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INFLUENCES OF MICROBIAL ACTIVITIES IN THE INSECTICIDES TREATED SOIL

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Abstract: *Globally, the consumption of nitrogen (N) fertilizer- is significantly increased day after day. The biological N_2-fixation is the main source of N input in agricultural soil. However, international emphasis on environmentally sustainable development with the use of renewable resources is likely to focus attention on the potential role of biological N_2-fixation in supplying N for agriculture. Several environmental conditions arelimiting factors to the growth and activity of the N_2-fixing root nodule rhizobia. One of the most important and potentially limiting factors to biological N_2 fixation is the use of pesticides including the insecticides. The objective of the study is to investigate the adverse effect of insecticides on N_2-fixing rhizobia. Experiments were conducted to study the impact of seven insecticides [Anthio 33 EC (Formotion (33%), Sevin 85 WP (Carbaryl (85%), Rogor L-40(Dimethoate (40%), Thiodan 50 WP (Endosulfan (50%), Phosphotion (Malathion (50%), Lannate 20 L (Methomyl (200 g/l), Pirimor 50 DP (Pirimicarb (50%)] on the growth of seven strains of N_2-fixing root-nodule rhizobia (four strains belonging to Rhizobium leguminosarum, one strain belonging to R. phaseoli, one strain belonging to R. trifolii and one strain belonging to R. loti) in vitro by measuring optical density. The rhizobial strains were grownin yeast extract mannitol broth medium treated each with different concentrations (0, 0.1, 1, 10, and 100 mg/litre ofactive ingredient) of each insecticide and incubated for 48 hours in microfermentor at 28°C The results illustrated that Phosphotion and Sevin had an adverse effect on the growth of the investigated rhizobial strains, whereas Thiodan was safe to these rhizobial strains. Whereas the strain Lóbab Z of R. leguminosarum was the most tolerantand R. trifolii Lo133/64 was the most sensitive one among the tested strains. The adverse effects of insecticides on rhizobia were observed at concentrations not normally expected to occur under field conditions. For further task, itis required to investigate the adverse effects of these insecticides on nodulation and N2-fixation capacity of specifichost plants with their rhizobia in pot and field experiments as well as other vital microbial processes in a wide range of soils.*

Keywords. *insecticides, N_2-fixing root-nodule rhizobia, in vitro, adverse effect*

INTRODUCTION

A tremendous amount of anthropogenic nitrogen (N) fertilizer has been applied to agricultural lands to sustain crop production all over the world. However, inappropriate N management applications have causednumerous ecological and environmental problems. Rather than anthropogenic nitrogen fertilizer, biological nitrogen fixation is more emphasized on supplying N for agriculture.
The main problem of using pesticides including insecticides is that the amount of applied pesticides which actually reaches the target organism is about 0.1 % and the rest usually ends up contaminating the soil ecosystem. [1] Invention of pesticides and insecticides has also promoted crop production by controlling the pests effectively which has also had negative impacts on environment due to its xenobiotics characteristics.

The negative impact of these chemicals on the soil environment and their processes has received more attention and has been described by many researchers. [2] Recent studies show that the applied pesticides may harm the natural microorganisms, disrupt soil ecosystem, and which eventually may affect human health by entering in the food chain. Soil has its beneficial microorganisms and their associated biotransformations. Soil microorganisms have the ability to perform biochemical transformations of different elements like nitrogen (N), phosphorus (P), sulfur (S), and carbon (C). Pesticides and insecticides may directly or indirectly has an effect on the important biochemical reactions such as mineralization of organic matter, N_2-fixation, nitrification, denitrification, and ammonification by activating/deactivating specific soil microorganisms and/or enzymes. [3,4] The fact that information on possible effects of pesticides on all biochemical processes is sparse proves that it deserves more emphasis from the researchers. The activity of the N_2-fixing root nodule rhizobia is limited by many factors such as increased use of pesticides and insecticides and even sewage sludge application. [5] The legume–rhizobia symbiosis has distinctive role in agriculture and it results in huge quantities of N_2-fixation throughout the world and any adverse effect on rhizobia results in decreased rates of biological nitrogen fixation. By infecting the roots of legumes, rhizobia bacteria can form nodules where N_2 fixation occurs. [6] Some pesticides can be detrimental to nitrogen fixing bacteria. It slows down the nitrogen fixation process in bacteria and reduces the bacterium's respiration rate which later prevents its positive effects. [7]

Insecticides

The term pesticide covers a wide range of compounds including insecticides, fungicides, herbicides, rodenticides, molluscicides, nematicides, plant growth regulators and others. [4] Pesticides and Insecticides are interchangeably used together which brings out a quite confusion within the community. To be specific, insecticide is just one type of pesticide, specifically for insects and it differs from pesticide by its formulation, chemical, organic and toxicity differences. Formulation is how these chemicals are used against certain pests. For example, the insecticide is effective at controlling insects in the larval or state of development. How the chemicals are derived and the toxicity level of the end product differs a lot due to their intended uses, targeted organisms and etc. This study has chosen seven different insecticides.

Formothion (Anthio 33 EC)

Formothion is a systemic and contact insecticide used for controlling spider mites, aphids, psyllids, mealy bugs, fruit flies and others. Formothion appears as viscous yellow oil or a crystalline mass. This insecticide is classified moderately toxic or listed in II toxicity class of U.S. EPA. However, oral and dermal ways of GHS hazard statement; it is considered as acute toxic compound. [8] It is available as an emulsifiable concentrate and an ultra-low volume spray. Formothion affects normal nervous system function because it is a cholinesterase inhibitor as well as organophosphate compound.

Carbaryl (Sewin 85 WP)

Carbaryl is an insecticide used on a variety of crops. Carbaryl is the first carbamate insecticide which was introduced in 1956, and more of it has been used throughout the world than all other carbamates combined. [9] These insecticides are either used as dusts or sprays. Carbamate pesticides are derived from carbamic acid and similarly kill insects as organophosphate insecticides. They are effective insecticides under their ability to inhibit acetylcholinesterase in the nervous system [10]. Acute (short-term) and chronic (long-term) occupational exposure of humans to carbaryl has perceived to be a source of cholinesterase inhibition. EPA has classified carbaryl as a Group D, not classifiable as to human carcinogenicity. General symptoms caused by extended low-level exposure to carbaryl are headaches, memory loss, muscle weakness, and cramps, and anorexia.

Dimethoate (Rogor L-40)

Dimethoate is a commonly used organophosphate insecticide. It was patented and introduced in the 1950s by American Cyanamid. Like other organophosphates, dimethoate is an acetylcholinesterase inhibitor which immobilizes cholinesterase, an enzyme necessary for central nervous system function. It works both by contact and through ingestion which means absorbance and distribution through the plant tissues are fast and is degraded relatively rapidly. Dimethoate appears as a white crystalline solid, with a camphor-like odor, white to grayish crystals for technical product. This material is a contact and systemic organophosphate insecticide effective against a broad range of insects and mites when applied on a wide range of crops. [11]

Endosulfan (Thiodan 50 WP)
Since the 1950s, endosulfan is one kind of organochlorine pesticide commonly used in agriculture to control a wide range of pests for crops, including cotton, rice, sorghum, and soy. [12] It causes worldwide concerns due to its environmentally persistent and semivolatile characters. Endosulfan can particularly accumulate in organism and cause neurotoxicity. [13] Endosulfan works as an endocrine disruption which also makes it become a controversial chemical in the market. Threat to human health and to the environment from this chemical is quite huge, thus the global ban of this chemical was negotiated under the name of Stockholm Convention in April 2011. [14]

Malathion (Phospotion)
Malathion is an insecticide that does not occur naturally. Pure malathion is a colorless liquid, and technical-grade malathion, which contains >90% malathion and impurities in a solvent, is a brownish-yellow liquid that smells like garlic. [15] Malathion is a synthetic phosphorous compound and cholinesterase inhibitorthat is strictly used as a topical pediculicide which is used to kill lice. [15] Malathion acts on the nervous system of the lice by irreversibly restraining the activity of cholinesterase enzyme. It eventually leads to the head lice's death.

Methomyl (Lannate 20L)
It is used as a nematocide, and an insecticide on vegetables, tobacco, cotton, alfalfa, soy beans, and corn. [16] Methomyl is classified as Restricted Use Pesticide (RUP) by the Environmental Protection Agency because of its high acute toxicity to humans. [17] Methomyl is a white crystalline solid with slight sulfurous smell. It was first introduced in 1966 as a broad-spectrum insecticide because it is pretty effective in two ways [17]. First of all, it kills the target insects upon direct contact as in a way of contact insecticide. Secondly, it is a systemic insecticide by causing systemic poisoning in target insects in a way that being transported throughout the pests which feed on treated plants. Methomyl is considered as a carbamate chemical by impeding cholinesterase, essential nervous system enzyme without being harmful to the plant.
[16] Toxicity of this chemical is quite high and is potential poisonous for humans. If ingestion or absorption happens, it is highly toxic, in case of inhalation it is considered moderate. [17]

Pirimicarb (Pirimor 50DP)
Pirimicarb is an insecticide for aphid control in a wide range of crops. *Aphids consists* has various types including woolly aphid, green peach aphid, and the cabbage aphid. The applications of this chemical are also wide starting from wheat, fruit, vegetables, to cotton, and tobacco. It was reported in 1969 and first marketed in 1970. It belongs to the carbamate substance group. [18]

Rhizobia strains
A group of bacteria which forms root nodules of leguminous plant roots among the soil bacteria is called Rhizobia. General characteristics of this group would be aerobic, gram-negative belonging to a class of Alphaproteobacteria. [19] Rhizobia are considered as plant growth promoting rhizobacteria in symbiosis with legumes. Rhizobial species belong to several genera such as: *Rhizobium, Mesorhizobium,Bradyrhizobium, Ensifer, Allorhizobium, Azorhizobium, Neorhizobium and Pararhizobium* and can exist as free-living soil saprophytes or as N_2-fixing endosymbionts of legume host plants roots that creating the root-nodules of host legumes or in close association with the plant roots [19].
Symbiotic relationship between host plants and rhizobia depends on the presence of nodulation (Nod) genes which are not always found in the species. Rhizobial strains are often located on plasmids, mobile genetic elements. Thus, rhizobial communities in the environment consist of both free-living cells lacking symbiotic

genes (Nod). Nod can replicate consistently in soil but does not form root nodules. [20]

Rhizobium leguminosarum

Rhizobium leguminosarum bv *trifolii* is a soil bacterium that creates a N_2-fixing symbiosis with clovers (*Trifolium* spp.). These plants belong to legumes (*Fabaceae*) and it makes up for 18000 species which are economically and industrially important plants. These species are the source of food, and biofuels. [21] The distinctive ability of rhizobia is to enter into a symbiosis with legumes. It eventually forms the special organs, called nodules, on host plant roots. [21] Inside nodules, rhizobial cells modify into bacteroids that convert atmospheric dinitrogen to ammonia, a compound that can be used by the plant [22]. Symbiosis plays an important role in many ecosystems, annually yielding 200 million tons of nitrogen, which is closer to the amount of artificial nitrogen fertilizers [23]. Lastly, from this group, 4 strains of *R. leguminosarum* were selected for this experiment to be investigated for the effects of insecticides.

MATERIALS AND METHODS

Insecticides

Seven types of insecticides (Table 2) which work effectively against a broad range of insects and mites when applied on a wide range of crops were used to determine of its adverse effects on microorganisms, rhizobia strains.

Table 2. Detailed data on inoculated insecticides [24]

Insecticide (commercial name)	Active Ingredients	Active Ingredients	Molecular Formula
Anthio 33 EC	Formothion	33%	C6H12NO4PS2
Sevin 85 WP	Carbaryl	85%	C12H11NO2
Rogor L-40	Dimethoate	40%	C5H12NO3PS2
Thiodan 50WP	Endosulfan	50%	C9H6Cl6O3S
Phosphotion	Malathion	50%	C10H19O6PS2
Lannate 20L	Methomyl	200g/L	C5H10N2O2S
Pirimor 50DP	Pirimicarb	50%	C11H18N4O2

Rhizobial Strains

Seven strains of N_2-fixing root-nodule rhizobil strainsa were used in the following investigations. Four strains of *R. leguminosarum,* one originated from Libya (HB-3841^{str+} streptomycin resistant mutant) strain, two Hungarian strains (Lóbab Z and Bükköny 75/4), and English strain (E1012) and three Hungarian strains (Feryol 1/4, Ló 133/64, Baltacim-3) belonging to *R. phaseoli, R. trifolii, R. loti* respectively as shownin Table 2.

Table 3.Detailed data on tested *Rhizobium* strains

Rhizobium strain	Laboratory code	Host plant	Origin
R, Leguminosarum	HB-3841^{str+}	*Vicia faba*	Libya
	E 1012		England
	Lóbab Z		Hungary
	Bükköny 75/4		Hungary
R. phaseoli	Feryol 1/4	*Phaseolus vulgaris*	Hungary
R. trifolii	Ló 133/64	*Trifolium pratense*	Hungary
R. loti	Baltacim-3	*Onobrychis viciifolia*	Hungary

Methods
An *in vitro* experiment was carried out to evaluate the response of four *R. leguminosarum* strains and each strain of *R. phaseoli, R. trifolii, R. loti* originated from different soil types to 7 insecticides using microfermentor technique. The microfermentor technique was carried out as following: Insecticides were applied at four concentrations (0.0, 0.1, 1.0, 10, and 100 mg/l).
These range of concentrations was tested in 5 ml yeast extract mannitol (YEM) broth medium inoculated with 125 µl bacterial suspension of cell density (10^6 CFU/ml). Sterilized, defined yeast mannitol broth medium contains the following ingredients in 1 litre of distilled water: yeast extract 1 g, mannitol 10 g. The growth was measured at 550 nm from a shaken culture using a Sanyo-Gallenkamp SP50 spectrophotometer
U.K after 48 hours incubation in rotary shaker (150 rpm) at 28°C. The results were presented as relative growth rate of control tubes.The results from this study provide critical baseline information for further examinations to optimize the growth of these root nodule fixing bacteria.

RESULTS AND DISCUSSION

From Figure 1, it can be concluded that growth of Lóbab Z and E1012 from England strains was higher than the rest of strains. Ló 133/64 is sensitive towards the toxicity of Phosphotion.

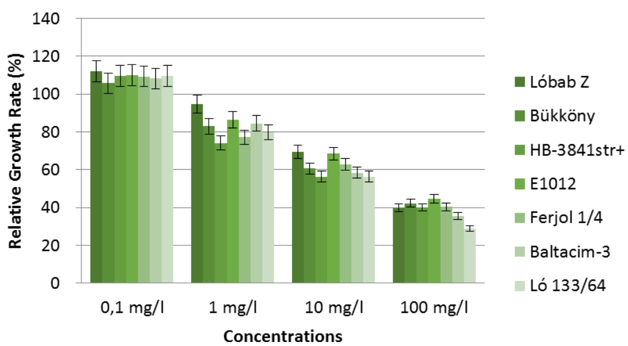

Figure 18. Effect of Phosphotion on relative growth of different rhizobial strains

Figure 2 shows that the strain Lóbab Z has higher tolerance against Sewin 85 WP insecticide, and Ló 133/64 was the sensitive one. Overall, there are gaps between the growths of each strain on each concentrations, it indicates that sewin 85 wp had a adverse effect on root-nodule bacteria.

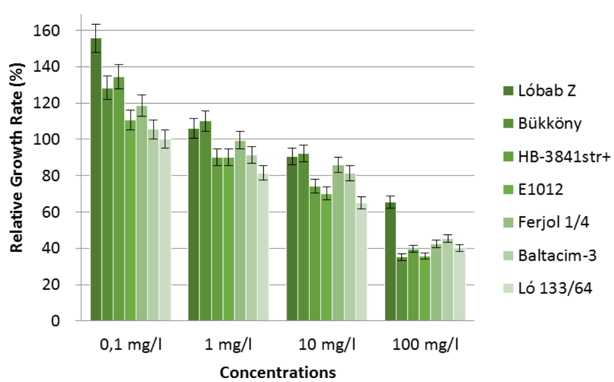

Figure 19.Effect of Sevin 85 WP on relative growth of different rhizobial strains

In Figure 3, there were some odd result from Rogor L-40 that shows HB-3841 str$^+$ has showed a higher growth at 10 mg/l of concentration than the rest of them and the growth decreased dramatically at 100mg/l of concentration. The tolerant strain was Lóbab Z and the sensitive strain was Baltacim-3.

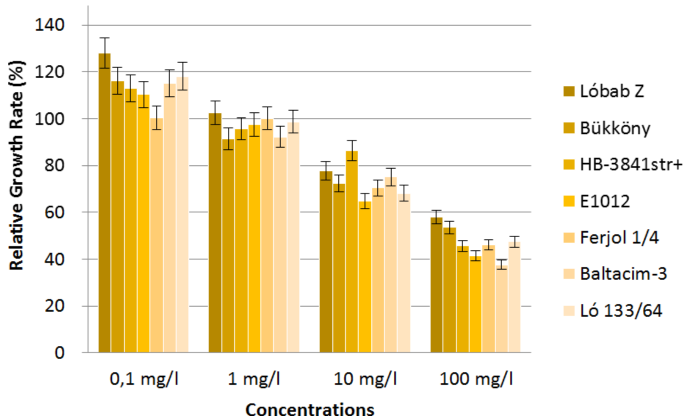

Figure 20.Effect of ROGOR L-40 on relative growth of different rhizobial strains

In Figure 4, it was found that Bükköny 75/4 had a highest growth at almost all concentration levels of Anthio 33 EC insecticides. Baltacim-3 and Ló 133/64 strains could not show high growth.

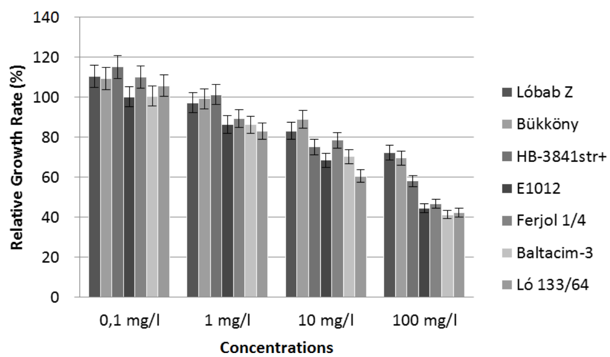

Figure 21.Effect of Anthio 33 EC on relative growth of different rhizobial strains

Regarding to the effect of Lannate 20L, Figure 5 illustrates that Baltacim-3 belonging to *R. loti* showed the lowest growth; while Bükköny 75/4 and Lóbab Z strains had shown relative high growth.

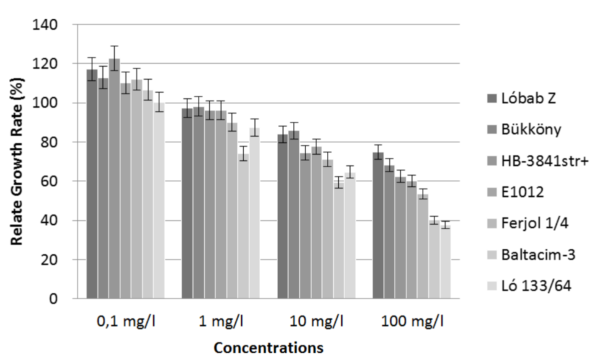

Figure 22.Effect of Lannate 20L on relative growth of different rhizobial strains

Figure 6 shows that Pirimor 50DP insecticide did not have adverse effect on E1012 and Lóbab Z strains. However, Ló 133/64 is the most sensitive one towards this insecticide as well.

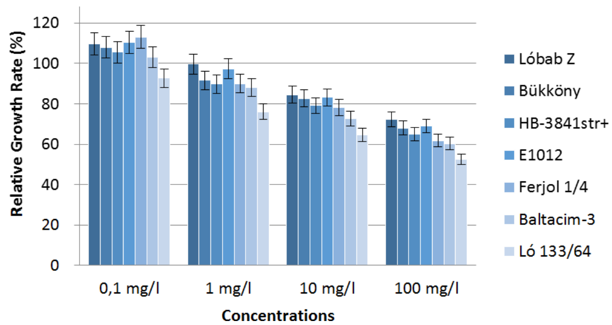

Figure 23.Effect of Piriimor 50 DP on relative growth of different Rhizobium strains

Figure 7 shows the effect of Thiodon insecticides on the different strains. Bükköny and Ferjol 1/4 showed bigger growth while this insecticide was harmful for strains Ló 133/64, E1012, Baltacim 3

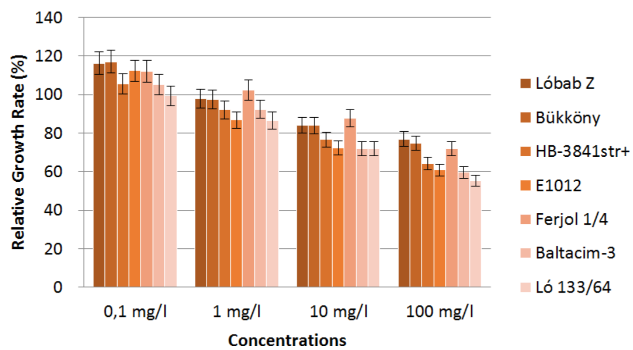

Figure 24. Effect of Thiodon 50 WP on relative growth of different rhizobial strains

Figure 8 summarizes the average growth of all rhizobial strains by each insecticidal effect, Phosphotion and Sevin 85 WP had an adverse effect on all strains. To contrast, Thiodon 50 WP, Piriimor 50 DP were safer to use. To summarize from the tolerance of strains, Ló 133/64, Baltacim-3 were sensitive to almost every . In comparison, Lóbab Z was the most tolerant and showed growth in this experiment, Bükköny 75/4 and Ferjol 1/4 were the following.

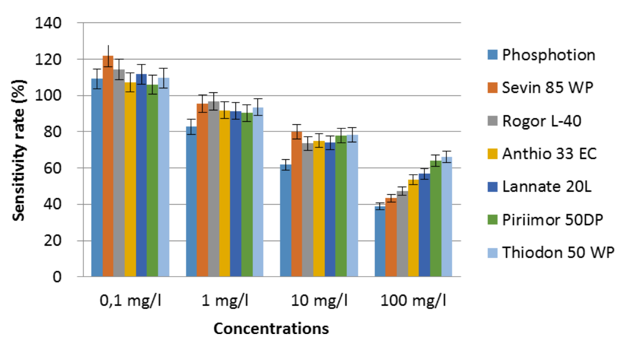

Figure 25. Comparison of effects of each insecticide on average growth of all strains

These results provided a partial explanation of the compatibility of *Rhizobium* strains and insecticides under laboratory conditions. As mentioned earlier, any adverse effect on legume-rhizobia symbiosis results in reduced rates of biological N_2-fixation which counts and contributes more than artificial N supply.

Therefore, the research with definite results and comparison to show how insecticides and pesticides affect these beneficial bacteria and its N_2-fixation process by decreasing the bacterial respiration rate should be carried out in the future.

CONCLUSION AND RECOMMENDATION

This study was designed to evaluate the effects of seven insecticides in vitro on the growth rate of seven rhizobial strains of N_2-fixing root-nodule. From this study, it can be concluded that the results obtained under *in vitro* experiment show the sensitivity of the studied *Rhizobium* strains was as described in the literature. It was found that the fungicides had similar effects on the investigated four *Rhizobium* strains. Itis concluded that *Rhizobium* strains vary in their sensitivity to the insecticides. The degree of growth inhibition at each concentration of insecticides tested shows the similar trends. The Lóbab Z strain (isolated from Hungarian soil) proved to be broad-tolerant (the most tolerant) to most of the insecticides investigated,Bükköny 75/4 (Hungarian) and HB 3841$^+$ (Libyan) strains were the following tolerant strains. Strain E1012 (of English origin) was relative sensitive strain which depended on the toxicity of insecticides. Thus, it can be said that insecticides react differently with the strains depending on their toxicity and concentration. It was found that Thiodon 50 WP, Piriimor 50 DP were the least toxic, compared with Phosphotion, Sevin 85 WP being the most toxic. Rogor L-40, Anthio 33 E, Lannate 20 L have moderately influenced strain survival and reproduction. More investigations should be carried out using different techniques and other species of symbiotic N_2-fixing bacteria. What other factors affect the strain tolerance towards any kind of pesticides and how insecticides specially affect N_2-fixing bacteria should be studied more in order to understand the relationship between the genes and symbiotic genes.

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