THE EFFECTS OF GLYPHOSATE ON THERMOTOLERANT *BACILLUS SUBTILIS*

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Abstract. Glyphosate is a widely used herbicide due to its minimal adverse effects. Consequently, its use has increased significantly over the past 35 years. Concerns about excessive glyphosate contamination have contributed to the World Health Organization (WHO) classifying it as a probable carcinogen. Although glyphosate primarily targets plants, its environmental contamination has had harmful effects on soil and aquatic organisms, microorganisms, animals, and humans. In this study, *Bacillus subtilis*, isolated from thermal spring waters, was exposed to sublethal doses of glyphosate to investigate its effects on exoenzyme production (amylase and protease), growth, and plasmid replication for 37°C and 47°C.
Keywords: *glyphosate tolerance in bacteria, glyphosate pollution, amylase and protease, plasmid amplification, Bacillus subtilis*

Introduction

The simultaneous increase in human population and food demand has created the need to obtain higher yields from a unit of agricultural land. As a result, the use of herbicides has significantly increased (Choudhury et al., 2016). Glyphosate (N-phosphonomethylglycine) is one of the most widely used herbicides worldwide, particularly since the 1980s (Bøhn et al., 2014). This substance inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is involved in the shikimic acid pathway during the synthesis of aromatic amino acids (Kocadal et al., 2023). Although the main target is weeds, many organisms, including microorganisms, in the environment can be exposed to the harmful effects of the chemical (Van Bruggen et al., 2000).

Glyphosate applications can lead to an increase in the amount of glyphosate and its degradation product, aminomethylphosphonic acid (AMPA), in the soil, water, and both target and non-target organisms (Myers et al., 2016). The negative effects of glyphosate and the surfactant polioksietilenamin on various microalgae, aquatic bacteria, and protozoa species have been demonstrated (Simranjeet et al., 2020; Sihtmäe et al., 2013; Leyva et al., 2018). The modes of action in aquatic microorganisms are similar to those in terrestrial plants and microorganisms: Glyphosate affects the synthesis of aromatic amino acids, chlorophyll production, photosynthesis, and respiration (Mensink and Janssen, 2002). The marine bacterium species *Vibrio fischeri* was sensitive to moderately low glyphosate concentrations in water, regardless of the formulation used (Sihtmäe et al., 2013). Although the *Bacillus subtilis* we are working on does not carry chlorophyll, it does not synthesize phenolic amino acids due to the inhibition of the shikimic acid pathway. Subsequently, adverse effects begin to emerge.

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A large number of articles have been published examining the effects of glyphosate on bacteria. Almost all of these studies have been conducted on soil bacteria, and there is very little research on spa bacteria. The shikimic acid pathway is found not only in plants but also in fungi and bacteria, making many microbial taxa sensitive to glyphosate. EPSPS is the target enzyme of glyphosate herbicides. However, not all organisms with the shikimic acid pathway are sensitive to glyphosate, depending on the class of EPSPS they produce: Class I EPSPS is sensitive to glyphosate, while class II EPSPS is tolerant to glyphosate (Funke et al., 2007; Priestman et al., 2007). For example, the *Agrobacterium tumefaciens* strain CP4 has a gene that encodes a class II version of EPSPS, which is not inhibited by glyphosate (Padgette et al., 1995). Similarly to plants, bacterial and fungal species with low sensitivity to glyphosate have been selected largely through the same mechanisms identified in plants (Priestman et al., 2007; Li et al., 2015; Liu et al., 2013; Staub et al., 2012).

As a result, the differences in sensitivity among microorganisms have affected the microbial composition of various habitats hosting glyphosate, including soil, plant surfaces, and animal intestinal tracts. Many examples can be given of the differences in glyphosate sensitivity among bacteria. For example, *Klebsiella variiocola* and *K. Pneumonice* are not significantly affected by the presence of glyphosate (Kurtoğlu et al., 2020). There are also some studies related to utilizing these types of microbial activities to clean up glyphosate and AMPA pollution in the environment (Lupi et al., 2015; Mercurio et al., 2014; Fan et al., 2012).

Generally, when bacteria are exposed to adverse environmental conditions, plasmid replications increase (Nadezhda et al., 2022; Nowick et al., 1976). It can be considered that glyphosate particularly inhibits the synthesis of aromatic amino acids and indirectly inhibits protein synthesis. This also reminds one of the mechanisms of action of many antibiotics (Edet et al., 2021; Otludil et al., 1992).

Although glyphosate contamination in thermal spring waters is highly unlikely, thermal wastewaters containing microorganisms are discharged into nature. The aim of our study is to investigate how thermotolerant microorganisms released into nature in this way are affected by glyphosate pollution. Additionally, the production and release of amylase, protease and plasmid replication by the bacteria under glyphosate stress conditions have been investigated.

Material and methods

Biological material and growth medium

B. subtilis, previously identified to the species level, was used, isolated from the thermal springs of Çermik, Diyarbakır, Turkey (Aytekin et al., 1993). Minimal mineral medium was used. The reason for using a mineral medium is to demonstrate that the bacterium is prototrophic. For this: 14 g $KHPO_4$, 6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 1 g $NaSO_4$, 0,2 g $MgSO_4$. 7 H_2O, dissolved in 1 liter of water, and autoclaved. To the cooled medium, glucose was added as a carbon source at a concentration of 5 g/L under sterile conditions. When the culture growing in the logarithmic phase reached OD:600: 0.5, 100 µl of the culture was added to 250 ml of the medium. Culture K (control) was prepared with glucose as the carbon source, culture A glyphosate 0.1 mg/ml, culture B glyphosate 0.2 mg/ml, and culture C glyphosate 0.3 mg/ml. The pH of all the culture media was adjusted to the pH of the thermal spring water, which is 7.06. The pH of all the culture media was adjusted to the pH of the thermal spring water, which is 7.06.

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Bacterial growth was performed at OD 600 nm using a Cinctra 6 UV spectrophotometer the experiments were repeated three times (*Fig. 1*).

Figure 1. *The laboratory environment and equipment in which the experimental stages were carried out*

Effect of glyphosate on B. subtilis

The minimal inhibitory concentration (MIC) of glyphosate for the studied bacterium was determined using microtiter plates and repeated three times. In each experiment, 100 µl of *B. subtilis* culture was added to 900 µl of broth medium containing glyphosate at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/ml. The experiments were conducted separately at 37°C and 47°C for 24 h. Bacterial growth was evaluated on blood agar medium. The MIC value was determined through quantitative analysis and microscopic examination.

Determination of amylase activity

Bacterial culture was centrifuged at 5000 rpm for 10 min. The supernatant is the crude enzyme fluid to be measured. Alpha-amylase activity was measured using 3,5-dinitrosalicylic acid (Hu et al., 2021). The 0.5 ml reaction mixture consisted of 1% starch (as the substrate), 50 mM sodium acetate buffer (pH 6.0), and an appropriately diluted enzyme solution. Following incubation at 37°C for 20 min, 0.5 ml of 3,5-dinitrosalicylic acid (DNS) was added to quantify the released sugars. The mixture was then boiled for 10 min to facilitate color development, and absorbance was measured at 540 nm on spectrophotometer.

Determination of protease activity

0.2 ml of the supernatant liquid was added to a 1% casein solution. 0.2 M Tris buffer 18 ml and 0.06 M $CaCl_2$ 1 ml. Incubated at 37ºC for 30 min. The reaction was stopped by adding 2 ml of 10% TCA and measured at 275 nm using a Cinctra 6 UV spectrophotometer (Müderriszade et al., 2001).

Determination of glyphosate amount

It was determined according to the method of Carnerio (Carneiro et al., 2015). Ninhydrin is dissolved in 5 g/100 ml in 96% ethanol and mixed with $Na_2MoO_4.2H_2O$

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prepared in 5 g/100 ml in distilled water. 100 µl of supernatant and 500 µl of the prepared solution are mixed and measured at 570 nm in a spectrophotometer.

Protein quantity determinations

It has been done according to the Bradford method (Bradford, 1976). A solution of 100 mg Coomassie Brilliant Blue G-250 was prepared by dissolving it in 50 mL of 95% ethanol (C_2H_5OH). Then, 100 mL of 85% phosphoric acid (H_3PO_4) was gradually added with gentle stirring. The final volume was brought to 1 L by adding H_2O. The solution was filtered and stored at 4°C. For measurements, 100 µL of extract was combined with 5 mL of Bradford reagent and incubated for 5 min. A standard curve of BSA (0, 0.0625, 0.125, 0.25, 0.5, and 1 g/L) was prepared, and absorbance was recorded at 595 nm in spectrophotometer.

Determination of total plasmid amount

To determine the total plasmid amount, the Rapid isolation method was applied, and plasmid amounts were found through spectrophotometric measurements (Hardy, 1987).

Chemicals

The $Na_2MoO_4.2H_2O$, Ninhydrin, and 3,5-Dinitrosalicylic acid used in the study were obtained from Merck, while other chemicals were sourced from Sigma.

Statistics

Kruskal-Wallis (Non-parametric ANOVA) multiple comparisons were evaluated using the Mann-Whitney U test. Significance was considered for $P < 0.05$.

Results

Bacterial growth

Figure 2a, b shows the effect of different glyphosate amounts on the reproductive performance of *B. subtilis* at 37°C and 47°C. When compared to the controls, the bacteria showed better reproductive performance at the spa water temperature of 47°C. However, at 37°C, bacteria were more affected by increasing sublethal glyphosate concentrations. The addition of aromatic amino acids to the medium (100 µg/ml tyrosine, tryptophan, and phenylalanine) has shown rapid recovery, especially in environments with low concentrations of glyphosate. This indicates that the shikimic acid pathway is significantly affected.

In comparison to the control group, thermotolerant *B. subtilis* exhibited high sensitivity to glyphosate, with an MIC value of approximately 0.2 mg/ml. The tested temperature conditions did not influence the MIC value. Regarding colony morphology, no notable differences were observed between the control group and the bacteria exposed to glyphosate. However, glyphosate concentrations of 0.4 mg/ml and higher significantly impacted lethality.

Determination of glyphosate amount in supernatant

When the supernatant glyphosate contents were measured (*Fig. 3*), we see that at 47°C, the bacteria produced less in the C medium containing glyphosate at a

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particularly high concentration. This indicates that the bacteria require more carbon sources at the optimal spa temperature for reproduction and that they have absorbed more glyphosate. The glyphosate doses we used were sublethal doses below the detected 0.5 mg/ml glyphosate dose. Additionally, no glyphosate contamination was detected in the spa water.

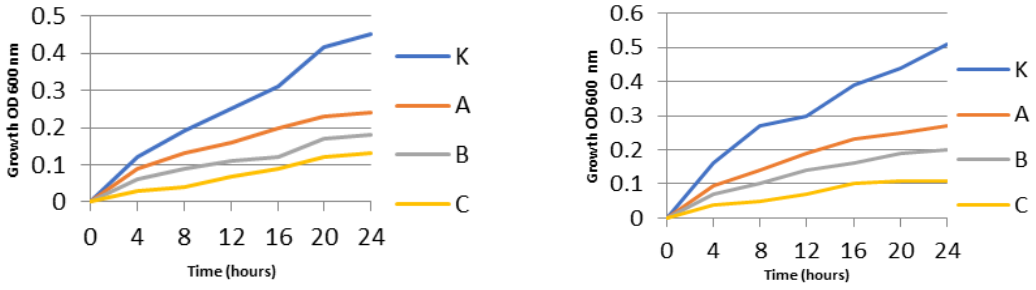

Figure 2. *(a)* B. subtilis *growth at 37°C in different glyphosate concentrations (K: control, A: 0.1 mg/ml, B: 0.2 mg/ml, C: 0.3 mg/ml). (b) B. subtilis growth at 47°C in different glyphosate concentrations (K: control, A: 0.1 mg/ml, B: 0.2 mg/ml, C: 0.3 mg/ml)*

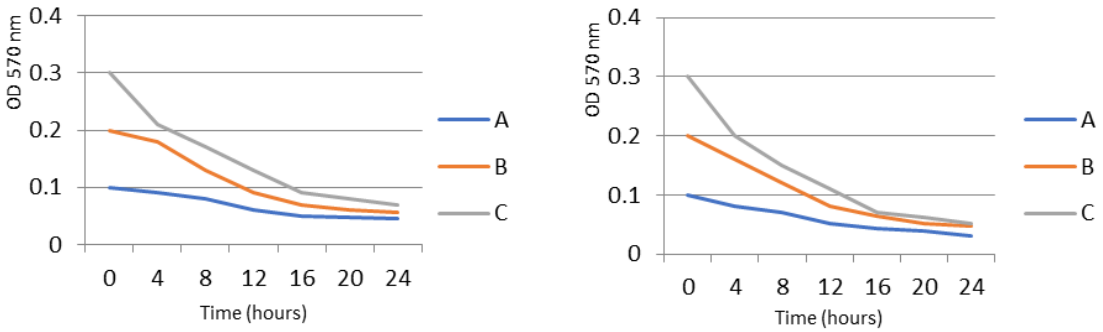

Figure 3. *(a) At 37°C, different Glyphosate concentrations (A: 0.1 mg/ml, B: 0.2 mg/ml, C: 0.3 mg/ml) the amounts of Glyphosate remaining in the supernatant after culturing B. subtilis. (b) At 47°C with different Glyphosate concentrations (A: 0.1 mg/ml, B: 0.2 mg/ml, C: 0.3 mg/ml) the amounts of Glyphosate remaining in the supernatant after culturing B. subtilis*

Effects of glyphosate on amylase and protease production in bacteria

When comparing the production performance of amylase and protease at different temperatures and glyphosate concentrations, the bacteria synthesized both enzymes slightly more at 47°C (*Fig. 4a, b*). However, increasing sublethal glyphosate concentrations negatively affected the synthesis of both enzymes at both 37°C and 47°C.

Effects of glyphosate on plasmid quantity in bacteria

In *B. subtilis*, the total plasmid amounts were examined under different glyphosate concentrations at 37°C and 47°C. Plasmid amplification was found to be higher at 37°C and with increasing sublethal glyphosate concentrations (*Table 1*). This suggests that adverse conditions affect the plasmid replication rates in bacteria (*Fig. 5*).

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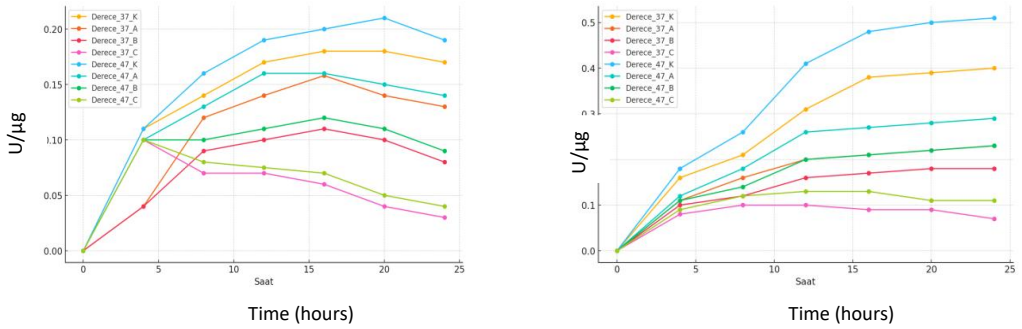

Figure 4. *(a) Amylase enzyme released into the environment at different glyphosate concentrations at 37°C and 47°C depending on the incubation time. (b) The protease enzyme released into the environment at different glyphosate concentrations at 37°C and 47°C, depending on the incubation time*

Table 1. *Change in plasmid amount at different temperatures and glyphosate concentrations at 24 h*

	Glyphosate mg/ml	37°C Plasmids µg/ml	47°C Plasmids µg/ml
K	0	0.180	0.165
A	0.1	0.265	0.200
B	0.2	0.302	0.220
C	0.3	0.356	0.286

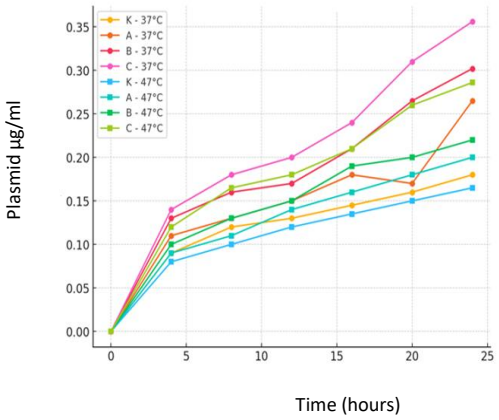

Figure 5. *Changes in plasmid quantity depending on incubation time at 37°C and 47°C under different glyphosate concentrations*

Statistics results

According to the Kruskal-Wallis (Non-parametric ANOVA) multiple comparison test, protease and amylase production were found to be statistically significant depending on temperature changes and different glyphosate concentrations. Additionally, plasmid amplification was also found to be statistically significant at sublethal glyphosate concentrations. Bacterial growth was also significantly affected by sublethal glyphosate concentrations.

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Discussion

There is very little research on the effects of glyphosate on bacteria living in thermal spring waters. Studies have generally focused on soil microorganisms (Myers et al., 2016; Torretta et al., 2018). However, considering that glyphosate pollution will also affect thermal microorganisms, there is a need to investigate the microorganisms living in these extreme environments.

The bacteria used in our study are highly sensitive to glyphosate applications. Our experiments showed that a glyphosate concentration of 0.4, 0.5 mg/ml. had a lethal effect. The minimum inhibitory concentration (MIC) was found to be 0.2 mg/ml. To observe the effects of glyphosate on *Bacillus subtilis*, sublethal concentrations (0.1–0.3 mg/ml.) were examined. Additionally, the optimal growth temperature of *Bacillus* species from soil (37°C) was compared with the temperature of the thermal spring (47°C) under laboratory conditions (*Fig. 2*). It was observed that increasing glyphosate concentrations significantly affected bacterial growth at both temperatures. Therefore, the bacteria we are studying may carry the Class I EPSPS enzyme system. These data are consistent with various studies (Funke et al., 2007). When aromatic amino acids were added to the medium, the improvement in bacterial conditions suggests that the toxic effect originates from the shikimic acid pathway.

Generally, bacteria exposed to stressful environmental conditions increase the synthesis of exoenzymes to more efficiently utilize extracellular carbon sources (Ajuna et al., 2023). When we compared reproduction, amylase, and protease activities under glyphosate applications in our experimental environments, we observed that reproduction decreased by approximately 50% at both temperatures, especially in the 0.1 and 0.2 mg/mL glyphosate environments. When examining amylase and protease levels, the decrease compared to the control was less than 50% (*Fig. 4a, b*). This suggests that, while glyphosate inhibits the synthesis of phenolic amino acids, the bacteria attempt to compensate for this deficiency by secreting amylase and protease into the extracellular environment. The inhibition of aromatic acid synthesis in bacteria reduces bacterial proliferation, similar to the effect of antibiotics. When examining *Fig. 3a, b*, it is observed that glyphosate levels in the supernatant were lower at 47°C. High temperatures may have increased the diffusion rate, accelerating the passage of glyphosate into the bacterial cytoplasm.

Another factor that changes in bacteria under environmental stress is the amount of plasmids. When *Table 1* and *Figure 5* is examined, it is observed that the plasmid amount at 37°C is slightly higher than at 47°C. Since the bacteria have adapted to live optimally at 47°C, a reproduction temperature of 37°C could create an unfavorable condition. An increase in plasmid replication has been observed in glyphosate-containing environments at both temperatures. In bacteria, when chromosomal DNA synthesis is inhibited, an increase in plasmid replication is observed (Nowick et al., 1976). Glyphosate may have caused this effect by inhibiting the synthesis of aromatic amino acids. Another point to consider is that changes in plasmid replication in bacteria can especially alter the development of antibiotic resistance in pathogenic bacteria (Van Bruggen et al., 2023). Glyphosate pollution may seem harmless, but it could have laid the groundwork for many unwanted changes in the ecosystem.

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