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Effect of Carbendazim, Imazetapir and Thiram on Nitrogenase Activity, the Number of Microorganisms in Soil and Yield of Red Clover (*Trifolium pratense* L.)

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Abstract

In field and pot experiments the effect of selected pesticides on the atmospheric nitrogen fixation, number of microorganisms in soil and yield of red clover has been investigated. The results obtained indicate that crop protection preparations applied in the experiments (Funaben T seed dressing compound and Pivot 100 SL herbicide) resulted in reduction of nitrogenase activity on the active strain of R*hizobium leguminosarum* bv. *trifolii* KGL both in pot and field experiment conditions. Moreover, the authors observed the toxic effect of the pesticides used on nodulation, root development and yield of clover. The herbicide and fungicide applied also inhibited the multiplication of the microorganisms in soil under red clover plantations in the first days upon application and, later on, stimulated their multiplication.

Keywords: R*hizobium leguminosarum* bv. *trifolii,* pesticides, N_2 fixation activity, inoculation, thiram, carbendazim, imazetapir, clover, bacteria, fungi

Introduction

Pesticides are used in agriculture for a variety of reasons and are applied in various ways, always resulting in their entering the soil in large or smaller amounts, where they may directly or indirectly influence the processes occurring therein. The pesticides differ by physical and chemical properties; therefore, their behaviour in soil may be different. Some accumulate, other are rapidly degraded. Due to the growth of pesticide use in various agricultural plantations, the issue of the impact of these chemicals on the qualitative and quantitative composition of soil microorganisms and the processes they direct, becomes more and more common, including that on the dinitrogen fixation in the relation *Rhizobiaceae* - leguminous plants [1].

Pesticides may directly affect the free-living populations of nodular bacteria in soil or indirectly influence the extent of infection and thus the number of nodules formed. The infection process can be changed either by pesticide influence on virulence of the attacking bacteria or by affecting the root fibres of the plants in which the infection occurs. The pesticides originating from the soil or from the plant's overground portion may influence the nodular growth and the effectiveness of nitrogen fixation through the effect inside the host plant.

The objective of this study was to recognize the effect of Funaben T fungicide seed dressing (a.i. - carbendazim and thiram,) and Pivot 100SL herbicide (a.i. imazetapir) on atmospheric nitrogen fixation, number of microorganisms in soil under cultivation and yield of red clover (*Trifolium pratense* L.*)*.

Materials and Methods

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

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Table 1. Characteristics of some soil physical - 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	<0.2	(<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 data for the periods April-September of 1977, 1998 and 1999 compared to mean from the period 1951-1993.

Months	1997		1998		1999		Mean from the period 1951-1993	
	Mean temp. (ºC)	Precipitation total (mm)	Mean temp. (ºC)	Precipitation total (mm)	Mean temp. (ºC)	Precipitation total (mm)	Mean temp. (ºC)	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

Pot Experiment

The pot experiment was carried out in a cold greenhouse. Each pot was filled with 7.3 kg of soil of 40% moisture content. The soil was taken from the same field that was used for the field experiment. Like the field experiment, the plants were cultivated in four combinations with five replications for each. The plants were grown from single grain seeding - six seeds in each pot. Seeds are coated before sowing with strains of root nodule bacteria, and where fungicide was used this was applied at this time as a seed dressing. Herbicide was applied by spraying onto the soil after sowing at a known quantity per unit area of pot.

Field Experiment

The plants were cultivated on 14m^2 plots in Złotniki Agruculture Experimental Station belonging to August Cieszkowski Agricultural University in Poznań. The plots were situated on soils of very good and good rye complex of grey-brown podzoic soils of humus sand (ps) texture (Table 1**).** The soil was of slightly acid reaction and moderate potassium, phosphorus and magnesium availability. During the experimental period, the suitability of soil-climate conditions was estimated as moderate. The meteorological conditions during the vegetation season are shown in Table 2.

The experiment was established in randomized block method in four replications (blocks). It was of a single-factorial character, the level of factor being three ways of plant protection: (herbicide, fungicide, fungicide + herbicide). Material without pesticides comprised the control.

The pesticides applied: Funaben T fungicide (a.i. carbendazim 20% and thiram 45%) and Pivot 100SL herbicide (a.i. imazetapir 100g/l) were manufactured by "Organika – Sarzyna" Chemical Works. The above pesticides were selected owing to their widespread use in agricultural cultivations, low toxicity level and no data on their possible impact on N_2 fixation by the nodule bacteria.

The clover seeds were dressed with the selected fungicide and inoculated with the effective strain of *Rhizobium leguminosarum* bv *trifolii* KGL root nodule bacteria directly before sowing. The bacteria originated from the collection of the Microbiology Department, Institute of Soil Science and Plant Cultivation IUNG in Puławy. The herbicide (0.8 l ha^{-1}) was applied into the soil directly after sowing. The control comprised plants inoculated with bacteria without the use of pesticides.

The following fertilization was applied: nitrogen as NH_4NO_3 – 25 kg ha^{-1}, phosphorus as P_2O_5 – 80 kg ha^{-1} applied before spring growth began. Potassium as K_2O – 120 kg ha^{-1} was applied twice in the amount of 60 kg before the first and second cuts.

Analyses

Four times during the vegetation season - in the field experiment (in the year of sowing and in the first year of utilization): at the beginning and at full plant flowering, before the first and second cut, was atmospheric nitrogen fixation determined directly in the field.

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

The quantity of biologically fixed nitrogen was determined based on nitrogenase activity using the acetylene to ethylene reduction method (ARA) [4].

Moreover, in the soil in which the plants were growing, the impact of pesticides on selected groups of microorganisms was determined on the third, seventh and fourteenth days after sowing and in the plant flowering phase, the microorganisms being: *Azotobacter*, fungi, the total number of bacteria, actinomycetes.

The additional indicator of the impact of the pesticides applied was the yield of the cultivated plant determined by the gravimetric method. .

Microbiological Analyses

In the soil samples collected from under the plants (in five replications), the total number of microorganisms (bacteria + actinomycetes and fungi) were determined in 1 g of dry soil by the dilution plate method on appropriate agar media (in three replications).

- The total amount of bacteria and actinomycetes (CFU g^{-1} dry soil) was identified by 2% agar soil extract upon 14-day incubation at 28^0C [5].
- The fungi were counted on Martin medium upon 5-day incubation at 24^0C [6].
- The number of *Azotobacter* (CFU g^{-1} dry soil) was determined by placing 0.5g of soil sample on Petri dishes and mixing with Jensen's medium. The plates were incubated at 24^0C for 3 days [7].

Statistical Analysis

All the results collected were subjected to formal evaluation in variance analyses adequate to the experiment configuration. The pot experiment results were evaluated in variance analyses for completely randomised single-factorial experiments, whereas the field experiment results - in analyses for multi-factorial experiments established in a configuration of completely randomised blocks. In the synthetic study of the field experiments' results, the full inter-subject variance testing procedure was applied to the mean experimental error and to the environmental interaction. All the F general tests and T specific ones were carried out at significance level $\lambda = 0.05$.

Results and Discussion

The Effect of Pesticides Applied on the Nitrogenase Activity and Numbers of Selected Groups of Microorganisms in the Soil Under the Clover in Pot Experiment Conditions

In all the pot experiment combinations with the application of pesticides, the impact of plant protection chemicals on the nitrogen fixation process, expressed as nitrogenase activity (moles of ethylene) (Fig. 1), was statistically significant.

The nitrogenase activity of *Rhizobium leguminosarum* bv. *trifolii* KGL strain under control conditions in symbiosis with red clover was 249.9 nMC_2H_4 x $plant^{-1}$ x $hour^{-1}$, while in the presence of fungicide, the activity dropped by 80% (Fig.1). Nitrogenase activity dropped by 95% compared to the control when applying the herbicide after sowing. When the combination of both plant protection preparations was applied, no nitrogenase activity was measured as all the plants were destroyed.

A significant drop of nitrogenase activity (on the level λ=0.05) in the pot experiment may be explained by strong phyto-toxicity of the pesticides applied. The low nitrogenase activity could have been caused by the effect of the preparations on the host plant instead of the *Rhizobium* strains. The nitrogenase results obtained could have been compliant with the appearance of the plants and roots reflecting the symbiosis with the bacteria. The preparations applied (fungicide and herbicide) conspicuously affected the condition of the plants and growth of roots as well as their nodulation (Photos 1,2). Many studies have indicated differentiated effect of pesticides on nodulation and dinitrogen fixation. In most cases it was found that applied herbicide doses affect nodulation and N_2 fixation [24, 26].

The plant protection preparations applied also affected other groups of soil microorganisms. It was recorded that both pesticides (fungicide and herbicide) applied significantly reduced the total number of bacteria in soil on the 7^{th} day upon sowing (compared to the control), while the herbicide (alone) applied significantly stimulated the bacteria multiplication on the 14^{th} day after sowing. The fungicide itself, however, appeared to be significantly stimulating the growth of bacteria under the clover in the plant flowering phase (Tab.3).

The crop protection preparations applied were not neutral to actinomycetes, either. The herbicide alone significantly stimulated the growth of actinomycetes, increasing their number as compared to the control, at all times of analyses (Tab. 3). The results clearly indicate that actinomycetes can use imazethapyr as an additional source of food. As noted in our experiment, and many other studies [24,25] soil microorganisms generally react to herbicides by increasing their biomass and activity.

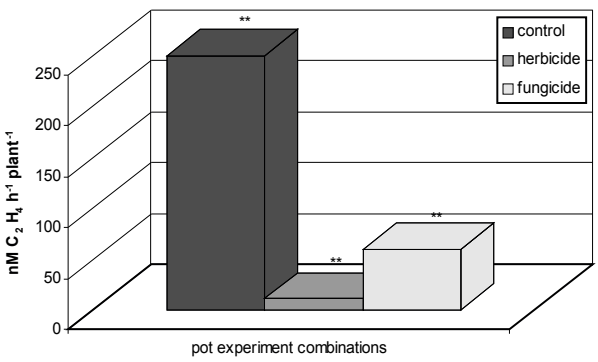
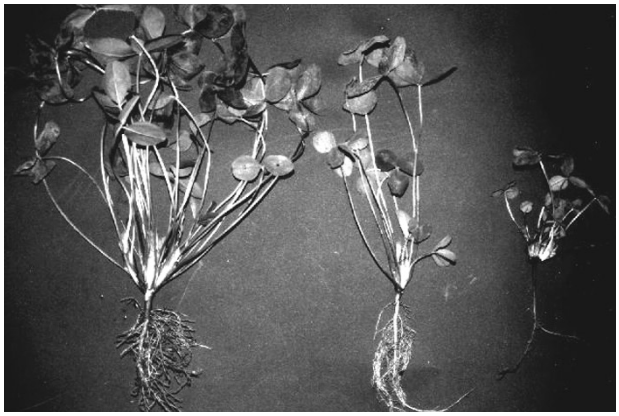


Fig.1. Effect of pesticides on nitrogenase activity of root nodule bacteria in symbiosis with clover in pot experiment.
**LSD (0.05)=42.31.



A B C

Photo 1. The appearance of red clover depending on the pesticide applied. A – control (no pesticide applied), B – herbicide (Pivot 100 SL) applied, C – fungicide (Funaben T) applied.

A B C

Photo. 2 The development of roots and root nodules in red clover, depending on the pesticide applied. A – control (no pesticide applied), B – herbicide (Pivot 100 SL) applied, C – fungicide (Funaben T) applied.

The number of actinomycetes in the soil dropped significantly upon 7 and 14 days following the sowing with the combined application of crop protection preparations.

The pesticides applied also influenced the *Azotobacter* genus bacteria multiplication, with carbendazim causing the number of bacteria growth by 100%, and imazetapir - by 34% (Tab. 3).

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

Time of analyses	Numbers of selected groups of microorganisms in 1 g of dry soil (CFU) depend on experimental combinations				
	control	herbicide	fungicide	herbicide+fungicide	LSDλ=0.05
Bacteria x 10^4					
3 days	323.3	380.0	280.0	276.6	n.s
7 days	276.6	253.3	256.6	143.3	73.1
14 days	286.6	520.0	286.6	260.7	128.4
Plant flowering	303.3	296.6	526.6	490.0	103.13
Actinomycetes x 10^4					
3 days	65.0	106.6	66.6	70.6	n.s
7 days	93.3	106.6	50.0	60.0	33.5
14 days	86.6	190.0	126.0	2.0	37.26
Plant flowering	110.0	150.0	123.3	156.6	n.s
Fungi x 10^3					
3 days	56.6	68.3	51.3	55.0	n.s
7 days	25.0	35.0	35.0	26.7	n.s
14 days	20.0	46.7	45.7	10.0	20.3
Plant flowering	18.3	21.6	13.3	28.3	n.s
Azotobacter					
3 days	289	180	139	143	n.s
7 days	151	145	101	138	n.s
14 days	63	85	112	1 9	33.5
Plant flowering	32	63	63	49	n.s

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

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

Plant developmental stage	Nitrogenase activity (nMC_2H_4 plant $^{-1}$ hour $^{-1}$)				
	Control	Herbicide	Fungicide	Herbicide+Fungicide	LSD(λ=0.05)
1997					
Initiation of flowering	587.1	206.2	250.0	289.6	n.s
Full flowering	201.1	38.8	100.7	57.6	n.s
1998					
Initiation of flowering	423.1	151.2	157.7	342.5	n.s
Full flowering	342.5	112.7	204.4	311.5	n.s

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

Table 5. Effect of pesticides on nitrogenase activity in clover in individual treatments in years 1997 and 1998 before the second cut on sowing years.

Plant developmental stage	Nitrogenase activity (nMC_2H_4 plant $^{-1}$ hour $^{-1}$)				
	Control	Herbicide	Fungicide	Herbicide+Fungicide	LSD(λ=0.05)
1997					
Initiation of flowering	359.6a	49.4b	42.4b	72.2b	244.4
Full flowering	188.5a	29.9b	25.9b	42.8b	91.5
1998					
Initiation of flowering	200.1	109.9	75.2	117.2	n.s
Full flowering	53.7	31.4	37.0	29.6	n.s

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

There are numerous studies on the effect of pesticides on the number of *Azotobacter* genus bacteria. Some of the pesticides applied may be totally neutral to the growth of its cells [14]. Others, however, significantly regulate the growth and development of these bacteria [15, 8] affecting, inter alia, the respiration processes.

The Effect of the Applied Pesticides on Nitrogen Fixation, the Number of Selected Groups of Microorganisms Under the Clover and Yield Under the Field Experiment Conditions

In the field experiment the crop protection preparations proved at some times of analysis, statistically significant effect on nitrogenase activity in the examined *Rhizobium leguminosarum* bv. *trifolii* strain. The most intensive nitrogenase activity in the investigated strain was always recorded at the beginning of the plant flowering phase (Tabs.4,5).

Although the applied plant protection preparations did not show any statistically significant effect at all times of analysis carried out, it was observed that in 1997 the addition of herbicide and fungicide (separately) reduced the nitrogenase activity in the strain investigated, whereas the combination of the applied plant protection preparations reduced the nitrogenase activity in the investigated strain by 51% at the beginning of the plant flowering phase. In 1997, before the second cut, the significant effect of pesticides was noted, both at early and full plant flowering phase (Tab.5). Results similar to those from 1997 were obtained in 1998 before the first cut; however, the pesticides applied also did not show any statistically significant effect on the nitrogenase activity under the clover, but the nitrogen fixation activity in combination with the crop protection preparations was observed to be lower (Tab.4).

A statistically significant effect on the nitrogenase activity was recorded in 1999 - the year of full utilisation of clover (Tab.6). The addition of the herbicide caused the reduction of nitrogen fixation under the clover cultivations by 44% at the flowering stage and by 63% at the full flowering stage.

The results obtained are confirmed in literature. The authors indicate that some of the pesticides applied in field doses significantly reduce nitrogen fixation, nodulation and growth of leguminous plants. Of the herbicides

Table 6. Effect of pesticides on nitrogenase activity in clover in individual treatments in years 1998 and 1999 before the first cut in years of full utilization.

Plant developmental stage	Nitrogenase activity (nMC_2H_4 plant $^{-1}$ hour $^{-1}$)				
	Control	Herbicide	Fungicide	Herbicide+Fungicide	LSD(λ =0.05)
1998					
Initiation of flowering	887.9	714.2	537.7	611.0	n.s
Full flowering	825.0	611.1	396.8	422.8	n.s
1999					
Initiation of flowering	606.8a	401.4b	333.8b	319.7b	132.9
Full flowering	343.0	127.1	118.2	101.6	46.24

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

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	H+F	LSD λ=0.05	C	H	F	H+F	LSD λ=0.05
Bacteria x 10^4										
3 days	234.1	193.9	210.7	170.0	n.s	421.5	393.6	395.3	383.2	n.s
7 days	241.4	241.1	210.5	261.1	n.s	405.4	356.8	397.5	395.7	n.s
14 days	156.8	185.5	325.1	189.4	106.03	180.0	202.2	422.9	189.6	n.s
Initiation of flowering	353.2	177.3	249.9	176.0	n.s	618.8	559.0	568.4	516.3	n.s
Full flowering	206.9	179.5	146.5	222.7	n.s	223.0	151.2	191.2	171.6	n.s
Actinomycetes x 10^4										
3 days	100.2	94.0	95.8	77.3	n.s	223.3	210.4	214.3	189.3	n.s
7 days	124.1	118.2	107.4	119.7	n.s	197.4	195.1	190.7	189.2	n.s
14 days	75.4	83.3	84.4	80.5	n.s	145.3	186.2	238.3	158.3	n.s
Initiation of flowering	111.5	88.6	92.2	75.1	n.s	242.6	208.3	208.3	167.7	n.s
Full flowering	82.2	72.9	80.3	81.4	n.s	124.6	66.3	55.3	81.9	n.s
Fungi x 10^3										
3 days	65.0	74.73	74.7	73.7	n.s	113.9	91.8	28.9	31.6	26.7
7 days	58.7	54.9	107.4	71.2	n.s	51.5	44.1	38.3	35.8	n.s
14 days	78.9	67.8	112.8	62.5	n.s	50.0	52.3	51.7	94.9	25.8
Initiation of flowering	49.0	47.8	68.5	48.7	n.s	60.5	37.5	76.7	52.5	n.s
Full flowering	45.4	44.4	50.0	39.4	n.s	37.8	45.9	43.4	54.0	n.s
Azotobacter										
3 days	8	5	2	4	n.s	66	34	50	59	n.s
7 days	30	28	21	25	n.s	122	98	58	60	n.s
14 days	25	18	23	24	n.s	63	108	65	72	n.s
Initiation of flowering	38	35	27	25	n.s	55	125	91	141	46.3
Full flowering	6	3	2	3	n.s	53	61	79	65	n.s

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

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

Time of analyses	Number of microorganisms (CFU g^{-1} DM of soil)									
	1998					1999				
	C	H	F	H+F	LSD λ=0.05	C	H	F	H+F	LSD λ=0.05
Bacteria x 10^4										
Initiation of flowering	758.7	440.2	374.2	613.5	n.s	1379.5	1068.3	1529.2	789.0	n.s
Full flowering	420	293.0	249.25	689.2	305.8	395.2	311.2	426.6	262.0	n.s
Actinomycetes x 10^4										
Initiation of flowering	574.2	212.5	130.0	207.5	n.s	285.5	229.2	224.0	230.2	n.s
Full flowering	207.5	135.0	95.0	186.3	n.s	139.9	134.2	105.3	134.4	n.s
Fungi x 10^3										
Initiation of flowering	124.6	101.3	95.4	138.2	n.s	167.8	134.7	142.7	151.0	n.s
Full flowering	102.0	89.0	70.8	150.o	39.9	62.4	47.1	54.6	29.1	n.s
Azotobacter										
Initiation of flowering	33	27	22	24	n.s	27	83	38	79	n.s
Full flowering	11	9	6	7	n.s	12	65	28	48	n.s

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

examined, a.o. 2,4,5-T (trichloroacetic acid) [22], 2,4 DB, balfin, profluralin [13], simazine [19, 20] appeared negative to the growth of leguminous plants and atmospheric nitrogen fixation.

In the field experiment carried out, the effect of the seed dressing and herbicide on the number of soil microorganisms was also recorded (Tabs.7,8).

The crop protection preparations applied were not neutral towards the number of *Azotobacter* genus bacteria. At the first two times, i.e. on the third and seventh day after sowing, a statistically insignificant decrease in the number of cells was recorded. However, at the other three times (i.e. on the 14th day after sowing and at the beginning and during the full flowering stage) the used pesticides stimulated its growth. However, only at the beginning of the clover flowering stage in 1998 the differences appeared to be statistically significant. The introduced herbicide caused an increase in the number of *Azotobacter* genus cells by 90% and the fungicide by 65%, compared to the control. The application of both of the crop protection preparations caused almost triple growth of the number of its cells compared to the control.

Based on the references, we may conclude that some of the used pesticides may be completely neutral to the growth of *Azotobacter* genus cells [14] while others significantly reduce its growth and development and some others even stimulate it [23].

In investigations of the crop protection preparations' effect on the number of the other groups of microorganisms, it was recorded that at most of the times it was statistically insignificant.

The effects of herbicide and fungicide impact on soil microorganisms are examined in many toxicological and environmental aspects of interaction pesticide - microorganism. The major problem raised by the research is the migration of pesticides in the soil, which causes the modification of plants and microorganisms. It is known that such migration is the effect of a number of factors, such as: physical-chemical properties of the compounds, their chemical degradability and biodegradability, sorption affinity towards soil colloids, soil properties and the extraction thereof by plant roots.

The tests prove that the crop protection preparations applied may inhibit or stimulate the growth of fungi and bacteria [10]. Upon being introduced in the soil the character of root secretions changes, which brings microbiological changes in the rhizosphere and around it. Examining the mechanisms of pesticide (mainly herbicide) action, it was observed that they change the biochemical processes of microorganisms, influencing their enzymatic systems [22]. The investigations also show that pesticides, depending on their chemical structure, constitute a more or less available source of energy to soil microorganisms, and are subject to biological and chemical degradability of various speeds. However, the microorganisms and plant roots extract the applied pesticides with large variability and their biological availability is also influenced by soil factors.

The crop protection preparations applied conspicuously change the qualitative composition of the soil microflora. The elimination of one group of organisms at the same time leads to the ecological succession of other spe-

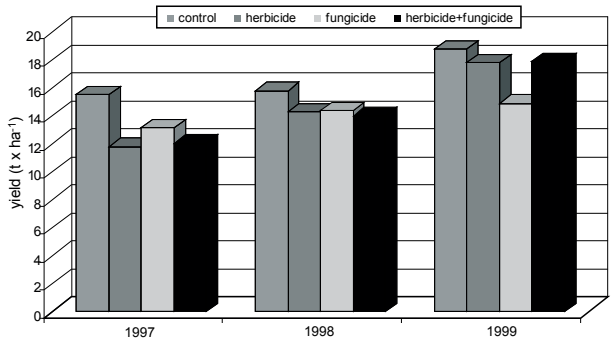


Fig.2. Effect of pesticides on clover yields in years 1997, 1998 and 1999. LSD (0.05)=n.s.

cies of fungi, bacteria [11]. In consequence, the number of the soil group of microorganisms changes.

Another manner of controlling the effect of the applied pesticides was the yield amount. The results obtained indicate that the crop protection plants did not show any statistically significant effect on the clover yield. Nevertheless, in the combinations where pesticides were applied, the plant yield was always lower when compared to control (Fig. 2). The negative effect of some pesticides on leguminous plant yields was also indicated in the papers by Misra [14], Gaur [14] and Fisher [2].

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