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Effect of Roundup 360 SL on Survival of *Pseudomonas* sp. SP0113 Strain and Effective Control of Phytopathogens

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ABSTRACT

Studies on determination of the effect of herbicides on survivability of Plant Growth Promoting Bacteria have a strategic usefulness in determination of plant health and the fate of applied agrochemicals in agroecosystem. Antimicrobial potential was assessed using estimation of a minimum inhibitory concentration of the Roundup 360 SL against *Pseudomonas* sp. A quantitative analysis of bacteria was performed, and the tendency of physicochemical changes in the mineral medium was evaluated during long-term exposure to the herbicide. Furthermore, the antagonism of the SP0113 strain against F. culmorum and F. oxysporum under stress conditions caused by Roundup® 360 SL was verified. It was demonstrated that use of the undiluted and 2.6-fold diluted product resulted in the inhibition of growth of the investigated strain. *Pseudomonas* sp. SP0113 showed survivability and resistance to near recommended dose concentration of Roundup® 360 SL. The possibility of bacterial development on the Tryptic Soy Agar (TSA) medium at contact concentrations of 14.4 and 5.4 mg mL^{-1}, as per the diluents quantity declared by the producer, indicates the role of cofactors such as: adiuvant or pH, redox potential (mV) or salinity. They comprise pH change, oxidation and salinity that may be due to the reaction of the active substance of the herbicide with mineral nutrient ingredients. The high salinity of environment, as a result of the reactions with the ingredients contained in the medium, is characteristic for concentrations higher than those recommended in practice. Furthermore, it was found that glyphosate limits the growth of fungi of the *Fusarium* genus, which support plant protection using strain SP0113.

Keywords: Biocontrol, Glyphosate, Plant growth promotion bacteria, PGPB.

INTRODUCTION

Roundup® 360 SL is a non-selective herbicide which contains glyphosate (N-(phosphonomethyl) glycine) in the form isopropylamine salt as active ingredient and surfactant, an ethoxylated fatty amine. Glyphosate belongs to aminophosphonate group compounds. It inhibits shikimic acid metabolic pathway in plants by inactivating EPSP synthase (5-EnolPyruvylShikimate-3-Phosphate synthase) enzyme activity. As shikimic acid metabolism also involves biosynthesis of certain amino acids, like tryptophan, tyrosine, and phenylalanine, permanent disturbance in synthesis of these amino acids may occur due to perturbance of

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the above pathway (Steinrücken and Amrhein, 1980; Duke *et al*., 2012).

In a natural environment exposed to the introduction of glyphosate, changes in the structural community of soil microorganisms were observed. In some cases, the use of glyphosate contributes to an increase in the microbial count or some microbial counts, causing a quantitative and qualitative imbalance among taxa. This phenomenon was observed in the soil environment (Wardle and Parkinson, 1990; Ratcliff *et al*., 2006; Saxton *et al*., 2011). Soil devastation resulting from the use of plant protection compounds can cause increased incidences of cereal diseases. Furthermore, the use of glyphosate increases the colonization of plant roots by *Fusarium* spp., and increased incidence of diseases such as FHB (Fusarium Head Blight) and root rot (Fernandeza *et al*., 2009; Kremer and Means, 2009), which can occur indirectly due to the colonization of plant roots by these pathogens, as a result of displacement of the agro-ecosystem bacteria such as *Pseudomonas* spp. naturally associated with the plant root system (Kremer and Means, 2009).

Along with the disturbed biological structure of the soil environment, physico-chemical changes in the soil solution, including the ability of rhizobacteria to share nutrients and natural sorption, is observed (Dick *et al*., 2010; Duke *et al*., 2012). Glyphosate has strong metal-chelating properties. This may result in immobilization of soil cations (Ca, Mg, S, and Cu), which are involved in plant metabolism, nodulation, biomass production, including those determining the formation of chlorophyll. The chelating properties of this active substance may also perturb or disrupt the plant-bacteria interactions particularly for nitrogen fixing symbiotic rhizobia. These bacteria require Nickel (Ni) for N-fixation activity, which is immobilized by glyphosate in soil, disrupting rhizobial nitrogen fixation by symbionts, which may in turn adversely affect the growth and development of host plants (Duke *et al*., 2012; Zobiole *et al*., 2010; Zobiole *et al*., 2012).

Despite the negative impact of glyphosate on the environment, many publications indicate a rapid degradation of this active substance in the soil by certain microorganisms. Furthermore, an increase in C and N mineralization in the soil environment was observed, despite the toxic effect of the herbicide found under laboratory conditions (Busse *et al*., 2001; Motavalli *et al*., 2004; Duke *et al*., 2012).

Glyphosate in soil is degraded by microorganisms to produce compounds such as AminoMethylPhosphonic Acid (AMPA), glycine, sarcosine and CO_2. Therefore, this compound may become a source of C, N and P for soil microorganisms (Jacob *et al*., 1988; Grundman *et al*., 2008; Moneke *et al*., 2010; Duke *et al*., 2012). Mineralization of glyphosate to CO_2 in soil is correlated with the occurrence of *Pseudomonas* sp. bacteria, so, it is believed that the fate of this herbicide in the soil environment depends on the mineralization potential by certain genera and species of the *Pseudomondaceae* family (Gimsing *et al*., 2004)

Due to an inverse correlation between *Pseudomonas* viable cell counts and presence of *Fusarium* in the plant root zone (Kremer and Means, 2009), the introduction of this bacteria may impart potentially positive yield and biocontrol effects on inoculated plants due to its ability to resist glyphosate or its products, thereby increasing crop yield as well as in reducing phytopathogenic attacks. In addition, it is noted that bio-fertilizing of crops exposed to pesticides is becoming increasingly common in order to bioremediation of soil (Romeh and Hendawi, 2014).This study aimed to determine the effect of the Roundup® 360 SL herbicide on survival and changes in the physicochemical parameters of liquid media inoculated with the *Pseudomonas* sp. SP0113 strain. This strain exhibits plant growth promoting properties and antagonism against phytopathogens (Przemieniecki *et al*., 2015).

MATERIALS AND METHODS

Identification and phenotypic characterization and PGP (Plant Growth Promotion) properties of the *Pseudomonas* sp. SP0113 strain were described in the previous paper (Przemieniecki *et al*., 2015).

Inhibition of Bacterial Growth by Roundup® 360 SL

A diffusion test on the TSA - Tryptic Soy Agar (Merck, Germany) - culture medium was used to determine the ability to inhibit growth. The medium was superficially inoculated using 100 µL of bacterial suspension with a density of 10^8 CFU mL^{-1}. Three paper discs per plate were soaked with 10 µL of Roundup® 360 SL solution with a concentration of 360 mg mL^{-1}, 100, 50, 10, 5, 1, 0.5, and 0.1 mg mL^{-1} of glyphosate and applied to a medium. The samples were incubated for 48 hours at 27°C. After 24 and 48 hours, the radius of the inhibition zone formed around the discs, which indicated the inhibition of bacterial growth, was measured. The experiment was performed in triplicate.

Determination of Minimal Inhibitory Concentration (MIC)

The results of the disc test were used to determine the concentrations applied in the experiment. The bacterial suspension in TSB (Merck, Germany) was adjusted to a density of OD_{600} A= 0.125 (1 10^8 CFU mL^{-1}). To 15 mL test tubes containing 9 mL of TSB (2X concentrated), 1 mL of Roundup® 360 SL diluted with appropriate amount of sterile demineralised water (v/v) to prepare final working concentrations of 180 mg mL^{-1}, 160, 140, 120, 100, and 80 mg mL^{-1} of glyphosate. Then, 100 µL of bacterial suspension was added to these test tubes. The samples were incubated for 48 hours at 27°C. After 24 and 48 hours, a qualitative assessment was made, indicating bacterial growth or lack of bacterial growth. The experiment was performed in triplicate.

Quantitative Analysis of Bacteria and Physicochemical Changes

The experiment was performed in 250 mL Erlenmeyer flasks containing 99 mL of liquid medium comprising 1.0 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.05 g of NaCl, 0.02 g of $CaCl_2$, 0.02 g of yeast extract, and 1.0 g of glucose in 1 L of deionized water containing adequate Roundup® 360 SL solutions. Aqueous solutions of Roundup® 360 SL were prepared, so that the content of glyphosate after mixing with the medium equalled 360 mg mL^{-1}, 100, 10, 1, and 0.1 mg mL^{-1} of glyphosate. Each flask was inoculated with 1 mL of concentrated bacterial culture. The initial density of bacteria after inoculation was 5×10^7 CFU (colony forming units) per mL. Incubation was carried out for 28 days at 27°C under continuous shaking at 150 rpm. To determine the number of colonies, every 24 hours, 1 mL of bacterial culture obtained from each liquid medium was diluted 10-fold and inoculated on Petri dishes containing TSA medium. Moreover, pH, conductivity, and salinity were measured. For the measurement of above-mentioned physicochemical parameters, a digital multimeter handylab multi12 Set (Schott, Germany) was used. The experiment was performed in triplicate.

Antagonism of PS0113 Strain against Pathogenic Fungi

The analysis was performed using a dual plate method on PDA (Potato Dextrose Agar, Difco, USA) medium. Initially, the adequately concentrated media were contaminated with a Roundup® 360 SL v/v solution, so that the final concentration of glyphosate were 14.4 mg mL^{-1} (high dose) and of 5, 4 mL^{-1} (low dose) and the

concentration of PDA medium components equalled 100%. The concentrations used in the analysis were contact concentrations calculated based on the recommended proportion of the product and water as a solvent (for most applications 0.33 to 1 litre of product per 100 L water).

Five millimetre agar media based discs containing mycelium of 14-day old culture of fast growing *Fusarium culmorum* and a slow-growing *F. oxysporum* was placed on one half of the solidified agar medium. On the other half, streak plating of an overnight culture of *Pseudomonas* sp. SP0113 was done about 3 cm away from the disc. The controls consisted of: media only, media with bacterial culture inoculation only, and media supplemented with Roundup® 360 SL only. The experiment was carried on for 7 days at incubation temperature of 27°C. After incubation, radial diameter of the mycelia growth was measured. Experiment was performed in four replicates.

All doses included in the study were converted to glyphosate concentration contained in herbicide Roundup 360 SL.

Statistical Analysis

Statistical analysis was performed using Statistica 10 software (ANOVA). Duncan's test at α= 0.01 was applied. SigmaPlot 12 software was used to draw the charts.

RESULTS AND DISCUSSION

This study used the *Pseudomonas sp.* SP0113 strain, which was characterized for plant growth promotion and protection properties, similar to other species of the *Pseudomonas* genus (Ramette *et al*., 2011; Stajković-Srbinović *et al*., 2014). Preliminary results of strain PS0113 culture in TSB medium (Triptic Soy Broth) has shown that Roundup 360 SL reduces mass gain of the bacteria only at the highest dose of 10 mg mL^{-1}, and limit of the increase in biomass was about 20% (Przemieniecki, *et al*., 2015). Therefore, in this study, the scope of analyses was expanded to research on the effect of the herbicide on the survivability test, and the analysis of changes in physical and chemical parameters of the medium containing Roundup, and to study the interaction of herbicide-antagonist-pathogen in order to determine the relationship between the herbicide containing glyphosate and the plant growth promoting bacterium.

The results of disc diffusion study demonstrated a high resistance of bacteria to the compounds contained in Roundup® 360 SL. No inhibition of bacteria was observed at 36-fold dilution of the pure product (10 mg mL^{-1}). However, after two days of incubation, the bacteria on a medium containing 100 mg mL^{-1} of glyphosate completely overgrew the bright zone around the disc. It was also determined that the minimal dose completely inhibiting bacterial growth was 140 mg mL^{-1}. The obtained results indicate that the strain used in the study is much more resistant to glyphosate (or other components contained in the herbicide) than other microorganisms, on which its effect was tested. Clair *et al*. (2012) observed that minimal inhibitory concentration for *Lactococcus lactis* subsp. *cremoris* amounted to 0.312 mg mL^{-1} and for *Lactobacillus delbrueckii* subsp. *bulgaricus* CF1 amounted to 1 mg mL^{-1}, and for fungus *Geotrichum candidum* amounted to 0.1 mg mL^{-1} (Roundup 400) and 0.615 (Roundup 450). Comparing the values from these results, it can be seen that, on the average, the *MIC* dose (140 mg mL^{-1}) of *Pseudomonas* sp. SP0113 was 400 times higher than the above-described three species of microorganisms (Table 1). In addition, the concentration was about 10 times higher than the dose used for the treatment.

These results may suggest that glyphosate present in the Roundup® product may not have a decisive effect on bacterial survival. The lethal effect of pure glyphosate at a dose of 10,000 ppm was not observed, while the lethal effect of the Roundup® product was revealed at a dose as low as below 100 ppm.

Table 1. Results of the diffusion test and *MIC*.

	Disc test		
	Inhibition value (mm)[a]		
Dose (mg mL^{-1})	After 24 h	After 48 h	MIC (mg mL^{-1})
360	3.2±0.6 A	3.0±0.6 A	
100	1.4±0.5 B	0 D	140
50	0.5±0.5 C	0 D	
Other doses	0 D	0 D	

[a] Values followed by the same letters do not differ significantly by the Tukey test ($P< 0.05$).

These results confirm our hypothesis on the influence of other factors or additives contained in the Roundup® product on microorganisms (Clair *et al*., 2012).

Analysis of bacterial count during the 28-day incubation with different concentrations of glyphosate showed a reduction in bacterial count. Undiluted Roundup® completely stopped the growth of microorganisms as indicated by the number of colonies grown on the medium. Diluted doses were relatively well tolerated by the *Pseudomonas* sp. strain used in the study, except for the variant of 100 mg mL^{-1} in the experiment using a liquid medium. The lowest inhibition was observed at a dose of 0.1 mg mL^{-1} with no significant reduction in the bacterial count as compared to the control. The interaction of the product with the mineral ingredients of the liquid medium, which led to significant changes in pH, oxidation/reduction potential and salinity, contributing to the change in the bacterial count, was observed (Figures 2-4). At a dose of 100 mg mL^{-1}, for the first 7 days of incubation, the bacterial count was significantly lower than in the control, while after 14 days of the experiment, complete elimination of bacteria was observed in the dilution (Figure 1).

According to the results of Wardle and Parkinson (1990), the increase in the bacterial count in soil may be associated with the increase in glyphosate dose. The authors observed that the count of fungi and actinomycetes did not change significantly for any of the doses, but at the same time, they observed a difference in the quantitative ratio of fungal species in the organic fraction of the soil and the inhibition of growth of one fungal species (*Cladosporium cladosporioides*) in the mineral fraction at higher doses of the product. However, under laboratory conditions, the authors observed an inverse correlation between the concentration of glyphosate and the count of fungal cultures on a solid medium. Ratcliff *et al*. (2006), in the study of microbial community in the soils of forest areas, demonstrated that the dose recommended by the manufacturer had no effect on the survival of microorganisms. In this study, a concentration of 100-fold higher than the recommended one and the undiluted product led to the growth of bacterial cells, change in the mass ratio of cells of bacteria and filamentous fungi, and increased use of organic carbon.

The results of the studies on the impact of glyphosate on *Aeromonas cavie*, as a bioindicator in the water of the Albufera lagoon (Valencia), revealed no toxicity for the doses of 50 and 100 mg mL^{-1}. The toxic impact of the chemical compound was demonstrated using the Microtox test, including *Vibrio fischeri* (Amorós *et al*., 2007). Analysis of the phytoplankton community performed in Lake Erie (Canada) by Saxton *et al*. (2011) showed both a positive and a negative impact of glyphosate. The two-way activity of this chemical compound in an aqueous environment is based on the reduction of competition in a given habitat due to the sensitivity of certain taxa of aqueous microorganisms, which allows for their displacement by the herbicide-tolerant taxa.

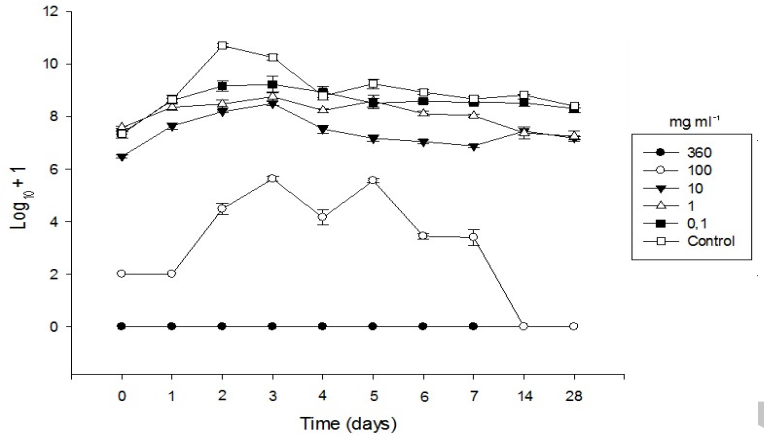

Figure 1. Changes in the bacterial count per 1 mL of culture during a 28-day exposure to different doses of Roundup® 360 SL.

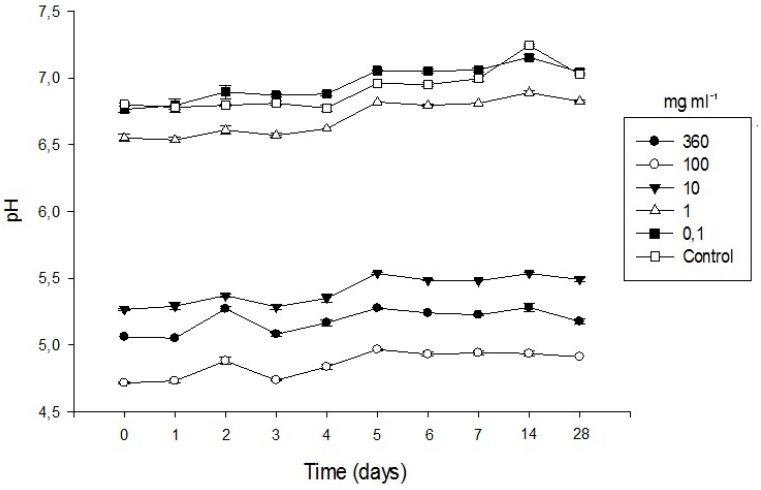

Figure 2. Changes in the pH of the environment (mineral medium) colonized by bacteria during a 28-day exposure to different doses of Roundup® 360 SL.

Furthermore, the decomposition of glyphosate in water can contribute to the supplying of water reservoir with nutrients, affecting its eutrophication.

During the 28-day incubation of *Pseudomonas* spp. culture in a minimal medium, the physicochemical parameters did not change significantly; however, value of parameters were varied depending on the concentration of glyphosate. A reverse ratio of hydrogen ions (pH) to the redox potential (E) was revealed. For the doses of 1, 0.1 mg mL^{-1}, and the control characterized by a pH close to 7, a low oxidation/reduction potential close to 0 mV was measured. In contrast, the doses of 10, 100 mg mL^{-1} and the undiluted product with a pH of approximately 5 were characterized by a redox potential of ~100 mV. In the conductometric measurement, it was observed that the variant containing glyphosate at a dose of 100 mg mL^{-1} had a 3-fold higher value of electrolytic potential in comparison to other cultures, exhibiting a low electrolytic potential, being evidence of low salinity (Figures 2-4).

The comparison of the measurement results of the physicochemical parameters, especially the electrolytic potential with bacterial survival, shows that the inhibition is in some way associated with these changes. The factor contributing to the growth inhibition of the *Pseudomonas* sp. at

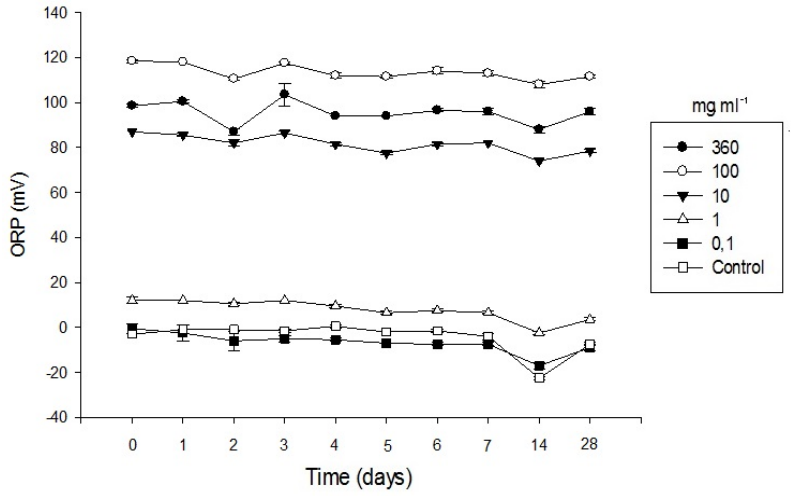

Figure 3. Changes in the Oxidation/Reduction Potential (ORP) of the environment (medium) colonized by bacteria during a 28-day exposure to different doses of Roundup® 360 SL.

100 mg mL^{-1} glyphosate is the result of a strong salinity of the liquid medium after the addition of the product, which caused a 5-fold increase in the electrolytic conductivity as compared to the control and its nearly 3.5-fold increase as compared to the undiluted product. However, during the 28 days of the experiment, no changes in these values within the individual doses were observed, suggesting the stability of the product and observed no influence of the tested bacterium on culture medium (Figure 4).

As indicated by Egamberdieva (2011), a high level of electrolytic conductivity adversely affects the survival of bacteria of the *Pseudomonas* genus. The study on the effect of different NaCl concentrations on the colonization of the growing tip of bean root by *P. extremorientalis* and *P. chlororaphis* showed a reduction in colonization by ~30% at a conductivity of 5

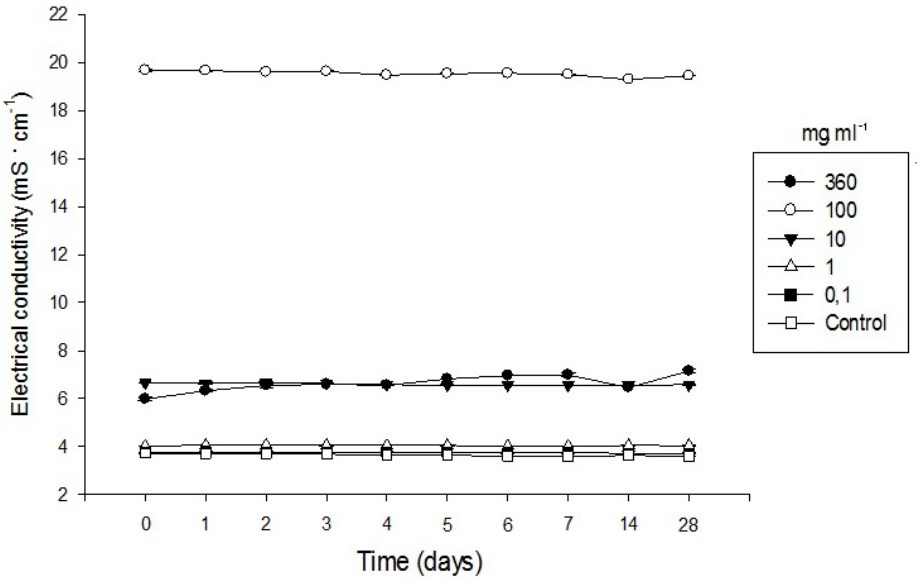

Figure 4. Changes in the electrolytic conductivity of the environment (medium) colonized by bacteria during a 28-day exposure to different doses of Roundup® 360 SL.

mS cm^{-1} and by over 50% at 12 mS cm^{-1}. In this experiment, the control was water with a conductivity of 0.5 mS cm^{-1}. In contrast, Goswami *et al*. (2013) analyzed the PGPB properties of the *Pseudomonas* strain isolated from sea water. This environment was characterized by a salinity close to the value of all doses applied in this study, except for the dose of 100 mg mL^{-1}.

Analysis of the interaction of the bacterium used in the study with phytopathogens demonstrated inhibition of mycelial growth in *Fusarium culmorum*, after application of *Pseudomonas* sp. SP0113 and both doses of glyphosate. As compared to the control, the antagonistic bacterium decreased the *F. culmorum* mycelium length by 40%, while in the variant of 5.4 mg mL^{-1} of glyphosate, mycelial growth was reduced by ~50%. In the combination of 5.4 mg mL^{-1} of glyphosate with *Pseudomonas* sp. SP0113, the greatest inhibition of ~79% was observed, and the mycelial length slightly exceeded 1 cm. On media containing glyphosate at a concentration of 14.4 mg mL^{-1}, no growth of the pathogen mycelium was observed. Analysis of the impact of the bacterial strain (on Roundup free medium) used in the study did not show inhibition of the *F. oxysporum* mycelium. However, after application of both doses of glyphosate, a reduction in the mycelium growth as compared to the control was observed, and the inhibition ratio exceeded 59% (Figure 5).

On the media supplemented with Roundup® 360 SL, the ability of the investigated bacterial strain to intensified swarming was observed, which resulted in the spread of bacteria to a much greater part of the Petri dish. This phenomenon could have an additional effect on growth inhibition and diffusion of active substances in the treatments containing glyphosate .

CONCLUSIONS

The strain *Pseudomonas* sp. SP0113 is a more resistant bacterium on glyphosate contained in herbicide Roundup 360 SL than some of the microorganisms previously described in scientific reports. The tested bacteria were characterized by their ability to survive in doses lower than 140 mg mL^{-1}, however, the long-term study showed that the growth of bacterial biomass in dose 100

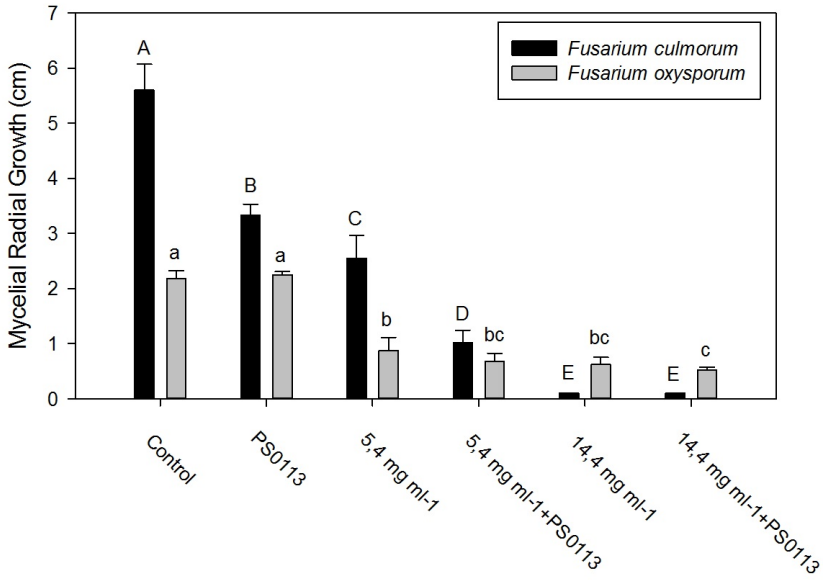

Figure 5. Mycelial growth of *Fusarium* spp. after 7 days of incubation at different concentrations of glyphosate and in the presence of *Pseudomonas* sp. SP0113. Values followed by the same letter do not differ significantly by the Tukey test (P< 0.05). Large letters (A to E) are assigned to *Fusarium culmorum*, small letters (a to c) are assigned to *Fusarium oxysporum.*

mg mL^{-1} was significantly lower than the control and was observed that live bacteria were not present after 7 days incubation. Further observations showed that in dose of 10 mg mL^{-1} (and other doses tested in the mineral medium experiment), bacteria grew well and were able to survive 28 days. The number of bacteria at this dose after 14 days of culture was approximately 10^7, while that of the control was approximately 10^8 and maintained until the end of the experiment. Analysis of physico-chemical parameters (pH and redox potential) showed high stability in the individual doses, despite the significant variation observed, in particular from 14 days of culture. Distinct differences were observed between the parameters studied of high (360 to 10 mg mL^{-1}) and low concentrations (1 to 0 mg mL^{-1}) which could have an impact on the survival of bacteria. However, a concentration of 10 mg ml^{-1} (having similar pH and ORP such as in 360 and 100 mg ml^{-1} concentrations) did not show inhibitory effect on *Pseudomonas* spp, unlike the other two doses of higher concentration. Study of the interactions between antagonist-pathogen-herbicide has shown that higher dose Roundup 360 causes almost total inhibition of mycelial growth of *Fusarium culmorum* and *F. oxysporum*. In case of the lower dose, a significant reduction of the mycelium *F. culmorum* was observed by both bacteria and addition of Roundup to the medium. However, at this dose (5.4 mg mL^{-1}), the most effective solution was combined effects of Roundup with antagonistic bacteria. This approach might be a new strategy for the protection against fitopatogenas agro-ecosystem of the antagonist that are resistant to chemical pesticides.

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اثر راندآپ 360 SL روی بقای ریسه SP0113 گونه سودوموناس و کنترل موثر عوامل بیماری زای گیاهی

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چکیده

مطالعات تعیین اثر علف کش ها روی بقای باکتری های افزاینده رشد گیاهان فایده ای راهبردی در تعیین سلامت گیاه و سرنوشت مواد شیمیایی کشاورزی به کار رفته در یک زیست بوم کشاورزی دارد. در این پژوهش، پتانسیل ضد باکتریایی با استفاده از برآورد کمینه غلظت بازدارنده (minimum inhibitory concentration) ماده راندآپ 360 SL بر علیه گونه سودوموناس ارزیابی شد. به این منظور، تحلیلی کمّی از باکتری انجام شد و تغییرات فیزیکوشیمیایی محیط رشد معدنی ناشی از قرار داشتن در معرض علف کش مزبور در طی یک دوره طولانی ارزیابی شد. افزون بر این، اثرات آنتاگونیستی ریسه SP0113بر علیه F. culmorumو F. oxysporumدر شرایط تنش ناشی از راندآپ 360 SL مشخص شد. نتایج نشان داد که کاربرد ماده رقیق نشده و رقیق شده در حد ۲/۶ برابر موجب جلوگیری از رشد ریسه مورد مطالعه شد. ریسه SP0113گونه سودوموناس در غلظت هایی از راندآپ که نزدیک به دُز توصیه شده مصرف بود زنده ماند و مقاومت نشان داد. امکان گسترش باکتری روی محیط رشد Tryptic Soy Agar (TSA)و در غلظت های تماس برابر ۱۴/۴ و ۵/۴ میلی گرم در میلی لیتر، (مقادیر مواد رقیق کننده طبق نظر سازنده مواد) به نقش عوامل کمکی مانند مواد کمکی (adiuvant) یا pH، پتانسیل اکسیداسیون-احیا، یا شوری اشاره دارد. این عوامل شامل تغییرات pH و واکنش با محیط رشد حاوی عناصر غذایی باکتری هاست. شوری زیاد محیط رشد که ازواکنش های شیمیایی مواد موجود در محیط رشد ناشی شده، از ویژگی های مواردی است که غلظت از حد توصیه شده بیشتر است. افزون بر این، چنین آشکار شد که glyphosateرشد قارچ های گونه فوزاریوم را که با ریسه SP0113 به حفظ گیاه کمک می کنند محدود می کند.