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Pesticide Side Effect on the Symbiotic Efficiency and Nitrogenase Activity of *Rhizobiaceae* Bacteria Family

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A b s t r a c t

The laboratory experiments tested the influence of selected pesticides on the symbiotic efficiency and nitrogenase activity of *Rhizobium leguminosarum* bv. *trifolii* KGL, *Sinorhizobium melilotii* Bp and *Bradyrhizobium* sp. *Ornithopus* B bacteria entering into symbiosis with clover, lucerne and serradella, respectively. The results obtained indicate that the pesticides used in the experiments (Funaben T seed dressing and Pivot 100SL herbicide) caused reduced nitrogenase activity in active strains tested. In addition, a toxic effect of the applied pesticides on the nodulation and root growth of the tested plants was observed.

K e y w o r d s: Rhizobiaceae, pesticide side affects, nitrogenase activity

Introduction

For years, the total cultivation area of leguminous plants has been kept on the same level and amounts to *ca.* 890 thousand hectares (of which *ca.* 670 thousand hectares are for red clover), which comprises 6.6% of the cultivation area. The large cultivation areas of these plants is associated with certain a role they play at increasing the soil fertility and improving the soil structure as well as the ability of the plants to produce high-protein green mass.

Pesticides used in leguminous plant crop plants often show side effects, influencing the symbiotic efficiency and nitrogenase activity. It was noted (Kumar 1981, Garcia and Jordan 1969) that the pesticides may indirectly influence the infection extent and the number of nodules formed. The infection process may be changed either by pesticide effect on the virulence of the attacking bacteria or by affecting the root fibers of the plants in which the infection occurs. In addition, the pesticides originating from the soil or from the overground portion of plants may influence the nodule development and the effectiveness of nitrogen fixation through the effect inside the host plant (Rup 1988).

The objective of this study was to recognize the effect of herbicide (Pivot 100SL a.i. imazethapyr) and fungicide (Funaben T a.i. 20% carbendazim and 45% thiram) on symbiosis efficiency and nitrogenase activity of *Rhizobium leguminosarum* bv. *trifolii* KGL and *Sinorhizobium meliloti* Bp and *Bradyrhizobium* sp. *Ornithopus* B.

Experimental

Materials and Methods

In laboratory experiments, three different plant species were used. Tested seeds were obtained from various experimental stations located in Wielkopolska. Seeds of hybrid lucerne (*Medicago media* L.) cultivar Radius, were obtained from Plant Breeding Station in Radzików, serradella (*Ornithopus sativus* L.) – from Plant Breeding Station in Wiatrów, whereas the red clover (*Trifolium pratense* L.) cultivar Tetra were obtained from Department of Genetics and Plant Breeding at Agricultural University of Poznań.

The following strains (of very high nitrogen fixation activity) were used for the analyses: *Rhizobium leguminosarum* bv. *trifolii* KGL for clover, *Sinorhizobium meliloti* Bp for lucerne and *Bradyrhizobium* sp. *Ornithopus* for serradella. The strains were obtained from the Microbiology Department Institute of Soil Science and Plant Cultivation in Puławy.

The pesticides used – Funaben T (a.i. Carbendazim 20% and thiram 45%) and Pivot 100SL (a. i. imazethapyr) were obtained from the Department of Soil and Plant Cultivation at Agricultural University of Poznań, and produced by „Organika – Sarzyna" Chemical Company. The selection of the above mentioned pesticides was based, most of all, on their common use in leguminous plant cultivation and their low-grade toxicity.

The analysis of the symbiotic efficiency with the use of pesticides was carried out in test tubes sealed with cotton plugs allowing for good air circulation (Somesegeran and Hoben, 1994). The plants were grown on semi-liquid medium for leguminous plants (Fähraeus, 1957).

Each plant species was tested in four combinations: Combination I (control) – plants inoculated with *Rhizobium* or *Bradyrhizobium* without pesticides; Combination II – plants inoculated with *Rhizobium* or *Bradyrhizobium* with addition of fungicide; Combination III – plants inoculated with *Rhizobium* or *Bradyrhizobium* with addition of herbicide; Combination IV – plants inoculated with *Rhizobium* or *Bradyrhizobium* with addition of fungicide and herbicide. 10 plants were applied to each treatment.

Prior to starting the culture, the plant seeds were sterilized in 5% sodium hypochlorite on the laboratory shaker for 20 minutes and then washed several times with sterilized water. After sterilization, they were germinated on a layer of lignin with one layer of absorbent paper, on Petri dishes, for 3–6 days (clover, lucerne) and 8–12 days (serradella). Additionally, for the combinations with the use of fungicide, the seeds were dressed with the agent directly after sterilization. Thus dishes on which the seeds germinated with the pesticides and those without such components were obtained.

During seed germination, the slants with medium for leguminous plants were prepared. For this purpose, test tubes of dimensions 250 mm × 25 mm, sealed with a cotton plug, were sterilized in a sterilizer at 160°C, and then 20 ml of prepared medium, cooled to 20°C was poured in a sterile way into each tube. The medium was cooled in order to obtain a proper slant with Ca CO_3 distributed evenly on the sufficiently thick slant. After preparing the slants, the germinated seeds (both dressed and non-dressed) were arranged on the medium in the tubes and after two days, they were infected with the suspension of three-day old bacteria strains in the amount of 0.5 ml and then, directly after the inoculation, herbicide was added in the amount corresponding to the field dose as recommended by the Plant Protection Institute.

The strains were multiplied on the slants on selective medium for *Rhizobiaceaae* acc. Thorthon (1976)**.** The inoculation was carried out using the suspension washed off from the slants, the plants were grown at 23°C in a vegetation room, the light exposure – 16 hours. The determined parameters of the effective symbiosis include: plant physiological status (morphology, nodulation, weight of the fresh matter of green parts), protein percentage content in the dry matter of the examined plants, nitrogenase activity.

The outside appearance of plants as well as the quantity and size of nodules were adopted as indices of the plants' physiological status. The weight of the fresh matter of green plant parts (mean from ten replications for each combination) was determined on the analytical balance. The protein percentage content in individual plant treatments was determined at the Chair of Soil and Plant Cultivation of the Agricultural University in Poznań on the basis of the near infrared (NIR) method using the Infralyzer 500 apparatus, Brau Luebbe Company.

The nitrogenase activity was determined after 6 weeks (clover, lucerne) and 8 weeks (serradella) of vegetation. The number and color of the nodules, the size of the plants and nitrogenase activity were assumed as the activity index (ARA), (Sawicka 1983). For this purpose, 10% of the gas phase volume of acetylene was injected into each tightly sealed test tube with the plant. After 1 hour 1 ml of gas phase was taken from test tube and analyzed on CHROM 5 gas chromatograph. Argon was used as a carrier. The nitrogenase activity was determined by virtue of the volume of acetylene reduced to ethylene (the average of 5 trials) and expressed in nmole C_2H_4 $plant^{-1}$ $hour^{-1}$, applying the theoretical conversion factor $N_2 : C_2H_4 = 1 : 3$.

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

Results and Discussion

Pesticide effect on symbiotic efficiency

Plant physiological status, nodulation and the weight of fresh matter of green parts. In the experiment carried out on clover, lucerne and serradella, inoculated with *Rhizobium* and *Bradyrhizobium* bacteria specific for the plants, the effect of the pesticides on the physiological conditions of the plants and their nodulation was studied.

The pesticides used (carbendazim thiram and imazethapyr) in recommended field doses appeared phytotoxic to the plants grown. The results are shown in Table I. Plants, which were treated as the control in the performed experiment and were not treated with the fungicide and herbicide, were characterised by a considerably stronger growth and more abundant green matter as evidenced by the weight of the fresh matter of green parts (Table II). In addition, the control plants were characterised by a very well developed root system.

In the cultures of plants where chemical preparations were used, single or in combinations, both carbendazim and imazethapyr impeded the growth of the lateral roots of the plants and the plants themselves were characterized with lower growth, yellowed cotyledons and the leaves (Table I).

The effect of the applied pesticides on nodulation was also observed (Table III). The strains in the control cultures (without pesticides) induced larger and more numerous nodules, while in the case of plants treated with the fungicide and herbicide, the nodules induced by the strains were smaller and not as numerous, which appears to confirm the effect of the chemicals of bacteria strains tested. In addition, the reduced

Table I
Pesticide effect on the appearance of the leguminous plants

Combination	Physiological condition of the host plant
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL**	Large, green plants
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL** + herbicide	Small plants with yellow discoloring
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL** + fungicide	Small plants with yellow discoloring
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL +** herbicide + fungicide	Small plants with yellow discoloring
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp**	Large, green plants
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp** +herbicide	Small plants with yellow discoloring
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp** +fungicide	Small plants with yellow discoloring
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp** + herbicide + fungicide	Small plants with yellow discoloring
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B**	Large, green plants
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B**+ herbicide	Small plants with yellow discoloring
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B** + fungicide	Small plants with yellow discoloring
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B** + herbicide + fungicide	Small plants with yellow discoloring

Table II
Effect of pesticides on the weight of the fresh mass of green plant

Plants	Weight of the fresh mass of the plant ($g \cdot plant^{-1}$)				
	control	herbicide	fungicide	herbicide+fungicide	NIR ($\lambda = 0.05$)
Lucerne	0.27	0.15	0.18	0.16	n.s.
clover	0.12	0.09	0.08	0.08	n.s.
Serradella	0.36	0.31	0.32	0.28	n.s.

Explanation: n.s. – non significant differences

Table III
Pesticide effect on the nodulation of the leguminous plants

Combination	Average number of nodules (of 10 plants) on the plant, color
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL**	8 (pink) large
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL** + herbicide	3 (pink) small
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL** + fungicide	5 (pink) small
Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* **KGL +** herbicide + fungicide	3 (pale pink) small
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp**	15 (pink) large
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp** + herbicide	7 (pink) small
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp** + fungicide	8 (pink) small
Hybrid lucerne inoculated with *Rhizobium melilotii* **Bp** + herbicide + fungicide	6 (pale pink) small
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B**	11 (pink) small
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B** + herbicide	7 (pink) small
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B** + fungicide	6 (pink) small
Serradella inoculated with *Bradyrhzobium* sp *Ornithopus* **B** + herbicide + fungicide	5 (pink) small

nodulation effects may be atributed to the carbendazim and imazetapir effect resulting in the inhibition of the lateral root growth in clover, lucerne and serradella.

The results obtained in non-inoculated plant cultures are reflected in reference literature, where the phytotoxic effect of various pesticides commonly used on leguminous plants was proved. Misra and Gaur (1974), in their investigations on the toxic herbicide effect on the plant, found that simazin used in peanut crops severely

affected the nodulation of roots with concentration as low as 2 ppm. The dosage of the herbicide even in normal field concentrations causes growth limitation, yellowing of the plants and their atrophy. The same herbicide examined by Kumar (1981) showed a significant reducing effect on nodulation, growth and development of pea plants. The investigations carried out by Pacewiczowa-Hauke (1969) on the toxic effect of simazin on lucerne, clover and serrradella proved, in turn, that the herbicide significantly affects the nodulation of the above mentioned leguminous plants and significantly changes the morphology of the root nodule. The investigations performed by Goring and Laskowski (1982) on leguminous plants prove that the nodulation may be reduced by denitroanilin (the active substance of the herbicide). They explain the nodulation reduction effects by the inhibition of the growth and dwarfing of the roots as the result of chemical action.

In field and pot experimental conditions, Niewiadomska (2002, 2004) observed a noxious influence of the applied pesticides (Funaben T, Pivot 100SL) on the nodulation, root development and yield of lucerne and clover.

Alachlor, linuron and prometryn also have a phytotoxic effect on leguminous plants, proven by Kao and Wang (1981). The above herbicides reduced the germination, development and flowering of soya. Alachlor inhibited the growth of lateral roots, whereas linuron and prometryn damaged the main root. Moreover, Nilson (1957**)** proved that the phenoxyl herbicide concentrations (2.4 D, MCPA) reaching 0.01–10 ppm, applied directly after rhizobia inoculation, reduced nodulation in beans. Garcia and Jordan (1969), in turn, analyzing the effect of 2.4 DB suggested that this herbicide directly affects the host plant and not the bacteria inducing nodulation, although in their earlier investigations, they reported that the herbicide significantly changed the metabolism of rhizobia in synthetic media. The direct side effect of the action of pesticides (Afugan, Brominal, Gramoxone, Selecron) on the nodulation and plant growth (peas, beans and lupine) was reported by Hemida *et al.* (2000).

The phytotoxic effect was observed among some fungicides used. Carboxyne and Captan significantly reduced the soybean root growth and the number of nodules, which was proven by Mallik and Tesfai (1985).

Proteins content. The results obtained from the analysis of the protein content in the green dry matter indicate that, the applied pesticides failed to exert a statistically significant influence on the discussed parameter (Table IV). Nevertheless, in combinations in which plant protection compounds were used a lower percentage protein content was recorded in the plant dry matter in relation to the control treatments

Table lV
Protein percentage content in DM of the examined plants

Plants	Protein percentage content in DM of the examined plants				
	control	herbicide	fungicide	herbicide+fungicide	NIR ($\lambda = 0.05$)
Lucerne	16.6	15.4	15.4	13.23	n.s.
Clover	17.8	15.27	16.07	16.49	n.s.
Serradella	18.9	17.16	17.66	16.33	n.s.

Explanation: n.s. – non significant differences

in which these preparations were not applied. This can be attributed, among others, to the reduced effectiveness of the process of nitrogen fixation caused by the action of pesticides. Some researchers reported that the decreased quantities of protein in plants could be attributed to the effect of plant protection agents on nitrogenase activity (Kao and Wang 1986).

Pesticide effect on nitrogenase activity

The pesticide active substances may also influence the biological activity of bacteria, including the nitrogenase activity. Much of literature is devoted to the effect of various pesticides on the diazotrophy process effected by *Rhizobium* and *Badyrhizobium* bacteria. Table V presents the results of nitrogenase activity analysis in particular combinations of plants, with or without pesticides.

The highest level of nitrogenase activity was observed in the *Sinorhizobium meliloti* Bp strain, which was used to inoculate hybrid lucerne seeds, whereas the strain of *Rhizobium leguminosarum* bv. *trifolii* KGL appeared to be less active (Table V). The *Sinorhizobium meliloti* strain appeared particularly low active in the presence of the applied fungicide and herbicide.

The nitrogenase activity in this strain, in the presence of carbendazim and thiram dropped by 93%, whereas in the presence of imazethapyr – by 91% compared to the control. When the combination of both pesticides was applied the nitrogenase was inactive (Table V). The applied chemicals also inhibited nitro-

Table V
Nitrogenase activity at in vitro cultures of nodular bacteria

Combinations	Nitrogenase activity (in $nMC_2H_4 \cdot plant^{-1} \cdot hour^{-1}$)		
	Red clover inoculated with *Rhizobium leguminosarum* bv. *trifolii* KGL	Hybrid lucerne inoculated with *Rhizobium meliloti* Bp	Serradella inoculated with *Bradyrhizobium* sp. *Ornithopus* B
Control	70.52	362.80	128.10
Pivot 100Sl (s.a. – imazethapir)	6.84	28.54	32.50
Funaben T (s.a. – carbendazim, thiram)	0.00	20.98	0.00
Pivot 100Sl (s.a. – imazethapir) + Funaben T (s.a. carbendazim, thiram)	6.23	0.00	13.36
NIR (0.05)	14.2252	149.2264	28.7091

genase activity in the case of the remaining two bacterial strains: *R. leguminosarum* bv *trifolii* KGL and *Bradyrhizobium* sp.*Ornithopus* B. In the case of *R. leguminosarum* bv *trifolii* KGL strain, a significant drop by 93.8% was noted in the presence of the herbicide alone, and in the presence of the fungicide alone – by 100%, compared to the control. In the case of the *Bradyrhizobium* sp.*Ornithopus* B. strain, the nitrogenase activity dropped by 100% in the presence of imazethapyr and by 70% – in the presence of carbendazim (Table V). In clover and serradella cultures, the nitrogenase activity drop was also noted by 91% compared to the control in *Rhizobium leguminosarum* bv. *trifolii* KGL strain and by 89% in *Bradyrhizobium* sp. *Ornithopus* strain in the combination of both pesticides. On the basis of the statistical calculations it can be concluded that pesticides significantly affected nitrogenase activity of the *Rhizobium* strains used and influenced the growth and appearance of leguminous plants which proved that the bacteria and the plants were sensitive to the chemical preparations applied.

It is difficult to determine without additional chemical or biochemical analyses whether a given pesticide affects directly the nodulation and nitrogenase activity of *Rhizobium* strains or indirectly inhibiting the plant growth.

Głowacka (1992) emphasizes out the influence of the environment, including herbicides on the structure of the surface polysaccharides of the *Rhizobium*. The defective nodulation (*i.e.* nodulation which is not typical for a given species) on lucerne, clover and serradella plants in pure cultures treated with chemical pesticides may be explained by the qualitative and quantitative change in the structure of the surface polysaccharides LPS and EPS caused by the applied chemicals. The investigations proved that these polysaccharides play a significant role in the bacteria symbiosis with the plant. In general it was stated that *Rhizobium* synthesizing changed LPS are defective in the infection process, *i.e.* do not create nodules or create ones not completely developed (Noel 1992). EPS in turn is considered as a specific signal particle in the symbiosis. It is neither a primary signal of the interaction between the bacteria and the plant, nor a signal determining the specificity of the symbiosis, but a significant particle in the invasion phase (Skorupska 1995). *Rhizobium* defective in EPS synthesis induced non-abortive nodules, without infectious threads or bacteroids, so-called empty nodules. In order to find whether the pesticides used in our experiment affected LPS and EPS we should certainly carry out a series of additional analyses confirming such assumption. The investigations described by Goring and Laskowski (1982) prove that the reduced nodulation process may not have any influence on nitrogen fixation and vice versa.

The numerous data on the effect of various pesticides on *Rhizobium* – leguminous plants symbiosis indicate that the pesticides having the phytotoxic effect on the plant, reducing its growth and root development, also reduce the nitrogenase activity. Garcia and Jordan (1969) during the investigations on the phenoxyl herbicides effect (2,4-D and MCPA) noted their negative effect on nitrogen fixation, nodulation and *Lotus corniculatus* growth.The negative impact of plant protection agents on nitrogenase activity is observed not only in the case of bacteria entering in symbiosis with leguminous plants but also in endophytes, which form associations with grasses capable of biological nitrogen fixation (Martinez-Toledo *et al*., 1998; Pozo *et al*., 2000). Dubetz and Rennie (1984) also proved the negative effect of post-germinating herbicides on nodulation, nitrogenase activity and development of soy.

It may be presumed that the pesticides used in the experiment could also cause genetic changes in the genome of bacteria inducing the nodules. In order to prove such hypothesis, additional analyses are required, which shall be subject to further investigations.

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