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Article: **Selenium Resistant Bacilli and Pseudomonas as Potential Candidate for Selenium and Iron Biofortification in Maize Plants**

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Selenium Resistant *Bacilli* and *Pseudomonas* as Potential Candidate for Selenium and Iron Biofortification in Maize Plants

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Abstract
Selenium is an essential element and is required in minute quantities for performing vital functions in living cells. Food is the ultimate source of selenium for animal and human populations. Crops, such as maize, which are used as food and feed, can be biofortified with selenium to alleviate selenium deficiency in both populations. The current study was conducted to isolate selenium-resistant bacteria from soil samples. Isolated bacteria were characterized on a morphological and biochemical basis. For specie level classification, 16S rRNA sequences were obtained. Isolated strains belonged to *Bacillus halotolerans* (TM3), *Pseudomonas protegens* (TM5), and *Bacillus endophyticus* (TM7). In-vitro PGPB characterization showed that some of the strains can produce IAA, Ammonia, HCN, and phosphate solubilization enzymes. Greenhouse pot experiments showed that the isolates enhanced seed germination rate, shoot length, and plant dry weight. Selenium supplementation caused decreased growth, but its effect was mitigated by the inoculation of isolated bacteria. Inoculation of these bacteria enhanced selenium content in maize leaves and shoots, ranging from 6-7%, while the addition of selenium to the soil increased selenium content by 300%. The iron content of maize leaves was also increased up to 17% in the inoculated strains.

1. Introduction

Selenium is an essential element with an extensive range of biological functions. They have catalytic, structural, and regulatory roles in living organisms. Selenium in the form of Selenoamino acid is incorporated into the proteins to assist other enzymes and hormones in physiological and biochemical reactions. More than 30 such selenium-containing proteins have been identified in living organisms, including glutathione peroxidase, thioredoxine reductase, 5-iodothyronine deiodinase, and selenoprotein P. These proteins help in reduction-oxidation homeostasis, thyroid hormone activation, protection from reactive oxygen species, and removal of heavy metals and their toxic effects [1]. In addition to the above roles, selenium is used as a supplement to increase the productivity of farm animals and poultry.

Selenium is required in trace amounts in the body. Daily consumption of less than 30 µg of selenium results in deficiency, whereas intake of greater than 400 µg causes toxicity in humans. Conversely, selenium intake of 100 µg/kg dry mass is recommended for cattle [2]. Iron is another micronutrient that is essential for living organisms. Iron in the body performs a large number of biological functions. Iron-containing proteins control the transport of oxygen, DNA synthesis, electron transport chain, and cell cycle progression. Iron deficiency is highly prevalent globally; more than 1.2 billion people are affected by anemia due to iron deficiency [3]. Hemoglobin and myoglobin are the two proteins with iron as the central functioning unit. Iron deficiency anemia is also prevalent in young calves due to low iron in milk, which is the only food source for them, while fodder is the major source of iron for cows [4]. Maize is one of the most important crops in the world. In 2020-21, the volume of corn production reached 1,125 million metric tons [5]. Maize is used to produce grain and fodder for human and animal consumption. In Pakistan, it is the third most important cereal crop after rice and wheat. It serves a multipurpose role in Pakistan, since it is used as food, feed, and fodder. In the last five years, the area of maize cultivation showed a rise of 5%; whereas, its production increased by 38% during the same period [6]. Maize is a nutritive fodder with a high amount of starch, proteins, oil, and fiber, so its use as fodder is increasing significantly in Pakistan. Punjab and KPK are the major producers of maize. The concentration of trace elements, such as iron and selenium, in plants depends on bioavailable ions in the soil. This bioavailability in the soil is affected by physiological factors such as pH, cation exchange capacity and redox potential [7]. Biofortification is the strategy to improve the nutritional status of crops and animal feed. The most common source of these trace elements is the plants, followed by meat [8]. So, in order to alleviate iron and selenium deficiency in humans and animals, crops must be biofortified with these elements. Fertilizers, plant breeding, biotechnological approaches, and beneficial microbes are used for biofortification. Research groups have reported biofortification of maize with zinc [9], selenium [10], provitamin A [11], and iron [12]. Fertilizers are expensive and lead to problems, such as eutrophication; whereas, plant breeding and biotechnological approaches need many studies before application in the field. In such conditions, microbe assisted biofortification can be applied for better yield. Millions of microbes are present in soil and are significant for plants. Free-living or symbiotic bacteria that stimulate plant growth directly or indirectly are called PGPB (Plant growth-promoting bacteria) [13]. These microbes assist plants in growth by different mechanisms such as IAA production, ammonia production, soil reclamation, suppression of pathogenic microorganisms, nutrient mobilization, and phosphate solubilization [14]. These microbes are an essential tool for producing biofortified crops. Many microbes have been used for producing biofortified foods, such as Bacillus *altitudinis* WR10 for iron fortification of wheat [15], zinc solubilizing bacteria BMRR126 and BMAR64 for zinc fortification of rice, and endophytic selenobacteria for selenium biofortification in *Glycine max* [16]. This study aimed to isolate selenium resistant bacteria from soil contaminated with insecticides. Subsequently, the bacteria are characterized morphologically, physiologically, biochemically and phylogenetically. Next, the bacteria are screened for PGPB characteristics and their

effect on maize plant growth, selenium content, and iron content of these plants.

2. Methodology

In the current study, selenium-resistant bacteria were isolated from soil samples collected from banks of waste drains and fields contaminated with insecticide (Figure 1). These bacteria were classified on a biochemical and molecular basis. PGP characters, such as IAA production, phosphate solubilization, HCN production, and ammonia production, were studied. Greenhouse experiments were performed by inoculating these isolated bacteria to the maize seeds. Growth parameters, such as seed germination, shoot length, and dry weight, were also studied.

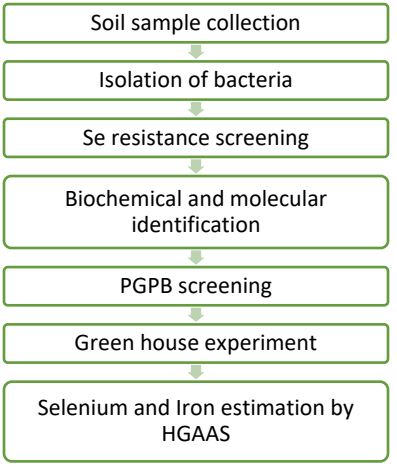


Figure 1: Graphical representation of methods followed in current study

2.1. Isolation of Bacteria from Soil

Five soil samples were collected from the citrus research institute, Sargodha (32°07'04.2"N 72°40'36.2"E) contaminated with insecticides and pesticides. These soil samples were transferred to the Institute of Microbiology and Molecular Genetics in sterile canisters. A 1g soil sample was diluted in distilled water and spread on LB agar plates. Well isolated colonies were further purified for assessing selenium resistance.

2.2. Qualitative and Quantitative Screening of Selenium Resistance

For qualitative screening of selenium resistance, isolated bacteria were streaked onto LB agar plates supplemented with 5mM sodium selenite. Only selenium resistant bacteria were able to grow on the supplemented agar, which formed characteristic red coloured colonies. These bacteria were further used to conduct a quantitative analysis by finding minimum inhibitory concentration, beyond which bacteria were unable to grow. LB broth tubes were prepared with sodium selenite concentrations 10, 20, 40, 80, 160, 320, 640, and 1280 mM, respectively.

2.3. Characterization of Isolates

Isolated strains were further classified based on morphological, physiological, biochemical, and molecular characteristics. Gram staining, catalase, oxidase, IMViC, starch hydrolysis and sugar fermentation tests were performed. After biochemical characterization, isolated strains were sent to Macrogen for partial 16S rDNA sequencing by NGS. Sequences with maximum homology were aligned using Clustal W, while the Neighbor-joining method was used to infer their evolutionary history [17]. MEGA X was used for this evolutionary analysis [18].

2.4. In-vitro Screening of Selenium Resistant Bacteria for Plant Growth Promotion

Plant growth-promoting traits such as Auxin bio-synthesis, Phosphate solubilization, Ammonia production, and HCN production were studied for the isolated strains. For IAA production, Auxin biosynthesis method described by Patten and Glick [19] was utilized. Overnight

fresh cultures were inoculated into DF salts minimal medium with and without L-tryptophan. After 42 hours of incubation supernatant from respective media was mixed with Salkowski's reagent. After 20 minutes of incubation, absorbance was measured at 535 nm wavelength. For phosphate solubilization, Gaur's method [20] was used with little modification. Fresh bacterial culture was streaked onto Pikovskaya's medium and incubated at 37 °C for 7 days. At the end of incubation, agar plates were observed for a clear zone surrounding the bacterial colonies. For Ammonia production, a method described by James and Natalie [21] was used. Bacterial cultures were inoculated in peptone water and incubated at 28 °C for 72 hours. After incubation, the culture was mixed with Nessler's reagent and colour change was observed. Brown to yellow color change indicated ammonia production due to bacterial culture. Lorck's method [22] was used to conduct the the HCN production test.

2.5. Pot Experiment in Green House

YH-1898 variety of *Zea mays* was obtained from Punjab Seed Corporation, Lahore, Pakistan for pot experiment. These experiments were conducted in the agricultural area of the Institute of Microbiology and Molecular Genetics at the University of the Punjab, Lahore (31.4932° N, 74.2972° E). These experiments started in March 2017. Eight kg of natural garden soil was filled in pots of diameter 12" and height 14". Bacterial cultures were inoculated in flasks containing 100 ml LB broth and incubated in a shaking incubator at 37 °C for 24 hours at 150 rpm. OD value of the culture was adjusted to 1 at a wavelength of 600 nm as mentioned by Yasin et al. [23]. This culture was then used to soak surface sterilized (01% $HgCl_2$) maize seeds. After preincubation of 20 min, these seeds were transferred to their respective pots. Pots were watered regularly, every second day, with 500 ml of water per pot. Similarly, for Selenium supplementation, sodium selenate, equivalent to 3 mg Se kg^{-1} of soil, was added to each pot. For the Second Se supplementation, one liter of 300 µM sodium selenate solution, equivalent to 3 mg Se kg^{-1} of soil, was added to each pot after spike formation as described by Yasin et al. [23].

2.6. Iron and Selenium Estimation

After 15 weeks of cultivation, plants were harvested and oven-dried at 50 °C until no further change in the mass was observed. These dried samples were then processed for acid digestion. 5 grams of maize leaves and stem were crushed in mortar and pestle to form a fine flour. One gram of this flour was soaked in 5ml of concentrated HNO_3 and left overnight. The next day mixture was warmed at 90 °C for 2 hours until no further brown fumes were released. Then, 4:1 mixture of HNO_3 and $HClO_4$ was added. This mixture was heated until white fumes of perchloric acid were released and the reduction of volume was up to 1ml. The remaining solution was diluted up to 10 ml with distilled deionized water. Subsequently, this digest was analyzed for Se content on HGAAS (Agilent 240AA) and Fe on AAS (Agilent 240AA).

2.7. Statistical Analysis

GraphPad Prism version 6.01 was used to perform variance analysis (one way). One-way ANOVA was followed by Dunnett's multiple comparisons test, which was used to determine significant differences between means. Normal distribution and equal variance were checked before the analysis.

3. Results

3.1. Isolation and Screening of Selenium Resistance

A total of 15 strains were isolated from 5 soil samples. These strains were purified and stored at low temperatures. These strains were further streaked on LB agar plates supplemented with selenite. Three of the strains were able to grow in the presence of selenite and formed intense red colonies. To determine MIC, these twelve strains were inoculated to LB broth amended with Na_2SO_3. All the strains were able to tolerate a 40mM concentration of selenite salt. The highest tolerance was shown by TM3, which was able to grow at 320mM of selenite in broth.

3.2. Biochemical and Molecular Identification

Three of the isolates which were able to grow in the presence of selenite were further characterized on the morphological, physiological, biochemical, and molecular basis. Two of the isolates were gram-positive, rod-shaped, and spore-forming; whereas, one strain was gram negative rod and non-spore-forming (Table 1). All three strains were catalase, oxidase, and citrate positive, but for indole test, they were negative (Table 2).

Table 1: Morphological characterization of isolated strains

Characteristics	Bacterial strains		
	TM5	TM7	TM3
Colony morphology	Irregular Slimy Grey	Round Regular White	Round Regular Creamy White
Grain straining	Gram -	Gram +	Gram +
Cell shape	Rod shape	Rod shape	Rod shape
Spore	-	+	+
Motility	+	-	+
Growth	Aerobic	Aerobic	Aerobic

Table 2: Biochemical characterization of isolates

Biochemical Tests	TM5	TM7	TM3
Oxidase	+	+	+
Catalase	+	+	+
Indole	-	-	-
Methyl Red	-	+	-

Biochemical Tests	TM5	TM7	TM3
Vogous-Proskeur	-	-	+
Citrate	+	+	+
Starch Hydrolysis	+	-	+
Gelatin Hydrolysis	+	-	-
Nitrate Reduction	+	-	-
Urea Hydrolysis	+	-	-
Arabinose	-	-	-
Glucose	+	+	+
Lactose	-	+	+
Mannitol	+	+	-
Maltose	-	+	-
Sorbitol	-	-	-

Table 3: Plant growth promotion attributes of isolates

Strains	Plant growth promoting characteristics			
	Phosphate solubilization	IAA production	Ammonia production	HCN production
TM5	-	+	+	-
TM7	+	-	+	+
TM3	+	+	+	+

Molecular identification was performed to identify species. BLAST results showed 99% strain homology TM-3 with *Bacillus halotolerans,* TM-5 with *Pseudomonas protegens*, and TM-7 with *Bacillus endophyticus.* Sequences of these strains were submitted to the NCBI database (accession numbers MT766904, MT767109, and MT767110). A phylogenetic tree of the obtained sequences was constructed, and each strain was branched with its respective group (Figure 2).

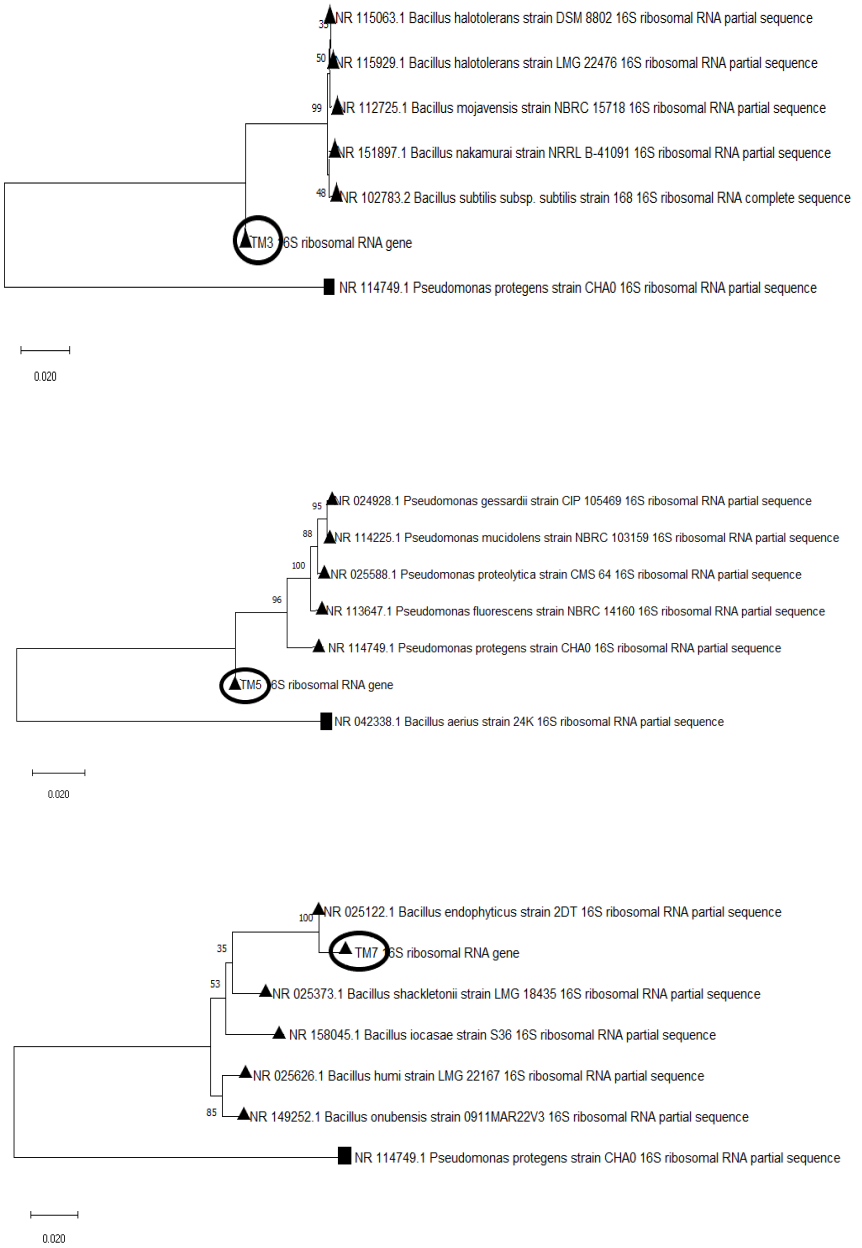


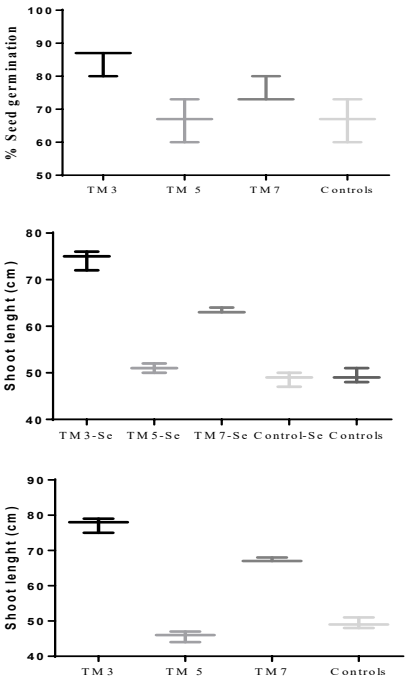
Figure 2: Phylogenetic analysis. Neighbor-joining tree of 16S rRNA gene sequence of bacterial isolate TM3, TM5 and TM7.Tree was constructed using MEGA X (boot-strap 500 replicates).

3.3. Plant Growth Promoting Traits

Bacterial isolates were screened to four plant growth-promoting characters such as IAA production, phosphate solubilization, ammonia production, and HCN production. All the isolated strains were able to produce ammonia, confirmed by the appearance of brown colour on the addition of Nessler's reagent to peptone water inoculated with bacteria. Strain TM3 *Bacillus halotolerans* and TM-7 *Bacillus endophyticus* were able to solubilize phosphate; whereas, Strain TM5-*Pseudomonas protegens* lacked enzymes that can solubilize phosphate. Strain TM-7 *Bacillus endophyticus* was unable to produce IAA, while strain TM5-*Pseudomonas protegens* and *TM3 Bacillus halotolerans* produced IAA in vitro. Similarly, two of the isolates, *TM3 Bacillus halotolerans,* and TM7-*Bacillus endophyticus* were positive for HCN production, while TM5-*Pseudomonas protegens* were negative for HCN production.

3.4. Effect on Plant Growth by the Isolated Strains

Percentage seed germination, plant dry weight, and shoot length of the maize plants in the presence and absence of bacteria were compared. Seed germination in pots inoculated with TM3-*Bacillus halotolerans* was significantly higher than the control pots; whereas, no change in seed germination was observed for strain TM5-*Pseudomonas protegens*. TM3-*Bacillus halotolerans* increased shoot length by 56% and TM7-*Bacillus endophyticus* increased it by 36%; whereas, TM5-*Pseudomonas protegens* resulted in a 7% increase when inoculated in pots containing natural garden soil. A slight decrease in shoot length was observed when garden soil was supplemented with sodium selenite. On the other hand, inoculation of isolates mitigated the toxic effect of selenium and enhanced shoot length. This increase was 52% for TM3-*Bacillus halotolerans*, 30% for TM7-*Bacillus endophyticus*, and 5% for TM5-*Pseudomonas protegens*. There was a significant rise in the dry weight for the pots inoculated with TM3-*Bacillus halotolerans* and TM7-*Bacillus endophyticus*; however, TM5-*Pseudomonas protegens* showed a 22% decrease in pots with natural garden soil. The addition of selenium decreased plant dry weight by 7%; however inoculation of bacterial isolates to the selenium supplemented pots resulted in the rise of dry weight for strains TM3-*Bacillus halotolerans* and TM7-*Bacillus endophyticus* by 44% and 40%, respectively (Figure 3).



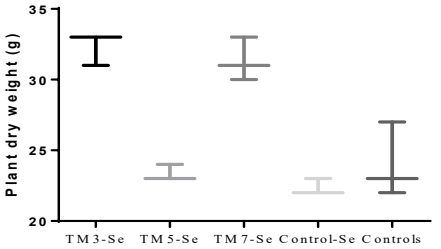
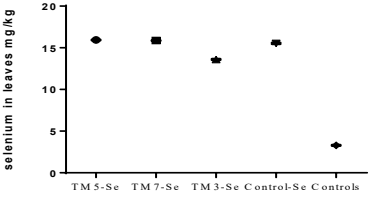
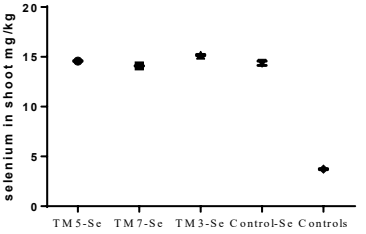


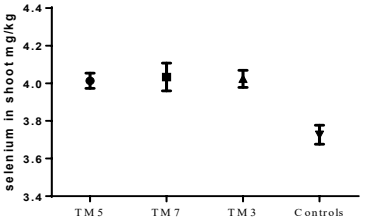
Figure 3: Pot experiment results. (A)Percentage seed germination (B) shoot length in pots grown in pots supplemented with selenium (C) shoot length in pots grown in pots with natural garden soil (D) plant dry weight in control and selenium supplemented soil.

3.5. Selenium and Iron Levels in Maize Leaves and Shoot

Selenium content in maize leaves and the shoot was measured by HGAAS. Selenium content in maize plants was lower in pots with natural garden soil. Conversely, a significant rise in selenium content was observed in selenium supplemented pots. The addition of selenium resulted in a 285% and 371% increase in the selenium content of shoot and leaves, respectively. Inoculation of the strains resulted in a 6-8 % rise in the selenium content of shoots and leaves. On the other hand, inoculation of strain TM7-*Bacillus endophyticus* resulted in a decrease in selenium content in the maize shoot. Similarly, inoculation of strain TM3-*Bacillus halotolerans* resulted in a decrease in selenium content in maize leaves (Figure 4).







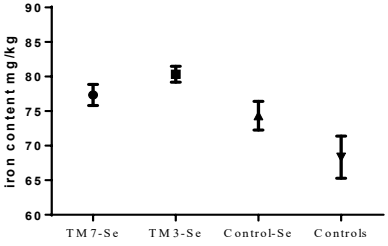


Figure 4: Selenium and Iron estimation in maize leaves and shoot (A) Selenium content of maize leaves in control and inoculated pots (B)Selenium content in maize shoot (C) selenium content of maize shoot in pots with natural soil (D) Iron content of maize leaves

Iron content in maize leaves was measured by AAS. Iron content showed an 8 % increase in pots supplemented with selenium as compared to pots with natural garden soil. Inoculation of strain TM7-*Bacillus endophyticus* and TM3-*Bacillus halotolerans* further increased iron content by 17% and 13%, respectively. However, inoculation of strain TM5-*Pseudomonas protegens* resulted in a 9% decrease in the iron content of maize leaves (Figure 4).

4. Discussion

Biofortification of selenium is the most important strategy used to reduce selenium deficiency in the population. It is preferred to food supplementation since organic forms of selenium in biofortified food are more bioavailable than supplemented ions. The addition of selenium to animal feed can produce beneficial effects on their health, performance, fertility, and meat quality [24]. A large number of studies reported higher selenium content in meat when animals were provided with selenium in different forms [25, 26]. Maize is a food and feed crop that can be used to improve selenium status in both humans and animals. Previous studies have reported that biofortified maize acts as a tool to reduce mineral deficiency in different populations [27, 28].

In this study, soil samples were collected from the citrus research institute Sargodha. Microbes were isolated from these samples. Physiological, morphological, biochemical, and molecular characteristics showed that these strains belong to *Bacillus halotolerans* (TM3), *Pseudomonas protegens* (TM5), and *Bacillus endophyticus* (TM7). A large number of studies have reported the isolation of bacteria from soil [29, 30, 31]. Yasmin et al. reported the isolation of *Pseudomonas protegens* from the agricultural field of maize. One similar study by Zhang et al. [32] reported the isolation of *Bacillus halotolerans* from drought-and salt-stressed rhizosphere soil. Another study by Das et al. [33] reported *Bacillus endophyticus* from the soil.

Isolated strains were screened for selenium resistance in LB broth supplemented with sodium selenite. All three strains showed high resistance against selenium and were able to resist selenite concentrations of 320mM, 40mM, and 160mm, respectively. Ghosh et al. [34] reported selenium-resistant Bacillus with MIC ranging from 300-750mM selenite. A study by Lusa et al. [35] reported selenium resistant Pseudomonas, which was able to withstand 6mM selenium ion concentration. Different selenium reduction mechanisms are applied by the bacteria to resist selenium and reduce it to selenium (0). Some bacteria can reduce selenium aerobically, while others can reduce it anaerobically. All the isolates from the current study reduced selenium aerobically. The proposed mechanism for aerobic reduction is by reductases and thiols present in the cytoplasm of bacteria. These enzymes include fumarate reductase, selenite reductase SerT, GSH, and BSH [36].

Plant growth-promoting bacteria (PGPB) can help plants by direct and indirect mechanisms. Direct mechanisms include the production of phytohormones and nutrient acquisition. For screening of these direct mechanisms, all the isolates were tested for IAA production, ammonia production, phosphate solubilization, and HCN production. TM3-*Bacillus halotolerans* possessed all four characteristics, while TM5-*Pseudomonas protegens* possessed IAA and Ammonia production only. Strain TM7-*Bacillus endophyticus* was able to produce ammonia. HCN and phosphate solubilizing enzymes in-vitro. Many studies reported bacillus bacteria with PGPB characteristics [37]. Hashem et al. [38] reported a bacillus with PGPB characters which can impact biotic stress as well. Similarly, Chitara et al. [39] reported selenium-resistant bacillus with PGPB characteristics. Zhang et al. [40] isolated pseudomonas having PGP characters. Inoculation of selenium-resistant bacteria to soil resulted in increased plant growth. Maize seeds treated

with TM3-*Bacillus halotolerans* resulted in 20% greater seed germination, 56% greater shoot length, and 48% higher plant dry weight as compared to plants in pots with natural garden soil. A similar rise in these factors was observed when this strain was inoculated into pots containing selenium-supplemented soil. On the other hand, TM7-*Bacillus endophyticus* caused decreased selenium content in selenium supplemented soils. Inoculation of these isolates also produced enhanced selenium content of maize leaves, both in the presence and absence of selenium supplementation. TM3-*Bacillus halotolerans* strain showed 12% lower selenium content in leaves when compared with plants in soil containing selenium. The use of microbes for selenium biofortification is an important strategy to improve the nutraceutical value of crops. A study by Durán et al. [41] reported the use of selenium-resistant bacteria for the biofortification of selenium in wheat. Similarly, Kaur et al. [42] reported the use of fungi for the biofortification of selenium in maize plants. Inoculation of these microbes increases the bioavailability of selenium in soil which results in increased uptake by the plants [43].

The addition of selenium to the soil resulted in a more than 300% increase in selenium accumulation by the maize leaves and shoot. Administrating selenium to the maize may result in increased uptake of selenium by maize plants. A study by De Feudis et al. [44] reported increased selenium accumulation in maize plants when selenium was provided at the rate of 200g ha-1. Another study by Ngigi et al. [10] showed increased selenium uptake by maize and beans when selenium was added to the soil or by foliar application. The reason for the increased selenium level in maize shoots and leaves is the greater bioavailability of selenate ions [45].

The addition of selenium to the soil resulted in increased uptake of different metals by the plants [46]. In the current study, the addition of selenate resulted in 9% higher iron content in maize leaves as compared to maize leaves grown in natural soil. This iron content was higher in plants inoculated with TM3-*Bacillus halotolerans* and TM7-*Bacillus endophyticus* strains; whereas, strain TM5-*Pseudomonas protegens* caused a 1% lower iron level in maize leaves. A study by Fang et al. [47] reported increased Zn, Se, and Fe content in rice on foliar application of fertilizer. The increase in iron content of maize leaves may be attributed to two approaches used by PGPB, which are siderophore production and iron solubility. A study by Mishra et al. [48] reported increased iron content in lentils by inoculation of Pseudomonas sp. strain. Another study by Khalid et al. [49] reported a significant increase in iron content of chickpea on inoculation with bacteria.

5. Conclusion

Plant growth-promoting bacteria have the potential to enhance the nutritional status of the population. Targeting food and feed crop like maize is a good strategy to overcome malnutrition in animals and humans both. The current study showed that the microorganisms isolated from soil have PGPB characteristics along with resistance against selenium. These bacteria enhanced maize growth and increased the Se and Fe content of maize leaves. Fodder produced from such crops can be used to increase selenium levels in cattle.

Conflict of Interest

The authors declare no conflict of interest.

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