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Effect of Carbendazim, Imazetapir and Thiram on Nitrogenase Activity, Number of Microorganisms in Soil and Yield of Hybrid Lucerne (*Medicago media*)

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Abstract

In field and pot experiments the effect of selected pesticides on the nitrogenase activity, number of soil microorganisms, and yield of hybrid lucerne was investigated. The obtained results show that crop protection preparations applied in the experiment (seed dressing compound Funaben T and Pivot 100SL herbicide) reduced the activity of nitrogenase in the active strain of *Sinorhizobium meliloti* both under conditions of pot and field experiments. In addition, the authors observed a noxious influence of the applied pesticides on nodulation, root development and yield of lucerne. Furthermore, in the first days after their application, the employed herbicide and fungicide inhibited multiplication of soil microorganisms under lucerne plantations, while later they were found to stimulate their multiplication.

Keywords: *Sinorhizobium meliloti*, pesticides, nitrogenase activity, inoculation, thiram, carbendazim, imazetapir, lucerne, bacteria, fungi.

Introduction

Numerous experiments have been carried out on the possible impact of pesticide activity on soil microorganisms. They include studies on problems associated with the movement in soil of herbicides and fungicides, possibilities of ground water contamination, and modification of plants and microorganisms.

Because of a steady increase in the application of pesticides on crop plants, it has become necessary to learn more about their impact on the symbiosis of *Rhizobiaceae* –leguminous plants. There is evidence to indicate that, when applied in field cultivations of leguminous plants, herbicides can have some influence on nodulation, dinitrogen fixation and development of growing plants [1].

It is believed that these compounds may also cause numerous side effects on growth and survival of microorganisms and, additionally, they can exert some impact on the balance between processes.

Sometimes, the doses of pesticides recommended by the Institute of Plant Protection reduced plant height and dry weight, and also reduced the number of nodules and nitrogen fixation rates. However, it was difficult to determine whether a given pesticide affected the process of nodulation or rather inhibited growth of the host plant [2; 3].

The objective of this study was to recognise the effect of the fungicide seed dressing Funaben T (a.i. - thiram, carbendazim) and Pivot 100 SL herbicide (a.i. – imazetapir) on nitrogenase activity, number of soil microorganisms under cultivation, and yield of hybrid lucerne (*Medicago media*).

Material and Methods

Investigations were carried out in two separate field experiments in 1997-1999, and one pot experiment conducted in a cold greenhouse in 1998.

Field Experiment

The lucerne plants were cultivated on plots of 14 m^2 area in the Złotniki Agriculture Experimental Station which belongs to the A. Cieszkowski Agricultural University in Poznań. The plots were situated on soils of very good and good rye complex of slightly acid reaction and moderate potassium, phosphorus and magnesium availability, belonging to grey-brown podzolic soils of humus sand (ps) texture (Tab.1). In addition, suitability of soil-climatic conditions for the cultivation of experimental plants was estimated as moderate throughout the experimental period. Table 2 shows meteorological conditions during the vegetation season.

The experiment was established as a single-factorial experiment using the method of randomized block in four replications. The level of the factor comprised three methods of plant protection: herbicide, fungicide and fungicide + herbicide. Treatment without pesticides was considered as control.

The applied pesticides: fungicide - Funaben T (active ingredients: carbendazim 20% and thiram 45%) and herbicide – Pivot 100SL (active ingredient: imazetapir 100 g/l) were obtained from the Organika–Sarzyna Chemical Company. The above-mentioned pesticides were selected because of their widespread use in agricultural cultivations, their low level of toxicity and absence of data concerning their impact on the root nodule bacteria – dinitrogen fixation activity.

Lucerne seeds were dressed with the chosen fungicide and, directly before sowing, they were inoculated with the effective strain of root nodule bacteria *Sinorhizobium meliloti* Bp derived from the collection of the Microbiology Department, Institute of Soil Science and Plant Cultivation in Puławy. The experimental herbicide was applied into the soil directly after sowing. The control comprised plants inoculated with bacteria but without pesticide application.

The following doses of fertilizers were applied: nitrogen as NH_4NO_3 – 25 kg · ha^{-1}, phosphorus as P_2O_5 – 80 kg · ha^{-1} which were applied before beginning of spring growth. Potassium in the form of K_2O was applied twice in the amount of 60 kg · ha^{-1} before the first and second cuts.

Pot Experiment

The pot experiment was carried out in a cold greenhouse. Each pot was filled with 7.3 kg of soil of 60% moisture content. The soil was taken from the field on which the field experiment was carried out. As in the case of the performed field experiment, plants were cultivated in four combinations in five replications. Lucerne seeds were sown as single grain seeding – six seeds per pot. They were dressed with the experimental fungicide and, directly before sowing, they were inoculated with the same effective strain of root nodule bacteria (*Sinorhizobium meliloti*) as in the field experiment. After sowing, the soil in pots was sprayed with the herbicide, the amount of which was calculated per pot area.

Analyses

Four times in a vegetation season (in the year of sowing and in the first year of utilisation): at the beginning and at full plant flowering and before the first and second cut,

Table 1. Characterization of some soil physico-chemical properties.

Soil level (cm)	pH	C g · kg^{-1}	N g · kg^{-1}	C:N	Percentage proportion of fraction with diameter in mm				Texture group*
					1-0.1	0.1-0.02	<0.02	(<0.02)	
0-30	5.1-6.2	5.93	0.611	9.70	75-80	18-24	7	(2)	ps(sand)

*According to BN-78/91 180-11

Table 2. Meteorological date for the periods April – September of 1997,1998 and 1999 to compare to mean from the period 1951-1993.

Months	1997		1998		1999		Mean from the period 1951-1993	
	Mean temp. (^{0}C)	Precipitation total (mm)	Mean temp. (^{0}C)	Precipitation total (mm)	Mean temp. (^{0}C)	Precipitation total (mm)	Mean temp. (^{0}C)	Precipitation total (mm)
April	7.3	49.7	11.8	34.9	11.5	60.6	7.8	31.0
May	15.6	72.5	16.9	40.6	16.1	44.4	13.6	47.6
June	19.5	20.2	19.4	70.2	18.3	75.4	16.9	62.3
July	20.2	194.8	19.4	60.2	22.3	31.8	18.5	73.0
August	23.1	22.9	18.1	60.0	23.2	22.8	17.8	54.4

dinitrogen fixation activity was determined directly in the field.

In the case of pot experiment, nitrogenase activity was determined only during the plant flowering phase directly in the pots.

The quantity of the biologically fixed nitrogen was estimated by nitrogenase activity measurement. For this purpose, acetylene to ethylene reduction method (ARA) modified by Sawicka [4] was used. Determinations for each experimental combination were made in three replications. Gas analysis were carried out on a gas chromatograph, type: CHROM 5.

In addition, in the soil in which the plants were growing, the impact of pesticides on the following selected groups of microorganisms on the third, seventh and fourteenth day after sowing and at plant flowering phase: *Azotobacter*, Actinomyces, total number of bacteria and fungi were determined.

The yield of the cultivated plant determined by the gravimetric method was used as an additional indicator of the influence of the applied pesticides.

Microbiological Analyses

In the samples collected from the soil under the plants, in five replications, the number of microorganisms was determined using the dilution plate method and appropriate agar media (in three replications). The number of colonies (average) was calculated for 1 g of dry soil (CFU· g^{-1}·DM):

·total amount of bacteria and actinomycetes was identified by 2% agar soil extract after 14 days of incubation at 28^0C [5],

·fungi were counted on Martin's medium after five days of incubation at 24^oC [6],

·the number of *Azotobacter* was determined by placing 0.5 g soil sample on Petri dishes and mixing it with Jensen's medium [7]. Plates were incubated for 3 days at 24^oC.

The obtained results were statistically evaluated using multidirectional analysis of variance, whereas the means were compared using Tukey's test.

Results and Discussion

Effect of the applied pesticides on nitrogenase activity and numbers of some selected groups of microorganisms in the soil under pot experiment conditions.

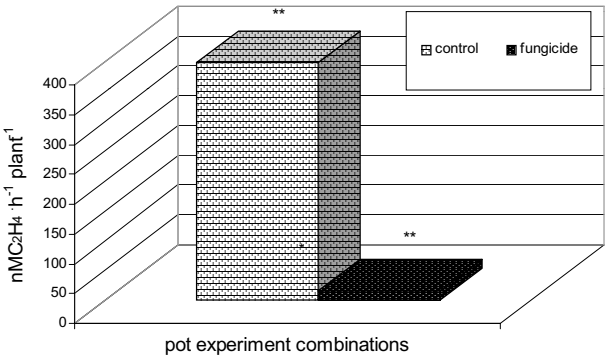
A statistically significant impact of plant protection chemicals on nitrogenase activity was recorded in all treatments of the pot experiment in which pesticides were applied (moles of ethylene) (Fig.1).

In the treatment in which both plant protection preparations were used jointly (fungicide and herbicide) as well as in the case of herbicide application, no nitrogenase activity was measured since all plants were destroyed because of the applied pesticides.

On the other hand, the application of the experimental fungicide alone resulted in a 95.5% decrease of the *Sinorhizobium meliloti* Bp strain activity in comparison with control (Fig.1.).

In the performed experiment it was observed that the applied plant protection compounds decreased not only nitrogenase activity but also inhibited plant germination and growth as well as their nodulation.

The applied pesticides were also found to affect the



**LSD (0.05)=149.3

Fig. 1. Effect of pesticides on nitrogenase activity of root nodule bacteria in symbiosis with lucerne in pot experiment.

growth of selected groups of soil microorganisms (Tab. 3). There is no doubt that the number of recorded microorganisms was influenced by the plant presence and their developmental stage.

It was observed that the applied pesticides reduced significantly the total count of bacteria in soil 7 days after sowing (in comparison to the control), while 14 days after sowing, the applied herbicide was found to stimulate significantly bacteria multiplication. On the other hand, the applied fungicide turned out to significantly stimulate the growth of bacteria under lucerne during the phase of plant flowering.

The applied crop protection preparations were not neutral to actinomycetes, too. The fungicide alone or when applied in combination with the herbicide exerted a significant stimulatory effect on the growth of actinomycetes during the phase of lucerne flowering leading, on average, to a 31% increase in their numbers in comparison with control. On the other hand, imazetapir (herbicide) caused a strong (up to 52%) decrease of their growth (in relation to control) (Tab. 3).

The application of pesticides also affected the multiplication of bacteria from the *Azotobacter* genus, resulting in the decrease of their number in soil at all dates of analyses in comparison with the control.

The effect of plant protection compounds on the growth of free-living nitrogen fixing bacteria, including *Azotobacter*, was also investigated by Langkramer [8]. He claims that the herbicide (Gramoxone) applied in his

experiments inhibited significantly the growth of *Azotobacter* cells. *Azotobacter* turned out to be sensitive to such active substances occurring in herbicides as mecoprop and dulorprop [9] when these pesticides were applied at recommended field doses.

The applied plant protection preparations inhibit the development of diseases and weeds, but, at the same time, they can either inhibit or stimulate the growth of fungi and bacteria [10]. Their introduction into soil alters the nature of substances secreted by roots causing microbiological changes in the rhizosphere and outside it. When studying ways of pesticide actions, it was observed that they also change biochemical processes of microorganisms by influencing their enzymatic systems. In addition, the applied plant protection agents frequently alter soil microfloral qualitative composition. Simultaneously, the elimination of certain groups of microorganisms results in the ecological succession of other species of fungi or bacteria [11]. Consequently, numbers of the soil microorganisms community undergo further alterations.

Effect of the applied pesticides on nitrogenase activity and numbers of some selected groups of microorganisms under lucerne and yield under field experiment conditions.

In the field experiment, the influence of selected pesticides on nitrogenase activity of the applied strain of *Sinorhizobium* in symbiosis with lucerne was also observed in individual years (1997, 1998, 1999) of experiments (Tabs. 4,5,6).

The highest nitrogenase activity was always observed in control treatments. Although the applied pesticides did not always reveal statistically significant impact on nitrogenase activity under the examined plants, the quantity of nitrogen fixation in combinations with plant protection preparations still showed a decreasing trend.

A statistically significant influence of selected pesticides on nitrogenase activity by bacteria from the *Sinorhizobium* genus was recorded, among others, at the stage of full flowering in 1997 under lucerne where nitrogenase activity was reduced significantly by the applied herbicide (87%) and fungicide (95%). When the two preparations were used jointly, the observed drop in nitrogenase activity, in relation to the control, was 88% (Tab.4).

Also in 1997 before the second cut (Tab.5) as well as in 1999 at the stage of full plant flowering (Tab.6) the applied pesticides were found to have had a significant effect on nitrogenase activity under lucerne both at the stage of early and full flowering. Application of pesticides in all treatments resulted in a strong reduction of nitrogenase activity of examined strains.

Results of field experiments were in keeping with results obtained in pot experiments in which a negative impact

Table 3. Effect of pesticides on the number of microorganisms in soil under lucerne grown in pots.

Time of analyses	Numbers of selected groups of microorganisms in 1g of dry soil (CFU) depend on experimental combinations				
	control	herbicide	fungicide	herbicide+fungicide	LSDλ=0.05
Bacteria x 10^4					
3 days	440.3	403.3	323.0	316.0	n.s
7 days	303.0	203.3	200.0	150.0	102.3
14 days	196.6	386.0	186.0	293.3	102.4
Plant flowering	386.8	110.0	420.0	303.3	133.4
Actinomycetes x 10^4					
3 days	120.0	106.6	106.6	70.0	n.s
7 days	93.3	76.6	93.3	63.3	n.s
14 days	100.0	153.0	90.0	143.0	n.s
Plant flowering	110.0	53.3	146.6	143.3	41.04
Fungi x 10^3					
3 days	56.6	48.3	41.6	25.0	n.s.
7 days	30.0	41.6	21.6	21.6	n.s.
14 days	33.3	50.0	35.0	41.6	n.s.
Plant flowering	25.0	5.0	33.3	18.3	n.s.
Azotobacter					
3 days	208	164	77	97	n.s.
7 days	121	119	67	75	29.5
14 days	97	86	72	71	n.s.
Plant flowering	56	58	31	32	n.s.

Explanations:LSD – lowest significant differences, n.s – non significant differences.

of the applied pesticides on nitrogenase activity was also demonstrated. The applied plant protection preparations did not only reduce nitrogenase activity in rhizobium bacteria but, additionally, they affected plant appearance. In field combinations in which carbendazim and imazetapir were used separately and together, plants were initially dwarfed and red. However, plants were not completely destroyed, as was the case in the pot experiment. This could be attributed to the partial degradation of the applied pesticides in field conditions as well as their migration to deeper soil layers or their leaching from topsoil.

Result discrepancies similar to ours were also obtained by other researchers who investigated the impact of selected active ingredients of pesticides on the process of dinitrophy and growth and development of leguminous plants under conditions of pot and field experiments [12,13].

The performed field experiments also revealed that the applied fungicide seed dressing and herbicide influenced the number of assayed groups of soil microorganisms (Tabs. 7, 8).

In both years of field experiments (1997 and 1998), a decrease, although statistically non-significant, in the number of *Azotobacter* cells was recorded, as it compared to the control, as the result of applied pesticides.

There are numerous studies devoted to the effect of pesticides on numbers of bacterial cells of *Azotobacter*

Table 4. Effect of pesticides on nitrogenase activity in lucerne in individual treatments in years 1997 and 1998 before the first cut.

Plant developmental stage	Nitrogenase activity (nMC_2H_4 $plant^{-1}$ $hour^{-1}$)				
	Control	Herbicide	Fungicide	Herbicide+Fungicide	LSD (=0.05)
1997					
Initiation of flowering	674.6	179.2	154.9	58.5	n.s.
Full flowering	134.2 a	21.9 b	6.7 b	16.7 b	71.0
1998					
Initiation of flowering	1277.5	633.5	948.0	2130.0	n.s.
Full flowering	450.7	226.4	252.7	306.6	n.s.

a,b,c – means with the same letters do not differ significantly.

Table 5. Effect of pesticides on nitrogenease activity in lucerne in individual treatments in years 1997 and 1998 before the second cut.

Plant developmental stage	Nitrogenase activity (nMC_2H_4 $plant^{-1}$ $hour^{-1}$)				
	Control	Herbicide	Fungicide	Herbicide+Fungicide	LSD (=0.05)
1997					
Initiation of flowering	395.2 a	62.5 b	133.0 b	22.8 b	64.2
Full flowering	200.9 a	25.5 b	88.7 b	17.8 b	71.3
1998					
Initiation of flowering	32.5	98.8	24.8	198.3	n.s.
Full flowering	306.5 a	83.8 b	244.9 a b	158.4 a b	166.0

a,b,c – means with the same letters do not differ significantly.

Table 6. Effect of pesticides on nitrogenease activity in lucerne in individual treatments in years 1998 and 1999 before the first cut.

Plant developmental stage	Nitrogenase activity (nMC_2H_4 $plant^{-1}$ $hour^{-1}$)				
	Control	Herbicide	Fungicide	Herbicide+Fungicide	LSD (=0.05)
1998					
Initiation of flowering	2858.3	2254.6	2217.0	2040.6	n.s.
Full flowering	1120.0	620.2	861.0	651.7	n.s.
1999					
Initiation of flowering	803.2	269.6	613.37	465.2	n.s.
Full flowering	451.7 a	116.3 c	311.4 a b	189.3 b c	179.3

a,b,c – means with the same letters do not differ significantly.

genus. Some preparations can be completely neutral to the growth of its cells [14], while others reduce significantly the growth and development of these bacteria [15] affecting, among others, the process of their respiration.

Analysing the impact of the applied plant protection preparations on numbers of the remaining groups of microorganisms, it was found that it was statistically non-significant in most cases. However, when comparing the two years of field experiments it can be noticed that the total numbers of bacteria and actinomycetes in the soil under cultivated plants in 1998 were higher in relation to those found in 1997 (Tab. 7). Similar results were achieved comparing fields in the year of full utilisation (Tab. 8), where the numbers of bacteria and actinomycetes were also higher in 1999 than in 1998. These differences can be attributed to different (in both years) weather conditions as well as to the effect of root secretions. The quantity and quality of soil microorganisms, for nutritional reasons, is strongly dependent on substances secreted by plant roots [16]. Literature data provides numerous inconsistent information concerning the total biomass of soil microorganisms, especially individual trophic or systematic groups. This can be attributed to inaccurate research methods as well as considerable variations in diurnal counts of microorganisms [17]. In addition, it is commonly accepted that the amount and composition of substances secreted by plant roots can also change following application of plant protection preparations.

Furthermore, depending on the applied pesticide, its breakdown by microorganisms can have either a metabolic or co-metabolic nature. If the pesticide is the source of C, N or supplies energy for microorganisms active in this process, then such breakdown is metabolic. In the case of a co-metabolic decomposition, the disappearance of the pesticide can be treated as a side effect of the action of enzymes synthesized by microorganisms for another purpose. Substrates produced in this way are not utilised by microorganisms to build their cells [18].

Table 7. Effect of selected pesticides on the number of microorganisms in soil in years of plant sowing.

Time of analyses	Number of microorganisms (CFU g^{-1} DM of soil)									
	1997					1998				
	C	H	F	F+H	LSD λ=0.05	C	H	F	F+H	LSD λ=0.05
Bacteria x 10^4										
3 days	199.3	162.3	190.8	161.7	n.s.	722.8	787.0	611.2	649.5	n.s.
7days	275.3	179.2	222.7	263.2	n.s.	408.0	387.0	390.8	365.1	n.s.
14 days	346.0	255.5	336.0	307.0	n.s.	322.6	261.2	260.2	256.8	n.s.
Initiation of flowering	274.6	174.4	284.0	435.2	n.s.	484.6	443.9	415.5	454.7	n.s.
Full flowering	242.0	203.3	116.0	183.7	50.2	243.4	186.6	122.5	217.1	n.s.
Actinomycetes x 10^4										
3 days	88.5	84.3	98.5	89.7	n.s.	220.7	251.1	249.7	302.1	45.1
7days	146.8	145.1	109.5	124.4	n.s.	244.8	199.7	219.6	220.8	n.s.
14 days	136.2	112.7	130.1	126.9	n.s.	174.7	139.8	142.6	163.5	n.s.
Initiation of flowering	123.5	62.3	103.5	123.0	n.s.	171.2	153.3	154.4	159.3	n.s.
Full flowering	114.1	85.2	65.5	113.3	n.s.	105.2	76.89	79.1	75.6	n.s.
Fungi x 10^3										
3 days	109.4	112.7	118.2	127.5	n.s.	134.4	82.3	110.8	66.0	n.s.
7days	84.3	82.8	170.1	233.6	n.s.	53.9	51.5	69.1	5.9	n.s.
14 days	143.4	115.8	120.7	126.1	n.s.	55.8	48.9	43.7	51.3	n.s.
Initiation of flowering	72.9	50.7	53.4	60.7	n.s.	68.6	41.8	58.8	64.7	n.s.
Full flowering	81.7	75.0	70.8	70.9	n.s.	52.5	29.0	37.9	56.8	n.s.
Azotobacter										
3 days	4	0	2	3	n.s.	36	19	21	25	n.s.
7days	31	23	18	22	n.s.	33	5	21	15	n.s.
14 days	18	15	13	15	n.s.	54	13	52	23	n.s.
Initiation of flowering	19	14	17	17	n.s.	24	10	17	20	n.s.
Full flowering	5	2	2	2	n.s.	21	6	13	6	n.s.

Explanations: as Table 3; C- control, H- herbicide, F – fungicide, H+F – herbicide + fungicide.

Table 8. Effect of pesticides on the number of microorganisms in soil under lucerne in years of full utilization.

Time of analyses	Number of microorganisms (CFU g^{-1} DM of soil)									
	1998					1999				
	C	H	F	F+H	LSD λ=0.05	C	H	F	F+H	LSD λ=0.05
Bacteria x 10^4										
Initiation of flowering	735.5	611.2	630.0	913.5	n.s.	669.9	976.8	1132.8	619.7	n.s.
Full flowering	144.0	346.7	189.0	228.7	n.s.	398.2	605.0	481.6	685.0	n.s.
Actinomycetes x 10^4										
Initiation of flowering	125.7	176.2	84.2	124.7	24.7	172.8	145.7	127.4	109.7	n.s.
Full flowering	80.0	171.7	83.0	98.0	63.7	136.5	148.6	123.4	98.7	n.s.
Fungi x 10^3										
Initiation of flowering	109.8	67.5	85.5	72.0	n.s.	120.6	118.7	94.17	117.7	n.s.
Full flowering	142.8	83.7	78.6	86.4	n.s.	29.0	29.0	35.6	59.4	n.s.
Azotobacter										
Initiation of flowering	25	20	21	20	n.s.	40	17	37	22	n.s.
Full flowering	3	2	1	1	n.s.	20	12	17	10	n.s.

Explanations: as Table 3; C- control, H- herbicide, F – fungicide, H+F – herbicide + fungicide.

Pesticide degradation activity exhibited by soil microorganisms can sometimes turn against these very organisms. This happens, primarily, in co-metabolic transformations when some metabolites are more toxic than indigenous compounds.

Yields were another way of controlling the impact of the applied pesticides (Fig. 2). It was observed that in treatments with pesticides plant yields were lower, but not statistically significant. This can be attributed to the fact that plant protection preparations used in field experiments, initially, caused a strong retardation of plant development weakening their growth or, in places, their complete diminishment. Considerable delay in plant development was observed in plots on which experimental pesticides were applied.

Studies carried out by Misra and Gaur [19] and Hauke-Pacewicz [20] on the effect of pesticides on symbiosis of legumes also indicated that field concentrations of plant protection preparations reduce yields, nodule numbers and dinitrogen fixation.

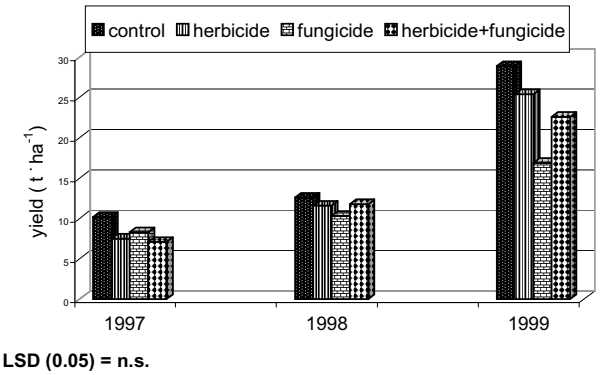


Fig.2. Effect of pesticides on lucerne yields in years 1997,1998 and 1999.

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