EFFECT OF NITRIFICATION INHIBITORS ON NITROGEN-FIXING BACTERIA

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ABSTRACT

The nitrification inhibitor dicyandiamide (DCD) did not inhibit the growth and respiration of N-fixing bacteria (Rhizobium leguminosarum Azotobacter chroococcum) in cell-suspensions with concentrations of 400 m gm^{-1} DCD. Growth of Rhizobium legumiinosarum was inhibited by 17% with 100 m gm^{-1} nitrapyrin (N serve), but respiration was not affected. [Growth of Azotobacter chroococcum was inhibited by 10 m gm^{-1} (10%) and 100 m gm^{-1} nitrapyrin (50%), in the latter case, respiration was also impaired (36%); thiourea only caused a minor growth-inhibition of Azotobacter chroococcum with 100 m gm^{-1} (8%) and had no effect on Rhizobium leguminosarum.

INTRODUCTION

One of the most important requirements for nitrification-inhibitors is specificity for bacteria of the species *Nitrosomonas europaea,* which perform the first step of nitrification. Impact on other soil-bacteria should be negligible or as low as possible. Symbiotic and non-symbiotic N-fixing bacteria of the genus Rhizobium and Azotobacter should especially not be impaired in their activity. The strong inhibiting effect of nitrification-inhibitors on N-fixing bacteria had been reported by different researchers: Hughes and Welch, 1970, Vannelli and Hooper 1993, and Bedard and Knowles 1997.

The objective of this study was to determine the effect of the nitrification-inhibitors, Dicyandiamide (DCD), N serve (NS) and Thiourea (TU), on growth and respiration of Rhizobium and Azotobacter.

Materials and Methods

The two strains, *Rhizobium leguminosarum* and *Azotobacter chroococcum,* were cultivated in the following nutrient solution:

D (-) Mannite	10.0 g
K_2HPO_4	0.5 g
$MgSO_4 + 7H_2$	0.2 g
NaCl	0.1 g
Yeast extract	1.5 g

pH 7.2 (adjusted after autoclaving).

The stock cultures grew on solid nutrient media. Petri dishes were prepared from the above nutrient-solution, after addition of 1.5 % agar, and inoculated by means of a loop.

Both microorganisms were cultivated in the above-mentioned nutrient solution. At first, inoculated medium was incubated at 25 ^{0}C on a rotary shaker; this preliminary culture was used as an inocolum after optical density was adjusted to 1.2 (578 nm). Five replicates of each treatment were started with 10ml nutrient solution plus respective amounts of inhibitors in flasks. The flasks were inoculated with 0.1 ml of the preliminary culture and shaken in the warm water-bath at 25 ^{0}C. After 20 h, the turbidity of the nutrient solution was measured, using a spectrometer (578nm).

Bacteria were pre-cultivated for 20h, then centrifuged (3500 rpm) and washed twice with 0.1 M phosphate buffer (pH 7.2). From this suspension, 1.5ml were pipetted together with 0.5 ml glucose (1 %) into the main part of warburg flasks. The center-well contained 0.2 ml KOH (5%). The nitrification inhibitors were pipetted into the side-arm and added after a preliminary run of 30 minutes. All measurements were done in 6 replicates at 28 ^{0}C.

RESULTS

1. Growth and respiration of Rhizobium

Among the tested inhibitors, only N-serve at the higher concentration of 100 m gm^{-1} affected growth of Rhizobium with significant depression of 17% (Figure-1). Respiration was not impaired in any case. The respiration rates of inhibitor-treatments and untreated controls were almost identical (Table-1). The untreated bacteria consumed 215

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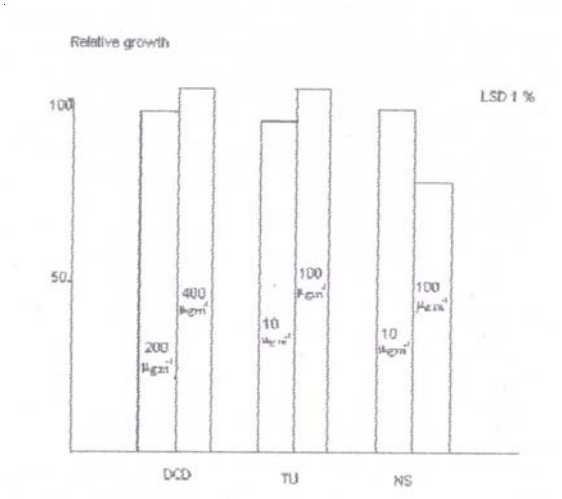

Figure - 1: Effects of inhibitors on relative growth of Rhizobium leguminosarum (control=100) (DCD=dicyandiamide, TU=thiourea, NS=nitrapyrin)

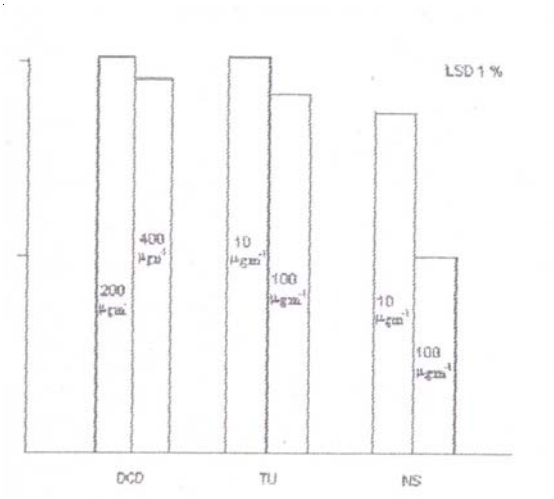

Figure - 2: Effects of inhibitors on relative growth of Azotobacter chroococcum (control=100) (DCD=dicyandiamide, TU=thiourea, NS=nitrapyrin)

ml oxygen during the experiment. All the 3 inhibitors led to a slightly increased respiration which, however, was never significant.

2. Growth and respiration of Azotobacter.

As compared to Rhizobia, the growth of asymbiotic Azotobacter bacteria was more sensitive to nitrification inhibitors (Figure-2). While 200 m gm^{-1} DCD, 400 m gm^{-1} DCD and 100 m gm^{-1} Tu did not affect the growth, the other treatments resulted in significant depressions of growth by 8% (100 m gm^{-1} TU). 10% (10 m gm^{-1} NS) and 50% (100 m gm^{-1} NS). Respiration was not affected to the same extent (Table-1). Dicyandiamide and thiourea did not reduce respiration, as compared to control. With 10 m gm^{-1} N-serve, however, total oxygen consumption over a 2h period was lowered by 36%, which was due to stagnating respiration at the beginning of the experiment. After half an hour, respiration increased similar to other treatments.

DISCUSSION

In this study, respiration was selected as the most sensitive parameter to be measured, because N-fixing bacteria have a very intensive respiration due to their high energy-demand to reduce N_2. Therefore, a depression in respiration-rate by a nitrification inhibitor would be expected to result in reduced N_2 fixation. The results of this study revealed their DCD is non-toxic to two important N_2 fixing bacterial genera, even at very high concentrations (400 m gm^{-1}). However, it delayed the bacterial oxidation of ammonium ions by depressing the activities of *Nitrosomonas* in the soil for a certain period of time. Thus, there is a need for further studies to be carried out on the manipulation of DCD doses to study the critical relationship of *R. leguminosarum* with host-plant included in the study. Stichlmair (1984) demonstrated that C-heterotrophic soil microoganisms (eg Arthrobacter sp., Bacillus sp.) and important soil-enzymes are also not affected by 400 m gm^{-1} DCD. In long-term field trials (53 years) with calcium cyanamide (containing 10% DCD), only positive effects on soil biological activities were observed by Bosch and Amberger (1983).

Thiourea surprisingly did not have a toxic effect on N-fixing bacteria in pure culture, even though it had been classified earlier as highly toxic for soil microorganisms by Frederick *et al* (1957) and Zacherd and Amberger (1990). N-serve, while having only a small effect on Rhizobium, strongly affected the non-symbiotic Azotobacter bacteria. The results are similar to Chamber *et al.* (1980), in which N-serve was defined as toxic for N-fixing bacteria and mycorrhizae. N-serve thus does not fulfill the ecological requirement of specifically inhibiting only on Nitrosomonas sp. Prevalence of nitrification inhibitor shows its control

Table – 1: Oxygen consumption of Rhizobium and Azotobacter within 2 hours

Treatment*	*Rhizobium leguminosarum*		*Azotobacter Chroococcum*
%	$\mu 10_2$	%	μlCo_2
Control	215	100	177
100			
400 μ gm^{-1} DCD	217	101	169
95			
100 μ gm^{-1} Tu	230	107	170
96			
100 μ gm^{-1} Ns	222	103	114
64			
L.S.D. 1%	18		15

* DCD - Dicyandiamide
Tu-Thiourea; Ns-Nitrapyrin (N-Serve)

on nitrogen-fixing bacteria in the soil, which transfer ammonia into nitrite and then these nitrities into nitrates.

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