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Research Article

Molecular Identification of Lead-Resistant Bacteria and Assessment of Their Effects on Vicia faba Planted in Lead-Contaminated Soil

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Abstract:

Bioremediation is a crucial tool for managing soil contamination. In this study, we aimed to reduce the side effects of lead (Pb) on *Vicia faba* seedling using Pb tolerant bacteria strains isolated from polluted places and to study these effects with Pb. Four Pb-tolerant isolates were selected from twenty-two strains. The isolated bacteria were identified using *16S-rRNA* gene sequence technique. Identified strains were classified as members of the genus *Bacilli*. The tolerant isolates were *Brevibacillus parabrevis, Brevibacillus reuszeri*, *Bacillus subtilis and Bacillus amyloliquefaciens*. The results indicated to the side effects of Pb on shoot fresh and dry weight, root fresh and dry weight, shoot and root length, proline content, activities of some antioxidant enzymes (catalase, peroxidase and polyphenol oxidase) as well as cytogenetical measurements. Lead inhibited the mitotic index and caused an increase in chromosomal aberrations. The treatment with four bacteria significantly reduced the side effects of Pb on the level of growth and physiological parameters. Also, it improved the mitotic index and reduced the chromosomal aberrations.

1. Introduction

Since heavy metals are not biodegradable and can only be transferred from one chemical state to another, they constitute a serious danger to agricultural production, food safety, and human health. Metals remain in the soil for a very long time (Naila et al., 2019; Sun et al., 2020).

Lead (Pb) is a heavy metal with an intensifying toxicant that devastates the human body. After arsenic (As), a heavy metal that plays no part in biological systems, Pb is the second most poisonous heavy metal. Lead toxicity harms plants in various ways, from germination to yield development, although its toxicity depends on time and concentration (Zulfiqar et al., 2019).

The biological remediation strategy uses organisms, such as plants (phytoremediation) or microbes (microbial remediation), to clean up metal-polluted soil. This form of soil remediation is promising and sustainable. It is a well-regarded natural and economical strategy (Chibuike and Obiora, 2014). Bioremediation is 50–65% more affordable in cleaning up Pb-polluted soil than traditional remediation methods, (Blaylock et al., 1997).

The present study aimed to isolate, identify, and characterize heavy metal-resistant bacteria from several polluted sources to be used as bioremediation agents of contaminated soil with lead (Pb) using *Vicia faba* as a biological system.

2. Materials and Methods

2.1. Isolation of bacteria

Several soil samples were collected from three different regions of contaminated soils (Talkha, Qalyubia Governorate, Nawag, Gharbia Governorate, and Quesna, Menoufia Governorate). Samples were mixed well, and ten grams of the mixture were taken and suspended in 90 ml of sterilized distilled water. Several dilutions were plated on nutrient agar media and incubated at 30°C for three days. Colonies that differed in morphology were isolated and cultured on nutrient agar plates.

2.2. Determination of minimum inhibitory concentration (MIC) for isolated bacteria

Heavy metal-resistant selected isolates were cultured on Pb-incorporated nutrient broth media with a gradual concentration of Pb to assess MIC. MIC was identified with the isolates failing to give growth on nutrient broth. The starting concentration of the heavy metals was 50 µg/mL, and the end was 3200 µg/mL (Marzan, et al., 2017).

2.3. Molecular identification of isolated strains

2.3.1. DNA Extraction and PCR reaction

DNA was extracted from the bacteria according to (Abed, 2013). A total of 50 µl of 1X reaction buffer, 1.5 mM $MgCl_2$, 1U Taq DNA polymerase (Promega), 2.5 mM dNTPs, 30 pmol of forward and reverse primers of *16SrRNA* gene (F:5'-, AGAGTTTGATCCTGGCTAG -3′ and R:5'- GGTTACCTTGTTACGACTT -3′), and 30 ng of genomic DNA were used as a template. An initial denaturation cycle lasting 5 minutes at 94°C was followed by 40 cycles of PCR amplification using a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) protocol. Each cycle included a denaturation phase lasting 30 seconds at 94 °C, an annealing step lasting 30 seconds at 45 °C, and an elongation stage lasting 1 minute at 72 °C. In the last cycle, the primer extension phase was prolonged to 7 min at 72°C. The PCR products were detected by electrophoresis on a 1.5% agarose gel. Amplified products were purified using an EZ-10 spin column according to user manual.

2.3.2.16S-rRNA amplified product sequencing analysis

Using Big Dye ™ Terminator Cycle Sequencing Kits according to user manual, the resultant PCR was sequenced in an automated sequencer ABI PRISM 3730XL Analyzer before being exposed to electrophoresis in an ABI 3730xl sequencer (Microgen Company), Korea. The sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned using Align Sequences Nucleotide BLAST. The phylogenetic tree was constructed by data matrix following the neighbor-joining method using MEGA 6.1 software (Rajeendran, et al., 2017).

2.4. Pots experiment

The experiments were conducted at the Department of Biological and Environmental Sciences, Faculty of Home Economics, Al-Azhar Univ. *V. faba* seeds were graciously provided by the Sakha Agricultural Research Station (SARS), Food Legumes Research Section, Kafr El-Sheikh, Egypt.

Three replicates ten seeds and is planted in plastic pots filled with peat moss were used for each treatment. Before planting, the soil was contaminated with Pb at the concentration of 30 mg Pb/ 120 g peat moss (El-Mahdy, et al., 2021) and inoculated with the selected tolerant bacteria strains (10 ml of 5×10^6 cfu). The experiment design include six groups. First group was negative control without any treatment. The second group was positive control (Pb). The third group was inoculated by *Brevibacillus reuszeri* with Pb. The fourth group was inoculated by *Bacillus subtilis* with Pb. The fifth group was inoculated by *Brevibacillus parabrevis* with Pb. The last group was inoculated by *Bacillus amyloliquefaciens* with Pb. After two weeks of soil inoculation with bacteria, *V. faba* (ten seeds) was planted in each replicate and inoculated with 10 ml of 5×10^6 cfu. Five seedlings of each replicate were taken after three weeks for morphological and physiological determination.

2.5. Morphological determinations

The morphological determinations included plant heights (cm), leaf area (cm^2), root fresh and dry weights (g), shoot length (cm), shoot fresh and dry weights (g).

2.6. Chlorophyll content

Chlorophyll levels were estimated by the spectrophotometric (Jenway 6305 UV/Visible) method according to **(**HK, 1985**)**; chlorophyll was expressed as µg/ml methanol

2.7. Antioxidants enzymes activity assay:

2.7.1. Catalase activity (CAT)

At 240 nm for the 60s, the absorbance was measured using a spectrophotometer (Jenway 6305 UV/Visible) by estimating the amount of H_2O_2 that has been broken down, the enzyme assay was carried out according to (Aebi, 1984).

2.7.2. Peroxidase activity (POD)

POD activity was measured at 420 nm according to described technique in (Chance and Maehly, 1955).

2.7.3. Polyphenol oxidase activity (PPO)

PPO activity was measured at 420 nm and 25°C in accordance with (Duckworth and Coleman, 1970).

2.8. Proline content

Proline content was determined spectrophotometrically at 520 nm. Proline concentration is expressed as mg/1g fresh weight (Bates et al., 1973).

2.9. Cytological analysis

For cytological preparations, 2% of the aceto-carmine stain was utilized, according to (Zedan and Omar, 2019).

2.10. Data Analysis

Data were presented as means and standard deviation (SD). The Duncan test was used to examine the significance of differences between means after the One-Way Analysis of Variance using SPSS software for Windows version 20. At a *p* value of 0.05, the results were significantly different.

3. Results

3.1. Determination of minimum inhibitory concentration (MIC) for isolated bacteria

The results of MIC for 22 bacteria isolated for Pb at concentrations 640, 1600, and 3200 µg/ml shown in Table (1). Four isolates were selected for identification and further studies. Three had MIC at 3200 µg/ml, and the fourth had MIC at 1600 µg/ml.

Table 1. Minimum inhibitory concentrations for bacterial isolates in media contain Pb heavy metal.

Sl. No.	Bacteria isolates	MIC for Pb (µg/ml)
1	isolate 1	1600
2	isolate 2	1600
3	isolate 3	1600
4	isolate 4	1600
5	isolate 5	3200
6	isolate 6	3200
7	isolate 7	3200
8	isolate 8	3200
9	isolate 9	1600
10	isolate 10	3200
11	isolate 11	3200
12	isolate 12	3200
13	isolate 13	3200
14	isolate 14	1600
15	isolate 15	1600
16	isolate 16	3200
17	isolate 17	640
18	isolate 18	640
19	isolate 19	640
20	isolate 20	640
21	isolate 21	1600
22	isolate 22	3200

3.2. Identification of bacterial isolates based on 16s RNA

*16S r*RNA encoding gene sequences has been used extensively in classifying and identifying bacteria. The strains 1, 2, 3, and 4 were long rod-shaped, gram-positive, spore-forming bacteria (Table 2). By sequencing the amplified product of *16S r*RNA gene of these strains and comparing them with previously published *16S r*RNA gene sequences, the strains were classified as members of the genus *Bacilli*. The sequence of the strain displayed the highest identity with the *16S r*RNA gene of a *Brevibacillus parabrevis* (88.77%, Fig. 1), *Brevibacillus reuszeri* (82.30%, Fig. 2), *Bacillus subtilis* (98.14%, Fig.3) *and Bacillus amyloliquefaciens* (93.71%, Fig. 4), respectively.

Table 2. Similarity percentage of each bacterial strains and the accession numbers.

Isolate	Accession no.	Similarity %
Brevibacillus parabrevis	KX832687.1	88.77
Brevibacillus reuszeri	KX350050.1	82.30
Bacillus subtilis	MN952609.1	98.14
Bacillus amyloliquefaciens	MH464958.1	93.71

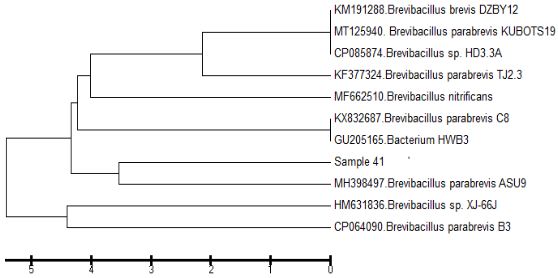

Figure 1. Phylogenetic tree using MEGA-5 of bacterial no. 1 using 16S rRNA, showing names of bacteria species and accession numbers.

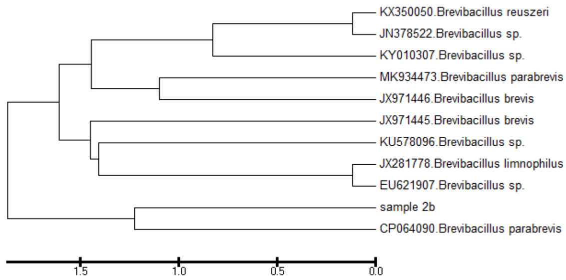

Figure 2. Phylogenetic tree using MEGA-5 of bacterial no. 2 using 16S rRNA, showing names of bacteria species and accession numbers.

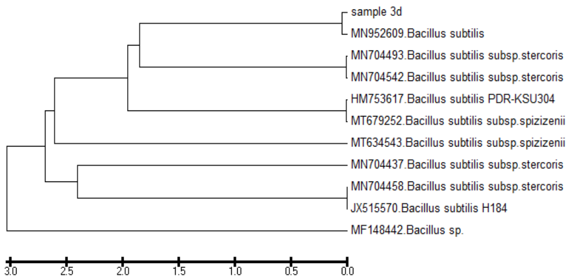

Figure 3. Phylogenetic tree using MEGA-5 of bacterial no. 3 using 16S rRNA, showing names of bacteria species and accession numbers.

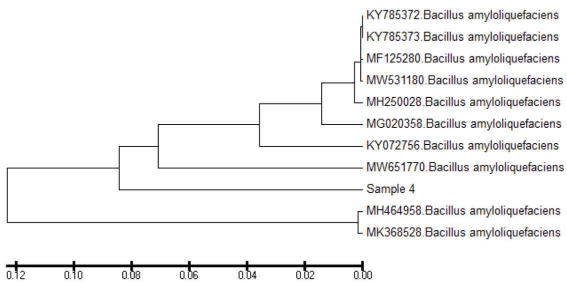

Figure 4. Phylogenetic tree using MEGA-5 of bacterial no. 4 using 16S rRNA, showing names of bacteria species and accession numbers.

3.3. Growth parameters

Figure (5) shown the effect of Pb on shoot and root length, shoot, and root fresh and dry weight.

There were significant differences between Pb group and the negative control. Lead led to an obvious decrease in all morphological parameters, except shoot fresh and dry weight, compared to the negative control. The treatments with some tolerant bacteria decreased the negative effects of Pb. The high effects of bacteria delete the significant differences between the negative and positive control. *Brevibacillus reuszeri* with Pb (BrPb) and *Brevibacillus parabrevis* with Pb (BpPb) treatments were the best in the most morphological traits.

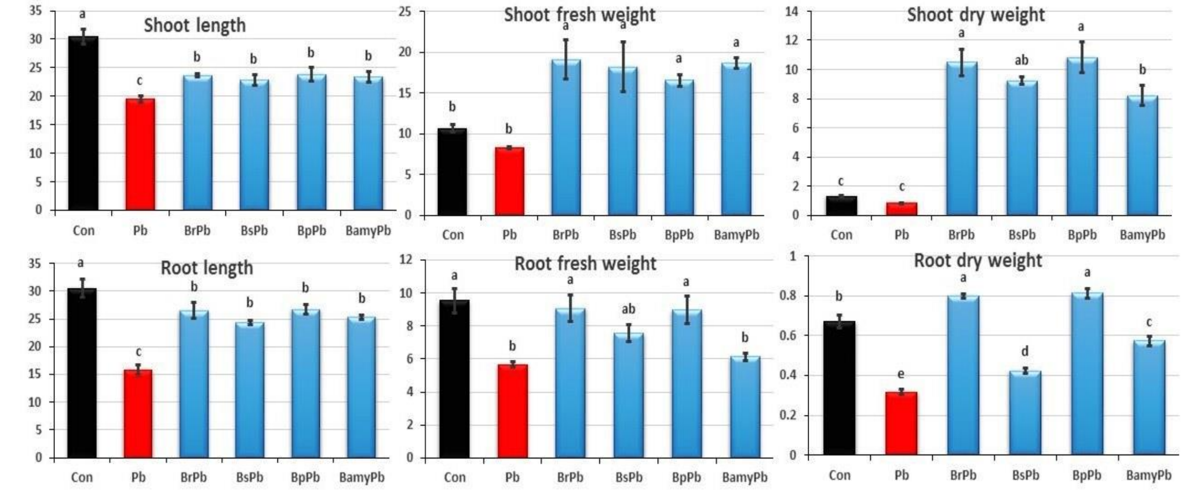

Figure 5. Changes in shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight and root dry weight seedlings of Vicia faba under control and Pb treatment without or with bacterial inoculum. Con, control; Pb, lead; BrPb, Brevibacillus reuszeri with Pb; BsPb, Bacillus subtilis with Pb; BpPb, Brevibacillus parabrevis with Pb; BamyPb, Bacillus amyloliquefaciens with Pb. Column with different letters are significant.

3.4. Physiological parameters

Figure (6) illustrates the effect of tolerant bacteria isolates on the recovery of the side effects of Pb on *Vicia faba* on the level of proline concentration. Pb significantly induced proline production in comparison with the negative control. On the other hand, *Brevibacillus reuszeri* treatment significantly recovery the natural content of proline in comparison with Pb and the other treatments.

Catalase, peroxidase and polyphenol oxidase significantly decreased in Pb treatment compared to the control (Fig. 6). The bacterial treatments significantly induced the activity of these antioxidant enzymes in comparison with the positive and negative control and reduced the side effects of Pb toxicity (Figure 6). The *Brevibacillus reuszeri* strain was the best treatment, followed by *Brevibacillus parabrevis* strain.

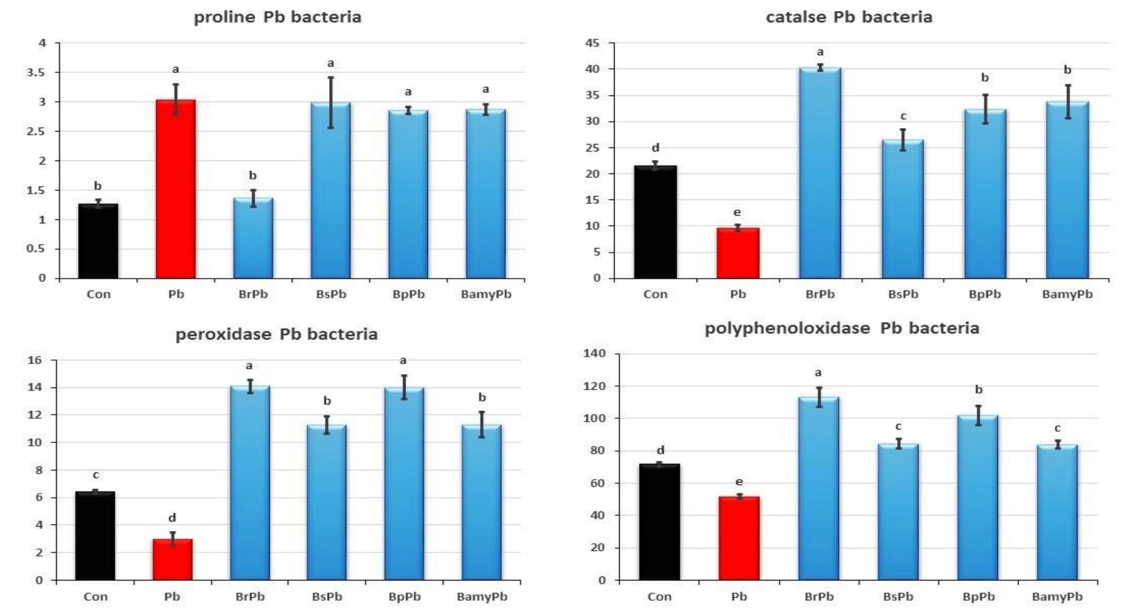

Figure 6: Changes in proline concentration, catalase, peroxidase and polyphenoloxidase in seedlings of Vicia faba under control and Pb treatment without or with bacterial inoculum. Con, control; Pb, lead; BrPb, Brevibacillus reuszeri with Pb; BsPb, Bacillus subtilis with Pb; BpPb, Brevibacillus parabrevis with Pb; BamyPb, Bacillus

amyloliquefaciens with Pb. Values with different letters are significant**.**

3.1. Cytological studies

In the current study, phases indix were calculated, Prophase is the dominant stage in all treatments. The highest percentage of prophase was recorded in the treatment pb + *Bacillus amyloliquefaciens*, whereas the highest percentage of metaphase, anaphase and telophase were recorded in pb + *Bacillus subtilis* treatment (Table 3). The results refer to that Pb significantly reduced the mitotic index (MI) compared with the negative control and other treatments. The bacteria strains can restore the normal MI except *Bacillus subtilis* (Table 4).

Chromosomal abnormalities recorded high ratio in Pb treatment. It showed abnormal morphological structure that includes C-mitosis, Pole-to-pole metaphase, bridges, disrupted, fragment, laggard, star anaphase and chromosome stickiness in the cells (Figure 7). On the other hand, inoculation with bacteria strains increase mitotic index and reduced chromosomal abnormalities (Table 4).

Table 3. Cytological indicators of examined root tips of V. faba treated with pb and inoculation with heavy metal-tolerant bacteria strains.

Treatments	No. of examined cells	No. of dividing cells	No. of abnormal cells	Mitotic phase (%)			
				Prophase	Metaphase	Anaphase	Telophase
Control	3252	310	-	70	14.83	11.29	3.37
Pb	3311	183	114	66.12	13.11	17.48	3.27
BrPb	3151	306	60	70.91	14.70	9.80	4.57
BsPb	3150	184	69	37.5	27.17	20.10	15.21
BpPb	3144	291	41	72.50	11.68	10.99	4.81
BamyPb	3137	351	78	74.35	11.68	7.69	6.26

BrPb, Brevibacillus reuszeri with Pb; BsPb, Bacillus subtilis with Pb; BpPb, Brevibacillus parabrevis with Pb; BamyPb, Bacillus amyloliquefaciens with Pb.

Table 4. Mitotic index, types and percentage of abnormalities of V. faba under pb stress and inoculated with bacteria.

Treatments	Mitotic aberration (%)								Mitotic index (%)	Abnormalities (%)
	S	D	F	B	C-M	PP	ST	L		
Control	-	-		-		-			9.60 ±1.96[a]	00.00±00.00[c]
Pb	55.26	21.92	9.64	5.26	6.14	-	0.87	0.87	5.50±0.50[c]	50.56±38.10[a]
BrPb	36.66	38.33	-	5	20	-	-	-	9.70±0.99[a]	19.44±4.76[bc]
BsPb	2.89	57.97	2.89	14.49	21.73	-	-	-	5.83±0.17[bc]	37.45±3.57[ab]
BpPb	12.19	51.21	12.19	4.87	14.63	-	-	4.87	9.25±2.30[ab]	14.13±0.45[bc]
BamyPb	26.92	42.30	5.12	3.84	14.10	1.28	1.28	5.12	11.21±3.50[a]	21.55±5.83[abc]
Sig									0.04	0.01

BrPb, Brevibacillus reuszeri with Pb; BsPb, Bacillus subtilis with Pb; BpPb, Brevibacillus parabrevis with Pb; BamyPb, Bacillus amyloliquefaciens with Pb. S: Stickiness, D: Disrupted, F, Fragment, B: bridge, C-M: C-metaphase, PP: Pole-to-pole metaphase, ST: Star anaphase, L: Laggard. BrPb, Brevibacillus reuszeri with Pb; BsPb, Bacillus subtilis with Pb; BpPb, Brevibacillus parabrevis with Pb; BamyPb, Bacillus amyloliquefaciens with Pb. Values with different letters are significant**.**

Discussion

The isolation of tolerant bacteria from polluted soils and water is one of the methods to select strains more tolerant to heavy metals and antibiotics (Safari and Younessi, 2017). The use of heavy metals-tolerant bacteria to remediation polluted soil from heavy metals was used in many research and given positive results (Yin, et al., 2019;Ahemad, 2019). In this study, four isolates were selected for identification and further studies. Three had MIC at 3200 µg/ml, and the fourth had MIC at 1600 µg/ml.

High similarity is currently acknowledged as the cutoff for distinguishing species. The comparison of almost full *16S r*RNA gene sequences has been routinely utilized to establish taxonomic relationships between bacterial strains. An accurate and practical method to regularly classify and identify prokaryotes is to compare an isolate's *16S r*RNA gene sequence against the sequences of type strains of all prokaryotic species (Harmsen, 2004). In this study *16S r*RNA gene sequence technique classified the strains as members of the genus *Bacilli*. The sequence of the strain displayed the highest identity with the *16S r*RNA gene of a *Brevibacillus parabrevis*, *Brevibacillus reuszeri*, *Bacillus subtilis and Bacillus amyloliquefaciens*

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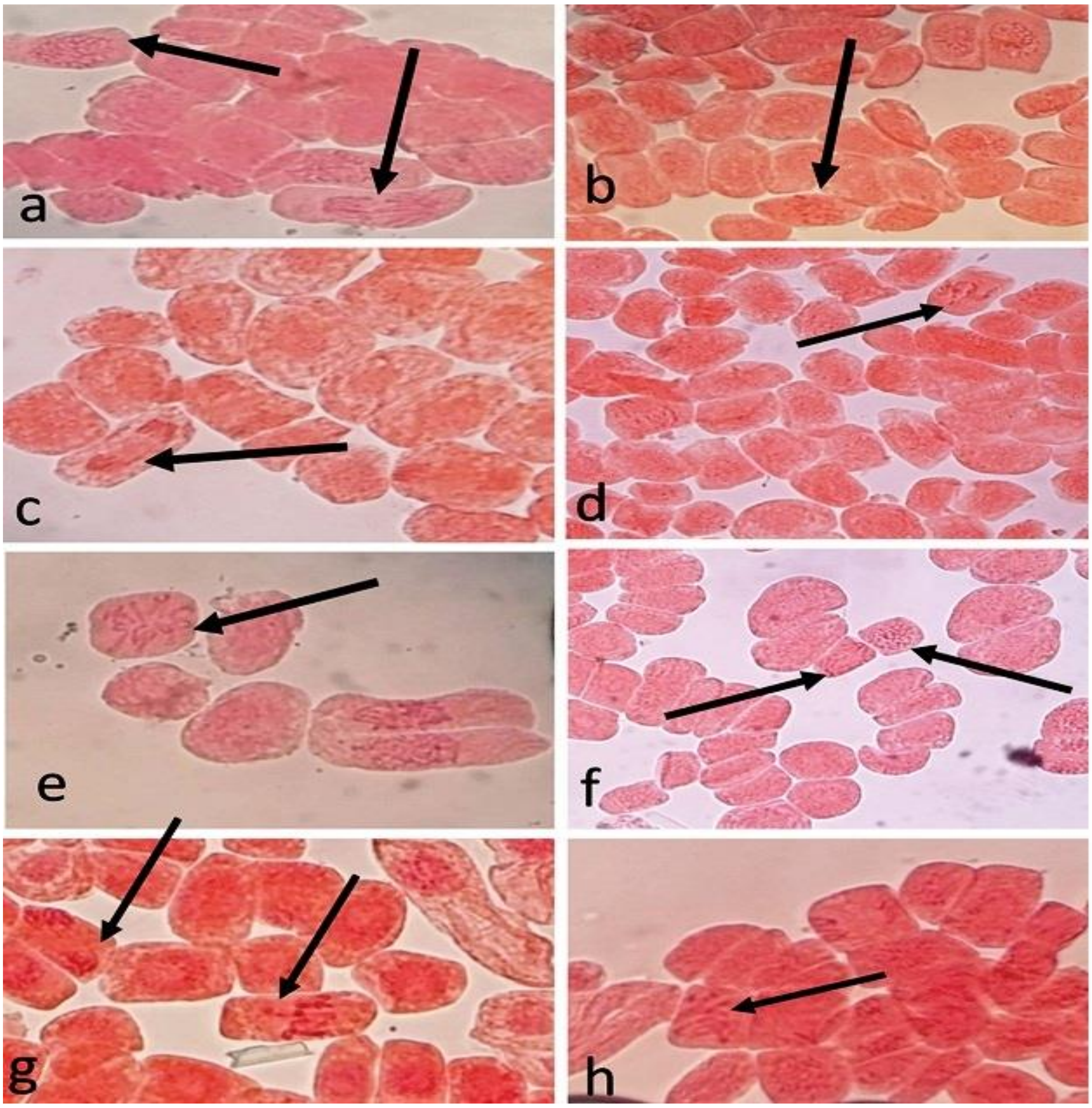

Figure 7. Types of abnormalities observed in V. faba root tips cells under lead stress. (a) Stickiness prophase and bridge in anaphase, (b) Disrupted anaphase, (c) Laggard chromosome in telophase,(d) C-metaphase,(e) Star anaphase,(f) Anaphase with fragment and stickiness prophase, (g) Stickiness telophase and vagrant chromosome in metaphase, (h) Pole-to-pole metaphase.

Bacterial strains were used in the pot experiment to remove the side effect of Pb on the plant. As expected, Pb had a negative effect on the morphological traits. When used bacterial strains, it reduced the side effect of Pb. *Brevibacillus reuszeri* with Pb (BrPb) and *Brevibacillus parabrevis* with Pb (BpPb) treatments were the best in the most morphological traits. Pb has side effects on plant growth. Due to reduced water potential, decreased nutritional content, and a blockage in the proton pumps, which further hinders cell division and elongation, the effect of heavy metals on plants resulted in a drop in growth. As a result, plants' length, fresh weight, and dry weight decreased. (Sarathambal et al., 2017). Similar findings have been obtained by (El-Mahdy, et al., 2021); they found that the dry biomass was decreased under the stress of Pb in *Vicia faba* plants. The inoculation of the polluted soil with bacteria reduced the effects of Pb on the plants, as shown in Fig. (5). These results agree with

(Sarma, et al., 2019), who reported that *Brevibacillus reuszeri* reduced the metals in microcosm's soil after 24 weeks of trial when compared to the control.

To measure the amount of heavy metal contamination, proline accumulation can be employed as a marker (Alia and Saradhi, 1991). Also, proline accumulates during abiotic stress (Kavi and Sreenivasulu, 2014). So, this was very important to measure as an indicator for Pb stress. In this study, there was an increase in proline with Pb treatment and *Brevibacillus reuszeri* treatment significantly recovery the natural content of proline.

The side effect of Pb on the activity of antioxidant enzymes was mentioned in many reports. (Wang, et al., 2012) reported that plants treated to 75 M Pb2+ saw an increase in superoxide dismutase and catalase activity within two days, and a subsequent decline. Also, (Shu, 2012) reported that when Pb was present, antioxidant enzyme activity was stimulated at low concentrations and inhibited at higher concentrations. Plants produce ROS scavengers including catalase, peroxidase, and polyphenol oxidase to combat oxidative stress (Thanwisai, et al., 2022). Bacterial inoculation decreases MDA levels while increasing protein and proline synthesis and enhancing the activity of antioxidant enzymes (Khan et al., 2018).

An increase in the cytotoxicity of any toxicant can be recognized by the decrease in the cell mitotic index (MI) (Pérez-de-Luque, 2017), it is a good indicator of cytotoxicity (Hu et al., 2017). The elevated Pb ion concentration resulted in more severe cytotoxic or genotoxic consequences (Lyu, et al., 2020). Pb interfered with plant mitosis (Shahid, et al., 2011).

These results agree with Lyu, et al., (2020) who showed that Pb adversely affected cell mitosis when cells were under Pb stress, causing chromosomal abnormalities and inhibiting the mitotic index. On the other hand, inoculation with bacteria strains increase mitotic index and reduced chromosomal abnormalities (Table 4). Endophytic microbes help plants resist a variety of environmental challenges. (Dhali, et al., 2021; Santoyo, et al., 2016; Smith et al., 2008). Combining the application of *Bacillus* sp. (AS03) and *Rhizobium* sp. (AS05) reduced the rate of chromosomal abnormalities and enhanced the root cells' capacity for mitosis (Dhali, et al., 2022).

Author Contributions:

"Conceptualization, Amina Zedan and Noha Sukar; methodology, Aisha Sharaf-Eldin and Sherifa Dawoud.; software, Noha Sukar; validation, Amina Zedan,; formal analysis, Aisha Sharaf-Eldin and Sherifa Dawoud.; investigation, Aisha Sharaf-Eldin, Noha Sukar and Sherifa Dawoud.; resources, Aisha Sharaf-Eldin, Noha Sukar and Sherifa Dawoud; data curation, Noha Sukar; writing—original draft preparation, Aisha Sharaf-Eldin, and Sherifa Dawoud.; writing—review and editing, Amina Zedan.; visualization, Noha Sukar.; supervision, Amina Zedan. All authors have read and agreed to the published version of the manuscript.

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