EFFECT OF SOME NITRIFICATION INHIBITORS ON *VICIA FABA* - *RHIZOBIUM* SYMBIOSIS

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ABSTRACT

The effects of nitrification inhibitors (Dicyandiamide, Nitrapyrin, and Thiourea) at 0, 0.1, 1, 10, and 100 mg/l on the growth and the respiratory activity of the Rhizobium leguminosarum bv. viciae strains were studied in vitro. In vivo, the study was done in acidic low humus content of brown forest soil in the presence of the host plant (Vicia faba). The results of in vivo were documented the in vitro that Dicyandiamide had relatively low effect on the growth and respiratory activity of microsymbionts and symbiotic characters, while, the Thiourea was toxic. Nitrapyrin had a moderate effect in both systems.

INTRODUCTION

Since nitrate is mobile and ammonia is an immobile in the soil, decreasing the time of nitrification and increasing the time of N availability for plant absorption should increase the efficiency of soil fertility as well as the biological N_2-fixation. Inhibiting nitrification offers the availability of nitrogen in the reduced form and thus may prove to be a useful tool in maximizing soil bioproductivity and minimizing water pollution with oxidized nitrogen forms. The only possibility to reduce losses of the amount of nitrogen is to inhibit the first step of nitrification by so-called nitrification inhibitors. The respiration rate of soil microorganisms is usually restricted by low concentrations of available materials in the soil. Nitrapyrin is specific inhibitor of the ammonium oxidation component of nitrification (Zacherl and Amberger 1990). Allievi and Gigliotti (2001) mentioned that the changes in the aerobic bacteria and autotrophic nitrifier numbers, and in the respiration and nitrification in two soil samples treated with Cinosulfuron at 42 (field rate) and 4200 µg/kg were determined after one and 4 weeks of incubation under laboratory conditions. Only nitrification at one week was slightly inhibited by Cinosulfuron, even at the field rate. *In vitro* toxicity tests carried out in agar media on representative aerobic bacteria, fungi and *Azotobacter* strains isolated from the two soil samples, as well as on 9 collection soil bacteria (*Arthrobacter* spp.,

Bacillus subtilis, *Bradyrhizobium japonicum*, *Erwinia carotovora*, *Pseudomonas aeruginosa, Rhizobium leguminosarum*, *Serratia marcescens*, *Streptococcus lactis* and *Streptomyces scabies*). The results showed that only a very high Cinosulfuron concentration (100 mg/litre) could have negative effects on the growth of a limited number of soil heterotrophic microorganisms, under conditions similar to those of soil environment. It is concluded that Cinosulfuron has a negative effect on only a few aspects of the microbial community in soil ecosystems, even at concentrations higher that those currently in use. The following investigations were conducted to examine the direct effects of the nitrification inhibitors on *Rhizobium* - *Vicia faba* symbiotic interaction.

MATERIALS AND METHODS

Sensitivity of *Rhizobium leguminosarum* bv. *viciae* strains: HB-23841*str*$^{+}$ (originated from Libya), E1012 (England), Bükköny 75/4, and Lóbab Z (Hungary) strains to the nitrification inhibitors: Dicyandiamide (1-cyanoguanidine), Nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] and Thiourea (Thiocarbamate) at 0, 0.1, 1, 10, and 100 mg/l and the respiratory activity of the *Rhizobium* cells in pure and nitrification inhibitors treated cultures using the microfermentor method and Warburg's respirometer technique, respectively were carried out according to the methods described by Bayoumi Hamuda et al. (1995). Data presented in term of relative growth rate comparing with controls.

An agroecosystem model was designed for symbiotic experiment. The model was conducted in the greenhouse using mesocosm filled with 2 kg of sterile loamy sand, acidic brown forest soil (pH 5.2) of low organic matter (1.22 %). The aim of the experiment was to study the direct effects of tested inhibitors at concentrations 0, 0.1, 1, 10, and 100 mg/kg soil on the survival of *Rhizobium* strains and their symbiotic interactions with the host plant faba bean (*Vicia faba*) *in vivo*. Seeds of faba bean were surface sterilized. Soil humidity was maintained at 45 - 50% of water holding capacity. Plants were harvested after 7 weeks, nodule number/plant, dry weight of plant and nodule/plant and total nitrogen content/plant (measured by micro-Kjeldahl method) were determined. Means of three replicates per treatment for each strain were analyzed using ANOVA to determine statistical differences among treatments and LSD at P = 0.05 was calculated.

RESULTS AND DISCUSSION

The effect Dicyandiamide, Nitrapyrin and Thiourea on *Rhizobium leguminosarum* bv. *viciae* strains was studied. Table 1. illustrates the moderate effects of Nitrapyrin on the growth of the strains. The strains were able to tolerate the lowest concentrations, while the strains were sensitive to 10 mg/l. The Libyan and English strains were completely inhibited at 100 mg/l. However, the Lóbab Z strain was more tolerant than the Bükköny 75/4 strain. Dicyandiamide and Thiourea had relatively low and toxic effect, respectively.

Table 1. Effect of some nitrification inhibitors on the growth of *Rhizobium* strains

Inhibitor	Dose (mg/l)	*Rhizobium* strains			
		HB-23841*str*$^+$	E 1012	Lóbab Z	Bükköny 75/4
Dicyandiamide	0.1	82.88±6.8	83.33±6.7	93.48±2.5	90.82±6.0
	1	62.16±5.1*	78.13±5.6	71.38±2.1*	75.63±5.0*
	10	49.10±10.1*	64.58±11.1*	67.39±4.3*	37.34±10*
	100	0.00*	0.00*	57.97±6.1*	15.19±15*
Nitrapyrin	0.1	88.41±2.7	78.65±2.2	91.15±3.1	89.6±5.4
	1	61.26±1.5*	61.68±3.6*	64.84±2.9*	71.43±2.4
	10	44.53±1.9*	39.44±1.5*	49.54±2.4*	66.30±2.8*
	100	0.00*	0.00*	12.34±1.3*	46.77±1.5*
Thiourea	0.1	62.41±2.5*	54.43±2.2*	73.48±2.1*	77.78±2.1
	1	48.65±3.1*	41.30±2.1*	55.43±2.7*	56.77±3.2*
	10	38.67±3.4*	22.34±2.6*	36.44±1.4*	39.13±2.6*
	100	0.00*	0.00*	16.04±1.7*	22.14±2.1*
$LSD_{(P=0.05)}$		**28.714**	**31.974**	**26.126**	**23.646**

* Significant at P = 0.05 level

A clear inhibition of respiration was observed with inhibitors as it is shown in Table 2. The table shows that an increase in the concentration of the inhibitor increased the inhibition of respiration by means of the relative O_2 consumption of the *Rhizobium* cells in nitrification inhibitors treated cultures. It was found that at lowest concentration, the relative O_2 consumed per cell dry weight was higher than the control and by increasing the concentration, the relative O_2 consumption decreased.

Table 2. Relative oxygen consumption of *Rhizobium* strains within three hours to control cultures using Warburg's respirometer.

Inhibitor	Dose (mg/l)	*Rhizobium* strains			
		HB-23841*str*$^+$	E 1012	Lóbab Z	Bükköny 75/4
Dicyandiamide	0.1	113.7	115.8	127.7	130.37
	1	83.63	96.65	80.55	91.1
	10	70.7	79.45	75.41	45.1*
	100	10.3*	11.7*	49.3*	22.7*
Nitrapyrin	0.1	95.2	98.4	112.6	104.5
	1	81.4	80.1	75.4	95.9
	10	65.1*	56.4*	33.7*	43.8*
	100	8.4*	6.3*	18.7*	27.1*
Thiourea	0.1	75.7	65.47*	97.58	112.57
	1	65.1*	48.38*	75.53	82.47
	10	31.4*	25.13*	36.44*	59.13*
	100	3.1*	12.1*	16.04*	14.64*
$LSD_{(P=0.05)}$		**34.495**	**32.452**	**35.335**	**37.406**

* Significant at P = 0.05 level

Fig. 1. demonstrates that by increasing the concentrations of Dicyandiamide the root-nodule number/plant increased (except in E1012). While it was decreased in case of Thiourea

treatment (except the two Hungarian strains). It is clear that Nitrapyrin has a moderate influence on the nodule number. Lóbab Z and Bükköny 75/4 strains were the most tolerant to applied inhibitors.

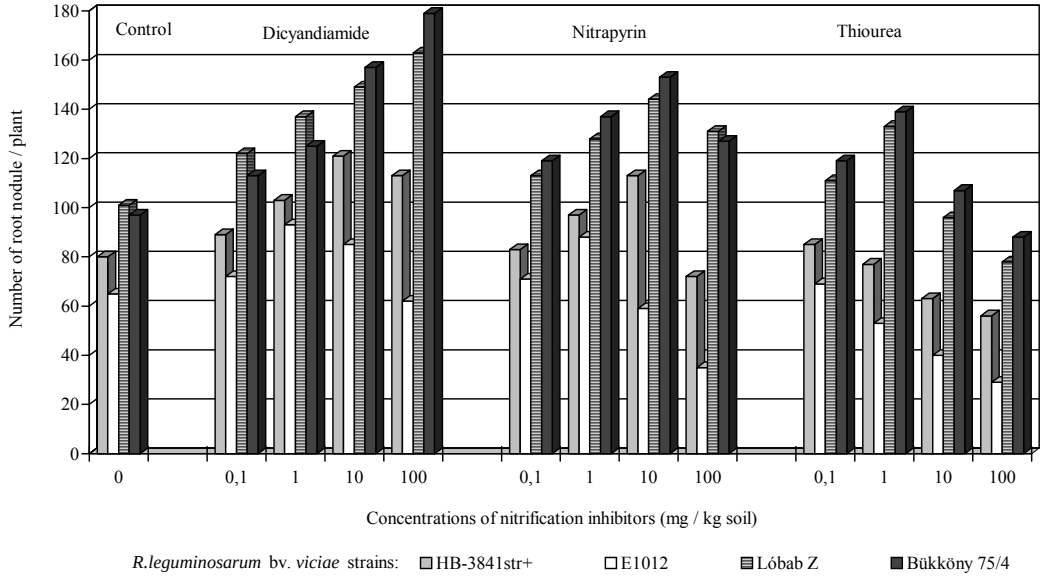

Fig. 1. Nitrification inhibitors affect root-nodule number of faba bean inoculated with *Rhizobium* in acidic brown forest soil

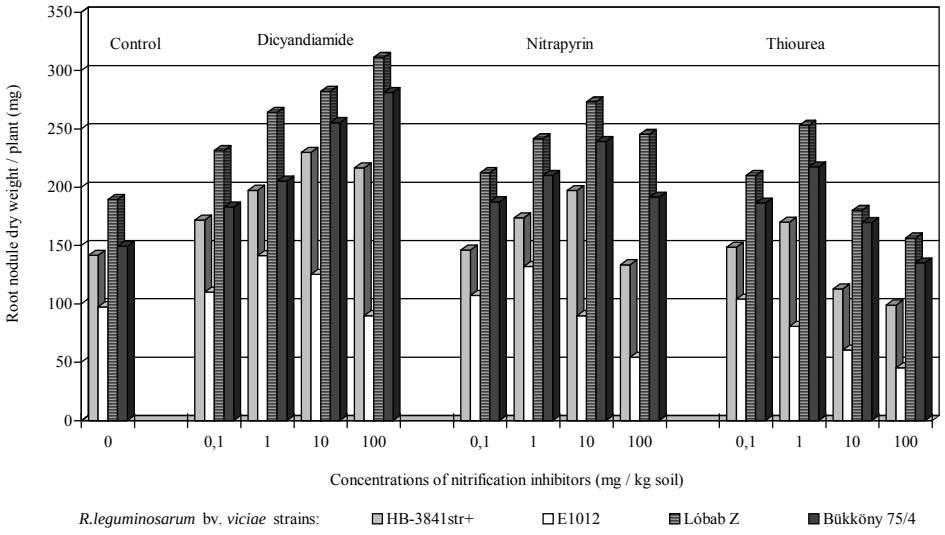

Fig. 2. Effect of Nitrification inhibitors on root nodule dry weight of faba bean inoculated with *Rhizobium* in acidic brown forest soil

Fig. 2. shows that the dry weight of root-nodule/plant was increased in case of Dicyandiamide for all strains, while, in case of Thiourea, the HB-3841*str*$^{+}$ was decreased at the concentration increased above 1 mg/l. Increasing the concentrations of the inhibitor decreased the root-nodule number of the plant inoculated by E1012 strain.

The same demonstration and conclusion can be made in case of total nitrogen content of faba bean plant (Fig. 3.).

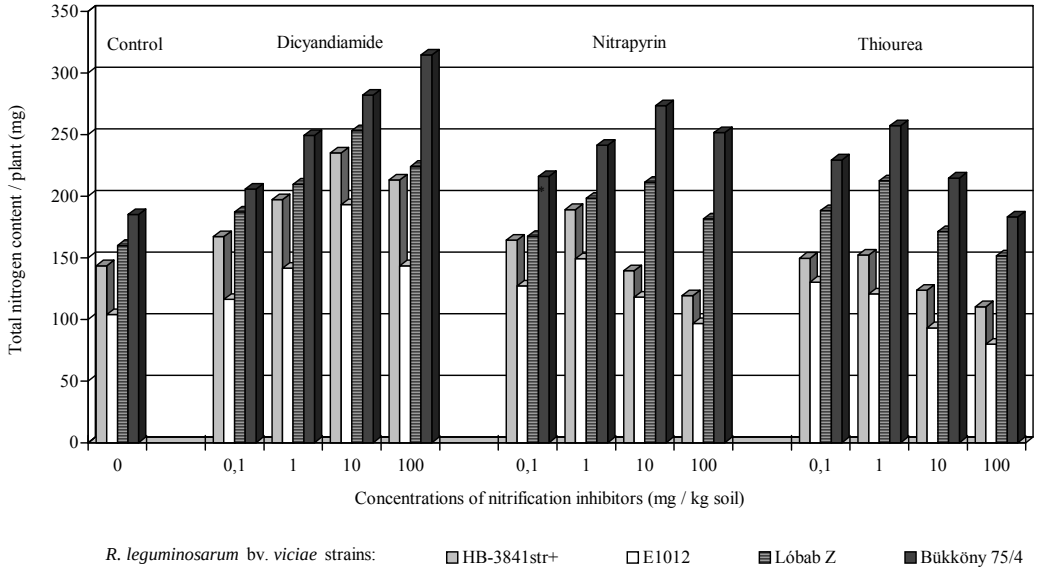

Fig. 3. Change of total nitrogen content of faba bean inoculated with *Rhizobium* due to the effect of nitrification inhibitors in acidic brown forest soil

The plants inoculated by the Hungarian strains have the highest total N-content and more significantly in case of Dicyandiamide. The investigated inhibitors differed in many respects, particularly with regard to effective concentrations and mode of action.

Nitrification is one of the most intensively studied microbiological components of the nitrogen cycle. A recent advancement in maintaining the desired NH_4^+ : NO_3^- ratio is the use of a nitrification inhibitor, which results in a desirable ratio of ammoniacal and nitrate nitrogen in the soil (Camberato and Bock 1990). Kucharski (1993) found that there is a negative effect of CMP, N-serve and ATC on horse bean growth and development, N-fixation, and bioactivity of some *Rhizobium*. In our studies, we found the negative effects at higher concentrations of Nitrapyrin and Thiourea at 1 mg/kg soil. Our observations indicate that high concentrations of Nitrapyrin and Thiourea were detrimental to all strains and these results were contradicted with the report of Zacherl and Amerger (1990), who showed that 100 ppm Nitrapyrin reduce 17% of *Rhizobium leguminosarum* growth. Our results show that the growth of the strains was inhibited by 100% (HB-3841*str*$^+$ and E1012), 42.03 % (Lóbab Z) and 84.81 % (Bükköny 75/4). Also, our results revealed that 0.1 and 1 mg/l are non-toxic, while 10 and 100 mg/l are highly toxic. We are in agreement with Jenson and Sörensen (1952) who mentioned that Thiourea has been classified earlier as very toxic for ammonia-oxidizing bacteria. According to our results, the effect whether positive or negative is depending on (i) the strain-type, (ii) culture medium, (iii) micro-environmental conditions of cultivation, and (iv) the doses of the compounds. The delay in lag phase in the activity at

highest concentration of inhibitors, might indicate the possibility of adaptation or toleration of the strains to these inhibitors, and this is clear in this case of *Rhizobium leguminosarum*.

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