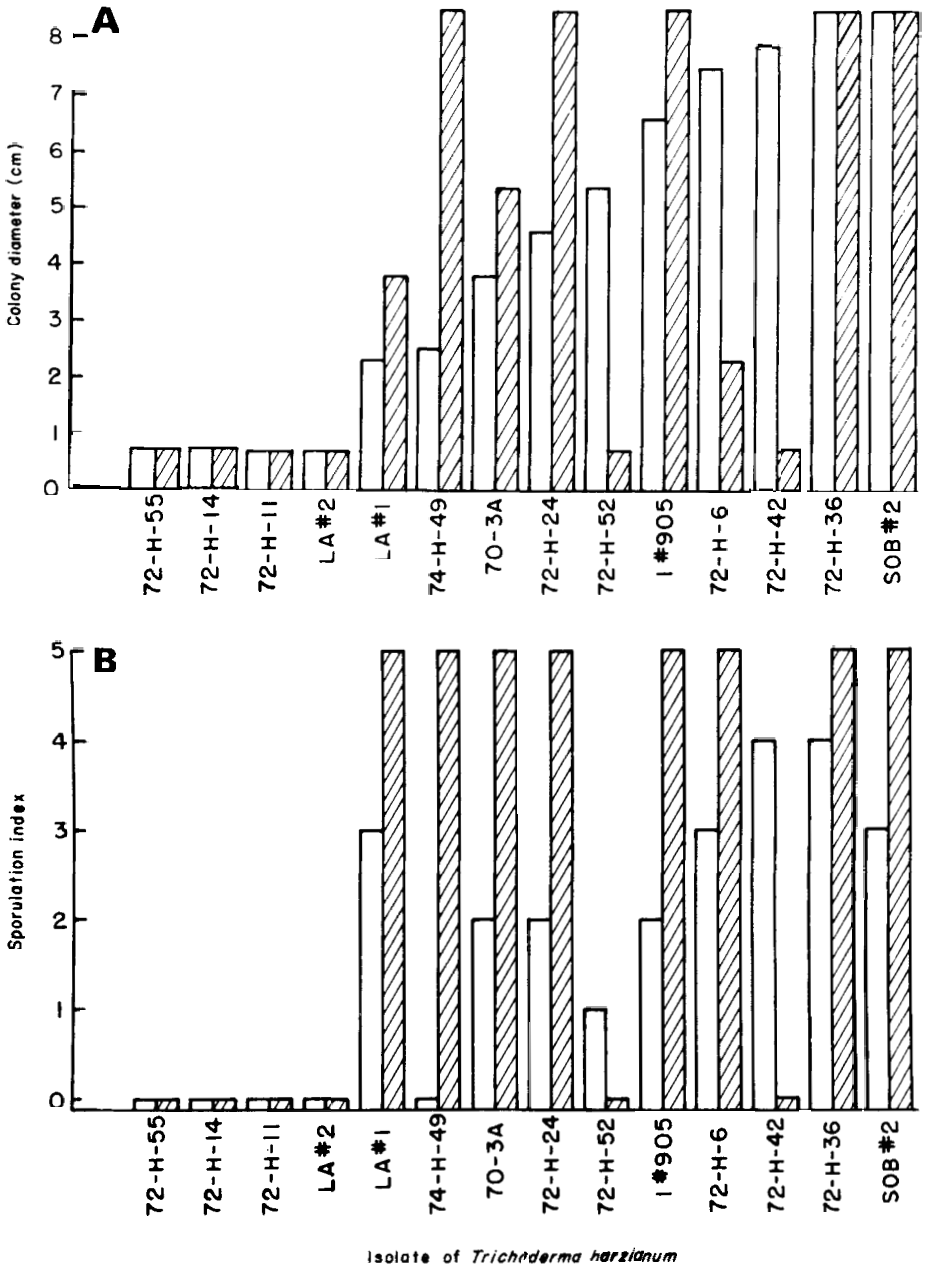

Figure 4. Effect of 50 μg sodium azide ml^{-1} on A, growth and B, sporulation of 14 *Trichoderma harzianum* isolates after 20 days in (□) potato–dextrose–agar and (▨) blackstrap molasses–agar. The sporulation index is determined subjectively as a range of indices from 0 to 5; 0 = no sporulation, 5 = profuse sporulation.

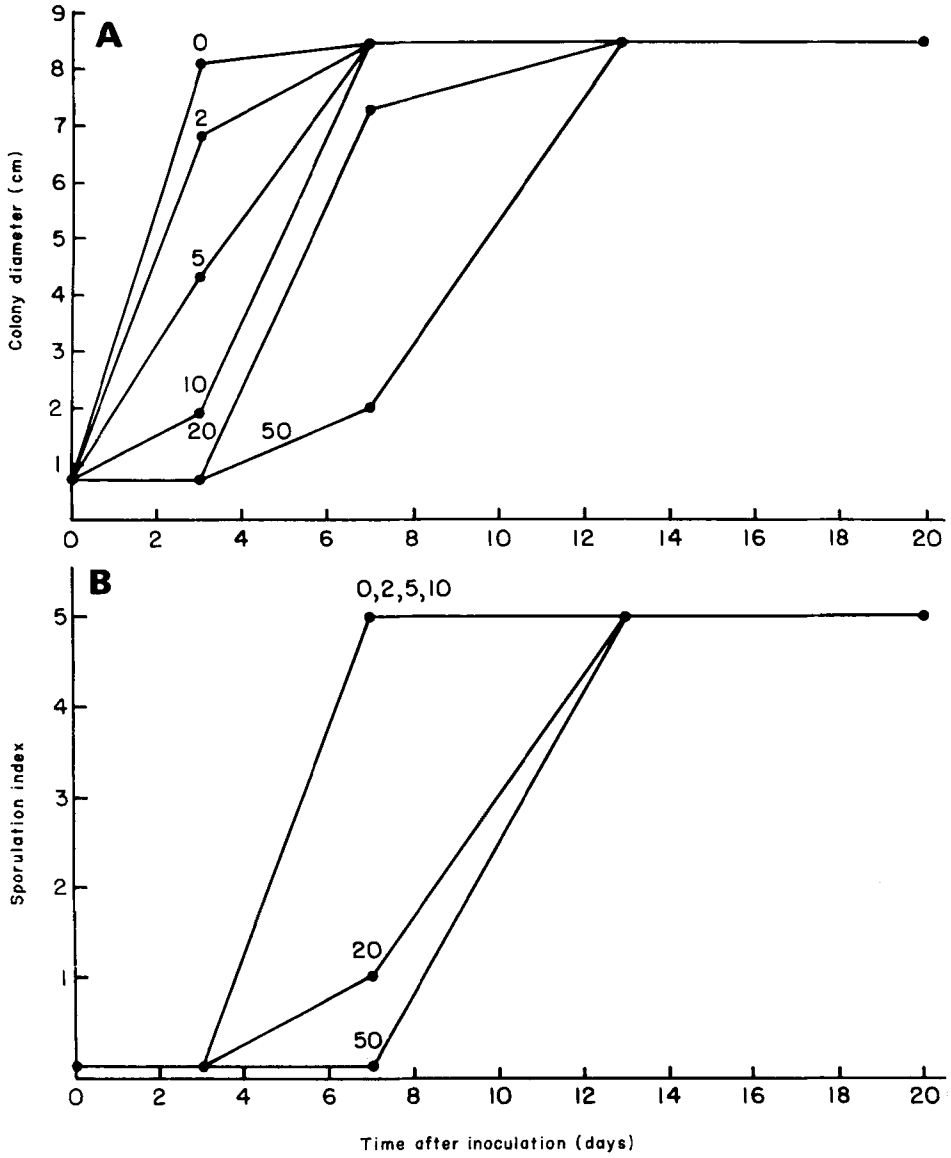

Figure 5. Effects of sodium azide at 0, 2, 5, 10, 20, and 50 μg ml^{-1} of blackstrap molasses-agar on A, growth and B, sporulation of *Trichoderma harzianum* isolate SOB No. 2 over a 20-day period. The sporulation index is determined subjectively as a range of indices from 0 to 5; 0 = no sporulation, 5 = profuse sporulation.

7 days, a sporulation index of 5.0 was assigned for the fungus on medium containing 0, 2, 5, and 10 μg sodium azide ml^{-1}. By the 13th day, a sporulation index of 5.0 was assigned to the fungus on medium containing 20 and 50 μg sodium azide ml^{-1}.

4. Discussion

Azides are classical inhibitors of enzymes in the electron-transport system, particularly of cytochrome oxidase and other metal-containing enzymes. Consequently, azides have been used in a number of selective media for some Gram-positive bacteria. It is, therefore, not surprising that

their activity on fungal populations in the present studies was one of depressing these populations immediately after azide application to the soil; this decrease in the number of fungi in the soil continued for some time. These results for fungal populations are in contrast with those reported for bacterial populations.[15] Bacterial populations were shown to increase, soon after treatment with sodium azide at 134.5 kg ha^{-1}, to levels much higher than in the controls, and then to decline with time to levels similar to those for the controls, resembling the classical response often described for methyl bromide.

This general pattern for fungal populations is not true for all species. Some, such as those in the genus *Trichoderma*, behave in a manner similar to bacteria. Because of their activity against respiratory enzymes, azides could be expected to be active only against forms that are actively growing at the time of treatment. Structures such as spores and other resting reproductive sources with low respiratory rates would be unaffected. As azide dissipates from the soil, spores of fungi can germinate, and those that are fast growing can be expected to recolonise the soil rapidly. The increase in numbers of *Trichoderma* colonies can be interpreted as partly the result of such mechanisms; however, the tolerance shown by *T. harzianum* in the in-vitro tests suggests that *Trichoderma* spp. may possess a cellular mechanism that either inactivates the azide or excludes it from sites of active respiration (mitochondria). Such mechanisms, coupled with the fast recolonisation rate, would give these fungi an ecological advantage over competing species in the soil.

This explanation is substantiated by the progressive increase in numbers of *Trichoderma* spp. with each succeeding year in plots treated with 134.5 kg sodium azide ha^{-1}. This is significant because *Trichoderma* spp. are classical antagonists[21] of several soil-borne pathogenic fungi, a fact that has been the basis for the development of successful biological control systems.

A corollary of the present findings is that many of the control effects attributed to azide may not be due to its direct activity against soil-borne pathogenic fungi. The results suggest that such effects may have been due to enhancement of antagonistic fungal populations. The activity of sodium azide thus resembles that of other chemicals used for soil treatments. Selectivity for *Trichoderma* spp. has been reported previously[6,22–25] for other broad-spectrum biocides, including formalin, carbon disulphide and allyl alcohol, and in many cases the beneficial effect of these treatments has been linked to such selectivity.

5. Conclusions

Sodium azide is a selective antifungal agent for soil treatment with potential for the development of management systems through enhancement of *Trichoderma* spp. for control of soil-borne fungal pathogens.

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