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Research Article

Effect of Different Metals on Synthesis of Siderophores by Endophyte Bacteria Isolated from Various Annual Plants

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Abstract: Endophyte bacteria are microorganisms that pass all or part of their life cycle in the tissues of healthy plants without causing any obvious signs of disease. Most siderophore-producing endophytic bacteria could improve the plant growth. Here, the effect of metals, iron (Fe), nickel (Ni), and cobalt (Co), on the growth and siderophore production profiles of 30 endophyte bacterial isolates were investigated. The results of the Minimum Inhibition Concentration (MIC) tests showed that endophytes exhibit varying degrees of tolerance to heavy metals and the metal tolerance decreased in the order Fe^{3+}>Ni^{2+}>Co^{2+}. It was revealed that while 10 isolates could not produce siderophores under any circumstances, 20 isolates produced siderophores at different degrees, and siderophore molecules synthesized and secreted by these 20 isolates had affinities for all three metals ($Fe^{3+,}$ Co^{2+}, and Ni^{2+}). In addition, siderophore production profiles of isolates under each heavy metal stress were investigated by adding these metals to the Chromium Azurol Sulfonate (CAS) medium at optimum concentration. The results suggested that siderophore synthesis could be one of the coping mechanisms of only two isolates with Co^{2+} and Ni^{2+} heavy metals. In the final stage of the study, molecular identification of a certain number of isolates selected according to their siderophore production values was carried out by 16S rRNA sequencing. As a result of the sequence analysis, 2 *Pseudomonas* sp., 4 *Bacillus* sp., 1 *Chryseobacterium* sp., 1 *Staphylococcus* sp., and 1 *Peribacillus* sp. were revealed.

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1. Introduction

Endophyte bacteria are microorganisms that spend all or part of their life cycle in/between plant tissues without causing disease symptoms in their host (Kumar et al., 2014). Endophytic bacteria (EBs) have been isolated and characterized from different plant parts, including roots, stems, leaves, seeds, fruits, and nodules of a wide variety of plants (Afzal et al., 2019). EBs can directly or indirectly contribute to plant health and development by improving nutrient and mineral cycles such as phosphate, nitrogen, and other nutrients (Santoyo et al., 2016). They are important for a variety of biotechnological

applications as they have the potential to produce a variety of secondary metabolites such as alkaloids, steroids, terpenoids, and flavonoids (Singh et al., 2017).

On the other hand, siderophores are extracellular secondary metabolites found in microorganisms, fungi, and plants that can contain very different chemical structures, generally known for their iron-binding properties. In other words, siderophores can be defined as iron-binding chelating agents synthesized by organisms in order to obtain iron from environments surrounded by iron starvation conditions (Hussein and Joo, 2014). It is also known that siderophores have the capacity to bind other metals besides iron (Hofmann et al., 2020). Previous studies have shown that siderophores can bind to metals other than iron such as silver, aluminum, cadmium, nickel, and mercury. Bacteria have developed various resistance mechanisms such as physical sequestration, exclusion, complexation, and detoxification, thus reducing their toxicity to tolerate heavy metal ion uptake (Rajkumar et al., 2010). There is also increasing evidence that metals other than iron can activate the synthesis of siderophores by bacteria, thereby implicating siderophores in the homeostasis of metals and especially heavy metal tolerance protecting bacteria against metal toxicity. (Schalk et al., 2011).

Biotechnological application areas of bacterial and fungal siderophore include medicine, pharmacology, bioremediation, biodegradation, and food industries besides agriculture. They can be used in agricultural applications to promote plant growth and also as biocontrol agents against plant pathogens (Rout et al., 2013). Heavy metals released from industrial plants to the environment pose an environmental threat if processes are not properly managed (Verma and Sharma, 2017). The use of siderophore-producing microorganisms in bioremediation practices, known as the use of organisms to clean polluted areas such as soil, water, and oceans, is gaining increasing attention (Braud et al ., 2009; Edberg et al., 2010). In addition, siderophores have the potential to be used to treat various diseases or improve human health. The first and most studied aspect of siderophore biotechnology is the treatment of iron overload during transfusion due to non-hemorrhagic conditions (Ribeiro et al., 2022). When siderophores combine with components such as metal ions, antibiotics, targeting ligands, and nanoparticles, they acquire many functions in imaging, sensors, or therapeutics (Fan and Fang, 2021).

The unique ability allows siderophores to remove heavy metals from contaminated environments, thereby facilitating their bioremediation. Therefore, it is important to screen and find the microorganisms producing siderophores from nature and to reveal the relations of these siderophores with non-ferrous metals. In this study, it was aimed to investigate the tolerance of 30 endophyte bacterial isolates, which were previously isolated from some cultivated and wild cereal plants (Poaceae family) against cobalt (Co^{2+}), nickel (Ni^{2+}), and iron (Fe^{3+}); to reveal the siderophore production profiles and capacities of these bacteria under cobalt (Co^{2+}) and nickel (Ni^{2+}) stress other than iron (Fe^{3+}).

2. Material and Methods

2.1. Bacterial isolates

In this study, endophyte bacteria isolated from some cultivated and wild cereal plants (Poaceae family) in and around Van province, which are in the stocks of Van Yuzuncu Yil University, Faculty of Agriculture, Department of Plant Protection, Bacteriology laboratory were used.

2.2. Determination of minimum inhibition concentration (MIC) values for cobalt (Co), nickel (Ni), and iron (Fe) elements of isolates

The Minimum Inhibitory Concentration (MIC) values for each metal ion of the isolates were determined by gradually increasing the heavy metal concentrations in the solid media medium until the isolates could not grow. For this, $CoCl_2$, $NiSO_4$, and $FeCl_3$ stock solutions were prepared and sterilized by filtration method. Nutrient Agar (NA) media containing $CoCl_2$, $NiSO_4$, and $FeCl_3$ increasing in 0.1 mM intervals were prepared by using these stocks. In order to prepare NA nutrient media containing the relevant concentrations of metals, the required volume of the relevant metal stock was added to the nutrient media sterilized by autoclave, cooled to 60-70 ºC, and mixed and poured into petri dishes under aseptic conditions. Suspensions of EB isolates were prepared at a density of 10^6 cfu/ml. 20 µl of these suspensions were taken and inoculated in the NA plates by dripping at points equidistant from each other. MIC values were noted when isolates did not grow in petri dishes even after 10 days of incubation.

2.3. Determination of siderophore activities

2.3.1. Chrome Azurol S (CAS) test

Before starting the experiment, the glassware was rinsed with 3 M hydrochloric acid (HCl) to remove iron and then washed in deionized water (Cabaj and Kosakowska, 2009). CAS reactive dye was prepared according to Schwyn and Neilands (1987). The ability of bacterial isolates to produce siderophores was determined using a modified version of the original universal CAS-siderophore test developed by Schwyn and Neilands (1987) (Arora and Verma, 2017). CAS agar plates were prepared by mixing 100 ml of separately sterilized CAS reagent solution into 900 ml of sterilized Luria Broth (LB) agar medium. The final pH of the CAS reactive dye and LB agar medium was slowly brought to 6.8 using NaOH and HCl before autoclaving.

The isolates obtained as a pure culture were transferred to CAS Agar medium by spot inoculation technique and incubated at 25ºC for 7 days. Orange colored zones formed around the colonies were evaluated as positive isolates for the CAS test (Payne, 1994). All analyses were performed in four replicates.

2.3.2. Determination of the ability of siderophores to bind Co^{2+} and Ni^{2+} metals other than Fe^{3+}

Chrome azurol S (CAS) reagent solution containing different metals (Co and Ni) was prepared to screen the ability of siderophores to bind cobalt and nickel metals other than iron (Mehnert et al., 2017; Hofmann et al., 2021). CAS reactive dye was prepared according to Schwyn and Neilands (1987), but instead of 1 mM $FeCl_3$ in the mixture, the same concentration (1 mM) $CoCl_2$ or $NiSO_4$ was used. Chrome Azurol Sulfonate (CAS) dye forms a complex with Co^{2+} or Ni^{2+} instead of $Fe^{3+,}$ and in the presence of a metal-chelating agent (siderophore), the specified reaction takes place, free dye is released and the blue color turns orange.

The isolates obtained as a pure culture were transferred to CAS Agar medium by spot inoculation technique and incubated at 25 ºC for 7 days. Orange colored zones formed around the colonies were evaluated as positive isolates for the CAS test (Payne, 1994). All analyses were performed in four replicates.

2.3.4. Determination of siderophore production profiles of isolates under metal stress

In order to examine the siderophore production profiles of the isolates under metal stress, the final concentrations of these metals in CAS media were determined by considering the MIC values of the isolates against each metal. Final concentrations in the media were determined as 0.3 mM for $CoCl_2$, 0.8 mM for $NiSO_4$, and 1 mM for $FeCl_3$. In order to prepare CAS-LB agar media containing metals at the specified concentrations, CAS-LB agar media were prepared by adding metals from previously prepared sterile stock solutions to the media to the final concentrations specified. In addition, CAS-Fe reactive dye was used for standardization in all the media prepared in this part of the study.

The isolates obtained as a pure culture were transferred to CAS Agar medium by spot inoculation technique and incubated at 25 ºC for 7 days. Orange colored zones formed around the colonies were evaluated as positive isolates for the CAS test (Payne, 1994). All analyses were performed in four replicates.

For isolates, the siderophore production index (SI) was calculated using measurements taken at the end of the incubation periods. Siderophore production indices were expressed as the ratio of the mean zone diameters measured in the relevant test to the mean colony diameters, based on the method first used as the extracellular enzyme production index (Carrim et al., 2006; Doğan and Taşkın, 2021).

2.4. Genotypic characterization of selected isolates

After determining siderophore activities, 10 isolates were selected for diagnosis processes, giving successful and different SI values. The selected strains were identified by 16S rRNA gene sequencing. DNA isolation was performed by the method modified from Govindarajan et al. (2007) and 16S rRNA was amplified in polymerase chain reaction (PCR) using genomic DNA as a template and bacterial universal primers, 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'- TAC GGT TAC CTT GTT ACG ACT T-3') (Frank et al., 2008). A 50 µL reaction mixture contained 2.5 U Taq polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.3 mM dNTPs, 25 mM $MgCl_2$, 20 pmol

of each primer, 5 µL of 10 x reaction buffer (Thermo Fisher Scientific), and 20 ng of template DNA. The step-up PCR procedure included denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. Amplification products were electrophoresed on a 1.5% agarose gel in 1 x TBE buffer.

The 16S rRNA gene sequencing was performed by BM Labosis Biotechnology Company (Türkiye) using the Sanger platform. The sequences obtained were analyzed using the database on the website "https://www.ezbiocloud.net/", and then the sequences were logged in to the GenBank site and accessed "Accession" numbers (Table 3).

2.5. Statistical Analysis

All enzyme measurement experiments were performed in four replicates and each petri measurement was repeated twice. Statistical Analysis System (SAS version 9.4 SAS, Cary, NC) was used to analyze the data. General linear model (GLM) analysis was used to determine differences between the averages of the groups, and Duncan multiple comparison test was used to determine differences between the groups. P values <0.05 were considered statistically different.

3. Results

3.1. Determination of minimum inhibition concentration (MIC) values for cobalt (Co), nickel (Ni), and iron (Fe) elements of isolates

The Minimum Inhibitory Concentration (MIC) values for each metal ion were investigated in an LB solid nutrient medium for a total of 30 isolates grown at 25 °C. Heavy metal concentrations in the solid media were determined by increasing them gradually in the range of 0.1 mM increments for each metal until the isolates could not grow. For all the isolates, the MIC values for each metal are given in Appendix A. The results revealed that no isolates could grow in the media containing 0.9 mM and above $CoCl_3$. Although the isolates showed different MIC values for cobalt metal, only one isolate was observed with the highest value of 0.9 mM, and 10 isolates with the lowest value of 0.4 mM. According to the results, 20 isolates could not grow at 1.1 mM and higher $NiSO_4$ concentrations. Only 2 isolates grew up to 2.6 mM, making them the most resistant to nickel. While the lowest inhibition value for iron belonged to only one isolate with 1.1 mM, 18 isolates could not grow at iron concentrations of 2.9 mM and above (Appendix A).

3.2. Determination of the ability of siderophores to bind Co^{2+} and Ni^{2+} metals other than Fe^{3+}

At this stage of the study, three types of solid LB media containing CAS reactive dye prepared with $FeCl_3$, $CoCl_2$, and $NiSO_4$ metals were prepared and the isolates were inoculated into these media. Yellow-orange zones formed around the colonies, which were incubated for 7 days at 25 ºC, were evaluated as siderophore formation and as positive isolates for the CAS test (Payne, 1994). At this stage, no metal was added to the nutrient media, except for the CAS reactive dye. Therefore, no metal stress was created.

Here, it was aimed to test the ability of the siderophore molecules produced by the isolates to meet their Fe^{3+} needs, to bind to Co^{2+} and Ni^{2+} heavy metals instead of Fe^{3+}. Instead of Fe^{3+}, Co^{2+} or Ni^{2+} metals form a complex with CAS dye and then the siderophore molecules secreted by the isolates bind the metals, resulting in the release of the free dye, which creates a yellow-orange zone around the isolate. (Figure 1). According to the siderophore production index (SI) data created using the zone and colony diameters obtained at the end of incubation, it was observed that 10 isolates with code numbers G119S1, G25K3, G79Y2, G35S1, G6Y2, G9Y2, G12S1, G101K4, G53Y2 and G135Y4 did not produce siderophores in all three media (Table 1). It was revealed that 20 isolates other than these isolates produced siderophores with different SI values (Table 1). In addition, it was seen that the siderophore molecules synthesized and secreted by these 20 isolates have affinities for all three metals ($Fe^{3+,}$ $Co^{2+,}$ and Ni^{2+}).

Table 1. Average siderophore production index (SI) values of isolates in LB medium containing Chrome azurol S (CAS) reagent solution (dye) prepared with $NiSO_4$, $CoCl_2$, and $FeCl_3$

		CAS-Fe	CAS-Co	CAS-Ni
Isolate No	**Codes of the Isolates**	**SI**	**SI**	**SI**
1	G119Y2T	1.51 ± 0.67^{g}	1.31 ± 0.02^{i}	1.52 ± 0.02^{hi}
2	G88K1	2.47 ± 0.02^{d}	3.33 ± 0.00^{ab}	3.21 ± 0.13^{abc}
3	G119S1	-	-	-
4	G120S3	1.55 ± 0.01^{g}	2.57 ± 0.08^{cdef}	3.76 ± 0.12^{a}
5	G32S2	2.00 ± 0.00^{ef}	2.83 ± 0.07^{bcd}	3.20 ± 0.02^{abc}
6	G25K3	-	-	-
7	G30S1	1.53 ± 0.03^{g}	1.33 ± 0.05^{i}	1.39 ± 0.06^{i}
8	G47K1	1.62 ± 0.13^{g}	2.88 ± 0.00^{bcd}	1.76 ± 0.19^{ghi}
9	G56Y1	2.96 ± 0.20^{ab}	3.05 ± 0.23^{bc}	2.31 ± 0.07^{efg}
10	G33Y3	3.18 ± 0.33^{ab}	3.07 ± 0.14^{bc}	3.15 ± 0.19^{abc}
11	G88S1	1.50 ± 0.00^{g}	2.30 ± 0.12^{efg}	2.45 ± 0.05^{def}
12	G20Y3	1.38 ± 0.01^{g}	1.67 ± 0.11^{hi}	1.40 ± 0.10^{i}
13	G115S1	2.33 ± 0.06^{de}	2.91 ± 0.09^{bcd}	3.23 ± 0.23^{abc}
14	G24Y1	1.66 ± 0.08^{fg}	2.62 ± 0.16^{cdef}	2.35 ± 0.49^{efg}
15	G79Y2	-	-	-
16	G35S1	-	-	-
17	G111K3	1.63 ± 0.04^{g}	1.67 ± 0.04^{hi}	1.86 ± 0.00^{fghi}
18	G37K1	2.23 ± 0.05^{de}	2.67 ± 0.17^{cde}	2.81 ± 0.38^{cde}
19	G6Y2	-	-	-
20	G45K1	1.56 ± 0.06^{g}	2.39 ± 0.11^{defg}	2.47 ± 0.13^{def}
21	G9Y2	-	-	-
22	G15S1	3.24 ± 0.10^{a}	3.59 ± 0.28^{a}	3.29 ± 0.10^{abc}
23	G12S1	-	-	-
24	G101K4	-	-	-
25	G99K3	3.00 ± 0.00^{ab}	2.00 ± 0.00^{gh}	3.00 ± 0.00^{bcd}
26	G53Y2	-	-	-
27	G111K1	2.83 ± 0.17^{bc}	3.00 ± 0.50^{bc}	3.33 ± 0.33^{abc}
28	G105Y1B	2.55 ± 0.22^{cd}	2.12 ± 0.13^{fgh}	2.01 ± 0.10^{fgh}
29	G45Y1	1.27 ± 0.14^{g}	2.05 ± 0.09^{gh}	3.55 ± 0.10^{ab}
30	G135Y4	-	-	-
p values		<0.001	<0.001	<0.001

* Siderophore Production Indices (SI) were calculated as the ratio of the mean zone diameters measured in the relevant test to the mean colony diameters. All measurements were made in triplicate.
**Within the same column, the difference between groups expressed with different letters is statistically significant ($p<0.05$) (Mean ± Std. Error).
***- means no siderophore production.

3.3. Determination of siderophore production profiles of isolates under metal stress

At this stage of the study, it was aimed to examine the siderophore production profiles of the isolates under metal stress. For this purpose, considering the MIC values of the isolates against each metal, the final concentrations in the nutrient media were determined as 0.4 mM for $CoCl_2$, 0.8 mM for $NiSO_4$, and 1 mM for $FeCl_3$. Siderophore production index (SI) data were created using the zone and colony diameters obtained at the end of the incubation (Table 2).

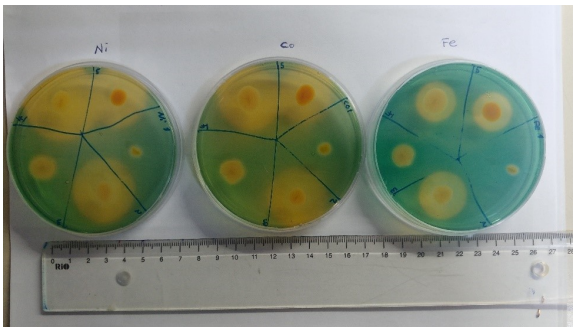

Figure 1. The sample photograph of the siderophore CAS test made with isolates with the same code number, from left to right, in a solid LB medium containing CAS reactive dye prepared with $NiSO_4$, $CoCl_2$, and $FeCl_3$, respectively.

1 mM $FeCl_3$ in CAS-LB+Fe, CAS-LB+Co+Fe, and CAS-LB+Ni+Fe nutrient media was used to eliminate siderophore production in the presence of ready, usable Fe^{3+} in the nutrient media for the isolates. In this way, siderophore production in these nutrient media would only be associated with heavy metals, Ni^{2+} and Co^{2+}. Interestingly, only two isolates, G56Y1 and G33Y3, were not affected by this rule and produced very high zones in all three media containing 1 mM usable Fe^{3+}, giving considerable SI values (Table 2). On the other hand, isolates with code numbers G111K1 and G105Y1B could not produce siderophores as expected in CAS-LB+Fe nutrient medium containing only 1mM $Fe^{3+,}$ but they gave significant SI values in CAS-LB+Co+Fe and CAS-LB+Ni+Fe medium containing the same amount of Fe^{3+} (Table 2). The isolates other than these did not produce siderophores, although they formed colonies in iron-containing media as expected.

3.4. Genotypic characterization of selected isolates

After the determination of the siderophore production indices (SI) given under heavy metal stress, 8 promising isolates (G32S2, G56Y1, G33Y3, G115S1, G111K1, G105Y1B, and G45Y1) and 1 non-siderophore producing isolate (G53Y2) were analyzed with 16S rRNA sequencing selected for identification. As a result of the comparative data analysis using the database, 16S rRNA gene region sequences of 8 isolates, 2 *Pseudomonas* sp. (G115S1, G45Y1), 3 *Bacillus* sp. (G15S1, G56Y1, G33Y3, G105Y1B), 1 *Chryseobacterium* sp. (G32S2), 1 *Staphylococcus* sp. (G111K1) and 1 *Peribacillus* sp. (G53Y2) gave results belonging to the genus (Table 3).

Table 2. Average siderophore production index (SI) values of isolates in LB media under different metal stress

		CAS-LB	CAS-LB+Co	CAS-LB+Ni	CAS-LB+Fe	CAS-LB+Co+Fe	CAS-LB+Ni+Fe
Isolate No	**Codes of the isolates**	**SI**	**SI**	**SI**	**SI**	**SI**	**SI**
1	G119Y2T	1.44 ± 0.06^{kl}	1.29 ± 0.08^{h}	1.42 ± 0.08^{hij}	-	-	-
2	G88K1	2.62 ± 0.09^{ef}	1.88 ± 0.37^{defgh}	3.71 ± 0.21^{abc}	-	-	-
3	G119S1	-	-	-	-	-	-
4	G120S3	2.01 ± 0.01^{ghi}	-	1.22 ± 0.06^{j}	-	-	-
5	G32S2	1.89 ± 0.03^{ghijk}	3.32 ± 0.19^{ab}	1.85 ± 0.14^{ghij}	-	-	-
6	G25K3	-	-	-	-	-	-
7	G30S1	1.73 ± 0.18^{hijkl}	1.47 ± 0.04^{fgh}	1.34 ± 0.08^{ij}	-	-	-
8	G47K1	2.17 ± 0.31^{gh}	2.21 ± 0.20^{cde}	2.00 ± 0.31^{fghi}	-	-	-
9	G56Y1	3.07 ± 0.04^{cd}	3.23 ± 0.29^{ab}	2.93 ± 0.61^{de}	3.49 ± 0.08^{a}	2.55 ± 0.31^{a}	2.97 ± 0.06^{a}
10	G33Y3	3.83 ± 0.21^{b}	3.67 ± 0.42^{a}	3.29 ± 0.42^{bcd}	3.31 ± 0.19^{a}	2.66 ± 0.11^{a}	2.57 ± 0.16^{ab}
11	G88S1	1.54 ± 0.00^{jkl}	1.51 ± 0.00^{fgh}	1.57 ± 0.00^{ghij}	-	-	-
12	G20Y3	1.66 ± 0.04^{ijkl}	2.05 ± 0.23^{defg}	1.34 ± 0.03^{ij}	-	-	-
13	G115S1	2.34 ± 0.04^{fg}	1.55 ± 0.32^{efgh}	3.89 ± 0.20^{ab}	-	-	-
14	G24Y1	1.71 ± 0.11^{hijkl}	2.15 ± 0.05^{def}	1.48 ± 0.11^{ghij}	-	-	-
15	G79Y2	-	-	-	-	-	-
16	G35S1	-	-	-	-	-	-
17	G111K3	1.72 ± 0.17^{hijkl}	1.88 ± 0.15^{defgh}	1.87 ± 0.08^{ghij}	-	-	-
18	G37K1	1.94 ± 0.21^{ghij}	2.27 ± 0.30^{cd}	2.07 ± 0.15^{fgh}	-	-	-
19	G6Y2	-	-	-	-	-	-
20	G45K1	2.02 ± 0.14^{ghi}	1.86 ± 0.07^{defgh}	1.83 ± 0.10^{ghij}	-	-	-
21	G9Y2	1.37 ± 0.01^{l}	-	1.61 ± 0.19^{ghij}	-	-	-
22	G15S1	3.46 ± 0.07^{bc}	2.84 ± 0.06^{bc}	3.23 ± 0.27^{cd}	-	-	-
23	G12S1	-	-	-	-	-	-
24	G101K4	-	-	-	-	-	-
25	G99K3	5.00 ± 0.00^{a}	2.33 ± 0.00^{cd}	4.00 ± 0.00^{a}	-	-	-
26	G53Y2	-	-	-	-	-	-
27	G111K1	3.00 ± 0.29^{de}	2.40 ± 0.10^{cd}	2.61 ± 0.39^{ef}	-	1.80 ± 0.00^{b}	2.03 ± 0.26^{bc}
28	G105Y1B	2.33 ± 0.15^{fg}	1.86 ± 0.24^{defgh}	2.11 ± 0.10^{fg}	-	1.51 ± 0.05^{b}	1.60 ± 0.24^{c}
29	G45Y1	2.06 ± 0.10^{ghi}	1.41 ± 0.06^{gh}	1.26 ± 0.03^{j}	-	-	-
30	G135Y4	-	-	-	-	-	-
p values		<0.001	<0.001	<0.001	0.442	0.002	0.005

* Siderophore Production Indices (SI) were calculated as the ratio of the mean zone diameters measured in the relevant test to the mean colony diameters. All measurements were made in triplicate.

**Within the same column, the difference between groups expressed with different letters is statistically significant ($p<0.05$) (Mean ± Std. Error).

***- means no siderophore production.

Table 3. Comparative analysis of sequence analysis results using the EzBioCloud database and GenBank accession numbers

Code of the Isolates	Top-hit reference species	Top-hit reference strain	Similarity (%)	Coverage (%)	GenBank Accession Numbers
G32S2	*Chryseobacterium shigense*	DSM 17126	97.23	70.2	ON571627
G56Y1	*Bacillus siamensis*	KCTC 13613	99.03	77.0	ON571630
G33Y3	*Bacillus siamensis*	KCTC 13613	98.59	28.9	ON571628
G115S1	*Pseudomonas orientalis*	CFML 96-170	99.36	75.4	ON571626
G53Y2	*Peribacillus simplex*	NBRC 15720	100.00	75.4	ON571625
G111K1	*Staphylococcus pasteuri*	ATCC 51129	99.91	76.1	ON571624
G105Y1B	*Bacillus halotolerans*	ATCC 25096	99.91	76.4	ON571623
G45Y1	*Pseudomonas kilonensis*	DSM 13647	99.82	76.7	ON571629

4. Discussion

In case of iron starvation, many microorganisms secrete at least one type of siderophore to make the limited amount of iron soluble in their habitat and take it into the cell (Haas, 2003). These secondary metabolites produced by bacteria, fungi, and plants are chelating agents that provide iron uptake. Iron exists as an insoluble oxide hydrate compound in many habitats where oxygen is present (Schalk et al., 2011).

In this study, siderophore production profiles of 30 endophyte bacterial isolates under iron (Fe^{3+}), cobalt (Co^{2+}), and nickel (Ni^{2+}) stress were revealed for the first time. In order to create a stress environment in the nutrient media by using the relevant heavy metals, it was first aimed to determine the minimum inhibition concentration (MIC) values for the Co^{2+}, Ni^{2+}, and Fe^{3+} elements of the isolates. According to the MIC values, while the isolates were most negatively affected by cobalt, they tolerated the increasing concentrations of iron the most among the metals (Appendix A). Although there is currently no acceptable standard concentration value, bacteria that can grow at concentrations of metal ions of 1.0 mM and above can be considered resistant to the relevant metal to distinguish metal resistivity (Malik and Jaiswal, 2000; Tomova et al., 2015). According to this inference, while none of the isolates were resistant to cobalt, all of them were resistant to nickel and iron.

Determining the ability of siderophores synthesized by the isolates and secreted out of the cell to bind Co^{2+} and Ni^{2+} metals other than Fe^{3+}, before creating heavy metal stress *in vitro*, was the next goal of this thesis study. For this, the universal CAS reactive dye was prepared separately for each of the three metals, using the same concentration of the relevant metals, and added to the solid nutrient media. No other metal additions were made to the prepared nutrient media. The results are consistent with data from studies showing that the universal CAS test originally developed by Schwyn and Neilands (1987) can be used not only to detect the siderophores with Fe-CAS solution but also to test siderophores for their ability to bind other metal ions (Mehnert et al., 2017; Hoffman et al., 2021). At this stage of the study, it was observed that the SI values of isolates such as G88K1, G32S2, and G88S1 with CAS reagents prepared with Co^{2+} and Ni^{2+} were higher than those with Fe^{3+} (Table 1). However, these data are insufficient to conclude that siderophores bind to Co^{2+} and Ni^{2+} ions with higher affinity. Since the siderophore molecules bound to these two metals cannot meet the iron requirement, the possibility of the cells synthesizing and secreting more siderophores into the environment should be considered. The hypothesis that the isolates synthesized a metal chelating agent other than the siderophore and that the SI values were high for this reason was also evaluated as low probability since metal was not added to the nutrient medium at this stage and did not create a stress environment.

In the third stage of the study, siderophore production profiles of isolates under metal stress were investigated. For this purpose, the maximum metal concentrations that caused stress but did not have a toxic effect on the isolates were determined by taking into account the MIC values against each metal, and CAS-LB agar media were prepared. As seen in Table 2, the reason for adding 1 mM iron in addition to cobalt and nickel to CAS-LB+Co+Fe and CAS-LB+Ni+Fe media was to keep the usable iron ion concentration high and thus to stop the production of siderophores. In this context, G111K1 and G105Y1B coded isolates gave extraordinary results; while the production of siderophores was stopped in CAS-LB+Fe medium, they showed the ability to produce a remarkable CAS reaction in CAS-

LB+Co+Fe and CAS-LB+Ni+Fe nutrient media (Table 2). These results suggest that one of the coping mechanisms of these two isolates with Co^{+2} and Ni^{+2} heavy metals may be siderophore synthesis.

Another interesting result of this experiment is the SI values of isolates with code numbers G56Y1 and G33Y3. These isolates continued to show CAS reaction without being affected by 1 mM Fe+3 metal ion added to CAS-LB broth and gave very high SI values (Table 2). However, there are many studies in which the addition of Fe^{3+} at much lower concentrations to the medium stopped/suppressed the synthesis of siderophores in many microorganisms. For example, in a study examining the effects of growth conditions on siderophore-producing bacteria, it was revealed that Fe^{3+} concentration increased up to 50 µM inhibited siderophore synthesis without affecting the growth rate in bacteria (Sinha et al., 2019). In a study conducted by Machuca and Milagres (2003), they observed that only *Aspergillus niger* fungi formed a CAS reaction (zone formation) in solid media even in the presence of 4 mM Fe^{3+} concentration in experiments conducted with various fungal species. It is known that the biosynthesis of siderophores is regulated by the iron content of the medium and inhibited in the presence of excess iron (Neilands, 1993; Machuca and Milagres, 2003). Positive results at high iron concentrations suggest the presence of a non-siderophore compound or a chelator other than a siderophore that reacts with the CAS reagent. For example, since *A. niger* is known to be a good producer of citric and oxalic acids, these organic acids might likely have reacted with CAS at high iron concentrations (Machuca and Milagres, 2003). In addition, the release of organic acids in response to iron-deficiency stress conditions has been documented for Neurospora crassa. It was thought that the acids secreted by these fungi interact with the iron concentrated on the cell surface, making the iron soluble, making it suitable for use by fungi (Winkleman, 1979; Guerinot et al., 1990). It should be taken into account that a similar scenario may also be valid for our isolates with code numbers G56Y1 and G33Y3.

At the last stage of our study, 8 isolates that we successfully molecularly identified were grouped into three main branches *Firmucutes* (*Bacillus* sp., *Peribacillus* sp., *Staphylococcus* sp.), *Proteobacteria* (*Pseudomonas* sp.), and *Bacteroidetes* (*Chryseobacterium* sp.). 16S rRNA gene sequences alone may not be sufficient to identify a new species, but it is the first and strongest indicator that a new species has been isolated (Tindall et al., 2010). Depending on the taxonomic group investigated, 16S rRNA sequence similarity between 98.2% and 99.0% seems reasonable as a threshold for discovering a new species (Meier-Kolthoff et al., 2013). It is well known that digital DNA hybridization (DDH) and 16S rRNA gene sequence similarities are not linear, and DDH values obtained for a given 16S rRNA gene sequence similarity value can differ significantly (Keswani and Whitman, 2001). In line with all these data obtained in recent years, it is a possibility that our G32S2 isolate, which matches *Chryseobacterium shigense* with a 97.23% similarity rate, may be a new species, and therefore polyphasic studies will be needed.

Conclusion

The detoxifying effect of siderophores or siderophores-producing MOs has been used for bioremediating metal pollution. After chelated by siderophores, metals can be sequestered through different extracellular mechanisms, such as biosorption and bioaccumulation. Also, siderophores have received much attention in recent years because of their potential roles and applications in various areas of environmental research such as biocontrols, biosensors, and bioremediation. Their ability to bind various metals in addition to iron makes siderophores important in a wide variety of biotechnological fields.

This study is the first study to characterize endophyte bacterial isolates isolated from some cultivated and wild cereal plants (Poaceae family) from certain regions of the Van Lake basin in terms of siderophores, which have important application areas in the biotechnological and health sector.

Ethical Statement

Ethical approval is not required for this study.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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Author Contributions

BT designed the research. BT and ŞA conducted experiments, analyzed data, wrote and revised the manuscript. Both authors read and approved the manuscript.

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