Diazotrophic Bacterial Response to Herbicide Toxicity: *In vitro* analysis

ABSTRACT

Diazotrophic bacteria play critical role in biological nitrogen fixation (BNF), and the application of herbiced significantly affect growth and activities of these bacteria. To investigate this, 1.0 ml of broth stock culture containing 2.9 x 10^5 cfu/ml and 2.6 x 10^6 cfu/ml of *Azotobacter* and *Bradyrhizobium*, respectively were inoculated into 10.0 ml of their respective growth medium [TDC broth, and Yeast extract mannitol broth (YEMB)] containing 0, 0.5, 1.0, 1.5 and 2.0 % v/v of the respective herbicides, atrazine, glyphosate, paraquat and 2,4-D. Thereafter, plate counts of the diazotrophs for each concentration was made at 24, 48, 72 and 120 h intervals using spread plate method on TDC agar and YEMA after incubation at room temperature (30 ± 2 ℃) for 72 h. The LC_{50} of the respective herbicides for *Azotobacter* and *Bradyrhizobium* was determined at 120 h using Probit analysis. Results showed that all tested concentrations except control, retarded diazotrophic bacterial population growth. Growth reduction increased progressively with increased concentrations of herbicides (P = 0.05). In general, herbicides was found to suppress the growth of diazotrophs by 29.7 – 100 %. The LC_{50} indicated symbiotic *Bradyrhizobium* displayed greater sensitivity to tested herbicides than free-living *Azotobacter* (P < 0.05). Conclusively, herbicides suppressed diazotrophic bacterial growth.

Keywords: *Azotobacter*, *Bradyrhizobium*, diazotrophs, herbicides, nitrogen fixation, toxicity

1. INTRODUCTION

Microorganisms that have the ability to fix molecular nitrogen (N_2) are collectively known as diazotrophs. Nitrogen fixation in the soil is critically important for substantial growth and yield of crop plants. Biological nitrogen fixation in soil involves microbial transformation of gaseous N_2 to organic forms such amino acids, pyrimidines and purines [1], which represents one of the most important source of nitrogen addition to the soil [2]. An estimated input of over 200 million tons of nitrogen is added to Earth's ecosystem annually via nitrogen fixation [3]. Biological nitrogen fixation is the second most significant biological phenomenon on earth after photosynthesis [4,21].

A number of diazotrophic bacteria such as *Azotobacter* and *Bradyrhizobium* are important microbial agents that contributed to nitrogen fixation in the ecosystem. While *Azotobacter* exist in soil as free-living nitrogen fixing agent, *Bradyrhizobium* fix atmospheric nitrogen in soil symbiotically in close association with leguminous plants. In addition to fixing nitrogen in soil, *Azotobacter* has been reported to enhanced the growth and wellbeing of many crop plants such as barley, wheat and potato [1]. Furthermore, they also enhance plant growth and yield via the production of plant growth hormones, synthesis of phytopathogenic inhibitors, stimulation of rhizospheric microorganisms and modification of nutrient uptake [5]. Nitrogen fixation through nodulation of legumes accounts for about 25 % of the total nitrogen fixed yearly on the earth. Nodulated legumes display remarkable ability to grow well in unfertilized soil lacking nitrogen where other group of plants grow poorly [6]. *Bradyrhizobium* is known to exhibit cosmopolitan existence in its nodulation ability over a broad variety of legumes such as groundnut (*Arachis*

hypogaea), cowpea (*Vigna unguiculata*), soybean (*Glycine max*), Bambara groundnut (*Vigna subterranean*), common bean (*Phaseolus vulgaris*) and Kersting's bean (*Macrotyloma geocarpum*) [7].

In recent time, exponetial increase human populaiion spurred y the need to increase food production , which has further incaresed the application of herbicides in the agriculture globally. The increase in herbicide usage has been linked to their ease of application, availability and effectiveness in weed control. Some of the most commonly applied herbicides includes, atrazine, glyphosate, butachlor, 2,4-D, paraquat among others. However, irrespective of their spectrum of activities on weed plants, they are non-discriminatory in their effect towards other unintended organisms in the ecosystem including microorganisms. In general, microbial populations of actinomycetes, bacteria, cyanobacteria, fungi and protozoa in the ecosystem have been reported to be negatively impacted by herbicides [8, 9]. This notwithstanding, knowledge of the specific response of certain important soil microbes such as diazotrophic bacteria whose roles is critical in maintaining soil fertility is required, as nitrogen is one of the major elements limiting crop growth and yield in agriculture. It is on the basis of ascertaining the specific toxicity response of two important diazotrophs, the free-living *Azotobacter* and symbiotic *Bradyrhizobium* to some commonly applied herbicides in agriculture that this study was carried out.

2. MATERIALS AND METHODS

2.1 Azotobacter and Bradyrhizobium isolation

Azotobacter was isolated from agricultural soil by collecting soil samples from the top layer (0-15 cm) . One gram of the soil sample (obtained from 10.0 g of the homogenized soil sample) was added to 9.0 ml physiological saline in a test tube. From this, ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were prepared and 0.1 ml aliquots of respective dilutions were subsequently plated out on TDC agar medium in triplicates using the spread plate method for *Azotobacter* isolation. The composition of TDC agar medium includes, glucose, 5.0 g; K_2HPHO_4, 1.0 g; $MgSO_4$, 1.0 g; $CaCO_3$, 10.0 g and agar, 20.0 g in 1000.0 ml of distilled water [10].

On the other hand, *Bradyrhizobium* for this study was isolated from root nodules of 3-months old *Arachis hypogaea* as described by Ubogu et al. [11]. Mature well-formed noodles were pulled off from roots. One g of the noodles was washed in tap water. This was then surface-sterilized in 70 % ethanol for 2 minutes and subsequently rinsed with distilled sterile water. Further surface-sterilization was carried out for 2 minutes using 3.5 % v/v sodium hypochlorite and immediately rinsed thrice with distilled sterile water after which noodles were crushed in a few drops of distilled sterile water in Mac Cartney bottle. After thorough crushing, this was made up to 10.0 ml using physiological saline. From this, ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) were prepared and 0.1 ml aliquots of respective dilutions were subsequently plated out on yeast extract mannitol agar (YEMA) in triplicates using the spread plate method for *Bradyrhizobium* isolation. The composition of the YEMA includes $MgSO_4$, 0.1 g; NaCl, 0.13 g; K_2HPO_4, 2.5 g; yeast extract, 0.5 g; agar powder, 20.0 g in 1000.0 ml of distilled water.

Both TDC agar and YEMA plates were incubated at 30 ± 2 ℃ (room temperature) for 72 h. Pure culture of isolated colonies obtained were then transferred to respective TDC agar and YEMA slant for further studies.

2.2 Characterization of isolated *Azotobacter* and *Bradyrhizobium*

Pure culture of bacterial isolates was identified based on cultural, morphological and biochemical properties employing Bergey's Mannual of Systemic Bacteriology (volume 2, Part B and C) [12 a and b].

2.3 *In vitro* toxicity assessment

In vitro toxicity response of *Azotobacter* and *Bradyrhizobium* to the herbicides, atrazine, glyphosate, 2,4-D and paraquat were evaluated in their respective growth medium (TDC broth and YEMB).

Using a sterile wire loop, bacterial growth from pure culture agar slant of *Azotobacter* was aseptically scrapped into 200 ml of TDC broth medium in 500 ml conical flask. Flask was then cocked with sterile cotton wool and incubated at 30 ± 2 ℃ for 72 hours with regular hand-shaking of flask for 30 minutes every 12 hours. This served as the stock culture from which toxicity study was conducted.

One milliliter from the stock culture containing 2.9 x 10^5 cfu/ml of *Azotobacter* were respectively inoculated into 10.0 mL of fresh TDC broth in a 15 ml test tube, containing the various concentrations of the respective test herbicides at 0, 0.5, 1.0, 1.5 and 2.0 % v/v. The TDC broth medium containing the respective test herbicides were then incubated at 30 ± 2 ℃ for 120 hours. The total *Azotobacter* counts (cfu/ml) for each tested herbicide concentration were determined in triplicates at time interval of 24, 48, 72 and 120 hours. Counts were determined by plating out 0.1 ml of serially diluted samples of each tested concentration of the tested herbicides on TDC agar medium using the spread plate method. Plates were incubation at 30 ± 2 ℃ for 72 hours. Thereafter, the total *Azotobacter* counts were taken and estimated in cfu/ml of sample.

Similar procedure employed for *Azotobacter* were repeated for *Bradyrhizobium*, except that YEMB and YEMA were the medium of growth, and the 1.0 mL from the broth stock culture inoculated into the respective broth containing the various concentrations of the tested herbicides and the control contained 2.6 x 10^6 cfu/ml of *Bradyrhizobium*.

The LC_{50} of the respective tested herbicides for *Azotobacter* and *Bradyrhizobium* in the presence of their respective growth medium were determined at 120 hours of the study. LC_{50} was determined using Finney Probit Analysis [13].

2.4 Statistical analysis

All the data garner from this study were analyzed using Microsoft Excel (Analysis Tool Pak). Triplicate data were analyzed using measures of central tendency and dispersion. The effect of herbicides concentration on *Azotobacter* and *Bradyrhizobium* populations were determined using analysis of variance (ANOVA), while the overall comparative sensitivity of *Azotobacter* and *Bradyrhizobium* to the test herbicides were determined using Student's *t*-test ($P < 0.05$). Finney Probit Analysis was used for determining LC_{50} of tested herbicides at 95 % confident level.

3. RESULTS

3.1 *In vitro* response of Azotobacter and Bradyrhizobium to herbicides

The populations of *Azotobacter* and *Bradyrhizobium* generally increased with time for the respective tested herbicides concentrations including the control. Nonetheless, there was a progressive reduction in population with increased concentrations of the herbicides ($P < 0.05$) (Fig. 1). No observable growth occurred for *Azotobacter* beyond 1.5 % v/v for the herbicide atrazine within the tested period. There was no growth of *Bradyrhizobium* at 2.0 % v/v in glyphosate within the first 24 hours. Similarly, *Azotobacter* and *Bradyrhizobium* did not manifest any form of growth at all the tested concentrations of paraquat within the first 24 h of the study. However, after 24 h, there was substantial growth of *Azotobacter* and *Bradyrhizobium* at all the tested concentrations of glyphosate and paraquat within the period of study. Generally, the growth of diazotrophs were suppressed between 29.7 – 100 % by the tested herbicides (Table 1).

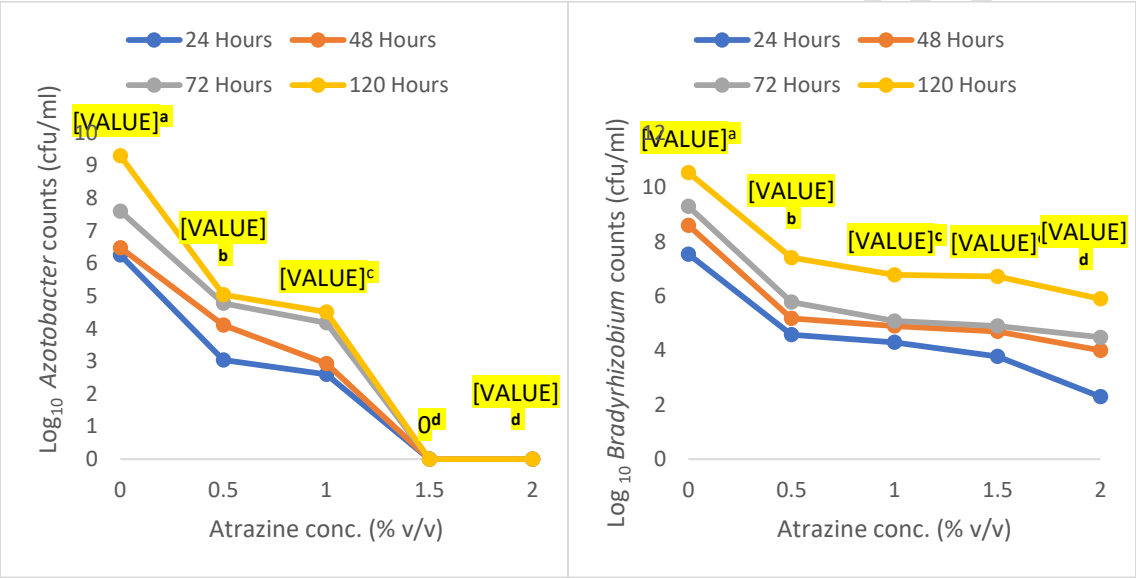

Figure 1a. Effect of different concentrations of atrazine on *Azotobacter* and *Bradyrhizobium* populations. *Values with same superscript alphabet (a, b, c, d, e) for different concentrations at 120 hours did not differ significantly for same tested organism (n = 15, ANOVA, $P < 0.05$).

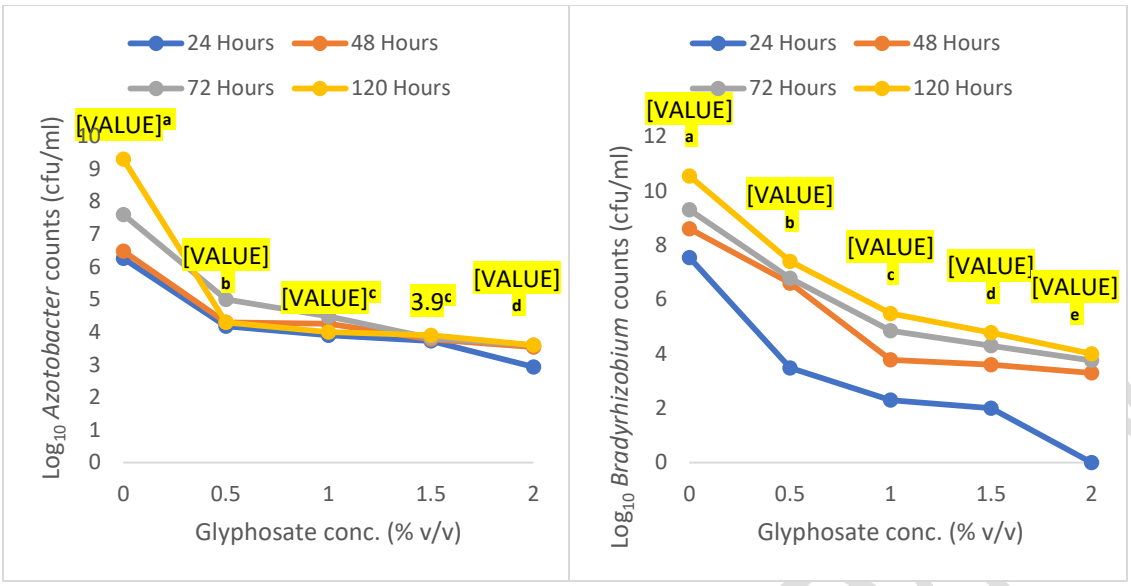

Figure 1b. Effect of different concentrations of glyphosate on *Azotobacter* and *Bradyrhizobium* populations. *Values with same superscript alphabet (a, b, c, d, e) for different concentrations at 120 hours did not differ significantly for same tested organism (n = 15, ANOVA, $P < 0.05$).

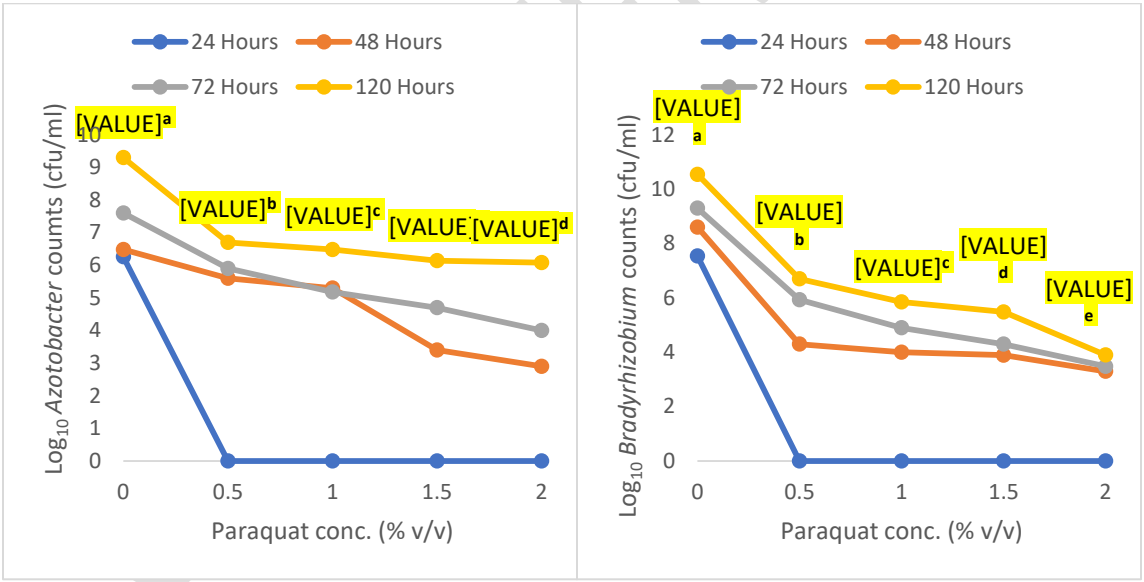

Figure 1c. Effect of different concentrations of paraquat on *Azotobacter* and *Bradyrhizobium* populations. *Values with same superscript alphabet (a, b, c, d, e) for different concentrations at 120 hours did not differ significantly for same tested organism (n = 15, ANOVA, $P < 0.05$).

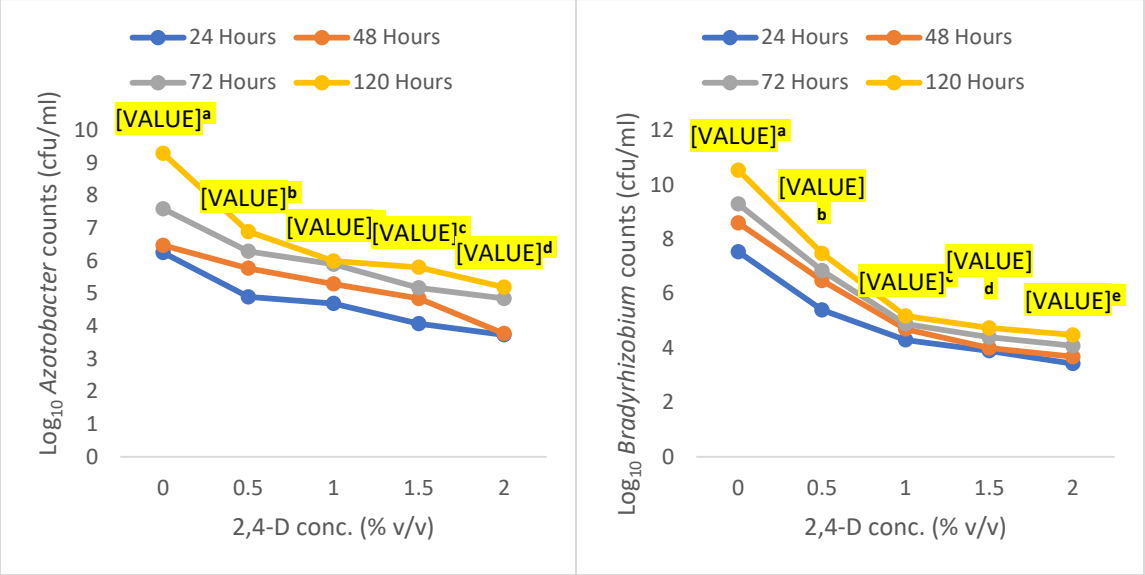

Figure 1d. Effect of different concentrations of 2,4-D on *Azotobacter* and *Bradyrhizobium* populations. *Values with same superscript alphabet (a, b, c, d, e) for different concentrations at 120 hours did not differ significantly for same tested organism (n = 15, ANOVA, $P < 0.05$).

Table 1. Percentage population growth suppression of *Azotobacter* and *Bradyrhizobium* by herbicides at 120 hours

		Percentage (%) population growth suppression			
		Herbicide conc. (% v/v)			
Bacteria	Herbicide	0.5	1.0	1.5	2.0
Azotobacter	Atrazine	45.8[a]	51.5[a]	100.0[a]	100.0[a]
Bradyrhizobium		29.7[b]	35.7[b]	36.2[b]	44.0[b]
Azotobacter	Glyphosate	53.8[a]	58.1[a]	58.1[a]	61.3[a]
Bradyrhizobium		29.8[b]	48.0[b]	54.6[b]	62.1[a]
Azotobacter	Paraquat	28.0[a]	30.3[a]	34.0[a]	34.6[a]
Bradyrhizobium		36.4[b]	46.9[b]	48.0[b]	63.0[b]
Azotobacter	2.4-D	25.8[a]	35.5[a]	37.5[a]	44.1[a]
Bradyrhizobium		29.0[b]	50.9[b]	55.0[b]	57.5[b]

*Values with different superscript alphabet (a, b), along the same column for same tested herbicide and concentration differ significantly (n = 3, Student's *t* test, $P < 0.05$)

3.2 LC_{50} of tested herbicides on *Azotobacter* and *Bradyrhizobium*

For *Azotobacter*, the LC_{50} of all the herbicides were greater than the highest tested concentrations of 2.0 % v/v except for atrazine. Conversely, for *Bradyrhizobium* the LC_{50} of all the herbicides were less than the highest tested concentrations except for atrazine (Table 2). implies that at the tested concentrations. Generally, *Bradyrhizobium* displayed more sensitivity to the tested herbicides than *Azotobacter* ($P < 0.05$).

Table 2. LC_{50} of tested herbicides on *Azotobacter* and *Bradyrhizobium* at 120 h.

Herbicide	LC_{50} (% v/v)	
	Azotobacter	*Bradyhizobium*
Atrazine	1.6	5.0
Glyphosate	4.5	1.1
Paraquat	28.7	1.3
2,4-D	3.13	1.0

*LC_{50} determined using Probit analysis at 95 % confident level

4. DISCUSSION

In this study, though there was a progressive rise in the population of the two diazotrophic bacteria (*Azotobacter* and *Bradyrhizobium*) with time, their growth rates were substantially retarded by the four tested herbicides (atrazine, glyphosate, paraquat and 2,4-D) in comparison to the control devoid of the herbicides. This finding is in concordance with that of Nahi et al. [14], who reported that all the tested herbicides (2,4-D, pretilachlor and paraquat) significantly decreased the growth of the diazotroph *Stenotrophomonas maltophilia*. Milosevia and Govedarica [15], also reported significant reduction in the populations of *Azotobacter* spp. and *Bradyrhizobium japonicum* with the application of a number of herbicides. Herbicides application to soil have been reported to reduce the number and diversity of diazotrophic bacteria [16].

Furthermore, studies have shown that herbicides application negatively impacted nitrogen fixation at certain concentrations [17]. The growth of the diazotrophs in this study generally decreased with increased concentrations of the tested herbicides. However, the degree to which the growth decreased varied with the diazotroph and type of herbicide. Similar to the finding in this study, Mohamed et al. [18], reported increased negative impacts of glyphosate and paraquat on the symbiotic nitrogen fixing bacteria, *Rhizobium nepotum*, *Rhizobium tibeticum*, *Rhizobium radiobacter* and *Pantoea agglomerans* as the concentrations of the herbicides increased.

The free-living (*Azotobacter*) and symbiotic (*Bradyrhizobium*) diazotrophs exhibited differential response to the tested herbicides. While the growth of *Azotobacter* was completely inhibited throughout the study period at concentration of above 1.5 % v/v of atrazine, *Bradyrhizobium* growth was inhibited by glyphosate at 2.0 % v/v only for the first 24 h. In the same vein, *Azotobacter* and *Bradyrhizobium* which were inhibited by all concentrations of paraquat within the first 24 h recovered from the growth inhibition thereafter. The findings here corroborate that of Latha and Gopal [8], who reported that pyrazosulfuron, butachlor, 2,4-D and pretilachlor initially decrease the population of *Azospirillum lipoferum* in comparison to the control but subsequently witnessed increased growth after 24 hours. Nahi et al. [14], also reported the recovery of the diazotroph *Stenotrophomonas maltophilia* after 7 days following initial inhibition

at higher concentration of tested herbicides. The diazotrophic bacterial recuperation following initial inhibition of growth may be attributed to their inherent resilience and capacity to adjust to the herbicides at specific concentrations over time. Comparatively, the symbiotic diazotroph *Bradyrhizobium*, displayed more sensitivity to the tested herbicides than the free-living *Azotobacter*. A number of studies lend credence to the finding here reported that diazotrophic bacterial response differ with the type of applied agrochemical pesticides [14, 19, 20]. The differential responses of the diazotrophic bacteria to the tested herbicides may be ascribed to the dissimilarity in the chemical composition of the herbicides and genetic make-up of the microorganisms.

5. CONCLUSION

This study showed that diazotrophic bacterial population growth was significantly retarded by atrazine, glyphosate, paraquat and 2,4-D at different tested concentrations. However, the degree of herbicides toxicity varied among the two tested diazotrophs with symbiotic *Bradyrhizobium* displaying more sensitivity to the tested herbicides than free-living *Azotobacter*. This indicates that the application of these herbicides in agriculture significantly suppress the biological nitrogen fixation, which strongly affects soil fertilty and crop productivity in agricultureal soils.

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