

## Effect of nitrification inhibitors on N-fixing bacteria *Rhizobium leguminosarum* and *Azotobacter chroococcum*

B. Zacherl & A. Amberger

Institute of Plant Nutrition, Technical University of Munich, Weihenstephan, BRD

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### Abstract

The nitrification inhibitor dicyandiamide [DCD] did not inhibit growth and respiration of N-fixing bacteria (*Rhizobium leguminosarum* and *Azotobacter chroococcum*) in cell suspensions with concentrations of 400 ppm DCD. Growth of *Rhizobium leguminosarum* was inhibited by 17% with 100 ppm nitrapyrin (N-Serve), but respiration was not affected. Growth of *Azotobacter chroococcum* was inhibited by 10 ppm (10%) and 100 ppm nitrapyrin (50%); in the latter case, respiration was also impaired (36%). Thiourea only caused a minor growth inhibition of *Azotobacter chroococcum* with 100 ppm (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 (oxidation of ammonia to nitrite). Impacts 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 objective of this study was to determine the effect of the nitrification inhibitors dicyandiamide (DCD), nitrapyrin (N-serve, NS) and thiourea (TU) on growth and respiration of *Rhizobium* and *Azotobacter chroococcum*. The strong inhibiting effect of these three substances on *Nitrosomonas europaea* had been demonstrated earlier [4, 5, 6].

### Material and methods

#### Stock cultures

The two strains, *R. leguminosarum* RL 1 and A.

*chroococcum* 9, were supplied by the Institute for Phytopathology, Technical University Munich - Weihenstephan and by the Bayerische Landesanstalt für Pflanzenbau und Bodenkultur (Munich). They were cultivated in the following nutrient solution:

D (-)-Mannite	10.0 g
K <sub>2</sub> HPO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> × 7 H <sub>2</sub> O	0.2 g
NaCl	0.1 g
Yeast extract	1.5 g

dissolved in 1 l dist. H<sub>2</sub>O  
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.

#### Bacterial growth

Both microorganisms were cultivated in the nutrient solution described above. At first, inoculated medium was incubated at 25°C on a rotary shaker; this preliminary culture was used as an

inoculum after optical density was adjusted to 1.2 (578 nm).

A five replicates of each treatment were started with 10 ml nutrient solution plus respective amounts of inhibitors in 25 ml Erlenmeyer flasks. The flasks were inoculated with 0.1 ml of the preliminary culture and shaken in a warm water bath (GFL Typ 1083) at 25°C. After 20 h, the turbidity of the nutrient solution was measured in a spectrometer (578 nm).

#### Respiration (Warburg-apparatus)

Bacteria were pre-cultivated for 20 h, then centrifuged (3500 rpm) and washed twice with 0.1 M phosphate buffer (pH 7.2). From this suspension, adjusted to an optical density of 1.5 (578 nm), 1.5 ml were pipetted together with 0.5 ml glucose (1%) into the main part of the 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 min. All measurements were done in 6 replicates at 28°C.

## Results

### 1. Growth and respiration of *Rhizobium leguminosarum*

Among the tested inhibitors, only N-serve at the higher concentration of 100 ppm affected growth of *R. leguminosarum* with a significant depression of 17% (Fig. 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

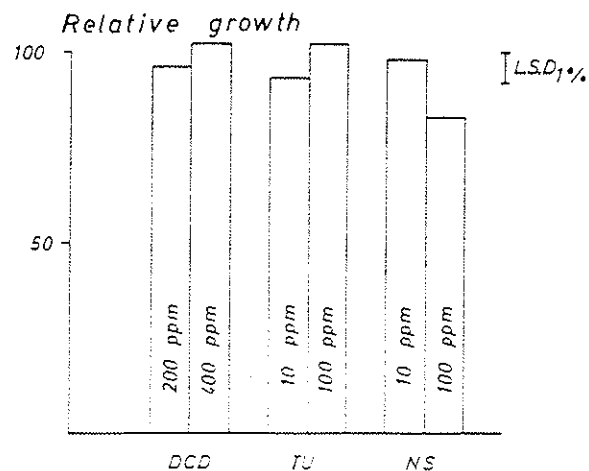


Fig. 1. Effects of inhibitors on relative growth of *Rhizobium leguminosarum* (control = 100) (DCD = dicyandiamide, TU = thiourea, and NS = nitrapyrin).

consumed 215 ml oxygen during the experiment: all 3 inhibitors led to a slightly increased respiration which, however, was never significant.

### 2. Growth and respiration of *Azotobacter chroococcum*

As compared to *Rhizobia*, the growth of asym-biotic *Azotobacter* bacteria was more sensitive to nitrification inhibitors (Fig. 2). While 200 ppm DCD, 400 ppm DCD, and 10 ppm TU did not affect growth, the other treatments resulted in significant depressions of growth by 8% (100 ppm TU), 10% (10 ppm NS), and 50% (100 ppm NS). Respiration was not affected to the same extent (Table 2). Dicyandiamide and thiourea did not reduce respiration as compared to control. With 100 ppm N-serve, however, total oxygen consumption over a 2 h period was lowered by 36%, which was due to a stagnating respiration at the beginning of the experiment.

Table 1. Oxygen consumption of *Rhizobium leguminosarum* and *Azotobacter chroococcum* within 2 hours

Treatment	Rhizobium leguminosarum		Azotobacter chroococcum	
	$\mu\text{l O}_2$	%	$\mu\text{l O}_2$	%
Control	215	100	177	100
400 ppm DCD	217	101	169	95
100 ppm TU	230	107	170	96
100 ppm NS	222	103	114	64
L.S.D. <sub>1%</sub>	18		15	

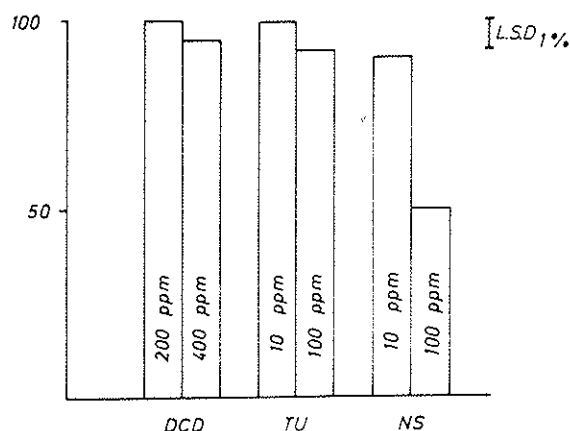


Fig. 2. Effects of inhibitors on relative growth of *Azotobacter chroococcum* (control = 100) (DCD = dicyandiamide, TU = thiourea, and NS = nitrapyrin).

After 30 min, respiration increased similar to the other treatments.

### Discussion

In our studies, 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. Our results revealed that dicyandiamide is non-toxic to two important N-fixing bacterial genera even at very high concentrations (400 ppm). However, it must be pointed out that the results can not directly be transferred to field conditions, as in our experiments *R. leguminosarum* was not in symbiosis with its host plants. Stichelmaier [7] demonstrated that C-heterotrophic soil microorganisms (e.g. *Arthrobacter sp.*, *Bacillus sp.*) and important soil enzymes are also not affected by 400 ppm DCD. In long-term field trials (53 years) with calcium cyanamide (containing 10% DCD), only positive effects on soil biological activity were observed [1].

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 [3].

N-serve, while having only a small effect on *Rhizobium*, strongly affected the non-symbiotic *Azotobacter* bacteria. This supports the findings of another study [2], 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.*

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