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Original scientific paper

Effects of different herbicides on the growth of *Rhizobium* sp. strains isolated from rhizosphere of legumes

Abstract

Sustainable agricultural production is frequently related with the use of agrochemicals, particularly pesticides, which are effective in combating plant infections and pests. Herbicides, a type of pesticide used to control weeds, can have a variety of effects on the growth of beneficial microbial populations in soil. The purpose of this paper was to investigate the effects of three pesticides on the growth of Rhizobium sp. strains. In this experiment, the growth of three bacterial strains (Rhizobium (Sinorhizobium) meliloti RM1, Rhizobium trifolii RT2, and RT3) was measured in the presence of three herbicides (Lumax 537.5, Agrodimark, and Siran 40 SC), which were added after impregnation of paper discs plated on Muller Hinton Agar. In this experiment, the recommended and doubled doses of each herbicide were employed. Resistance, susceptibility - increased exposure, and susceptibility - standard dosage were assessed following inhibition zone diameter measurement. Our findings demonstrated that the diameter of the inhibitory zone varies with herbicide type, dose, and rhizobial species. The findings revealed that treatments with increasing herbicide concentrations produced a larger inhibition zone diameter. The use of Lumax 537.5 SE led to higher inhibition zone diameter compared with other herbicides. In general, Rhizobium trifolii was more sensitive to Lumax 537.5 SE and Agrodimark than Rhizobium (Sinorhizobium) meliloti. This work may have scientific and applicative relevance for plant farmers, improving their knowledge of soil microbiology and pesticide application.

Keywords: *herbicides, Rhizobium sp., inhibition zone, nodule bacteria*

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Introduction

In the period of sustainable plant production, the interactions of plants and microorganisms are of increasing importance in the transformation and mobilization of nutrients from limited supplies in the soil, and then in the uptake of these nutrients so that plants realize their maximum genetic potential. As the world's population grows more and more, agriculture tries to keep up with the increased demands for the production of plant and animal feed (van Dijk et al., 2021). For adequate crop yields, there are numerous obstacles such as lack of arable land, phytophagous insects, microorganisms that cause plant diseases, weeds and other pests whose occurrence or risk of occurrence often requires the use of pesticides (Burul et al., 2022).

Although pesticides are useful, excessive and inadequate application can represent a potential danger for the entire environment (Zhou et al., 2025). A group of phytopharmaceuticals, herbicides, act on non-target organisms, especially those that are useful for agricultural production (Fishel, 2005). If symbiotic nitrogen fixation is adversely affected, crop yields will also be adversely affected. Herbicides are among the potential factors that can affect symbiotic nitrogen fixation (Brochado et al., 2023) and growth of nodule bacteria (Fox et al., 2007; Anderson et al., 2004). These bacteria participate in the symbiosis with legumes, such as soybean (*Glycine max* L.), bean (*Phaseolus vulgaris* L.), chickpea (*Cicer arietinum* L.), peanut (*Arachis hypogaea* L.), and forage legumes such as alfalfa (*Medicago sativa* L.) and clover (*Trifolium* sp.).

Rhizobia and legume symbiosis has garnered interest and is currently being studied more and more. Legumes' capacity to use rhizobium to fix atmospheric nitrogen is extremely significant from an energetic, ecological, and financial standpoint. The process of seed inoculation is required to establish a symbiotic relationship between rhizobia and legume roots (Buntić et al., 2019). Another crucial factor is how well a particular bacterial strain adapts to the culture being injected (Ilangumaran et al., 2021). By applying nitrogen-fixing bacteria to the seeds or soil, a process known as inoculation, each plant interacts with the rhizobia that live in the legume's root. In this manner, the plant supplies nitrogen that the bacteria have fixed from the atmosphere (Fustec et al., 2010), and the bacteria receive nourishment from the plant (Afzal et al., 2019). Legumes improve the physical, chemical, and biological properties of the soil, supply nitrogen, inhibit weeds, and lessen the likelihood of pests and illnesses affecting other crops (Kebede, 2021). Thus, interest in learning more about the activities of the genus *Rhizobium* sp. and their useful applications in environmentally friendly and sustainable agriculture has grown as a result of recent discoveries on the detrimental side effects of bacteria brought on by the use of chemicals in agriculture.

This study sought to determine how the herbicides Lumax, Agrodimark, and Siran 40 SC affected the growth of nodule bacteria from the genus *Rhizobium* sp. isolated from alfalfa (*Medicago sativa* L.), white clover (*Trifolium repens* L.), and red clover (*Trifolium pratense* L.).

Material and methods

Rhizobium (*Sinorhizobium*) *meliloti* RM1, which was isolated from alfalfa (*Medicago sativa* L.), and *Rhizobium trifolii* RT2 and RT3, which were obtained from white (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.), respectively, were the three nodule bacteria employed in this study. These strains were utilized for dilution in saline after being kept on nutrient agar (Torlak, Serbia) at 27°C for four days. Following four days of incubation at 27°C, the

microbial cell concentration was $1x10^8$ CFU/ml. The bacterial inoculum was thus produced for additional analysis.

Lumax 537.5 SE (Syngenta), Agrodimark (Agromarket, Serbia) and Siran 40 SC (Agromarket, Serbia) were used in this research. Lumax (Mesotrion + S-metolacholor + Terbuthylazine) is a selective herbicide used for the control of annual grasses and broadleaf weeds (Hassannejad et al., 2023). Agrodimark (Dikamba) is applied for the growth inhibition of broadleaf weeds in cereal production (Ransom et al., 2014), while Siran 40 SC (Nicosulfuron) is used for the suppression of annual and perennial narrow-leaved weeds in the corn production (Zhong et al., 2023). These herbicides were applied in two doses. Recommended doses (according to the manufacturer`s instructions) for Lumax was 12 g/l, for Agrodimark 2 g/l and for Siran 40 SC 5 g/l. In this experiment, double dose of each herbicide was also used in analysis. All herbicides were prepared by dillution in water in above described concentrations.

A modification of Deb et al.'s (2015) approach was utilized to determine rhizobia sensitivity/persistance to herbicides. Muller Hinton Agar, previously sterilized in an autoclave at 120°C for 20 minutes, was inoculated with the previously described bacterial inoculum. After 15 minutes of agar plate inoculation, 6 filter paper disks (6 mm diameter) with 10 µl herbicides were placed on each Mueller Hinton agar surface. In control, distilled water was used for The Petri dishes were incubated at 37°C for 24 hours in the dark.

The diameter of the inhibitory zone was measured and reported in millimeters. The extracts' effects are divided into three categories: resistant (R), susceptible - increased exposure (I), and susceptible - standard dose.

After determining the zone of inhibition, statistical significance ($P<0.05$) between treatments was determined using SPSS software (v. 22, SPSS Inc. Chicago, IL, USA): ANOVA, post hoc, Tuckey test and T-test.

Results and discussion

Based on the measured zones of inhibition, the effect of certain herbicides on the growth of nodule bacteria from the genus *Rhizobium* sp. was investigated, specifically *Rhizobium* (*Sinorhizobium*) *meliloti* isolated from alfalfa (*Medicago sativa* L.) and *Rhizobium trifolii* isolated from white (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.). Our findings revealed that the diameter of the inhibition zone varies depending on the herbicide type and dose, as well as the rhizobial species.

Table 1 shows data on the effects of herbicides on the growth of *Rhizobium (Sinorhizobium) meliloti* RM1. The most pronounced inhibition zone was detected following Lumax application, whereas the smallest diameter of inhibition zone was reported with Agrodimark treatments. Increased initial herbicide concentration resulted in a significant increase in inhibition zone diameter as compared to the manufacturer's suggested concentration. When the prescribed quantities of Agrodimark and Siran 40 SC were used, *Rhizobium (Sinorhizobium) meliloti* RM1 growth was lightly inhibited.

Rhizobium trifolii RT2 demonstrated the significantly highest susceptibility to the herbicide Lumax 537.5SE (Table 2). Its suggested dosage of 12 g/l resulted in the establishment of a 1.50 mm inhibition zone diameter, which was raised fivefold compared to the recommended dose. After applying the prescribed doses of Agrodimark and Siran 40 SC, there was a light suppression of bacterial growth, but increasing the concentration of both herbicides resulted in a significant rise in the inhibition zone diameter.

Table 1. Diameter of inhibition zone for *Rhizobium* (*Sinorhizobium*) *meliloti* RM1 against herbicides / **Tablica 1.** Promjer zone inhibicije *Rhizobium* (*Sinorhizobium*) *meliloti* RM1 u prisustvu herbicida

DIZ (mm)	Control	Lumax 537.5 SE concentration (g/l)		Agrodimark concentration (g/l)		Siran 40 SC concentration (g/l)	
		12	24	2	4	5	10
Disc 1	0	2	4	0	2	1	3
Disc 2	0	2.5	4.5	2	2	1	4
Disc 3	0	1	5	1	3	2	3.5
Disc 4	0	3	6	1.5	2	0.5	5
Disc 5	0	2	5	0.5	3	1	4
Disc 6	0	1.5	4	0	2	0	3
Average	**0**[a]	**2.00**[bA1] ± 0.75	**4.75**[bX2] ± 0.71	**0.83**[bB1] ± 0.76	**2.33**[bY2] ± 0.49	**0.92**[bC1] ± 0.54	**3.75**[bZ2] ± 0.85

Legend: DIZ – Diameter of inhibition zone (mm); ***a, b*** *– values marked with different lowercase letters between the control and different concentrations of treatments show a statistically significant difference (p<0.05), T-test;* ***A, B, C*** *– values marked with different uppercase letters among the lower concentrations show a statistically significant difference (p<0.05), ANOVA post hoc Tukey test;* ***X, Y, Z*** *– values marked with different uppercase letters among the higher concentrations show a statistically significant difference (p<0.05), ANOVA post hoc Tukey test;* ***1, 2*** *– values marked with different numbers among different concentrations of the same treatment show a statistically significant difference (p<0.05), T-test.*

Lumax treatment resulted in the largest diameter of the inhibitory zone for *Rhizobium trifolii* RT3. The use of 24 g/l of this herbicide resulted in a significant increase in inhibition zone compared to the recommended concentration (Table 3). Other herbicides had a smaller influence on inhibition zone diameter: 0.75 and 3.92 mm for Agrodimark, and 1.25 and 4.92 mm for Siran 40 SC. In general, *Rhizobium trifolii* showed greater sensitivity after applying Lumax 537.5 SE and Agrodimark than *Rhizobium (Sinorhizobium) meliloti*. Drouin et al. (2010) studied the susceptibility of nitrogen fixing strains to several herbicides. These scientists discovered that *Rhizobium* and *Sinorhizobium* strains were unresponsive to metolachlor (a component of Lumax) treatment. The same authors claimed that *Rhizobium* strains were more sensitive to dicamba (a component of Agrodimark) than *Sinorhizobium* strains, which our analysis validated.

Table 2. Diameter of inhibition zone for *Rhizobium trifolii* RT2 against herbicides
Tablica 2. Promjer zone inhibicije *Rhizobium trifolii* RT2 u prisustvu herbicida

DIZ (mm)	Control	Lumax 537.5 SE concentration (g/l)		Agrodimark concentration (g/l)		Siran 40 SC concentration (g/l)	
		12	24	2	4	5	10
Disc 1	0	2	7.5	1	3	0	5
Disc 2	0	1	12.5	0.5	5	0	6
Disc 3	0	1	7	1	4	1	5.5
Disc 4	0	0.5	4	2	3	1.5	4
Disc 5	0	2	5	0	2	1	3
Disc 6	0	2.5	6	1	4	1	5
Average	**0**[a]	**1.50**[bA1] ± 0.75	**7.00**[bX2] ± 3.31	**0.92**[bB1] ± 0.64	**3.50**[bY2] ± 1.10	**0.75**[bC1] ± 0.56	**4.75**[bZ2] ± 1.05

Legend: DIZ – Diameter of inhibition zone (mm); ***a, b*** *– values marked with different lowercase letters between the control and different concentrations of treatments show a statistically significant difference (p<0.05), T-test;* ***A, B, C*** *– values marked with different uppercase letters among the lower concentrations show a statistically significant difference (p<0.05), ANOVA post hoc Tukey test;* ***X, Y, Z*** *– values marked with different uppercase letters among the higher concentrations show a statistically significant difference (p<0.05), ANOVA post hoc Tukey test;* ***1, 2*** *– values marked with different numbers among different concentrations of the same treatment show a statistically significant difference (p<0.05), T-test.*

In most samples, there was no discernible change in the diameter of the inhibitory zone at the indicated herbicide concentration. Mohamad and Al-Naser (2018) found that several herbicides taken at prescribed dosages had no negative influence on rhizobial development in vitro. However, in all studies, increasing the herbicide dose decreased bacterial growth and increased the diameter of the inhibitory zone. Furthermore, Paniagua-Lopez et al. (2023) suggest that using fewer pesticides enhances the performance of rhizobia strains. Burul et al. (2022) discovered that rhizobia can use herbicides as a carbon and energy source(s), reducing their potential negative effects, which is consistent with our results.

Table 3. Diameter of inhibition zone for *Rhizobium trifolii* RT3 against herbicides
Tablica 3. Promjer zone inhibicije *Rhizobium trifolii* RT3 u prisustvu herbicida

DIZ (mm)	Control	Lumax 537.5 SE concentration (g/l)		Agrodimark concentration (g/l)		Siran 40 SC concentration (g/l)	
		12	24	2	4	5	10
Disc 1	0	6	15	0	4	2	6
Disc 2	0	7.5	12	1.5	3	1.5	5
Disc 3	0	2.5	22.5	1	3.5	2.5	4
Disc 4	0	3	16	0	5	1	4.5
Disc 5	0	5	15.5	1.5	4	0	7
Disc 6	0	1.5	14	0.5	4	0.5	3
Average	**0**	**4.25**^{bA1} ± 2.28	**15.83**^{bX2} ± 3.74	**0.75**^{bB1} ± 0.66	**3.92**^{bY2} ± 0.75	**1.25**^{bC1} ± 0.87	**4.92**^{bZ2} ± 1.41

Legend: DIZ – Diameter of inhibition zone (mm); ***a, b*** *– values marked with different lowercase letters between the control and different concentrations of treatments show a statistically significant difference (p<0.05), T-test;* ***A, B, C*** *– values marked with different uppercase letters among the lower concentrations show a statistically significant difference (p<0.05), ANOVA post hoc Tukey test;* ***X, Y, Z*** *– values marked with different uppercase letters among the higher concentrations show a statistically significant difference (p<0.05), ANOVA post hoc Tukey test;* ***1, 2*** *– values marked with different numbers among different concentrations of the same treatment show a statistically significant difference (p<0.05), T-test.*

Although Potera (2007) pointed out that pesticides hinder nitrogen fixation, from our results it is evident that 2-fold increase in herbicide concentration did not had lethal effects on nodule bacteria. This can be linked with ability of nodule bacteria to establish adaptive mechanisms to the herbicides, followed by modification of vital cell processes, which contributes to overcoming of environmental stress (Kots and Kukol, 2021). Walder et al. (2022) found strong interaction between microbial communities and pesticide residues in agricultural ecosystems. These interactions are more expressed between pesticides residua and pesticide-degrading microbes.

Conclusion

Our findings revealed that the influence of the tested herbicides on the growth of the tested nodule bacteria was dependent on the type of herbicide, the applied dose, and the rhizobia strain. In most treatments, the herbicides used had a minor inhibitory effect on *Rhizobium (Sinorhizobium) meliloti* RM1, but a larger inhibitory effect on *Rhizobium trifolii* strains. Increased herbicide concentration resulted in a more pronounced inhibitory effect on rhizobia growth. Lumax 537.5 showed highest sensitivity of nodule bacteria. Other herbicides (Agrodimark and Siran 40 SC) had a smaller influence on inhibition zone diameter. Further research will focus on the effects of reduced pesticide concentrations on diverse microorganisms involved in the nitrogen and carbon cycles.

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Izvorni znanstveni rad

Učinak različitih herbicida na rast sojeva Rhizobium sp. izoliranih iz rizosfere leguminoza

Sažetak

Održiva poljoprivredna proizvodnja često je povezana s upotrebom agrokemikalija, posebno pesticida, koji su učinkoviti u borbi protiv biljnih bolesti i štetnika. Herbicidi, vrsta pesticida koji se koristi za suzbijanje korova, mogu imati različite učinke na rast korisnih mikrobnih populacija u tlu. Cilj ovog rada bila je istražiti učinke tri pesticida na rast sojeva Rhizobium sp. U ovom pokusu, rast tri bakterijska soja (Rhizobium (Sinorhizobium) meliloti RM1, Rhizobium trifolii RT2 i RT3) mjeren je u prisutnosti tri herbicida (Lumax 537.5, Agrodimark i Siran 40 SC), koji su dodani nakon impregnacije papirnatih diskova nanijetih na Muller Hinton Agar. U ovom pokusu korištene su preporučene i dvostruke doze svakog herbicida. Otpornost, osjetljivost - povećana izloženost i osjetljivost - standardna doza procijenjene su nakon mjerenja promjera zone inhibicije. Naši nalazi pokazali su da se promjer inhibitorne zone mijenja ovisno o vrsti herbicida, dozi i vrsti rizobija. Rezultati su otkrili da tretmani s rastućim koncentracijama herbicida proizvode veći promjer zone inhibicije. Upotreba Lumaxa 537.5 SE dovela je do većeg promjera zone inhibicije u usporedbi s drugim herbicidima. Općenito, Rhizobium trifolii bio je osjetljiviji na Lumax 537.5 SE i Agrodimark nego Rhizobium (Sinorhizobium) meliloti. Ovaj rad može imati znanstvenu i aplikativnu važnost za uzgajivače biljaka, poboljšavajući njihovo znanje o mikrobiologiji tla i primjeni pesticida.

Ključne riječi: herbicidi, Rhizobium sp., zona inhibicije, kvržične bakterije