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Research Article

Determination of plant growth promoting properties and bioremediation potentials of *Bacillus mycoides* Flügge and *B. thuringiensis* Berliner

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Abstract: Industrial activities destroying natural resources for decades have been one of the most significant factors in environmental destruction. As a result of industrialization, environmental pollutants became one of the biggest threats for the biosphere. Heavy metals, one of these environmental pollutants, have become a significant health threat for organisms by forming metal accumulations in water and soil. In addition to the existing ones, most researchers believe that there is a great need for alternative biological processes to be used in the control of heavy metal pollution. Bioremediation is the process of removing various toxic pollutants, such as heavy metals from the environment, especially with the help of fungal and bacterial microorganisms, sometimes plants and earthworms. The use of bacteria in the bioremediation process is prevalent. In this study, the metal tolerance and plant growth-promoting properties of *Bacillus mycoides* and *Bacillus thuringiensis* isolated from the root soil of and orchid plant were investigated. The abilities of both bacteria to tolerate copper, lead, iron, silver, and zinc were tested in addition to and their indole acetic acid production (IAA), siderophore production, phosphate solubility and Aminocyclopropane-1-Carboxylate-deaminase (ACC-deaminase) activity were determined. The two isolates exhibited a high level of tolerance towards different pH levels, temperature ranges and metal concentrations. The results showed that *B. mycoides* and *B. thuringiensis* isolates can be used as bioremidant agents in metal-contaminated soils and also as biological fertilizers due to their plant growth-promoting properties.

Özet: Endüstriyel faaliyetler, onlarca yıldır doğal kaynakları yok eden çevresel tahribatın en önemli faktörlerinden biri olmuştur. Sanayileşmenin bir sonucu olarak, çevre kirleticileri biyosfer için en büyük tehditlerden biridir. Çevre kirleticilerinden biri olan ağır metaller, su ve toprakta metal birikimleri oluşturarak canlılar için önemli bir sağlık tehdidi haline gelmiştir. Bu nedenle ağır metal kirliliği ile mücadelede alternatif biyolojik süreçlere büyük ihtiyaç duyulmaktadır. Biyoremediasyon, ağır metal gibi çeşitli toksik kirleticilerin özellikle fungal ve bakteriyel mikroorganizmalar, bazen bitkiler ve toprak solucanları yardımıyla ortamdan uzaklaştırılması işlemidir. Biyoremediasyon sürecinde bakterilerin kullanımı yaygındır. Bu çalışmada, orkide kök toprağından izole edilen *Bacillus mycoides* ve *Bacillus thuringiensis*'in metal toleransı ve bitki büyümesini destekleyici özellikleri araştırıldı. Spesifik olarak, bakır, kurşun, demir, gümüş ve çinko metallerini tolere etme yetenekleri ve indol asetik asit üretimi, siderofor üretimi, fosfat çözünürlüğü ve 1-Aminosiklopropan-1-karboksilat-deaminaz (ACC-deaminaz) aktivitesi belirlendi. İki izolatın farklı pH seviyelerine, sıcaklık aralıklarına ve metal konsantrasyonlarına karşı yüksek düzeyde tolerans gösterdiği bulundu. Sonuçlar, *B. mycoides* ve *B. thuringiensis* izolatlarının metalle kontamine topraklarda biyoremidant ajanlar olarak ve ayrıca bitki gelişimlerini teşvik edici özellikleri nedeniyle biyolojik gübre olarak da kullanılabileceğini göstermiştir.

Introduction

Advanced treatment methods that can effectively eliminate the deficiencies in existing treatment systems are being developed in order to achive restoring of the disturbed natural balance and cleaning soil and water resources. The development of environmentally friendly alternatives and establishment of biological control applications instead of use of chemicals in the fight against environmental pollutants and plant pathogens have recently been a popular issue (Ongena & Jacques 2008). Bioremediation, among these, is a globally applied environmental restoration method that aims to reduce environmental pollution (Vural *et al.* 2018). This method is based on destroying organic and inorganic pollution with the help of microorganisms (Nie *et al*. 2020). Bioremediation agents include members of the genus *Bacillus*, and the best-described species in terms of the bioremediation potential. are *B. subtilis, B. cereus* and *B. thuringiensis. Bacillus* spp. strains have the ability to reduce heavy metals such as lead, mercury, chromium, arsenic and nickel in the environment through various strategies (Wróbel e*t al.* 2023).

Recent research reported that among microorganisms used for bioremediation, the use of plant growth-promoting rhizobacteria (PGPR) is becoming more common due to their diverse ability to detoxify and break down toxins and their significant effects on promoting plant growth (Haider *et al*. 2021). These PGPR are successfully used in developing countries, and their market share is expected to increase in the future. PGPR is known to contain different species of various genus one of which is *Bacillus* (Ejaz *et al.* 2021). The properties that make *Bacillus* species biotechnologically effective are the variety of their secondary metabolites and their ability to produce various structurally different antagonistic substances (Kalaycı *et al*. 2021). The genus *Bacillus*, which includes species often isolated in soil and water, draws the attention of researchers with its growth-promoting properties on plants. Recently, studies have focused on a high diversity of lipopeptides from *Bacillus subtilis* with high efficiency and direct action against plant pathogens (Fira *et al.* 2018). Qi *et al.* (2016) showed in their study that *B. thuringiensis* strains are among the PGPRs that can promote plant growth. In the same study, seed germination and shoot elongation were promoted by treating the tomato seeds with a bacterial culture filtrate and a bacterial suspension.

All organisms need certain heavy metals in trace amounts, but the high intake of these metals leads to occurence of serious toxic cases, i.e. physiological damage, not only in humans but also in other animals and plants. Lead, gold, chromium, cadmium, silver, nickel, zinc and copper are among the heavy metals reported to reach dangerous levels in humans (Pandian *et al.* 2014, Rama Jyothi, 2021). In order to eliminate or reduce the risk of plants grown in soils contaminated with heavy metals in terms of animal and human health, bioremediation studies, especially those performed with the use of *Bacillus* species, are important.

In a study heavy on metal bioremediation by a multi-metal resistant endophytic bacteria L14 (EB L14) isolated from a cadmium hyperaccumulator, *Solanum nigrum* L. was characterized for its potential application in metal treatment. 16S rDNA analysis revealed that this endophyte belonged to *Bacillus* sp. The hormesis of EB L14 were observed in presence of divalent heavy metals [(Cu^{+2} (21.25%), Cd^{+2} (75.78%) and Pb^{+2} (80.48%)] at a relatively low concentration (10 mg/L) (Guo *et al*. 2010).

In addition to the bioremidant properties of *Bacillus* species, their plant growth promoting properties led us to study on this subject.

In the present study, we aimed to morphologically and biochemically identify the *Bacillus* bacteria isolated from the root soil environment of an orchid (*Phalaenopsis* spp.) plant, determine their heavy metal tolerance values, and reveal their plant growth-promoting properties.

Materials and Methods

The isolation and diagnosis of the strains

The bacterial strains used in the study were isolated from the root soil of a *Phalaenopsis* spp. plant obatined in Ovit plateau, Rize, Türkiye. For the isolation process and diagnosis, Bergey's Guide to Systematic and Determinative Bacteriology was used (Bergey, 1994).

Two bacteria (5O5Y1 and 5O9 isolates) were isolated from the orchid root soil. The isolates were identified by both morphological and macroscopic investigations and biochemical evaluations as *Bacillus* mycoides (the 5O5Y1 isolate) and *Bacillus thuringiensis* (the 5O9 isolate),. Fresh cultures were prepared on Muller-Hinton agar (MHA) to continue the study. Both isolates' pH and salt tolerances were tested to determine the optimal value ranges for their growth and development.

For this purpose, Mueller Hinton Broth (MHB) media containing different pH values (4.5-5.0, 6.8-8.0) and salt (10%, 15%) concentrations were prepared. A single colony was taken from fresh cultures of the isolates in MHA medium, inoculated into MHB and incubated at 36°C for 24 hours in a shaking incubator (Memmert, Model 600). McFarland 0.5 turbidity bacterial suspension was prepared from overnight grown bacterial cultures. 200 µL of each of the MHB medium containing different pH values and salt concentrations was dispensed into wells of a 96-well plate. Then, 10 µL of bacterial suspensions for both isolates were dropped into each well of the plate (Mladenović *et al.* 2018). The pH and salt concentration values in the plate wells with or without bacterial growth were determined. The optical density of the growth liquid was measured at 600 nm absorbance with a UV-VIS spectrophotometer (SpectraMax® M, Molecular Devices, USA). Experiments were carried out in 3 repetitions.

Determination of Plant Growth Promoting Properties of the Isolates

The presence of ammonium, Indole Acetic Acid (IAA) production, 1-Aminocyclopropane-1-Carboxylate (ACC)-deaminase activity, phosphate dissolution and siderophore production of the isolates were investigated. A colorimetric method was used with pepton water broth medium and Nessler reagent to measure the ammonium production of the isolates. The presence of ammonium was confirmed through a positive evaluation of color change from light yellow to red (Aydoğan *et al*. 2013). IAA production test was performed to determine whether the isolates produce the plant growth-promoting factor

IAA extracellularly (Fürnkranz *et al*. 2009). ACC-deaminase activity was measured using the basic method of Dworken & Foster (1958). Phosphate solubilization activity was performed according to the methods of Fürnkranz *et al.* (2009) and Aydoğan *et al.* (2013). The formation of a transparent zone around the bacterial colonies growing in the petri dish containing phosphate medium and the color change from green to yellow in the broth phosphate medium were considered positive for phosphate solubility. The Chromium Azurol S Agar Assay (Cas-AGAR), as described by Dworken & Foster (1958) and Alexander & Zuberer (1991), was used to assess the siderophore production of the bacteria. The formation of a yellow zone around the bacterial colonies was considered a positive result for siderophore production. Each experiment was performed in triplicate.

Determination of Heavy Metal Tolerances of the Isolates

MHA medium containing different concentrations (1, 2.5, 5 ve 10 mM/L) of metal salts ($AgNO_3$, $CuSO_4.5H_2O$, $FeCl_3.6H_2O$, $ZnCl_2$ ve Pb $(NO_3)_2$) was used to determine metal tolerances of the isolates (Velásquez & Dussan 2009). The overnight cultures of the isolates were inoculated in Petri dishes on a MHA medium containing different metal salts and incubated at 36±1°C for 5 days. Observation of bacterial growth in Petri dishes was evaluated as positive for metal resistance.

To determine the concentration value of copper sulfate salt ($CuSO_4$) that prevents bacterial growth was determined by minimum inhibition (MIC) and minimal bactericidal (MBC) tests (NCCLS 1999). Growth curves of the isolates were determined at different pH (5.0, 5.5, 6.0, 6.5, 7, and 7.5) ranges in broth containing 3 mM $CuSO_4$. The growth curves were created by measuring MIC and under MIC values every hour at 36±1°C on a bacterial growth monitoring device (Bioscreen C, UK) for 24 hours.

Results

Identification of Bacteria by Traditional Methods

The isolates was identified at species level by macroscopic and microscopic investigations and with the use of relevant biochemical characteristics (Fig. 1 and Table 1). According to the traditional and molecular definitions, isolate 5O5Y1 was identified as *Bacillus mycoides* and isolate 5O9 was identified as *B. thuringiensis.*

The study examined some physical properties of the isolates (ability to grow at different temperatures, presence of salt, and pH). It was observed that the isolates could grow at 10% salt concentration but did not grow at 15%. *Bacillus mycoides* 5O5Y1 isolate had better growth potential at 10°C, and the *B. thuringiensis* 5O9 isolate had better growth potential at 45°C. The *Bacillus mycoides* 5O5Y1 isolate showed good growth potential in all pH ranges (4.5-8.5) tested, while the *B. thuringiensis* 5O9 isolate had better growth potential in all tested pH ranges (4.5-8.5) (Table 1).

Investigation of Plant Growth-Promoting Properties of the Isolates

Our study determined that *Bacillus* isolates had plant growth-promoting properties, except for ACC-deaminase. A number of plant growth-promoting factors of the identified bacteria (Siderophore production, Phosphate solubility, ACC deaminase activity and Ammonium production) were tested in liquid or solid media (agar plate) (Table 2). It was observed that siderophore production, phosphate solubility and IAA production of the *B. thuringiensis* 5O9 isolate were better than the other isolate, while ammonium production was lower. It was observed that the ammonium and siderophore production abilities, which are important for plant development, were high in our *Bacillus* isolates, in line with the data.

Table 1. Morphological and biochemical characteristics of the 5O5Y1 and 5O9 isolates used in the study.

Tests	5O5Y1	5O9	Tests	5O5Y1	5O9
Colony types	R	R	%10 NaCl	+	+
O_2 utilization	FA	FA	%15 NaCl	-	-
Oxidase	+	-	10°C	2+	+
Catalase	+	+	45°C	±	2+
Motility	-	-	pH 4.5	2+	+
Gram	+	+	pH 5.5	2+	+
Spore	Subterminal	Central	pH 8.5	2+	+
Indole	+	-	Lec	4+	4+
Methyl red	+	+	Gel	+	-
Citrate	-	-	Urease	-	-
KIA	A/A	Al/Al	Amylase	-	-
H_2S	-	-	Nitrate	-	+
Gas	-	-	Species	*B. mycoides*	*B. thuringiensis*

R; Rough colony, FA; Facultative anaerobe, A/A; Acid/Acid, Al/Al; Alkalide/Alkalide, In; Indole, MR; Methylred, Cit; Citrate, KIA: Three sugars, G; Gas, Nit; Nitrate, Lec: Lecithinase, Gel; Gelatinase, -/-; It does not reproduce in the nutrient medium. -; There is growth, but the test result is negative, ±; weak positivity. +; The result is positive, 4+; The result is very good

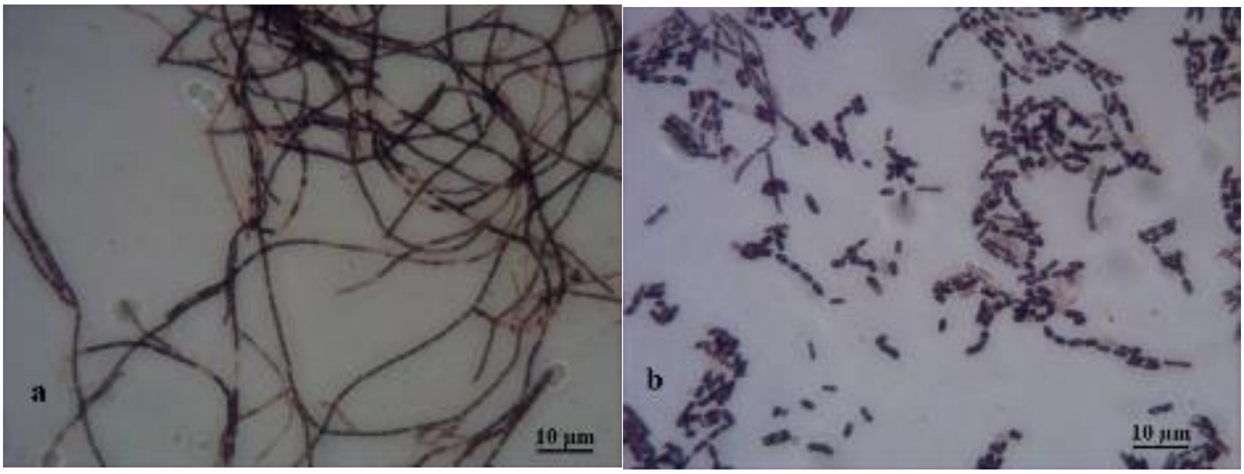

Fig. 1. Gram staining images of the isolates. **a.** *Bacillus mycoides* 5O5Y1, **b.** *B. thuringiensis* 5O9

Determination of Heavy Metal Tolerances of the Isolates

The growth characteristics of the strains in the study were tested on agar medium (1, 2.5, 5 and 10 mM) containing different concentrations of metal salts (Cu, Pb, Fe, Ag, and Zn). The isolates were found to have good growth potential even in environments with our highest metal concentration of 10 mM Cu, Pb, Fe, Ag, and Zn. It was determined that they showed the ability to grow only in lower concentrations in zinc environment (Table 3).

Our study observed heavy metal resistance in both 5O5Y11 and 5O9 isolates as Cu > Fe > Ag and Pb >Zn, respectively (Table 3). Agar wells and MIC methods determined copper salt's minimal inhibition concentration values in the isolates. It was observed that the tested isolates generally started to be affected in the presence of 6-12 mM Cu in solid media and were not affected at 3 mM. The MIC of the Cu concentration, which was observed to be the most resistant, was 12.5 mM (Table 4).

The study investigated the growth characteristics of the isolates in different pH environments (pH range 5.0-7.5) in the presence and absence of copper. The bacterial growth curve was not affected in the presence of 3 mM Cu in the medium, but it was observed that the growth decreased as the Cu concentration increased. Although the presence of Cu^{+2} slowed the mid-log stage of bacteria under the same medium conditions (pH: 7, 36±1°C), it did not affect the final bacterial concentration (Figs 2, 3).

Table 2. The results of the experiments for determination of plant growth-promoting properties of the two isolates.

Strain No	Sid(R/Z)	Phos(R/Z)	ACC	Amo	IAA acitivity
5O5Y1	12/16	4/-	-	3+	5.03±0.98
5O9	30/34	5/-	-	2+	11.66±0.29

R: bacteria reproduction; Z: halo zone diameter; (-); no activity; (++); positive activity; (+++); activity is very good for ammonium production. Siderophore (Sid.) production and phosphate (Phos.) solubility: 0-9 mm zone diameter; positive activity: 10-19 mm zone diameter; activity good: ≥20 mm zone diameter; activity excellent. IAA activity ≤0; No activity, 0-9; Activity positive, 10-15; Activity good, ≥16; Activity very good.

Table 3. The ability of two isolates to grow at different metal concentrations (1, 2.5, 5, and 10 mM).

Strain No	Ag	Fe	Cu	Pb	Zn
5O5Y1	≤10/ +	≤10/ 2+	≤10/ +	≤10/ +	≤5/ +
5O9	≤10/ +	≤10/2 +	≤10/ 3+	≤10/ +	≤2.5/ 2+

Bacterial growth in the presence of metal salts in the 1-10 mM range: (-); No bacterial growth (+); weak growth (2+); good growth (3+); excellent growth

Table 4. Determination of Cu^{+2} tolerance of *Bacillus* sp. strains used in the study.

	IZ (mm)						IV (mM/L)	
Strain	100 mM	50mM	25mM	12.5 mM	6.25mM	3.12 mM	MIC	MBC
5O5Y1	15	10	8	6	6	-	12.5±0	100
5O9	16	13	10	6	6	-	12.5±0	50
**B. subtilis* W168	16	14	14	10	6	-	6.3±0	50

MIC; Minimal inhibitory concentration, MBC; Minimal bactericidal concentration, IZ; Inhibition Zone, IV; Inhibition Values, *; Control Bacteria.

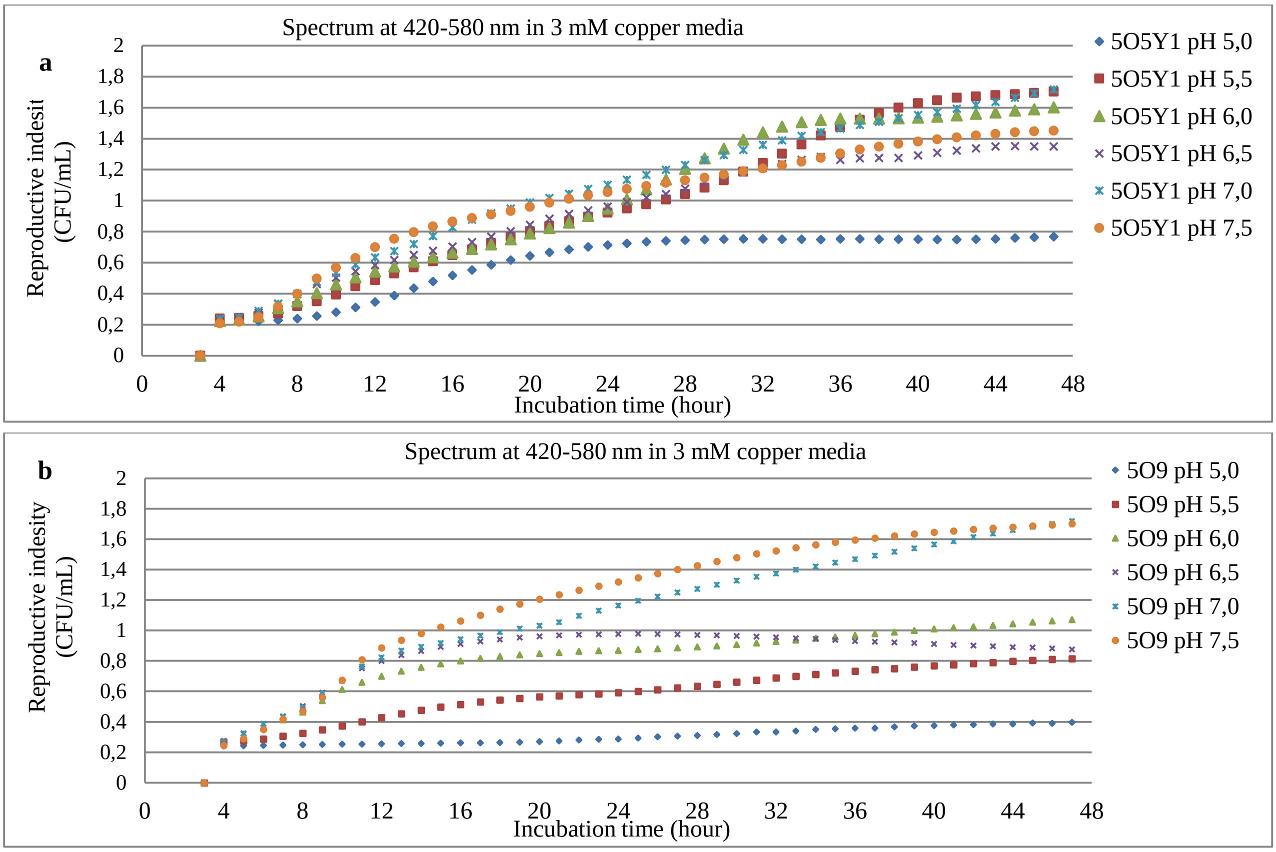

Fig. 2. Growth graph of *B. thuringiensis* 5O9 strain at different pH concentrations. **a.** Presence of Cu^{+2}, **b.** absence of Cu^{+2}.

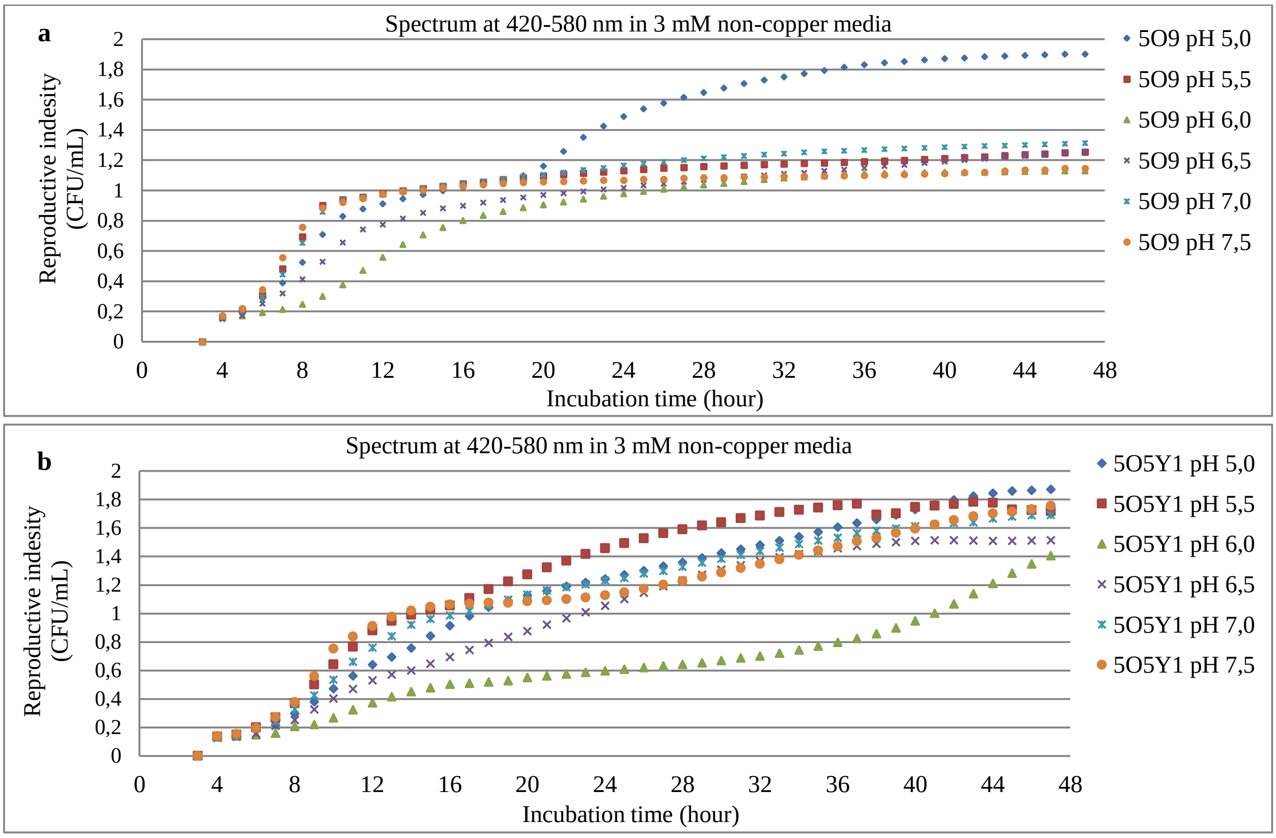

Fig. 3. Growth graph of *B. mycoides* 5O5Y1 strain at different pH concentrations. **a.** Presence of Cu^{+2}, **b.** absence of Cu^{+2}.

When the growth curve of the *B. thuringiensis* 5O9 strain was examined, it was observed that the growth curve at pH 6.0-6.5 in a metal-free medium reached the logarithmic stage late and the stagnation stage in 12-18 hours. At other pH values, it was observed that the logarithmic phase was completed in the first 8 hours, and the best growth was at pH 5.0, 5.5, and 7.0. The best growth was at 7.0-7.5 in the presence of metal, the logarithmic phase was prolonged compared to the metal-free environment, but the pause phase reached 36-40 hours. It was determined that the strain was highly negatively affected at other pH values, the most severely at low pH (5.0 and 5.5).

In our study, it was observed that the *B. mycoides* 5O5Y1 strain had a low growth density in the pH range of 6.0-6.5, and it reproduced better at other pH ranges. The logarithmic phase was fast in the first 10 hours, while it entered the stationary phase after the 40th hour. In the presence of metal, it was observed that little growth was at low pH (pH 5.0), and the logarithmic phase did not change much in density, reaching 40 hours at other pH values.

Discussion

Plant-associated bacteria are essential in adapting the host to the changing environment by altering plant cell metabolism or promoting plant growth (Ma *et al*. 2011). Features such as IAA production, ACC deaminase activity, siderophore production, phosphate solubility, ammonium production, and nitrogen fixation are among the mechanisms that promote plant growth under heavy metal stress conditions (Ullah *et al.* 2015).

Soil microorganisms can affect trace metal mobility and plant availability (Idris *et al.* 2004). Previous studies reported that metal-resistant isolates from different genera, such as *Pseudomonas* and *Bacillus*, produce IAA and plant growth-promoting factors (Rajkumar *et al*. 2012). In the study of Ahmad et. al (2008) on 72 PGPR isolates, solubilization of phosphate was commonly detected in 80% of those belonging to *Bacillus* sp., 74.47% of those belonging to *Azotobacter* sp., 55.56% of those belonging to *Pseudomonas* sp. and of those belonging to *Mesorhizobium* (16.67%). The authors also found that 12.77% of the *Azotobacter* isolates produced siderophores, followed by *Pseudomonas* and *Bacillus*, respectively (Ahmad et al. 2008). Similarly, our study showed that our metal-resistant *Bacillus* isolates produced important factors that promote plant growth, such as siderophore production, phosphate solubility, ammonium production and IAA production (Table 2).

The fact that the isolates included in the study have plant growth-promoting properties promises the potential to be local PGPR strains. Using bacterial strains in plant rhizospheres in agricultural applications represents a sustainable technology in many developed countries (Dupuy *et al*. 2018).

Many studies have investigated whether bacterial biosorbents effectively purify heavy metal-contaminated areas. Accordingly, PGPRs are widely used to inoculate plants as a biologically defined approach to increase the phytoremediation efficiency of toxic metals (Chen *et al*. 2017, Zornoza *et al*. 2017).

Microorganisms use various strategies to remove heavy metals from the environment. The preferred heavy metal removal methods of the genus *Bacillus* are biosorption, bioaccumulation and bioprecipitation (Shao *et al*. 2019). Allievi *et al*. (2011), subjected *Bacillus* species known to produce the S layer to a biosorption assay at high copper metal concentrations. As a result of the experiment, they observed that *B. sphaericus* and *B. thuringiensis* could absorb copper metal in high concentrations. They reported that both species absorb 40% of copper per milligram of bacteria. The *B. thuringiensis* 5O9 isolate we used in our study yielded similar results in terms of metal tolerance. Our *B. thuringiensis* 5O9 isolate grown on media containing heavy metals such as silver, iron, copper, lead, and zinc at different concentrations (1-10 mM). Similar to the study above, the isolate showed much better growth properties than other metals, especially in the presence of 10 mM copper (Table 3).

The growth curves of *B. altitudinis* MT422188 were examined in the presence and absence of copper. The presence of copper (0.1 mM Cu^{+2}) in the medium briefly prolonged the lag phase of bacterial cells and was reported not to stop growth (Khan *et al*. 2022). Another study with different metals also showed that members of the genus *Bacillus* can aim for the phytoremediation of nickel and support plant growth in nickel-contaminated soils (Ma *et al.* 2009).

Oves *et al*. (2013) investigated the metal absorption ability of *Bacillus thuringiensis* OSM29 isolate at varying concentrations of cadmium, chromium, copper, lead and nickel. The effect of some physicochemical factors, such as medium pH, initial metal concentration and contact time, on biosorption was evaluated. The optimum pH for removing nickel and chromium was 7, while an optimum pH of 6 was observed for cadmium, copper and lead. Similarly, it was observed that the *B.thuringiensis* 5O9 isolate grew best at pH 6.0-6.5 in copper metal-free medium and pH 7.0-7.5 in copper metal-containing medium. These studies revealed the importance of determining the effect of different pH and metal concentrations on the growth of bacteria.

The pH conditions can significantly affect the solubility of heavy metal ions and the charge in the cell. This effect significantly helps to remove heavy metal ions from the contaminated environment (Jin *et al*. 2018). It is reported that the pH (4-8) range is significant for all biomass (Sánchez-Clemente *et al*. 2020). In their study, Kalaycı *et al.* (2021) reported that approaching neutral pH values accelerates metal uptake by bacteria. Our study observed that both isolates could grow in broth media with pH values between 5.0 - 7.5. However, as seen from the Figs 2, 3, the presence of 3 mM Cu in the medium prolonged the logarithmic growth phase of the bacteria. The presence of Cu in the medium and a neutral pH positively affected bacterial growth.

Conclusion

It is known that bacteria that promote plant growth are one of the most critical components for agricultural applications in today's conditions. These plant growth-promoting bacteria are used successfully in various countries, and their agricultural market share is also expected to increase. According to our results, it was determined that our isolated bacteria could be resistant to different metal salts and environmental conditions and could also support plant growth. These bacteria may have significant potential for re-remediation of heavy metal-contaminated areas, but more research is needed.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

Author Contributions: Concept: Ü.Z.Ü.E., Ş.A.K., Design: Ş.A.K., Execution: Ü.Z.Ü.E., A.B., Ş.A.K., Material supplying: Ü.Z.Ü.E., E.U., Data acquisition: Ü.Z.Ü.E., A.B., Ş.A.K., Data analysis/interpretation: Ü.Z.Ü.E., A.B., Writing: Ü.Z.Ü.E., A.B., Ş.A.K., Critical review: A.B., Ş.A.K.

Conflict of Interest: The authors have no conflicts of interest to declare.

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