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# IMPACT OF GLYPHOSATE HERBICIDE ON SOME SOIL BIOLOGY AND FUNCTION

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***Abstract:*** *Soil microbiotas regulate the bioprocesses in soil that are essential for plant growth, soil health and sustained productivity. Despite an increasing concern of consequences of using vast amounts of Glyphosate-based herbicides in agroecosystems, their potential effects on non-target soil organisms and soil functioning are mostly unknown. Application of herbicides may not affect the overall size of the soil microbiotas but selectively can effect on specific microbial groups which may result in changing the balance of bioactivity and consequently nutrient availability, disease incidence and plant growth. Glyphosate is a broad-spectrum, non-selective and post-emergence herbicide, used as an active ingredient in several weed killing products since 1970s. Glyphosate is also vulnerable to microbial degradation and its main degradation product, aminomethylphosphonic acid, is strongly adsorbed to soil solids. For these reasons, Glyphosate has generally been regarded as an environmentally safe herbicide. In the current study, $N_2$-fixing soil microbial populations and their activities were determined in microcosms under the stress of Glyphosate herbicide were applied at 3 doses based on the active ingredients and incubated for 10 weeks at 28°C. Results indicated that: Application of Glyphosate had negative impact on symbiotic $N_2$-fixation by legumes in rotation and application of a post-emergent herbicide caused significant changes in the microbial community and the activities of some hydrolytic enzymes in the rhizosphere. Impacts of Glyphosate on rhizosphere microorganisms and activities were investigated. Information on the effects of herbicide application on useful soil microbiotas and their bioprocesses is necessary for the successful crop production. A particular practices may have the desired result in one situation but have little effect in another because biological communities respond to the interaction of multiple factors including food sources, physical habitat, moisture, and impacts of historical land use. Therefore, before a new product is applied to a field soil, it should be tested on a limited area and the results should be monitored in comparison to an untreated one.*

***Keywords:*** *Herbicides, Glyphosate, soil bioprocesses, alfalfa rhizosphere*

## INTRODUCTION

Soil is a natural environment required for the growth and development of plants, consists of a mixture of organic and inorganic components occurring in gaseous, aqueous and solid states. Genetically and environmentally, soils differ significantly; they tend to perform the function of natural reservoirs of water and nutrients necessary for a suitable development of the plant root system and soil microbiomes. Soil has the capacity of retaining various pollutants, such as pesticides; and therefore, it

functions as a pollution absorber. They contribute to the contamination of the food chain, which can potentially threaten human health.

The goal of managing the soil biology is to improve biofunctions, e.g., forming and stabilizing soil structure, cycling nutrients, controlling pests and disease, and degrading or detoxifying contaminants. New climatic change and agricultural practice have led to an emerging pressure from weeds and phytopathogens, which complicate farming practice and have resulted in the increased use of agrochemicals worldwide. The term "Pollution" has many definitions, one being the presence of a substance in the environment whose chemical composition or quantity prevents the functioning of natural processes and produces undesirable environmental and health effects [1]. Pollution prevention is defined as the reduction of pollutants at the source. Moreover, the technological advancement has also given rise to new pollutants which are increasing at an alarming rate and are above the self-remediating ability of the environment. There is an urgent need to find technologies that would reduce these rates/pollution levels to risk-free status in quick and easy manner [2]. Soil microbiomes are very important for agroecosystem function and sustainability due to their contributions to nutrient cycling and to soil structure maintenance [3, 4].

Soil microbiomes habitat, their activity and interactions with each other are interdependent and they may vary even within a micro-scale. The most important soil microbiomess are: bacteria, fungi, algae, protozoa and nematodes, because their metabolism is associated with the flow of energy and the circulation of elements in ecosystems in the term of biogeochemical cycles.

Microbial properties like microbial diversity and biomass are useful for predicting changes in the soil functioning and for providing an integrated and relevant vision of soil quality. Little information is currently available for preventing the loss of microbial diversity and activity through the use of sustainable farming practices from the first cultivation year. Plants are able to select their own microbiome based on their specific root exudates or some other strategies such as root physical structure and mineral nutrients preference [5].

Activities of soil enzymes and the amount of hormones produced by soil microbiomes are important indicators of soil quality and its health because of their immediate response to natural or anthropogenic changes in soil. The analysis of microbial distribution in soil environment is a key factor determining the optimal conditions for the crop development and productivity.

The exposure to pesticide has detrimental effects on health and might contribute to an increased risk of long-term diseases, including cancer and neurodegenerative diseases, reproductive and developmental disturbances, and emerging risks such as developmental neurotoxicity and immunotoxicological effects [6]. Soil organisms can be grouped by size [7], or by functions as described by Wardle [8].

Herbicide use is a vital component of modern agriculture; in particular under reduced till systems. With increased adoption of stubble retention and reduced till practices and the introduction of new herbicides, herbicide use will remain as an essential practice in the near future. Application of herbicides may not affect the overall size of the microbial pool but selectively affect specific groups of microbiotas which may result in altering the balance of biological activity (soil biological health) and consequently nutrient availability, disease incidence and plant growth.

Glyphosate is one of the most widely applied herbicides globally but its persistence in seawater has not been reported. The primary function of herbicides is to protect agricultural crops from infestation with weeds and to prevent arable soil from being overgrown by plant cover indigenous to the ecosystem. The chemicals known as herbicides are mainly synthetic organic compounds with broad molecular configurations having as a common property the ability of selectivity killing or inhibiting the growth of plants. The effects of 2,4-D, a potent herbicide, on the rhizosphere associated N-ase, $N_2$-fixing bacteria and plant growth characters are little understood [9]. Glyphosate, a widely used broad spectrum, non-selective, post-emergence herbicide has been shown to reduce soil bacterial populations in field soils [10]. Bayoumi [11] studied the sensitivity of three strains of *R. leguminosarum* bv. *viceae* comparing with strains of *R. leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *trifolii*, and *R. loti* to 8 herbicides. Among them, Paraquat was the most toxic one. Bayoumi et al. [12] reported that Acetochlor was the least inhibitor on tested *Rhizobium* strains. Bayoumi & Kecskés [13, 14, 15] in pot experiments established that microsymbionts were more sensitive to Trifluralin than macrosymbionts, whereas are sensitive to Paraquat, which reduced the size, number and dry weight of root-nodules, phytobiomass, height, and total N-content / plant. Eberbach & Douglas [16] found Paraquat and Trifluralin affected the nodulation potential of *Rhizobium*-legume. Quantitative and representative

recovery of microbiomes from environmental soil samples is essential in understanding ecosystem function. Microbial communities play essential roles in the earth's ecology. A powerful concept in modern biology is that of the ecosystem. Soil enzymes are present in important cycles, such as C (invertase), N (urease and protease), and P (phosphatase) cycles. Soil enzyme activities are used as indices of microbial activity (Bergstrom et al., 1998) and react quickly to environment change. The biological activity in soil under the impacts of herbicides is an important issue. The present review is a comprehensive presentation of the studies discussing the state of art on the activity of soil enzymes and microorganisms. Hydrolytic enzymes activities (phosphatase, β-glucosidase), oxidoreductase activities (dehydrogenase) and indole acetic acid production, were used as measures of soil perturbation by Benitez et al. (2004). The hypothesis in this study is to estimate the effects of applied Glyphosate on (1) survival of some symbiotic $N_2$-fixing bacteria before and after herbicide application and (2) activities of some soil enzymes.

## MATERIALS AND METHODS

The soil samples used for the present study were collected from a non-cultivated field (without previous treatments) of the experimental station at Szent István University, Gödöllő, Hungary. The soil of the sampling area was sandy brown forest of general properties are given in Table (1).

Table (1): Physical and chemical analysis of used soil

| Different parameters | Value of contents |
|---|---|
| $pH_{KCl}$ | 5.33 |
| $K_A$ | 24.6 |
| Total Salt % | 0.03 |
| $CaCO_3$ % | 0.01 |
| Humus % | 1.22 |
| $NH_4$-N mg/kg | 1.69 |
| $NO_3$-N mg/kg | 3.08 |
| AL-$K_2O$ mg/kg | 107 |
| AL-$P_2O_5$ mg/kg | 106.1 |
| Oxalate Fe mg/kg | 789 |
| Na mg/kg | 36 |
| Mg mg/kg | 203 |
| Zn mg/kg | 6.9 |
| Cu mg/kg | 2.7 |
| Mn mg/kg | 36 |
| $SO_4$ mg/kg | 4.8 |

The first portion was stored at 4°C for enzymatic activities and microbiological analyses. The second portion was stored at cool room for plant-microbe interaction. Herbicides Sys 67 B 80%, Dikamin D 40%, Ro-Neet 6E 74%, Gliaka 20%,, Linuron 50%, Sys 67-ME 80%, Gramexone A 25%, and Treflan 26% were used in the *in vitro* while Glyphosate used *in vivo* experiments. The characterisations of the tested herbicides are mentioned in Table (2).

Eight herbicides were subjected to establish their side-effects on the growth rate of 8 *S. meliloti* strains in yeast mannitol broth (YMB) medium using microfermentor technique [12, 15] at 5 concentrations of active ingredients (0.1, 1.0, 10.0 and 100 $mgl^{-1}$) of each herbicide. The experiment was designed to detect the interactions between the herbicides amendment and the biological $N_2$-fixation in brown forest soil as well as the potential activities of some soil enzymes in alfalfa rhizosphere soil. The study was conducted in pot experiment predicated to low and high herbicide doses. The herbicide was applied to the soil before (post-emergence) and after (pre-emergence) plantation and plant inoculation in two separated sets of pots.

Table (2): Some characteristics of herbicides used in laboratory and greenhouse investigations

| Group | Common name | Active substance | Field Recommended |
|---|---|---|---|
| Aliphatics | Gliaka | Glyphosate (20%) | 1.2 l |
| Bipyridylims | Gramexone A | Paraquat (25%) | 3.5 l |
| Carbamates | Sabet 72 EC | Cycloate (72%) | 4.0 l |
| Dinitroanilines | Treflan | Trifluralin (26%) | 3.5 l |
| Phenoxys | Dikamin D | 2,4-D (40%) | 2.4 l |
| | Sys 67 B | 2,4-DB (80%) | 2.0 kg |
| | Sys 67-ME 80 | MCPA (80%) | 2.0 kg |
| Substituted Ureas | Linuron 50 | Linuron (50%) | 2.0 kg |

Generally, the following experiments of predicating the impacts of herbicide on the plant-microbe interactions were carried out using one Hungarian cultivar of alfalfa, three new isolated strains of *S. meliloti* (GHF-162, GHF-281 and GHF-3153), one standard strain (GHR-94) and one herbicide Glyphosate at 3 concentrations (Table 3). Sterile plastic pots of 3 Kg capacity were used, filled with 2 kg sterile (steamed 100°C for 1 h on three consecutive days) brown forest soil. Seeds of alfalfa (*M. sativa)* were surface-sterilized and germinated in the pots. The post-emergence or pre-emergence application of the Glyphosate was applied to soil accordance with the fact that one hectare containing 3.6 x $10^6$ kg soil, $10^4$ $m^2$ (surface area), 0.25 m (depth) and 1.44 kg $m^{-3}$ (soil density). The commercial formulation of the tested herbicides diluted with sterile distilled water to the appropriate concentration of each one according to its active ingredient (a.i.) on top of 25 cm of field soil.

Table (3): Herbicide treated doses

| Active ingredient (a.i) | Rate (liter a.i/ha) | Relevance to field application rate |
|---|---|---|
| Glyphosate | 0.60 | ½ Recommended |
| | 1.20 | Recommended |
| | 2.40 | 2 x Recommended |

Seeds were selected for healthy and uniformity without any injury, and surface sterilized with 70% ethanol followed by acidified 0.2% $HgCl_2$ for 5 minutes, and thoroughly washed in several changes of sterile distilled water. Seeds were then soaked for 8 h at room temperature in sterile distilled water (soaking water was changed every 2 h) and then seeds were germinated on sterile wetted filter paper in large Petri dish for 72 h in the dark at 28°C according to Franco & Vincent (1976). Seedling rhizosphere was inoculated with 10 ml of a suspension of biofertilizer *Sinorhizobium* inoculum (GHR-94, GHF-162, GHF-281, or GHF-3153) prepared as follows: each one of the five biofertilizers *S. meliloti* strains was grown in YMB for 48 h at 28°C, to give a final cell concentration 2.5 x $10^8$ cell capacity $ml^{-1}$, using haemocytometer for calibration. Seedlings were watered with sterile tap water when required, and the plants grown under natural illumination (14 h) at around 28 ± 2°C.

At the end, plants were carefully uprooted, and washed several times in tap water for farther investigations. Data recorded / plant as follows: Height of plant shoot in cm, number of root nodules. Dry weight of plants biomass and nodules were determined after oven dried at 75°C to a constant weight and the values were expressed as g $plant^{-1}$ and mg root nodules $plant^{-1}$. Total N-content (mg $plant^{-1}$) was measured using micro-Kjeldahl method as a criterion of $N_2$-fixation [17]. Therefore, all visible plant fragments were carefully removed from the soil sample prior to the enzyme assays. The potential activities of some enzymes in the alfalfa rhizosphere and soil bulk were measured under the stress at different concentrations of herbicides, and inoculated different biofertilizers strains of *S. meliloti*.

The following enzymes were determined: Dehydrogenase activity was determined according to García et al. [18]. Dehydrogenase activity is expressed as μg of INTF per gram dry soil. Protease activity on N-α-benzoyl-L-argininamide (Protease-BAA) was measured using the method of Nannipieri et al. [19]. Protease activity is expressed as μmol of $NH_4^+$-N released per gram dry soil per hour.

Phosphatase activity was carried out using the method of Tabatabai & Bermner [20] and the phosphatase activity is expressed as μmol of PNP per gram dry soil and incubation time (hour). β-glucosidase activity is expressed as μmol of PNP per gram dry soil and incubation time (hours) after applying the method of Masciandaro et al. [21].
The experiments were conducted in three replicates and some statistical analyses are carried out to determine the efficiency of the application of herbicide on the soil biology.

## RESULTS AND DISCUSSION

Pesticides are xenobiotic molecules necessary to control pests in agriculture, home, and industry. However, water and soil can become contaminated as a consequence of their extensive use. Therefore, because of its eco-friendly characteristics and efficiency, bioremediation of contaminated sites is a powerful tool with advantages over other kinds of treatments [22].

### Selection of bacterial strains and herbicides

Table (4) summarizes the relative growth rates and the tolerance ability of the *S. meliloti* strains toward the potent effect of herbicides in compared with control growth of the strains in herbicide-free broth medium. These investigations showed that herbicide MCPA had no harmful influence on *S. meliloti* strains GHR-94, GHF-162, GHF-281, and GHF-3153. However, the other strains were tolerated the herbicide-amended YMB up to 10 $mgl^{-1}$. Herbicides Paraquat and Trifluralin were highly toxic to the all tested strains and their effect was rapid. The investigated strains can be grouped in three groups according to the effect of Paraquat, first group contained tolerant strains to 10 $mgl^{-1}$ (GHR-94, GHF-162, GHF-281 and GHF-3153), the second group included the tolerant strains to 1 $mgl^{-1}$ (GHF-1141 and GHF-353), the third group of strains tolerant 0.1 $mgl^{-1}$ (GH-130 and GHF-2100). Herbicides Linuron, Cycloate and Glyphosate were toxic to all strains but at 100 $mgl^{-1}$. But their effects on the growth rate of strains were more gradual compare with the effect of Paraquat and Trifluralin. Herbicides 2,4-D, 2,4-DB, had stimulated effect on the growth of the strains GHR-94, GHF-162, GHF-281 and GHF-3153 throughout all investigated concentrations. These herbicides stimulated the growth rates of the all tested strains at different patterns at 1 $mgl^{-1}$ and 10 $mgl^{-1}$ but at 100 $mgl^{-1}$, strains GH-130, GHF-1141, GHF-2100, and GHF-353 were inhibited. The results indicated that the most tolerant strains of *S. meliloti* were GHR-94, GHF-162, GHF-281 and GHF-3153. Also, most of statistically significance values as regard between the relative growth rate of the different investigated strains and the low and high of herbicidal concentrations.

Table (4): Tolerance of the selected strains to concentrations of herbicides

| Strain | Herbicides (mg/l) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Glyphosate* | *Paraquat* | *Cycloate* | *Trifluralin* | *2,4-D* | *2,4-DB* | *MCPA* | *Linuron* |
| GHR-94 | *100* | *10* | *100* | *10* | *> 100* | *> 100* | *> 100* | *100* |
| GH-130 | *100* | *0.1* | *100* | *1* | *> 100* | *> 100* | *10* | *100* |
| GHF-162 | *100* | *10* | *100* | *10* | *> 100* | *> 100* | *> 100* | *100* |
| GHF-1141 | *100* | *1* | *100* | *1* | *> 100* | *> 100* | *10* | *100* |
| GHF-281 | *100* | *10* | *100* | *10* | *> 100* | *> 100* | *> 100* | *100* |
| GHF-2100 | *100* | *0.1* | *100* | *1* | *> 100* | *> 100* | *10* | *100* |
| GHF-353 | *100* | *1* | *100* | *1* | *> 100* | *> 100* | *10* | *100* |
| GHF-3153 | *100* | *10* | *100* | *10* | *> 100* | *> 100* | *> 100* | *100* |

### Effect of the applied herbicide on symbiotic relationship

Depending on the received results, and in addition, to determine the impacts of Glyphosate (selected herbicide) on the effectiveness (dry matter and total N content), „saprophytic competence" and competitiveness to evaluate the nodulation potential of the strains, *in vivo* experiment was conducted in a pot model under several factors. This investigation was done to show the influence of application

of different factors such as herbicide applied in post- and pre-emergence of young alfalfa seedlings inoculated with the selected biofertilizer of *S. meliloti* strains (GHR-94, GHF-162, GHF-281 and GHF-3153). The results showed that reduction in nodulation was usually associated with reduced plant growth caused by herbicidal injury. The double of field recommended applied dose of herbicide in pre-emergence phase reduced the morphological characterization and plant dry weight of the plant as well as the symbiotic properties. These investigations show that herbicide Glyphosate had no harmful influence on the investigated *S. meliloti* strains if used as recommended. Generally, herbicide is presently used for establishment of alfalfa. The herbicide at three applied rates (0.5x, x and 2x, x = FRD) did not cause any visual phytotoxicity to the plant crop. Glyphosate had significantly improved the number and size of nodules especially those plants inoculated by the new isolated strains (GHF-162, GHF-281 and GHF-3153) compared with standard strains GHR-94.

Table (5) shows the impacts of soil treated by Glyphosate on the nodule numbers formed by different strains of biofertilizer.

In Table (5), results indicated a significance differences between the controls plants grown in soil free from herbicide treatment and those plant grown in soil treated with herbicide at post-emergence at 0.5x dose applied to the soil. But when the herbicide was applied as pre-emergence, no statistical differences were recognized throughout all treatments.

The nodule formation at post-emergence was more than at the pre-emergence condition. Only one case showed statistically difference between the plant inoculated by all biofertilizers and Glyphosate at 0.5x under post-emergence. The results indicated that the new isolated strains were more effective to modulate the plant than the standard strain (GHR-94) did. Here, the nodulation potential of the strains in decreasing order can be GHF-3153 > GHF-281 > GHF-162 > GHR 94.

Table (5): Impacts of post- and pre-emergence of Glyphosate on nodule number (number plant$^{-1}$) of alfalfa root inoculated by different biofertilizers

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| GHR-94 | *0* | *25* | *25* |
| | *0.5x* | *33** | *31* |
| | *x* | *29* | *28* |
| | *2x* | *27* | *25* |
| GHF-162 | *0* | *34* | *34* |
| | *0.5x* | *42** | *36* |
| | *x* | *39* | *32* |
| | *2x* | *35* | *30* |
| GHF-281 | *0* | *40* | *40* |
| | *0.5x* | *47** | *42* |
| | *x* | *42* | *41* |
| | *2x* | *40* | *37* |
| GHF-3153 | *0* | *44* | *44* |
| | *0.5x* | *51** | *46* |
| | *x* | *47* | *45* |
| | *2x* | *43* | *41* |
| S.D. ($P$ = 0.05) | | *6.733* | *6.970* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

Table (6) shows the relationship between the application of Glyphosate and the nodule dry weight, under the artificial inoculation by different biofertilizers. It was found that, the nodule dry weight in the case of post-emergence was more than those in the pre-emergence, and the results showed a statistically differences in the nodule dry weight on at the 0.5x level of herbicide applied dose. Similar results can be obtained based on the nodule dry weight in which the efficiency of the strain towards the effect of Glyphosate in decreasing order is GHF-3153 > GHF-281 > GHF-162 > GHR 94.

Table (6): Impacts of post- and pre-emergence of Glyphosate on nodule dry weight (mg plant$^{-1}$) of alfalfa root inoculated by different biofertilizers

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *GHR-94* | *0* | *132* | *113* |
| | *0.5x* | *163** | *138** |
| | *x* | *145* | *134* |
| | *2x* | *128* | *107* |
| *GHF-162* | *0* | *172* | *144* |
| | *0.5x* | *201** | *169** |
| | *x* | *196* | *157* |
| | *2x* | *175* | *132* |
| *GHF-281* | *0* | *174* | *146* |
| | *0.5x* | *204** | *172** |
| | *x* | *182* | *154* |
| | *2x* | *174* | *139* |
| *GHF-3153* | *0* | *197* | *163* |
| | *0.5x* | *226** | *191** |
| | *x* | *217* | *179* |
| | *2x* | *196* | *163* |
| *S.D. (P = 0.05)* | | *27.203* | *20.940* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

The effect of Glyphosate on the plant growth can be shown in Table (7), which summarized the action of the herbicide on the plant height. The results of the present study indicated that the plant inoculated with different biofertilizers increased the plant height significantly under the post-emergence treatment at all treated doses (0.5x, x, and 2x of FRD). While, under the effect of pre-emergence treatments, the plant height was increased but not statistically differences with the control treatment. The biofertilization of the plant with the new isolated strains of *S. meliloti* improved the plant height than the standard strain GHR-94. The effective strain was GHF-3153.

Table (7): Impacts of post- and pre-emergence of Glyphosate on plant height (cm) of alfalfa root inoculated by different biofertilizers

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *40.1* | *40.1* |
| | *0.5x* | *46.5** | *39.1* |
| | *x* | *47.2** | *41.1* |
| | *2x* | *44.2* | *38.3* |
| *GHR-94* | *0* | *52.3* | *46.2* |
| | *0.5x* | *61.4** | *51.1* |
| | *x* | *60.3** | *54.8* |
| | *2x* | *58.7** | *49.5* |
| *GHF-162* | *0* | *55.7* | *51.2* |
| | *0.5x* | *64.3** | *57.9* |
| | *x* | *62.7** | *55.3* |
| | *2x* | *61.3** | *52.6* |
| *GHF-281* | *0* | *59.6* | *54.3* |
| | *0.5x* | *70.5** | *61.3* |
| | *x* | *68.5** | *57.8* |
| | *2x* | *65.2** | *54.8* |

| | | | |
|---|---|---|---|
| *GHF-3153* | *0* | *61.7* | *56.4* |
| | *0.5x* | *74.5** | *64.7* |
| | *x* | *70.4** | *60.4* |
| | *2x* | *68.6** | *57.6* |
| *S.D. (P = 0.05)* | | *5.75* | *4.69* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

Table (8) explains the beneficial inoculation of the plant by the biofertilizer strains of *S. meliloti*. The plant dry weight is a fact of the symbiosis take place and the process was efficient. This means that the biological nitrogen fixation was happened, and the plant can accumulate the dry matter.
The application of the Glyphosate at post-emergence to the soil significantly increased the plant dry matter compared with the control. At pre-emergence application, it was significant differences when the plant inoculated by GHF-162.

Table (8): Impacts of post- and pre-emergence of Glyphosate on plant dry weight (g plant$^{-1}$) of alfalfa root inoculated by different biofertilizers

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *3.4* | *3.4* |
| | *0.5x* | *3.6* | *3.5* |
| | *x* | *3.4* | *3.2* |
| | *2x* | *3.1* | *3.2* |
| *GHR-94* | *0* | *3.7* | *3.7* |
| | *0.5x* | *4.1** | *3.8* |
| | *x* | *4.7** | *3.9* |
| | *2x* | *4.5** | *3.7* |
| *GHF-162* | *0* | *3.9* | *3.9* |
| | *0.5x* | *4.4** | *4.2** |
| | *x* | *4.5** | *4.2** |
| | *2x* | *4.3** | *4.1** |
| *GHF-281* | *0* | *4.1* | *4.1* |
| | *0.5x* | *4.5** | *4.2* |
| | *x* | *4.7** | *4.3* |
| | *2x* | *4.6** | *4.3* |
| *GHF-3153* | *0* | *4.2* | *4.2* |
| | *0.5x* | *4.8** | *4.4* |
| | *x* | *4.7** | *4.3* |
| | *2x* | *4.5** | *4.3* |
| *S.D. (P = 0.05)* | | *0.308* | *0.235* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

The present study showed that there were no statistically differences between the post- and pre-emergence application of herbicide in term of plant dry weight. The results indicated that GHF-3153 is the most effective strain. The concept of total N content/plant gives an idea about the $N_2$-fixed by the association between the symbiotic partners as shown in Table (9). It was found that the nitrogen accumulated in plant after inoculation with biofertilizers was increased compared with the control uninoculated plants. Results were indicated that the strain GHF-3153 is the most effective strain and economically can be industrially formulated as biofertilizer with high potent to increase the plant growth, plant dry matter and able to fix the atmospheric nitrogen more than the other investigated strains.

It was found that the application of herbicide at the x and 2x of FRD increased significantly the amount of nitrogen accumulated in the plant; also the amount of nitrogen measured in plant grown at post-emergence application of herbicide was higher than the amount measured at pre-emergence. In pre-emergence, the application of double FRD was significantly increased the amount of nitrogen in the plants. The results directly demonstrated high amount of nitrogen content in the plant and indirectly showed the ability of the strains to multiply and survive in the soil.

Table (9): Impacts of post- and pre-emergence of Glyphosate on total N content (mg N plant$^{-1}$)

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *214* | *214* |
| | *0.5x* | *236* | *233* |
| | *x* | *273* | *256* |
| | *2x* | *299** | *285** |
| *GHR-94* | *0* | *278* | *278* |
| | *0.5x* | *317* | *305* |
| | *x* | *366** | *346** |
| | *2x* | *379** | *369** |
| *GHF-162* | *0* | *315* | *315* |
| | *0.5x* | *338* | *324* |
| | *x* | *389** | *383** |
| | *2x* | *401** | *389** |
| *GHF-281* | *0* | *394* | *394* |
| | *0.5x* | *445* | *399* |
| | *x* | *468** | *462** |
| | *2x* | *477** | *475** |
| *GHF-3153* | *0* | *427* | *427* |
| | *0.5x* | *487* | *466* |
| | *x* | *501** | *496** |
| | *2x* | *527** | *502** |
| *S.D. (P = 0.05)* | | *73.330* | *67.112* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

**Effect of the applied herbicide on the function of some soil enzymes**

In the second part of the alfalfa *S. meliloti* strains in symbiotic relationship in the presence or absence of herbicide enhanced soil enzyme activities in the rhizosphere soil. In this study, soil treated with Glyphosate at 0.5 x had greater dehydrogenase, protease, β-glucosidase and phosphatase activities than soil treated with higher concentrations (Tables 10-13).

Table (10) indicates that the inoculation promoted the dehydrogenase activity and enhanced the enzyme activity at post-emergence over the activity at the pre-emergence. The results showed that application of Glyphosate at x and 2x of FRD were more statistically significant with the control. The inoculation of plants with GHF-3153 more effective to enhanced the activity of the enzyme.

Table (11) shows that the biofertilization of plants with *S. meliloti* strains promoted the protease activity and enhanced the enzyme activity at post-emergence over the activity at the pre-emergence. Similar mode of interactions between the symbionts and the application of herbicide were observed in case of determining the activity of protease. The results showed that application of Glyphosate at x and 2x of FRD were more statistically significant with the control and the incoculation of plants with GHF-3153 more effective to enhance the activity of the enzyme. Statistically, significant differences between the FRD of the herbicide at higher doses and the enzyme activity were found.

Table (10): Impacts of post- and pre-emergence of Glyphosate on the activity of dehydrogenase (μg INTF/g soil) in the rhizosphere

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *70,4* | *70,4* |
| | *0.5x* | *84,3* | *71,8* |
| | *x* | *91,6* | *80,7* |
| | *2x* | *115,8** | *100,6** |
| *GHR-94* | *0* | *88,1* | *88,1* |
| | *0.5x* | *117,3* | *97,5* |
| | *x* | *128,5** | *105,4* |
| | *2x* | *157** | *134,5** |
| *GHF-162* | *0* | *97,9* | *97,9* |
| | *0.5x* | *124,7* | *104,3* |
| | *x* | *144,9** | *127,1** |
| | *2x* | *178,9** | *144,7** |
| *GHF-281* | *0* | *104,1* | *104,1* |
| | *0.5x* | *147.3** | *133,1** |
| | *x* | *186,9** | *165,7** |
| | *2x* | *201,1** | *178,5** |
| *GHF-3153* | *0* | *117,9* | *117,9* |
| | *0.5x* | *159,5** | *138,9** |
| | *x* | *194,1** | *154,4** |
| | *2x* | *212,8** | *177,9** |
| *S.D. (P = 0.05)* | | *40.07* | *29.08* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

Table (12) illustrates that the *S. meliloti* strains promoted the phosphatase activity and increased the enzyme activity at post-emergence over the activity at the pre-emergence. Similar results were found as in the cases of dehydrogenase and protease that the effect of the plant inoculation with different strains of *S. meliloti* of interactions between the symbionts and the application of herbicide were observed in case of determining the activity of phosphatase. These results showed that application of Glyphosate at x and 2x of FRD were more statistically significant with the control and the incoculation of plants with GHF-3153 more effective to enhance the activity of the enzyme.

Table (13) shows that the activity of β-glucosidase activity in the rhizosphere of the alfalfa plants were inoculated by different strains of *S. meliloti* was increased with the increasing the concentrations of the Glyphosate, especially at higher concentrations which show a statistically significant. The strain GHF-3153 proved to be ecological important for increasing the activities of the soil enzymes and reduce the toxicity of the herbicide. Also, the selection of the *S. meliloti* strains for the monitoring the effect of herbicide applied in the field of symbiotic $N_2$-fixation was successful and suitable for applied in the field after industrial process to be commercial for market.

Many pesticides are used around the world to protect crops against insects, fungi, weeds, and other pests [23]. In addition to their agricultural application, they are also used for preserving wood and controlling home garden pests [24].

Adegas et al. [25] mentioned that the intensive use of Glyphosate has resulted in the selection of seven resistant species: ryegrass (*Lolium multiflorum*), buva (*Conyza bonariensis, C. canadensis, C. sumatrensis*), bittergrass (*Digitaria insularis*), caruru- palmeri (*Amaranthus palmeri*) and the wing-foot grass (*Eleusine indica*). To some extent, our results are in agree with the results of Aynalem1 and Assefa [26] when they investigate the effect of Glyphosate on the Rhizobia Isolated from Nodules of *Vicia faba*.

Table (11): Impacts of post- and pre-emergence of Glyphosate on the activity of protease (μmol $NH_4^+$-N/g soil/h) in the rhizosphere

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *1,39* | *1,39* |
| | *0.5x* | *1,47* | *1,25* |
| | *X* | *1,66* | *1,43* |
| | *2x* | *1,87** | *1,65** |
| *GHR-94* | *0* | *1,54* | *1,43* |
| | *0.5x* | *1,88** | *1,67** |
| | *X* | *1,94** | *1,74** |
| | *2x* | *2,2** | *1,98** |
| *GHF-162* | *0* | *1,63* | *1,45* |
| | *0.5x* | *1,97** | *1,89** |
| | *X* | *2,06** | *1,95** |
| | *2x* | *2,28** | *2,03** |
| *GHF-281* | *0* | *1,71* | *1,57* |
| | *0.5x* | *2,05** | *1,83** |
| | *X* | *2,34** | *1,99** |
| | *2x* | *2,65** | *2,07** |
| *GHF-3153* | *0* | *1,76* | *1,66* |
| | *0.5x* | *2,03* | *1,90** |
| | *X* | *2,45** | *2,01** |
| | *2x* | *2,61** | *2,25** |
| *S.D. (P = 0.05)* | | *0.335* | *0.233* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

Herbicides can exert collateral effects on soil microbiotas and important functions such as N cycling, decomposition, hydrolytic enzyme activities, etc. Some of these compounds represent a source of N to microbial communities through mineralization. Soil microbiotas regulate a majority of processes in soil that are essential for plant growth, soil health and sustained productivity.

A large, diverse and active soil microbiotas could provide soil conditions for sustainable crop production through (i) crop residue decomposition and improvement of nutrients, (ii) preventing aggressive phytopathogens taking hold and improve plants ability to withstand disease effects, (iii) reducing the loss of inorganic fertilizers through erosion and leaching by short-term immobilization (iv) stabilizing soil structure and (v) reducing the reliance for agrochemicals and reduced persistence of pesticides in soil and thus less off-site impacts.

Application of herbicides may not affect the overall size of the soil microbiotas but selectively affect specific groups of microbiota which may result in altering the balance of biological activity and consequently nutrient availability, disease incidence and plant growth. Non-target effects of herbicides could be either positive or negative.

Herbicides are widely used in agriculture to control unwanted organisms including weeds. However, they can also threaten the environment and human health through spray drift, runoff, leaching, disposal, spills, and leakage [26, 27]. Thus, their dissipation, elimination, and distribution behaviors under environmental conditions must be comprehensively assessed, especially in the soil and aquatic sediments (Davis et al. 2005) that tend to be the environmental compartments most at risk for herbicide accumulation.

Management of herbicides usage that cause reversible inhibitions is difficult, as reaching a balance between high herbicide efficiency and minimum non-target effects requires a better understanding of herbicide-microbial communities-environment interactions.

Table (12): Impacts of post- and pre-emergence of Glyphosate on the activity of phosphatase (µmol PNP/g soil /h) in the rhizosphere

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *54,3* | *44,4* |
| | *0.5x* | *76,3* | *67,3* |
| | *x* | *89,1** | *78,9** |
| | *2x* | *107,6** | *92,6** |
| *GHR-94* | *0* | *74,7* | *56,6* |
| | *0.5x* | *67,4* | *66,1* |
| | *x* | *100,1** | *86,3** |
| | *2x* | *115,7** | *92,3** |
| *GHF-162* | *0* | *88,9* | *74,6* |
| | *0.5x* | *97,6* | *87,5* |
| | *x* | *114,5* | *99,6** |
| | *2x* | *132,1** | *112,1** |
| *GHF-281* | *0* | *89,8* | *78,6* |
| | *0.5x* | *103,5* | *84,5* |
| | *x* | *132,1** | *104,1** |
| | *2x* | *144,3** | *115,3** |
| *GHF-3153* | *0* | *95,7* | *88,5* |
| | *0.5x* | *115,6* | *104,6* |
| | *x* | *134,5** | *123,3** |
| | *2x* | *156,3** | *134,2** |
| *S.D. (P = 0.05)* | | *26.67* | *22.39* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

Table (13): Impacts of post- and pre-emergence of Glyphosate on the activity of β-glucosidase (µmol PNP/g soil /h) in the rhizosphere

| *Biofertilizers* | *Herbicide dose* | *Emergence* | |
|---|---|---|---|
| | | *Post-* | *Pre-* |
| *Uninoculated* | *0* | *114* | *98* |
| | *0.5x* | *123* | *115* |
| | *x* | *148* | *132* |
| | *2x* | *178** | *154** |
| *GHR-94* | *0* | *167* | *145* |
| | *0.5x* | *183* | *163* |
| | *x* | *212** | *189** |
| | *2x* | *241** | *203** |
| *GHF-162* | *0* | *177* | *145* |
| | *0.5x* | *197* | *165* |
| | *x* | *223** | *189* |
| | *2x* | *254** | *214** |
| *GHF-281* | *0* | *179* | *156* |
| | *0.5x* | *201* | *189* |
| | *x* | *254** | *221** |
| | *2x* | *266** | *235** |
| *GHF-3153* | *0* | *174* | *162* |
| | *0.5x* | *204* | *187* |
| | *x* | *277** | *211** |

| | | | |
|---|---|---|---|
| | *2x* | *287** | *267** |
| *S.D. (P = 0.05)* | | *48.9* | *41.6* |

Values are means of three replicates, and values labelled with * shows statistically significance with the control (untreated) plants

Different soil microbial populations and their activities were determined in a microcosms of 3 kg capacity under the stress of 8 herbicides were applied at 3 doses based on the active ingredients and incubated for 10 weeks at 25°C. Results from these investigations on the effects of a single application of selected herbicides in alfalfa growing soils indicated that: Some of herbicides currently used in alfalfa soils have a negative impact on key groups of microorganisms; Most of the negative effects were reversible partly or fully within 10-weeks after herbicide application. Some herbicides caused a significant shift in bacteria : fungi ratio, reduced the rate of cellulose-decomposition; Some herbicides applied had negative impact on symbiotic $N_2$-fixation by legumes in rotation and application of a post-emergent herbicide caused significant changes in the rhizosphere microbial activity and the composition of microbial community as well as the activities of some hydrolytic enzymes. It was found that Glyphosate causes activation in soil urease and invertase soil enzymes but suppression of phosphatase [29].

Finally, this study was designed to assess the effect of Glyphosate on growth of alfalfa sinorhizobial isolates in vitro and on their $N_2$-fixation performance. Hence, four selected isolates were isolated using plant-soil trap method from soil samples collected from farm lands. Those isolates were morphologically characterized using YMA medium and authenticated as nodulating sinorhizobial isolates using sand culture. These isolates were treated with 0.5X, X and 2X herbicide doses. The result showed that almost all isolates were not affected. For *in vivo* experiment, alfalfa seedlings in brown forest soil culture were inoculated with four relatively *in vitro* test tolerant isolates. The inoculated isolates were treated with half, double and field recommended concentration of Glyphosate,. Thus, experimental plants almost all showed normal nodulation and $N_2$-fixation performance as compared to the control.

## CONCLUSIONS AND RECOMMENDATIONS

The consistent application of agrochemical herbicides has been reported to impact negatively on human health, environment, and food safety, and facilitated the emergence of weed resistances. A generalized relationship between injury level and yield response can be established in weed-free experiments by observing the injury patterns which result from the application of various rates of herbicides. Glyphosate did not significantly interfere in plant growth with a gradual reduction in height increase relative to the control, with increasing Glyphosate doses. Post-emergence increase total N content, total root-nodules, root-nodule dry weight, plant height, plant dry weight and all investigated soil enzymatic activities at all applied doses. It was observed that there is still a need for research evaluating the physiological suitability of specific soybean cultivars to determine the tolerance of the crop to different mechanisms of action in order to minimize the frequent use of herbicides and weed control strategies, good performance and productivity of the crop. This is promising result to identify and obtain agrochemical resistant inoculum for more $N_2$-harvest under no-tillage agriculture system, but further actual field experiment will be required.

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