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NITRIFICATION INHIBITOR AND NITRATE AFFECT THE ROOT COLONIZATION OF *VICIA FABA* BY *RHIZOBIUM LEGUMINOSARUM*

Hosam E.A.F. BAYOUMI HAMUDA[1], Ibrahim ISSA[2], H. ABDORHIM[3]

[1]Environmental Protection Engineering Institute, Óbuda University, Budapest, Hungary

[2]Soil and Water Department, Faculty of Agriculture, Sirte University, Sirte, Libya

[3]Botany Department, Faculty of Science, Sebha University, Sebha, Libya

ABSTRACT:

Little informations concern the effects of different nitrate sources at certain concentrations known in the presence of nitrification inhibitors of herbicide origin to inhibit the nodule formation and functioning on root colonization of legume plant by microsymbiont Rhizobium. Laboratory and greenhouse pot experimental studies were conducted to determine the effect of two nitrification inhibitors: 2-chloro-6-(trichloromethyl) pyridine (Nitrapyrin) and ammonium thiosulfate on symbiotic N_2*-fixation in faba bean (Vicia faba) nodules. Plant dry weight was used to assess the effect of the nitrification inhibitors on the Rhizobium - faba bean symbiosis. The results of pot experiment indicated that this study investigated the potential differences between two R. leguminosarum strains in the presence of two herbicides which functioning as nitrification inhibitors on root colonization of faba bean at different levels of various nitrate sources. Secondly, this study evaluated the effects of 20 and 50 mg N/kg soil on root colonization of faba bean by the rhizobial strains. The presence of low concentration of* NO_3^-*-N increased the rhizobial population, the root length colonized and nodule number as well. The strain GH130 was more sensitive to the two nitrification inhibitors and inorganic N forms except calcium nitrate than Lóbab Z strain. Results demonstrate that the two herbicides decreased the concentration of nitrate in the soil.*

***Keywords*:**

Nitrification inhibitors, nitrate sources, root colonization, Vicia faba, Rhizobium leguminosarum

1 INTRODUCTION

Biological oxidation of ammonia to nitrate is known as nitrification. Nitrate is also sensitive to denitrification, whereby N is converted and lost from the cropping system in the gaseous forms N_2, NO and NO_2. These gases are partially responsible for global warming. Inhibition of nitrification in agricultural systems would therefore be a considerable gain both economically and environmentally.

Since nitrate is mobile and ammonia is 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.

SPYROU et al. (2009) stated that the effects of synthetic pesticides on the soil microbial community have been thoroughly investigated in the past mostly by culture-dependent methods and only few recent studies have used culture-independent approaches for this purpose. However, it should be noted that most of these studies have been conducted in microcosms where the soil microbial community is exposed to unrealistic concentrations of the pesticides, providing an unrealistic exposure scheme for soil microorganism.

Synthetic nitrification inhibitors are commercially available. Some plants, such as the tropical grass *Brachiaria humidicola*, were proposed to release compounds from their roots that suppress nitrification (ISHIKAWA et al., 2003).

A highly sensitive bioassay was developed using a recombinant *Nitrosomonas europaea* that can detect nitrification inhibitors released from roots (IIZUMI et al., 1998; SUBBARAO et al., 2006). The presence of ammonium is the stimulus for release of inhibitors from *B. humidicola* roots (SUBBARAO et al., 2007). Inter- and intra-specific differences among plant species in the capacity to release inhibitory compounds exist (SUBBARAO et al., 2009).

ALI et al. (2008) stated that increasing the nitrapyrin application rate to 8.32 mg kg^{-1} caused a 50% reduction in nitrification up to 4 weeks. It has been reported recently that nitrification inhibitor Nitrapyrin increased NO, retention with NO, fertilization in a soil-peat-sand medium (MILLS et al., 1976), a pine bark-sand medium (MILLS & POKORNY, 1978), and a peat-vermiculite medium (PILL, 1981). Nitrapyrin was hypothesized to exert an inhibitory effect on biodenitrification as well as on nitrification (MILLS et al., 1976).

CALDERON et al. (2005) established that excessive application of manure may lead to NO_3^- leaching to groundwater and fluxes of nitrogen oxides to the atmosphere. Nitrification inhibitors such as nitrapyrin (N-serve; 2-chloro-6-(trichloromethyl)pyridine) may help to conserve manure N in the root zone by limiting NO_3^- supply to denitrifiers. Also, the observed reductions in microbial biomass may affect N availability beyond the time frame of the experiment because less N will be available for remineralization.

Inhibiting nitrification offers the availability of N in the reduced form and thus may prove to be a useful tool in maximizing soil bioproductivity and minimizing water pollution with oxidized N forms. The only possibility to reduce losses of the amount of N is to inhibit the first step of nitrification by nitrification inhibitors (HAUCK, 1984).

The respiration rate of soil microorganisms is usually restricted by low concentrations of available materials in the soil. Nitrapyrin (N-Serve) is specific inhibitor of the ammonium oxidation component of nitrification (ZACHERL & AMBERGER, 1990). ROBERTS et al. (2003) mentioned that a 28 d N transformation test was developed in the laboratory with a suitable soil which was amended with powdered plant meal as an organic N source.

Soil samples of 1 kg treated with five concentrations of Nitrapyrin, in the range 1.0 and 100 mg kg^{-1} dry weight were incubated for 28 d at 20 ± 2 °C. A dose response was produced and the N mineralization EC_{50} (95% C.I.) for nitrapyrin was 3.1 mg kg^{-1} dry soil. The determined EC_{50} was compared with literature figures for similar end points but using different methodology. Lack of O_2 limits nitrification in the soil; therefore an increase in nitrate production indicates good soil aeration. Also, pH is an important factor in the nitrification (LÅNG, 2003).

Influence of various chemicals such as nitrification inhibitors on the agrochemical and microbiological parameters of a soddy-podzolic soil was investigated by VAKKEROV-KOUZOVA (2010). It was found that the toxic effect of these compounds on the soil microbial content was higher with their increasing concentrations.

The objective of these investigations was to measure the impacts of different forms of inorganic N and various N-transformation inhibitors on the symbiotic interaction between *Rhizobium – Vicia faba*.

2 MATERIALS AND METHODS

Random samples were taken from the surface layer (0–15 cm) after removing the first 2 cm of unfertilized sandy low organic matter (1.22) content of acidic (pH 5.2) brown forest soil in Gödöllő, Hungary.

The treatments consisted of thirty six combinations of three rates of the Nitrapyrin (2-chloro-6-(trichloromethyl)-pyridine) and ammonium thiosulfate (0.5, 5, and 50 mg/kg soil), two rates (20 and 50 mg/kg soil) of three inorganic N forms (calcium nitrate, potassium nitrate and sodium nitrate).

Two fast growing strains of *R. leguminosarum* (Lóbab Z and GH130) which form effective nodules on *Vicia faba* plant roots were used as inoculants for root nodulation in pots containing one of the above combinations. There was also a control treatment either with no N or inoculants nor both. The water holding capacity of the soil approximately kept at constant (45%).

The experiment was carried out in the greenhouse at 25°C ± 3°C. Sensitivity of *R. leguminosarum* strains to Nitrapyrin and Thiosulfate and the respiratory activity of the *Rhizobium* cells in pure and nitrification inhibitors treated cultures using the microfermentor method and Warburg's respirometric technique respectively were carried out according to the methods described by BAYOUMI HAMUDA et al. (1996).

Root colonization of horse bean under each combination was examined after 2 weeks of sowing the seeds in 200 g pots. Data presented in term of relative growth rate comparing with controls. Plants were harvested after 7 weeks, nodule number/plant, dry weight of plant and total nitrogen content/plant (using Kjeldahl method) were determined. Means of three replicates per treatment were analyzed using ANOVA to determine statistical differences among treatments at P = 0.05.

3 RESULTS AND DISCUSSION

The effects of different nitrate sources and nitrification inhibitors (Nitrapyrin and Thiosulfate) on the relative growth rates and O_2 consumed per cell dry weight *R. leguminosarum* strains were 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 at 50 mg/l the strains were sensitive. The Thiosulfate had relatively low effect. The relative O_2 consumption of the *Rhizobium* cells in nitrate sources or nitrification inhibitors treated cultures was measured.

Table 1. Effect of different concentrations of nitrate sources, Nitrapyrin and Thiosulfate on the relative growth rates and oxygen consumption of R. leguminosarum strains

Nitrate sources and N inhibitors	Level (mg/l)	*Rhizobium leguminosarum* strains			
		Lóbab Z		GH130	
		Relative growth	Relative O_2 consumption	Relative growth	Relative O_2 consumption
Calcium	20	125	116	108	104
	50	89	82	76	69
Potassium	20	113	109	104	101
	50	81	83	77	79
Sodium	20	110	106	101	101
	50	79	80	73	77
Nitrapyrin	0.5	96	113	89	104
	5	64	71	68	68
	50	53	49	49	44
Thiosulfate	0.5	101	107	94	102
	5	81	87	75	81
	50	69	73	59	65

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 especially in case of the strain GH130.

The influences of faba bean root colonization due to the inoculation by *R. leguminosarum* strains in soil treated with different combinations of various nitrate sources and N inhibitors at 21 days after plantation are given in Table 2.

It was found that Nitrapyrin more toxic regarding the root length than Thiosulfate, and Lóbab Z *Rhizobium* strain more tolerant to Nitrapyrin than the strain GH130. The most toxic combination was found is the combination which contained $NaNO_3$ followed by KNO_3 and $Ca(NO_3)_2$.

Table 2. Effect of different combinations of nitrate sources, Nitrapyrin and Thiosulfate on root length (cm) due to the Rhizobium colonization after 21 days of plantation

Nitrification Inhibitors	Level (mg/kg)	*Rhizobium leguminosarum* strains											
		Lóbab Z						GH130					
		Concentrations (mg/kg soil) of nitrate sources applied to the soil samples											
		Ca		K		Na		Ca		K		Na	
		20	50	20	50	20	50	20	50	20	50	20	50
Control		11.9						10.3					
Nitrapyrin	0	12.5	11.3	12.2	11.3	11.9	11.6	11.4	11.1	10.8	10.5	10.2	9.8
	0.5	12.1	11.7	11.5	11.1	11.2	10.4	11.1	10.8	10.4	10.1	9.9	9.6
	5	11.8	11.2	10.9	10.6	10.7	10.1	10.7	10.4	10.1	9.7	9.6	9.3
	50	10.9	10.6	10.3	9.8	10.1	9.5	10.3	10.1	9.6	9.2	9.1	8.5
Thiosulfate	0	12.9	12.3	12.4	11.7	9.6	9.1	9.7	9.3	9.1	8.9	8.8	8.4
	0.5	12.6	12.1	11.8	11.1	9.2	8.7	9.2	8.8	8.8	8.3	8.2	7.8
	5	12.1	11.7	11.2	10.4	8.8	8.5	8.7	8.1	8.3	7.9	7.8	7.4
	50	11.4	11.1	10.6	10.1	8.4	8.1	8.2	7.6	7.9	7.4	7.2	6.8

Table 3. illustrates that nodule number and plant biomass were reduced at all concentrations of Nitrapyrin in the presence of different concentrations of various nitrate sources in comparison with control.

Table 3. Effect of different combinations between nitrate sources and Nitrapyrin on nodule number per plant, plant dry weight and total nitrogen content

Nitrate source	Level (mg/kg)	Nitrapyrin Level (mg/kg)	*Rhizobium leguminosarum* strains					
			Lóbab Z			GH130		
			Nodule NO/plant	Plant dry weight (g)	TNC (mg)	Nodule NO/plant	Plant dry weight (g)	TNC (mg)
Ca^{2+}	20	0	83	6.9	461	71	6.3	408
		0.5	76	6.7	467	73	6.1	415
		5	53	5.9	475	50	5.5	424
		50	37	5.1	488	29	4.7	439
	50	0	42	6.3	493	38	5.8	475
		0.5	35	6.1	499	28	5.7	481
		5	22	5.7	512	19	5.5	493

		50	8	5.1	534	3	5.1	503
K^+	20	0	78	6.8	454	71	6.3	397
		0.5	68	6.3	467	62	5.8	407
		5	49	5.8	477	43	5.2	426
		50	33	4.7	481	28	5.1	445
	50	0	39	6.2	489	31	4.9	461
		0.5	31	5.4	499	27	4.7	477
		5	20	4.6	501	16	4.3	485
		50	7	4.1	517	5	4.1	491
Na^+	20	0	63	6.5	421	54	5.8	384
		0.5	52	5.9	434	41	5.6	393
		5	39	4.6	446	29	5.3	404
		50	28	4.4	452	16	4.9	419
	50	0	31	5.9	466	11	4.6	432
		0.5	27	5.4	478	7	4.3	448
		5	19	4.8	481	3	4.2	452
		50	3	4.2	493	0	3.9	460

The rate of reduction was increased by increasing the concentrations. Total nitrogen content was found to be increased by increasing the concentrations of Nitrapyrin in presence of all various nitrate sources.

Our results are not in agreement with PARKIN et al. (2010) when the nitrification inhibitor, Nitrapyrin has been shown to decrease soil N losses during the fall and spring, and maintain fertilizer-N availability to the crop. Additionally, nitrification inhibitors have shown promise in reducing soil N_2O emissions. This observation is not confirmed by the results in Table 3 which showed that the decreasing in nodule number as a result of reduction in rhizobial population will indirectly increase the N content in soil.

Table 4. Effect of different combinations between nitrate sources and Thiosulfate on nodule number per plant, plant dry weight and total nitrogen content

Nitrate source	Level (mg/kg)	Thiosulfate Level (mg/kg)	*Rhizobium leguminosarum* strains					
			Lóbab Z			GH130		
			Nodule NO/plant	Plant dry weight (g)	TNC (mg)	Nodule NO/plant	Plant dry weight (g)	TNC (mg)
Ca^{2+}	20	0	83	6.9	461	71	6.3	408
		0.5	79	6.8	469	77	6.1	419

		5	62	6.1	487	65	5.9	432
		50	54	5.7	498	37	4.8	443
	50	0	47	6.5	499	48	5.9	487
		0.5	37	6.3	509	37	5.8	489
		5	25	5.8	531	21	5.6	504
		50	11	5.2	554	7	5.4	524
K^+	20	0	78	6.8	454	71	6.3	397
		0.5	76	6.5	476	67	5.9	422
		5	53	5.9	491	46	5.4	446
		50	37	4.8	498	32	5.3	451
	50	0	43	6.3	493	38	5.1	469
		0.5	34	5.6	509	29	4.9	497
		5	26	4.9	521	19	4.6	498
		50	12	4.4	531	10	4.4	499
Na^+	20	0	63	6.5	421	54	5.8	384
		0.5	56	6.1	443	43	5.7	399
		5	43	4.7	456	32	5.4	423
		50	32	4.5	465	18	5.1	441
	50	0	39	6.1	478	17	4.7	453
		0.5	29	5.5	481	9	4.4	464
		5	21	4.9	487	5	4.3	469
		50	0	4.4	499	0	4.1	467

RICE & OLSEN (1988) found that Nitrapyrin at 2 μg ml^{-1} increased nodule numbers on alfalfa plants grown in nutrient solution. High rates of the two compounds significantly reduced nodule numbers. They suggested that the nitrification inhibitors have the potential to affect N_2-fixation processes in soil and nodules, but that the effect is not likely to be of practical significance when the compounds are used at rates normally required to inhibit nitrification.

Our results showed that all Nitrapyrin concentrations reduced nodule numbers (Table 3) on faba bean roots in the presences of all concentrations of various nitrate sources. Table 4 demonstrates that by increasing the concentrations of Thiosulfate, the root-nodule number/plant increased in the presence of calcium nitrate, while it was decreased in all cases of Nitrapyrin treatments. It is clear that Nitrapyrin has a moderate influence on the nodule number. Lóbab Z strain was more tolerant to applied inhibitors than GH130 strain. Our results revealed that 0.5 and 5 mg/l are non-toxic, while 50 mg/l are highly toxic. We are in agreement with JENSON & SÖRENSEN (1952) who mentioned that Thiosulfate has been classified earlier as very toxic for ammonia-oxidizing bacteria.

Regarding to the total nitrogen content per plant dry matter, the results indicate that the increase in nitrate form with or without N inhibitor the TNC per plant dry matter increased. At the same time the plant dry matter decreased when the high concentrations of various nitrate form were applied in the presence or absence of N inhibitors. Similarly, the root-nodule number was decreased by increasing the treatment doses. The *Rhizobium* population of the two strains was also, decreased when the more nitrate applied to the soil, and Nitrapyrin was more toxic to *Rhizobium* population than Thiosulfate (unpublished data). Our results in agreement with the conclusion of SAAD et al. (1996) that Thiosulfate slightly inhibits the nitrate without involving the formation of volatile S compounds as potential nitrification inhibitors. Denitrification was not affected by the addition of Thiosulfate. DOLEN et al. (1980) found that plant dry weight, total N, and total Ca was increased at 0.1 and 1 ppm N-serve. They indicated that at greater 10 ppm the plants showed visual symptoms of a stunted growth, stem elongation, flowers and pods failed to form or were aborted, young leaves were curled, and roots were club shaped with many branches. These symptoms were increasingly evident with increasing N-serve application rates. Dry wt and total N in the plant was less than the control at the higher N-serve applications. Table 2 shows that the dry weight of plant was decreased in case of Nitrapyrin in case GH130 more than as in case of Lóbab Z and lower than when soil treated with Thiosulfate.

The total nitrogen content in soil treatments with Nitrapyrin or Thiosulfate in the presence of sodium nitrate or potassium nitrate were lower than in the presence of calcium nitrate. Whereas the total nitrogen content higher in plant dry matter which grown in Thiosulfate in the presence of calcium nitrate were higher in any other treatments. KUCHARSKI (1993) found that there is a negative effect of CMP, N-serve and ATC on horse bean growth and development, N_2-fixation, and bioactivity of some *Rhizobium*. In our studies, we found the negative effects at higher concentrations of Nitrapyrin and Thiosulfate at 5 mg/kg soil. Our observations indicate that high concentrations of Nitrapyrin and Thiosulfate were detrimental to both strains and these results were contradicted with the report of ZACHERL & AMERGER (1990), who showed reduction in growth of *R. leguminosarum,* was only 17% with 100 ppm Nitrapyrin.

Similarly, negative effects were found e.g., in contrast the microbes targeted by DCD, the ammonium-oxidising bacteria, were significantly affected by DCD with reductions in population size and altered activity (O'CALLAGHAN et al., 2010). Heightened concerns for pollution from N and its ecological effects, and a promising new nitrification inhibitor on the horizon most likely will renew interest in using nitrification inhibitors as a management tool, making their future the brightest ever. For further investigations, the rate of nitrate mobilization and ammonia formation will be measured under each combination according to the results obtained.

4. CONCLUSION

The addition of $NaNO_3$ or KNO_3 at 50 mg/kg soil in the presence of Nitrapyrin or Thiosulfate strongly inhibited the symbiotic relationship (nodule number, total nitrogen content and plant dry weight), while, $Ca(NO_3)_2$ at increased the rhizobial population, especially in the presence of low doses of Thiosulfate (0.5 and 5 mg/kg) and the root length.

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Corresponding author:

Prof. Dr. habil Hosam E.A.F. BAYOUMI HAMUDA
Environmental Protection Engineering Institute,
Rejtő Sándor Faculty of Light Industry and
Environmental Protection Engineering,
Óbuda University,
H-1034 Budapest, Doberdó út 6. Hungary
Phone: 361-666-5946 fax: 361-666-5909
E-mail: bayoumi.hosam@rkk.uni-obuda.hu