Atlas Journal of Biology 2018, pp. 546–550

Assessment of Resistance of four Nitrogen-Fixing Bacteria to Glyphosate

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Received: May 21, 2018 / Accepted: August 4, 2018

Abstract

The presence of residual pesticides in the soil affect the microbial Communities, as well the continuous use of pesticides exacerbates this problem. Glyphosate is one of the most used herbicides in the world. Up to date several studies have evaluated the tolerance and resistance of bacteria to glyphosate. Nitrogen-fixing bacteria play an important role in soil fertility; thus, the alteration of these bacterial communities decrease soil fertility. The objective of this study was to evaluate the effect of glyphosate application on four bacterial strains *Pantoea agglomerans*, *Rhizobium nepotum*, *Rhizobium radiobacter,* and *Rhizobium tibeticum*. Glyphosate was applied as the sole source of carbon at the rate (0 g/l, 0.5 g/l, 1g/l, 3g/l, 6g/l and 12 g/l) with two methods. Microbial growth was measured by the Colony Forming Units (CFUs /ml) method. Comparing with the control, our results showed that the growth of the four strains decreased by increasing the concentration of glyphosate. The four strains have shown resistance to glyphosate in the direct enrichment compared to the continued enrichment method. Comparing strains with each other, *Rhizobium radiobacter* is the most resistant strain to glyphosate.

Keywords: Glyphosate; Pesticide; Nitrogen-fixing bacteria; Tolerance.

Introduction

Glyphosate [N-(phosphonomethyl) glycine], the active ingredient of the herbicide Roundup, is a systemic, post-emergent, broad-spectrum herbicide, it is an inhibitor of aromatic amino acid synthesis (Calvet et al., 2005). Is Introduced in agriculture in the 1970s (Munira et al., 2016). The high efficiency in protecting crops against weeds has enabled the glyphosate to become the most widely used herbicide in the world (Chlopecka et al., 2017), knowing that it occupied a quarter of the world's herbicide sales (Munira et al., 2016).

Glyphosate is foliarly applied. However, an important quantity of the herbicide may reach the soil. Moreover, it will be leaching to groundwater. Thereby, the study of interaction microorganisms/glyphosate in soil is very important. The soil is one of the largest reserves of the planet biodiversity. The nitrogen-fixing and phosphate-solubilizing bacteria are among the important bacteria in the soil, they play an important role in the nitrogen and phosphorus availability in the soil, and consequently on soil fertility. The bacterial nitrogen fixation phenomenon is an alternative to the high use of chemical fertilizers (Bhattacharjee et al., 2008).

Previous studies have indicated that the glyphosate may modify natural ecosystem by affecting different components of the soil microbial community (Carlise and Trevors, 1988; Ermakova et al., 2010). Glyphosate can affect the fungi and bacteria in soil (Imparato et al., 2015). Whereas, numerous studies have shown microorganisms can degrade that glyphosate. Several potential glyphosate degrading microorganisms have been isolated from organophosphates contaminated soils (Shushkova et al., 2010) such as *Enterobacter cloacae* (Kryuchkova et al., 2014), *Geobacillus caldoxylosilyticus* (Obojska et al., 2002), *Pseudomonas* spp. (Dick and Quinn, 1995), *Rhizobium* sp. and

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Atlas Journal of Biology - ISSN 2158-9151. Published By Atlas Publishing, LP (www.atlas-publishing.org)

Agrobacterium sp. (Liu et al., 1991).

Due to its great impacts mainly on the ecology and the health, and based on previous studies in this field, we carried out this study whose objectives are to evaluate the glyphosate tolerance using two enrichment methods of four nitrogen-fixing bacteria, which were isolated from the nodules of a legume (*Bituminaria bituminosa)*. As well to assess the effects of glyphosate concentration on the growth of these four bacterial strains, using glyphosate as a sole source of carbon.

Materials and Methods

Chemicals Used

The glyphosate used was a commercial RoundUp® (containing 360 g active ingredient/L of glyphosate, Monsanto) purchased from a local dealer's store in Tanger, Morocco. All the other chemicals were characterized by a high purity commercially available.

To evaluate the tolerance or resistance of our bacterial strains to the glyphosate we have used it as a sole source of carbon and phosphorus, we used a mineral salt medium without carbon source (MSMC). The composition of MSMC in grams per liter of distilled water pH (7.0-7.2). was KH2PO4 (1.5), Na2HPO4 12H2O (1.5), NH4SO4 (2), MgSO4 7H2O (0.2), CaCl2 (0.01), FeSO4 and 7H2O (0.001). The media was supplemented with glyphosate sterilized by filtration (0.2 µm filter). Mineral salt medium (MSM) with glucose as carbon source was used as control, the composition of MSM Control in grams per liter of distilled water pH (7.0-7.2) was C6H12O6 (10). KH2PO4 (1.5), Na2HPO4 12H2O (1.5), NH4SO4 (2), MgSO4 7H2O (0.2), CaCl2 (0.01) and FeSO4 7H2O (0.001).

Bacterial Strains

Four bacterial strains were selected to test their tolerance or resistance to glyphosate. These strains were originally isolated from nodules root of legume "*Bituminaria bituminosa*" cultivated in the experimental station of the faculty of sciences, Moulay Ismail University, Meknes. The selection of strains was based on their ability to fix nitrogen. The selected strains are *Pantoea agglomerans, Rhizobium nepotum, Rhizobium radiobacter* and *Rhizobium tibeticum*.

Preparation of Inoculum

Inocula were prepared for the four strains by culturing the strains in 50 ml of nutrient medium for three days at 30°C under stirring conditions (150 rpm) until growth reached late exponential phase. Cells were harvested by centrifugation at 4, 600 g for 5 min, washed with sterile saline solution 0.9% and were resuspended in 0.5 McFarland standard (Optical density of 0.108 at 625 nm), this suspension was used as inoculum.

Treatments of Used Bacteria

Continuous Enrichment

Glyphosate tolerance experiments were performed in flasks (250 ml) containing 100 mL of sterile MSMC with 0.5 g /l of glyphosate. 2 ml of each inoculum was added to a sterilized flask and for each sstrain, three replicates were done, then all the flasks were incubated on a rotary shaker at 150 rpm for 7 days at 30 ° C. After 7 days 5 mL of each flask were transferred to fresh MSMC containing 1g/L glyphosate and incubated for 7 days. Three additional and successive transfers were made into media successively containing 3, 6 and 12 g/L of glyphosate.

Direct Enrichment

Two (2) ml of each inoculum were added in flasks (250 ml) containing 100 ml of sterile MSMC with 0.5g/l, 1g/l, 3g/l, 6g/l and 12g/l of glyphosate in each one. Triplicate culture were incubated on a rotary shaker at 150rpm for 7 days at 30°C.

Enumeration of Bacterial Strains

The bacteria were counted for each concentration (0.5, 1, 3, 6, 12 g/l) including the control, after 7 days of incubation; 1 ml of each sample was used to provide a series of dilutions (10-1, 10-2, 10-3, 10-4 and 10-5). Moreover, 0.1 ml of each dilution was added to the plates containing the Plate Count Agar (PCA) medium, which its composition in gram per liter of distilled water, pH (7.0, 7.2) is: Tryptone (5), yeast extract (2.5), glucose (1), Agar (15). The plates were incubated at 28±2 °C, for 72h.

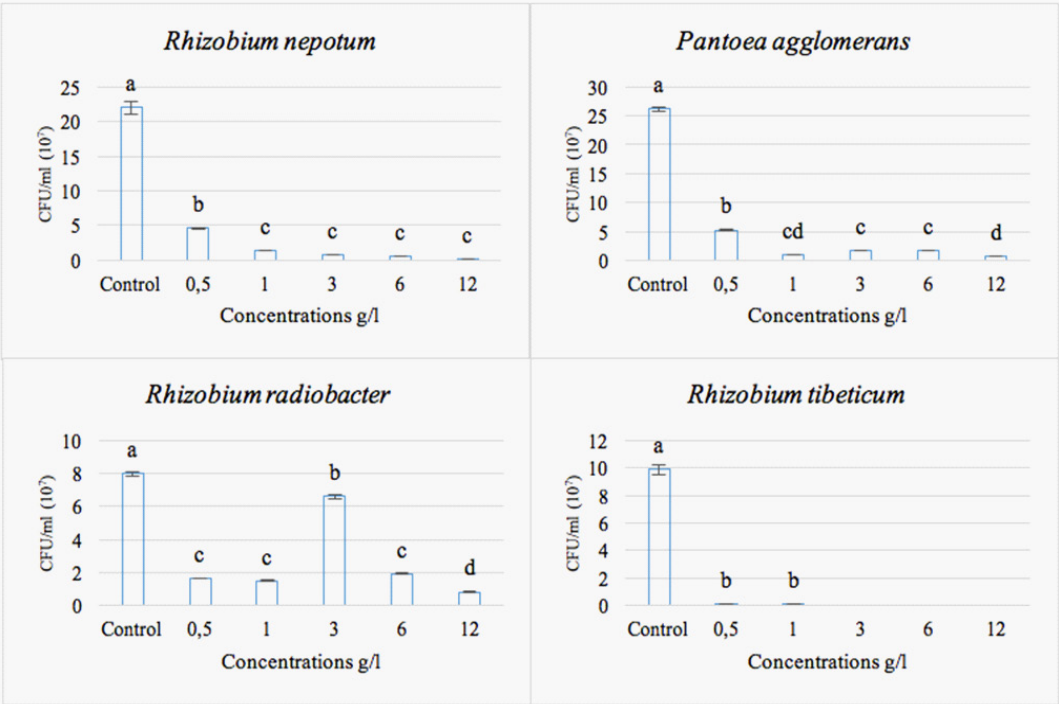
Statistical Data Analysis

All variables were analyzed by ANOVA (analysis of variance) and significant differences among treatments were determined using Tukey post-hoc test. Differences between treatments were considered statistically significant at $p < 0.05$. IBM SPSS statistics 20 was used for all above statistical analysis.

Results and Discussion

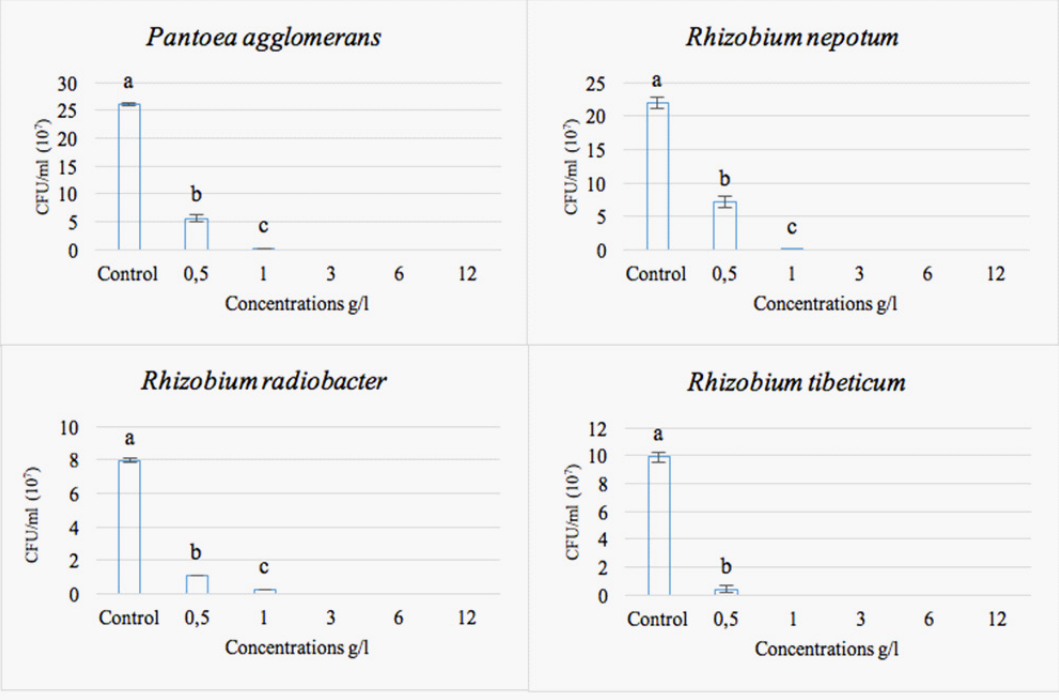
In order to assess the tolerance of bacterial strains (*Pantoea agglomerans, Rhizobium nepotum, Rhizobium radiobacter,* and *Rhizobium tibeticum*) to glyphosate, we evaluated the growth of each strain while increasing glyphosate concentration with two methods: continuous and direct enrichment.

Statistically, and comparing with the control, significant differences ($p< 0.05$) were observed for the four strains using both methods: direct enrichment (Figure 1) and continuous enrichment (Figure 2). Bacterial load decreased highly while increasing glyphosate concentration in continuous enrichment, whereas in direct enrichment the bacteria showed a tolerance to glyphosate except *Rhizobium tibeticum* strain. Moreover, comparing the strains with each other in the continuous enrichment, no resistance was observed in the concentrations greater than or equal to 1 g/l of glyphosate, which is the recommended



Each bar represents the mean average (n=3), the error bars represent standard deviations. Means followed by different letters are significantly different at $p<0.05$ according to Tukey test.

Figure 1. Effect of increasing glyphosate concentration on the growth of Bacterial strains in direct enrichment.



Each bar represents the mean average (n=3), the error bars represent standard deviations. Mean followed by different letters are significantly different at $p<0.05$ according to Tukey test.

Figure 2. Effect of increasing glyphosate concentration on the growth of Bacterial strains in continuous enrichment.

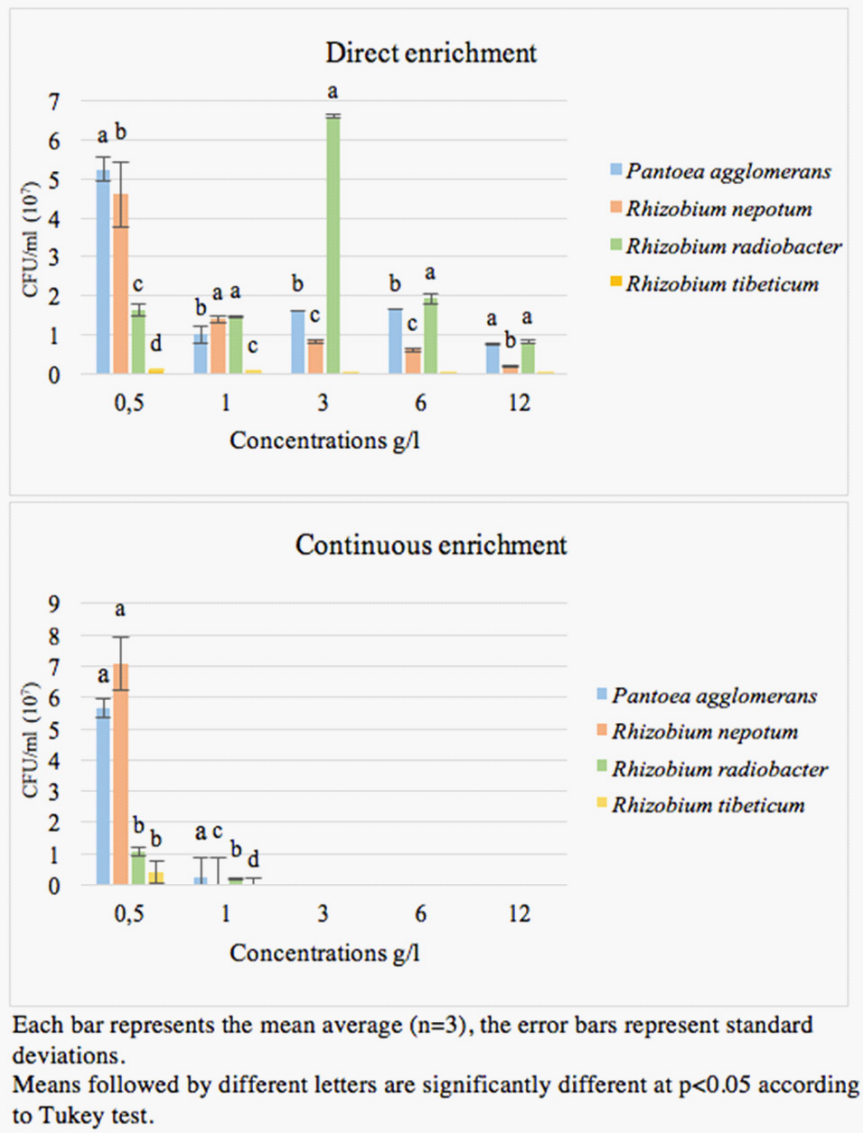


Figure 3. Comparison of glyphosate effect on four strains in two methods.

dose in Morocco (Figure 3). Whereas in direct enrichment the behavior of the four strains was different (Figure 3), *Rhizobium radiobacter* and *Pantoea agglomerans* strains tolerated concentrations up to 12 g/l (Figure 1), while *Rhizobium nepotum* tolerated concentrations up to 6 g/l (Figure 1). Whilst *Rhizobium tibeticum* had no activity in all treatment (Figure 1). The microorganism's physiology and genetic's play a key role on their tolerance or resistance to pesticides. Generally, the microorganisms resistant to pesticide have a great potential to break the pesticides into simple products, which may be used by them as nutrient sources, such as carbon and phosphorus (Cassigneul et al., 2016; Myresiotis et al., 2012; Bellinaso et al., 2003). According to the results of (Wijekoon et al., 2018) which have shown that *Pseudomonas* sp. and *Bacillus* sp. are able to degrade glyphosate even at its high concentrations, these results are consistent with ours mainly for *Rhizobium radiobacter* and *Pantoea agglomerans* in direct enrichment and without carbon in the medium. As so, it can be suggested that these two strains used glyphosate as a source of carbon, while the behavior of these two strains was different in continuous enrichment. This result can be explained by the accumulation of the glyphosate, which make the medium toxic. Thus, several studies have reported the eco-toxicity of glyphosate on specific soil microorganisms (Allegrini et al., 2015; Sihtmäe et al., 2013). On the other hand, according to the study made by (Ahemad et al., 2012), the glyphosate affects negatively the growth of *Mesorhizobium* strain MRC4, these results are consistent with those obtained for *Rhizobium tibeticum* strain, as well as those of (Ermakova et al., 2010) who claimed that none of the two *Achromobacter* sp. and *Ochrobactrum anthropi* bacteria could degraded glyphosate.

Overall, the effect of glyphosate on the bacterial community is different from one bacterial species to another. Some strains are able to resist or tolerate glyphosate and use its metabolites as a source of nutrients and/or energy, whereas the glyphosate may be toxic for other strains; these findings was proved by several studies (Sihtmäe et al., 2013; Araujo et al., 2003; Tsui et al., 2003).

Our results showed that the continuous use of glyphosate affect nitrogen-fixing bacteria which play an important role in soil fertility. however, more research is needed in the field to better understand the effect of glyphosate on soil fertility. Therefore, perspective research projects can focus on the genetics of the soil microbial community and its behavior towards glyphosate application.

Acknowledgments

Our special thanks to Environment & Soil Microbiology Unit, Department of Biology, Faculty of Sciences, Moulay Ismail University, Meknes, Morocco and all those who helped us to accomplish this work.

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