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DEVELOPMENT AND CHARACTERIZATION OF LEAD-TOLERANT RHIZOBIUM MUTANTS FOR ENHANCING PEA GROWTH

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ABSTRACT: The increased concentrations of lead (Pb) are found to be highly toxic to soil microorganisms and can affect their structural diversity. Therefore, the present study aimed to induce superior strains of the bacterium *Rhizobium* by EMS mutagenesis of wild-type RL16 and examined the effects of developed mutants on the crop yield of pea plants in the experimental greenhouse. Nine superior mutants of *Rhizobium* were developed to tolerate the wild-type strain's LC95 concentration (6.3 mM Pb (No3) 2). This study demonstrated that the mutants could produce more extracellular proline than the wild-type, increasing their ability to survive in lead heavy metal stress conditions. *Rhizobium* application improves the pea characters and *Rhizobium* mutants indicate better recovery because the mutants were more tolerant to Pb than the wild-type strain. Also, the results demonstrated that *Rhizobium* mutants' inoculation showed more proline production by pea plants which agrees with their ability to recover the negative effect of Pb contamination in pea characters. PCR amplification of the *pbrA* gene showed that all wild-type and mutants contained the Pb-resistant gene.

Keywords: *Rhizobium* mutants, proline, IAA, bioremediation, pea, *pbrA* gene.

INTRODUCTION

Rhizobia is a significant class of Gram-negative bacteria that saves about 90 million tons of total biological nitrogen required for agriculture via symbiosis with leguminous plants (Hakim *et al*., 2020; Khalid *et al*., 2020; AlKurtany *et al*., 2023). Biological nitrogen fixation (BNF) is an important nitrogen source, and the various legume crops and their host species often fix as much as 200 to 300 kg of nitrogen per hectare per year (Peoples *et al*., 1995). BNF has become one of the most attractive strategies for developing sustainable agriculture

(Rouhrazi *et al*., 2016). At the same time, legumes are one of the most significant crops worldwide after cereals. They are rich in vitamins, protein (20–45%), carbohydrates (±60%), and fiber (5–37%) and are distinguished with low levels of fats (Maphosa and Jideani, 2017).

However, with the huge industrial revolution and increasing human activities, the accumulation of heavy metals has developed rapidly in the soil (Abdel-lateif 2017; Jach *et al*., 2022; Shen *et al*., 2023). This heavy metal soil contamination has mainly resulted from applying metal pesticides and fertilizers and using sewage sludge. The elevated levels of heavy metals such as Pb, Ni, and Zn are toxic for both soil microorganisms and plants and lead to the disruption of genes responsible for symbiotic processes (Chaudri *et al*., 2008; Stan *et al*., 2011). All these metals could be toxic at low concentrations (Gadd 1992). When exposed to moderate heavy metal concentrations, soil microorganisms were found to be extremely sensitive (Giller *et al*., 1998). Several studies have shown that metals adversely influence microorganisms (Shi *et al*., 2002), affecting their growth, morphology, and activities (Baath *et al*., 1998; Lakzian *et al*., 2002; Khan and Scullion 2002), including symbiotic N2 fixation (McGrath *et al*., 1988). Lead toxicity is one of the most

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widely studied heavy metal poisonings (Järup 2003). It reported that the increased concentrations of lead (Pb) were highly toxic to soil microorganisms and could affect their structural diversity (Abdel-lateif *et al*., 2018). Consequently, research has focused on the impact of heavy metals, particularly lead, on Rhizobium (Ibekwe *et al.,* 1995). Additionally, Rhizobium can serve as an indicator organism for various harmful substances, including heavy metals, as mentioned by Botsford (1999).

However, several studies stated that *Rhizobia* has several strategies to tolerate and sequester the negative effects of heavy metals through adsorption, accumulation, and secretion of enzymes and metabolites (Hao *et al*., 2014; Carrasco *et al*., 2005; Teng *et al*., 2015). Phytoremediation is another alternative strategy to clean heavy metals-contaminated soil using certain plants (Ali *et al.,* 2013; Neilson and Rajakaruna, 2015). Several legumes such as *Cytisus, Lotus, Lupinus, Genista, Glycine,* and *Pisum* have been suggested as pioneer species for phytoremediation (Gómez-Sagasti and Marino 2015). Hence, the employment of appropriate legumes and their *Rhizobia* partners to sequester the toxic effects of soil heavy metals in an eco-friendly way is a global demand (Glick 2010; Jach *et al*., 2022). Thus, incorporating heavy metal tolerant strains of *Rhizobium* for sustainable agricultural practices can reduce reliance on chemical fertilizers while mitigating the adverse effects on the environment. Therefore, numerous attempts were made to induce genetic mutations in *Rhizobia* to obtain strains with high tolerance to biotic stresses using chemical mutagens (Haggran *et al*., 2011; Gnanachitra and Sridar 2019).

Therefore, the specific objectives of this study were to (i) develop mutants of *Rhizobium* tolerant more than to lead (Pb) more than wild-type RL16 using EMS (ii) study the effect of *Rhizobium* mutants on pea plants parameters i.e. chlorophyll content, root characters, shoot characters, nodule characters, and the nitrogen content (iii) detect the Pb tolerant *pbrA* gene using PCR-based specific primers.

MATERIALS AND METHODS

Bacterial strain

This research used the wild-type bacterial *Rhizobium leguminosarum* biovar Vicia strain RL16 (Hegazy WN *et al*., 2024), which was previously identified as a heavy metal-tolerant strain.

Development of *Rhizobium leguminosarum* mutants by EMS

Ethyl methane sulphonate (EMS) at a concentration of 0.5M was used as a chemical mutagenic agent to mutagenize the *Rhizobium* strain as described by O'Connell *et al*. (1990) as follows: the wild-type RL16 strain was grown in YEM broth for two days, centrifuged at 10,000 rpm for 5 min and the cell pellets were washed with phosphate-buffered saline and resuspended in sterile water. The cell suspension was distributed into 1.5 ml Eppendorf tubes and the appropriate volume of EMS solution was added to obtain 0.5M EMS as the final concentration. The cells were treated with EMS for 30, 60, 90, and 120-min EMS, in addition to cells without EMS treatment. The reaction was stopped at the appropriate time interval by adding 1 ml of 6% sodium thiosulfate. The tubes were centrifuged, and the pellets were washed twice with sterile water. The suspension was diluted to 10^{-2} and 25 microliters were spread on YEMA plates containing 6.3 mM $Pb(No_3)_2$ and the resulting colonies were counted. Each colony was picked and grown on YEMA media supplemented with 6.3 mM $Pb(No_3)_2$ for seven consecutive generations to determine the stable mutants. Nine obtained *Rhizobium* mutants were coded RLM1, RLM2, RLM3, RLM4, RLM5, RLM6, RLM7, RLM8, and RLM9.

Screening of *Rhizobium* for indoleacetic acid (IAA) production

IAA production by bacterial wild-type and mutants was evaluated using the method described by Ehmann (1977). Each strain was cultured in yeast extract mannitol (YEM) broth medium supplemented with 500 µg/ml tryptophan and containing lead as a heavy metal at a concentration of 6.3 mmol. Also, a

tryptophan-free culture medium containing lead was kept as a control for each isolate, with the experiment performed in triplicate. The culture was incubated at 28 ± 2 °C for 3 days. After incubation, about 1.5 ml of the culture was transferred to a sterile Eppendorf tube and centrifuged at 10,000 rpm for 10 min. The production of indole acetic acid was then measured by adding 2ml Salkowski's reagent in a ratio of 2:1(50 ml of 35% perchloric acid ($HClO_4$) and 1 ml of 0.5 M iron trichloride ($FeCl_3$) and incubate the reaction at room temperature in a dark condition for 30 min., and the absorbance at 530 nm were recorded using a spectrophotometer. The IAA concentration was ascertained using a standard curve correlating IAA concentrations with absorbance.

Screening of *Rhizobium* for extracellular proline

The amount of extracellular proline secreted by the bacterial wild-type and mutants was determined by growing at 37 °C for 6 h in 50 mL YEM broth medium containing lead (6.3mM) adjusted to pH 7.0 under shaking conditions (150 rpm). Each culture strain was harvested by centrifugation at 10,000 rpm at 4 °C for 10 min, the supernatant was used for measuring the proline concentration as described by Bates *et al*. (1973) with some modifications. 2 mL of the culture supernatant was mixed with 1200 µL of sulphosalicylic acid (3% aqueous solution) and centrifuged at 13,000 rpm for 10 min at 4 °C. 500 µL of supernatant was transferred into a clean test tube and the volume was made to 1 mL using sterile H_2O. This step was followed by adding 1 mL of glacial acetic acid and 1 mL of ninhydrin (2% solution prepared in acetone) and incubating for 1 h in a water bath at 90 °C. It was cooled down in an ice bath, followed by adding 2 mL toluene and mixing for two min using a vortex mixture. The supernatant was collected in a fresh tube and the absorbance at 520 nm was measured on a spectrophotometer and toluene was used as a blank. The proline concentration was determined from a standard curve.

Greenhouse experiment

A pot study with three replications and a randomized block design was conducted in the greenhouse to examine the growth of Pea (*Pisum sativum*) plants under heavy metal lead stress and inoculation with *Rhizobium* mutants. Plastic pots with a diameter of thirty centimeters, each was filled with four kilograms by a mixture of clay and sand at a ratio of 3:1. To cultivate the strains, 100 milliliters of YEM broth were infused with the corresponding *Rhizobia* left to incubate for 48 hours, or until a homogenous population of 10^{-2} was reached. Before planting, the soil was polluted with lead metal at a concentration of 6.3 mM as Pb $(No_3)_2$. Ten cultures, RL16 as wild-type, and RLM1, RLM2, RLM3, RLM4, RLM5, RLM6, RLM7, RLM8 and RLM9 were used in the study. Pea seeds were surface sterilized and then soaked in 35% sugar solution with *Rhizobium* for 5 min to help the inoculants adhere to the seeds and three pea seeds were planted in each pot. The first treatment was used as a control (wild-type *Rhizobia* and pea plants without heavy metal stress), while the second treatment included wild-type *Rhizobia* strain with heavy metal stress. The soil moisture was maintained at 60-70% of the total moisture capacity during the experiment by randomly weighing the pots and adding deionized water as needed. After two months, the plants were harvested and collected. The plant measurements determined were root length (cm), root fresh weight (gm), root dry weight (gm), stem length (cm), stem fresh weight (gm), stem dry weight (gm), number of nodules, nodule fresh weight (gm), nodule dry weight (gm). In addition, chemical analysis was used to evaluate the amounts of nitrogen (N) present in whole plant samples. The Kjeldahl method is used to determine the nitrogen content. Leaf chlorophyll content was estimated from the youngest fully developed leaf using a chlorophyll content meter (SPAD 502 chlorophyll meter). Also, proline concentration was determined according to the methods of Ortiz *et al.* (2015) with slight modifications. Briefly, 1.25 g ninhydrin was dissolved in 20 ml of 6 M phosphoric acid and 30 ml of glacial acetic acid by heating on a hot plate while stirring. The solution was allowed to cool and kept at 4 °C, which became stable after 24 h. About 500 mg of fresh pea leaf sample was ground in 10 ml of 3% aqueous sulfosalicylic

acid and centrifuged at 10,000 × g for 10 min. 2 ml of the upper liquid was reacted with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin solution in Falcon tubes at 100 °C in a water bath for 60 min and the reaction was stopped in an ice box. 4 ml of toluene was added to the extract mixture and stirred vigorously for 15–20 s in a shaking incubator at 250 rpm. The mixture was kept in the dark for 30 min, the toluene-containing chromophore was aspirated from the aqueous phase, and the absorbance was read at 520 nm using toluene as a blank sample. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:

[(ug proline/ml x ml toluene) / 115.5 µg/µmole]/[(g sample)/5] = µmoles proline/g of fresh weight material. (Bates 1973)

PCR Amplification of Pb-resistant gene

DNA was extracted from wild-type strains and mutants using the method reported by Wilson (2001). The primers for the amplification of a Pb-resistant gene (*pbrA*) were pbrA1; 5-ATGAGCGAATGTGGCTCGAAG-3 (forward) and pbrA2; 5-TCATCGACGC AACAGCCTCAA-3 (reverse) (Borremans *et al.*, 2001). PCRs were conducted in a 50 µl reaction volume containing 100 ng DNA, 25 µl PCR Master Mix (2x My TaqTM Red Mix, Thermo Scientific Fisher, USA), and 20 µM of forward and reverse primers, using the following conditions: Initial denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 3 min at 72°C. A final extension was done at 72°C for 7 min. PCR products were separated on 2% agarose gels at 100 V for 1 h in TBE buffer, stained with ethidium bromide, and photographed under UV light.

Statical Analysis

Data were analyzed statistically using ANOVA at a 5% significance level. The software Costat version 6.3. copyright 2008 Cohort Software (798 Lighthouse Ave. PMB 320, Monterey, CA, 93940, USA) was used.

Results

1. Development of Rhizobium mutants:

In this research, the wild-type bacterial *Rhizobium leguminosarum* biovar Vicia strain RL16 (Weam NH *et al*., 2024 unpublished data) was used as wild-type to develop lead high tolerant mutants. It was previously identified as a lead-tolerant strain to LC_{90} concentration. It was shown that it tolerates the concentration of lead of 6.0 mM which equals LC_{90} of its growth. To develop better *Rhizobium* mutants, the strain RL16 was exposed to EMS. The cells were treated with 0.5M EMS concentration for 30-, 60-, 90-, and 120-min EMS. Following these treatments, the selection of viable cells was counted on media containing 6.3 mM $Pb(No_3)_2$ concentration. This concentration equals LC_{95} of RL16 strain viability. The bacterial counts of all treatments were performed and represented as the percentage of survival colonies (Table 1). The survival percentage rate of these isolates went down as time increased. The maximum growth resulted in the treatment exposed to EMS for 30 min. The live bacteria were further screened for stability as a mutant for seven generations on the YEMA medium containing 6.3 mM $Pb(No_3)_2$. The live bacterial mutants (n = 9) were screened for growth on the YEMA medium containing 6.3 mM $Pb(No_3)_2$. These mutants showed better growth than the RL16 strain (Table 2). This study revealed that the Rhizobium mutants grew better than the wild Rhizobium strain in the presence of lead LC95.

Table 1: Effect of EMS treatment on survival of wild-type *Rhizobium* strain RL16 on YEMA media supplemented with 6.3 mM $Pb(No_3)_2$.

EMS exposure time	Survival%
30 min	42.4
60 min	36.4
90 min	27.3
120 min	15.2

Table 2: Survival of wild-type *Rhizobium* strain RL16 and RLM mutants on YEMA media supplemented with 6.3 mM $Pb(No_3)_2$.

Strain	Survival %
RL16 wild-type	38.39
RL M1	44.64
RL M2	40.18
RL M3	42.86
RL M4	47.32
RL M5	53.57
RL M6	41.96
RL M7	57.14
RL M8	62.5
RL M9	60.71

2. Production of extracellular proline by Rhizobium mutants under the lead conditions:

The extracellular secretion of proline by *Rhizobium* strains in the culture media is presented in Figure 1. The culture supernatant of mutants grown at lead stress accumulated proline to a high concentration of up to 58 µM/ml. In contrast, the culture supernatant of a wild-type strain grown on lead stress had an accumulation of only 24 µM/ml proline (Figure 1). The bacterial cultures of grown wild-type accumulated 10 µM/ml proline under no-stress conditions. All mutants accumulated proline concentrations significantly higher than the wild-type under stress conditions.

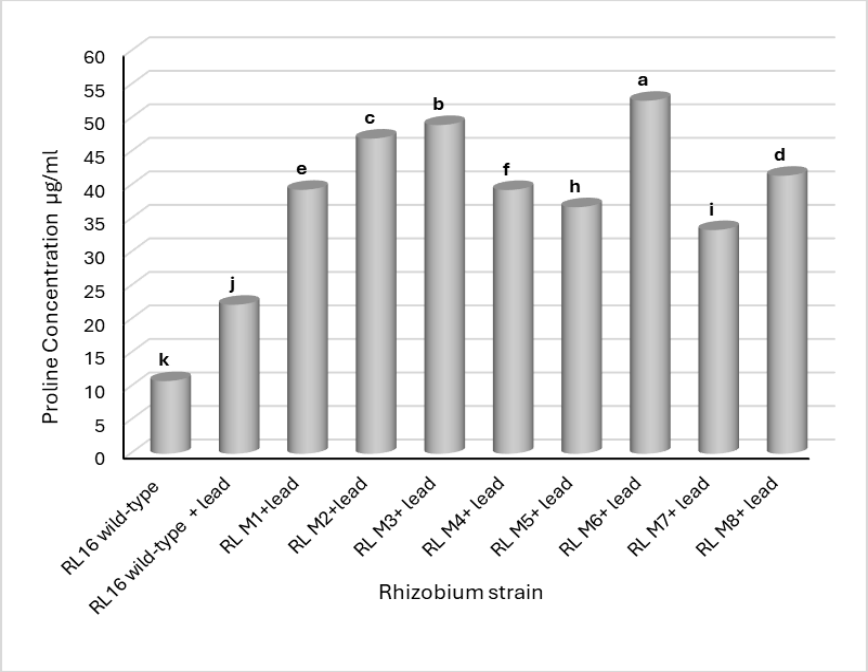
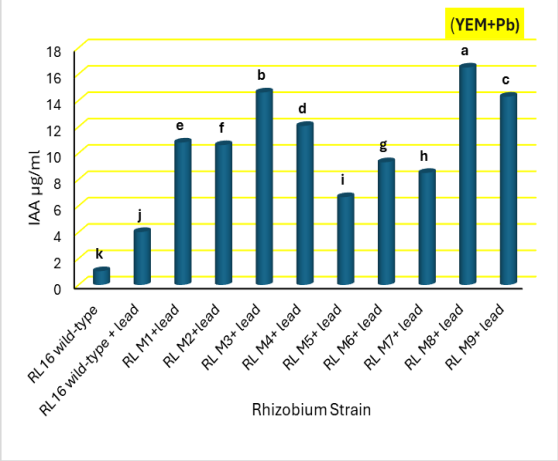


Fig (1): Concentration of extracellular proline production by wild-type strain and mutants after growing in YEMA media supplemented with 6.3 mM $Pb(No_3)_2$. Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

3. IAA production by Rhizobium mutants under the lead conditions in the presence and absence of L-tryptophan

Wild-type and mutants were screened for IAA production by comparing the stranded curve of known IAA concentrations with the Salkowski reagent. At the same time, the effect of L-tryptophan positively induced the biosynthesis of IAA from *Rhizobium* strains was studied. The colorimetric assay showed that all strains were able to produce IAA on a YEM medium supplemented with or without tryptophan at varying concentrations depending on their efficiency and enzymatic potency (Figure 2). However, the production of IAA in the presence of L-tryptophan was more than in the absence of L-tryptophan absence in all cases. However, the wild-type strain produced proline without lead heavy metal at a significantly low concentration. Furthermore, all RLM mutants produced significantly higher proline concentrations than the wild-type under stress conditions (Figure 2a). Indeed, a relatively high concentration was found in the culture filtrate of strain RLM8 in the presence of lead. In the presence of L-tryptophan, the data presented in Figure 2b showed increasing IAA production in the mutants treated with L-tryptophan. However, RLM5 demonstrated the highest significant value of IAA production and RLM6 was the lowest significant.

a)



b)

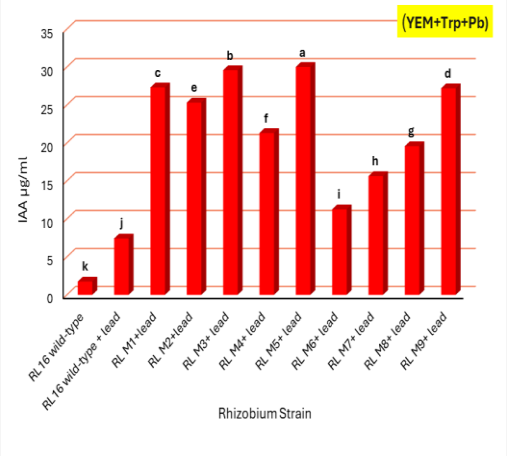


Fig. (2): Production of IAA by wild-type strain and mutants after growing in YEMA media supplemented with 6.3 mM Pb $(No_3)_2$ without L-tryptophan (a), with L-tryptophan (b). Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

4. Analysis of the Interaction between Pisum sativum L. and Rhizobium mutants under the lead conditions

The effects of inoculation with different *Rhizobium mutants* on the morphology and morphometric measurements of *P*isum *sativum* grown under lead stress conditions were investigated.

4.1 Chlorophyll content

Chlorophyll content in the fresh plant leaves was estimated (mg Chlorophyll/gm of fresh leaf weight). In the first observation, the chlorophyll concentration was increased in most treatments compared to the control under stress conditions except RLM6 (Figure 3). In other words, the chlorophyll content of *P. sativum* was positively significantly affected by all RLM mutants' inoculations except RLM6 which showed a negative effect compared to wild-type strain under stress conditions. The best effect was due to RLM9. In the second observation, the chlorophyll contents estimated were significantly lower with some strains than the control with no stress.

However, the stem length demonstrated a negative significant effect with all strains except strain RLM9. Stem fresh weight exhibited significant excess with all strains and RLM9 exhibited the largest excess. Also, all mutant treatments performed significantly higher stem dry weight values than wild-type under stress conditions and RLM7 was the best. The same significantly greater direction was observed for the number of nodules where RLM9 was highest. The values were even higher than the wild-type strain without stress. Nodule fresh weight indicated higher significant performance for all mutants except RLM3 and RLM9 than wild-type under stress conditions with RLM4 being the highest. Nodule dry weight gave higher significant values except LRM9 and RLM3 was the highest. As for nitrogen percentage, all mutants revealed higher significant percentages than wild-type under stress conditions and RLM9 was the highest.

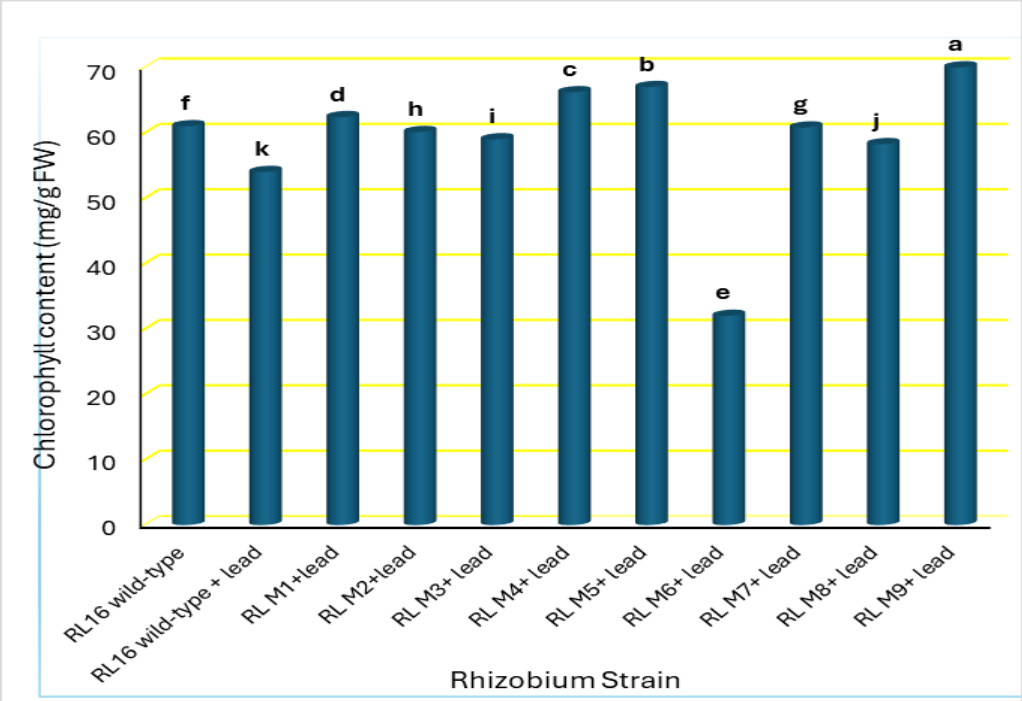


Fig. (3): The chlorophyll concentration in *Pisum sativum* plants grown in soil contaminated with 6.3 mM as Pb (No3)2 and inoculated with wild-type strain and mutants. Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

4.2. Root Characteristics

Root fresh weight, dry weight, and length indicated significant differences with all strains (Figure 4). It is obvious that the heavy metal lead significantly suppressed the fresh weight of pea plant roots as in inoculation with wild-type strain with/without stress. However, inoculation with all mutants significantly increased the values of root fresh weight. Five of them RLM1, RLM2, RLM3, RLM6, and RLM8 increased values of root fresh weight significantly even more than the wild-type with no stress (Figure 4a). Also, it is evident (Table 4b) that there was a significant difference among the isolates in recording the dry weight of the root of the pea. However, all the isolates produced higher root dry weight than the wild-type control under stress conditions except RLM5 and RLM7. The highest dry weight of the root was recorded with the strain RLM1 even more than the wild-type with no stress and the lowest was obtained from RLM5 and RLM7. At the same time, there was a

significant difference among the strains in recording the pea's root length (Table 4c). However, all the strains produced higher root lengths than the control under stress conditions except RLM2 and RLM7. The highest root length was obtained with the strain RLM4, and the lowest root length was obtained with the RLM2. Root length increased significantly with seven isolates, excluding RLM2 and RLM7. The wild-type strain inoculation under no stress showed a highly significant value of pea root length. However, RLM9 showed the highest value in pea root length.

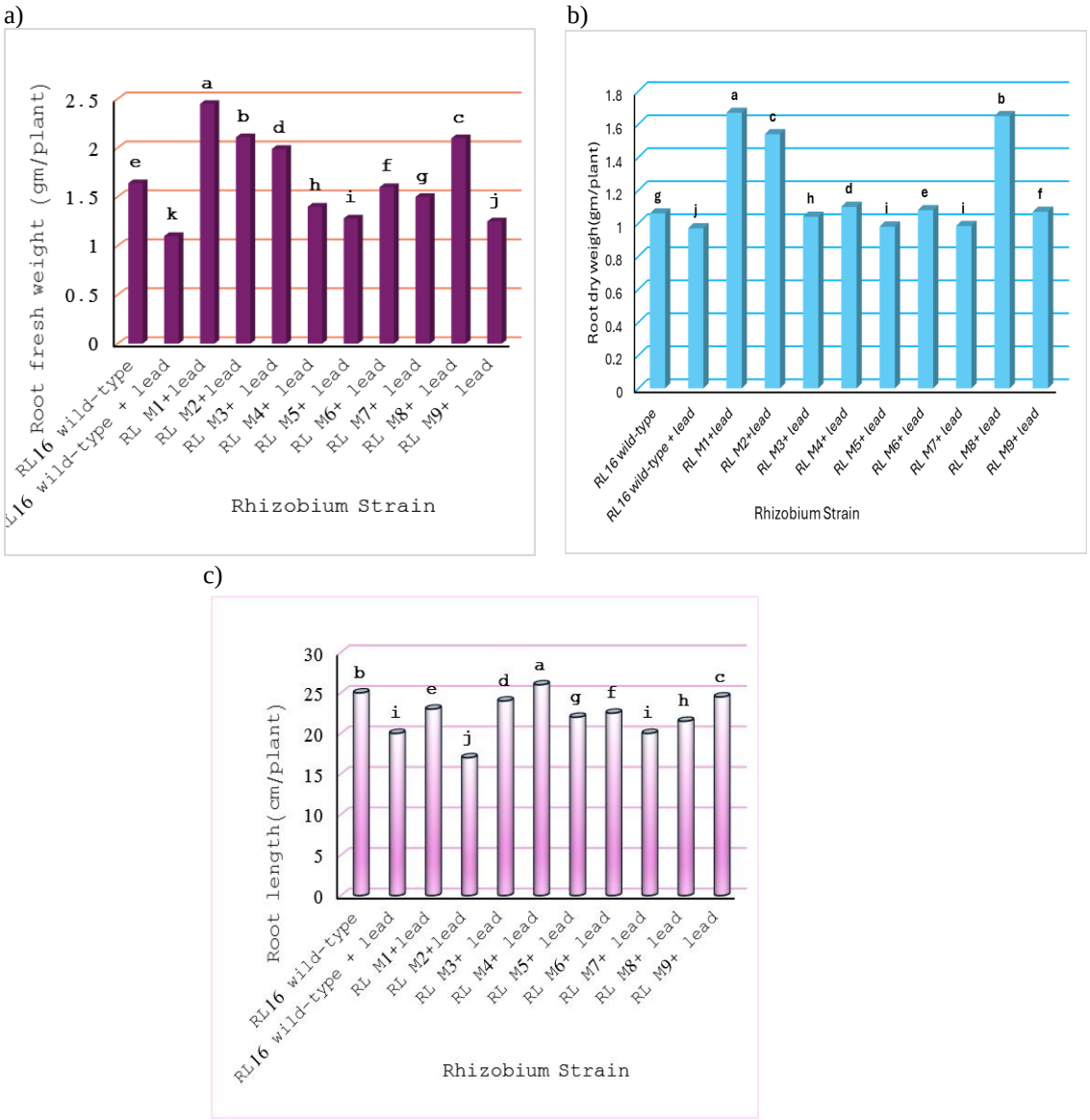


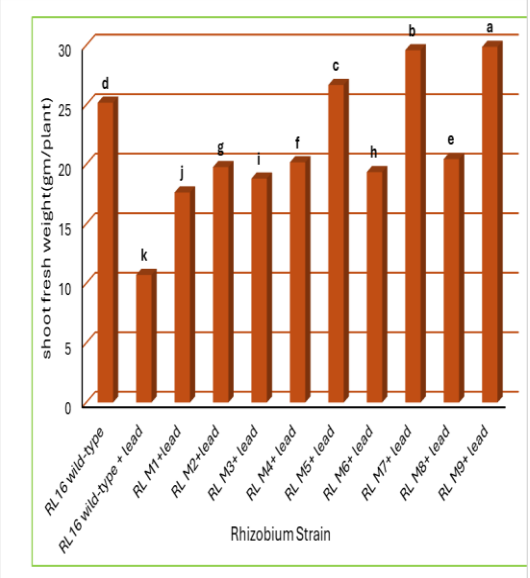
Fig. (4): Root characters of *Pisum sativum* plants contaminated with lead metal at a concentration of 6.3 mM as $Pb(No_3)_2$ and inoculated with wild-type strain and mutants (a) Root fresh weight (b) Root dry weight (c) Root length). Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

4.3 Shoot Characteristics

The investigated heavy metal significantly reduced the shoot fresh weight of the evaluated pea plants as shown in Figure (5a) when inoculated with wild-type strain. However, the mutants were able to overcome the negative

effect of lead. RLM5, RLM7, and RLM9 exhibited markedly elevated values compared to wild-type inoculation under non-stress conditions. A similar pattern was observed for root dry weight (Figure 5b), with RLM4, RLM5, RLM7, and RLM9 exhibiting superior values compared to wild-type inoculation without lead. However, the reduction in shoot length and the negative effect of lead were not there (Figure 5c). Furthermore, the inoculation of all mutants did not significantly enhance pea shoot length compared to the wild-type, except RLM9.

a)



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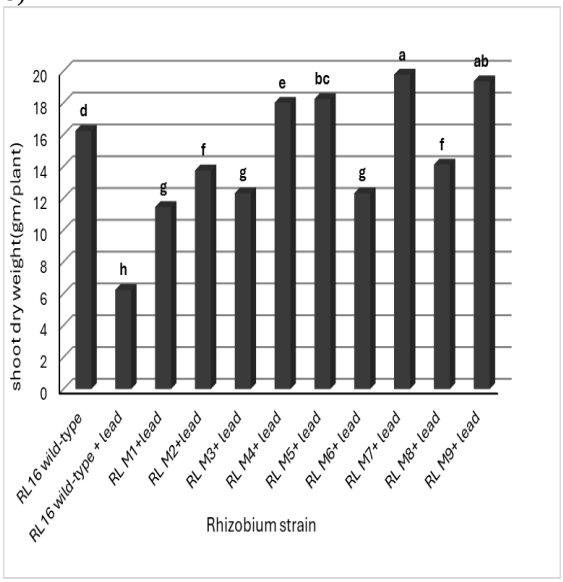

c)

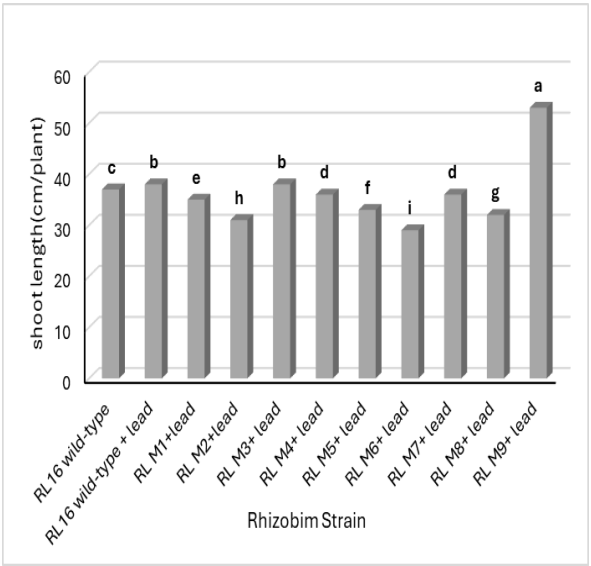

Fig. (5): Shoot characters of *Pisum sativum* plants contaminated with lead metal at a concentration of 6.3 mM as $Pb(No_3)_2$ and inoculated with wild-type strain and mutants. (a) Shoot fresh weight, (b) Shoot dry weight, and (c) Shoot length. Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

4.4 Nodule Characteristics

An analysis of the effects of different inoculations with different *Rhizobium* strains on plant nodule characters is shown in Figure 6. The nodule fresh weight of the pea was not affected by lead treatment. Inoculation with all mutant

strains except RLM3 and RLM9 also increased the nodule fresh weight values (Figure 6a). RLM4 showed a remarkably high significant value. The nodule dry weight proved the same direction (Figure 6b). However, nodule numbers were significantly affected by lead treatment. Inoculation with *Rhizobium* mutants recovered the harmful effect (Figure 6c).

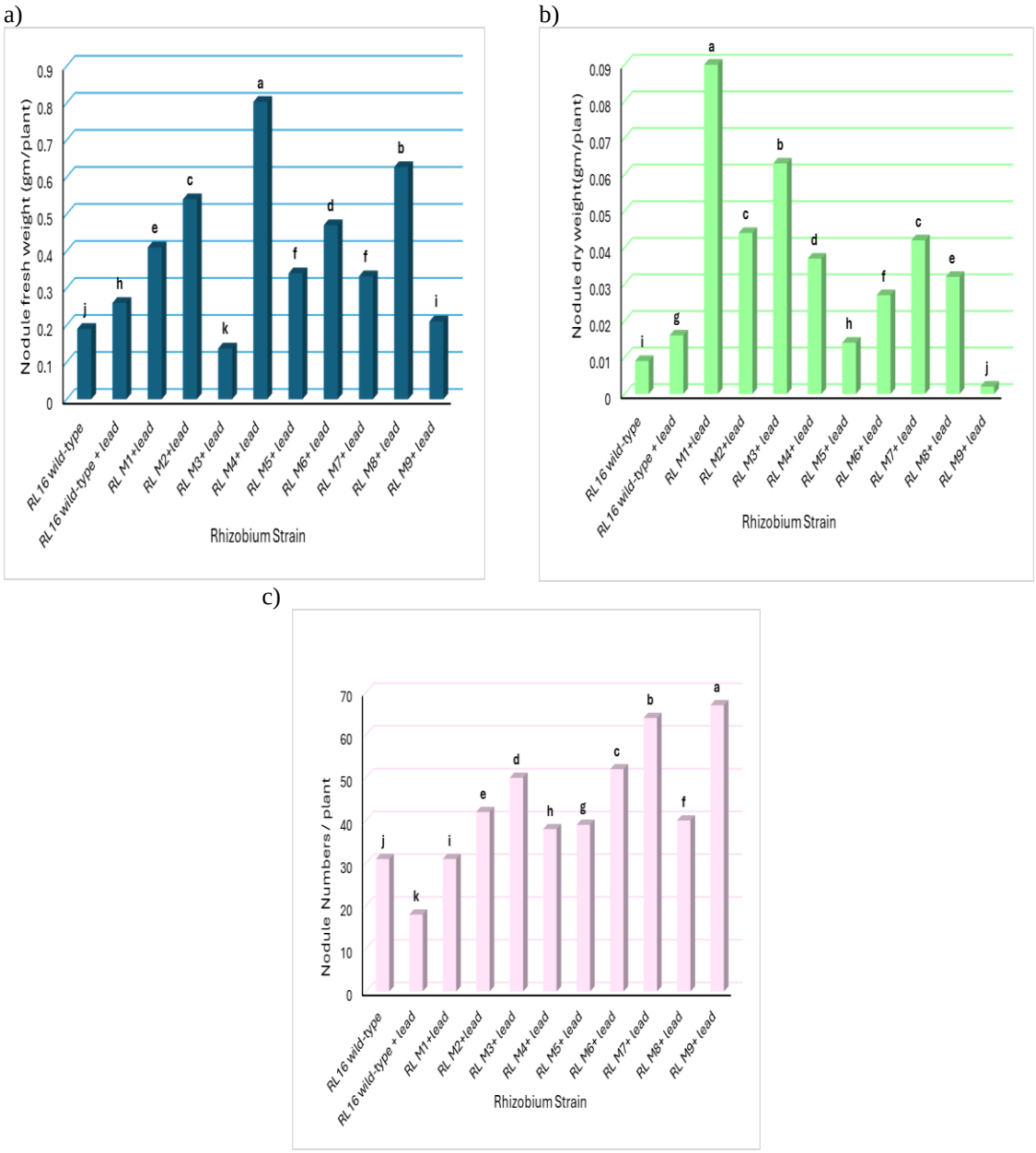

Fig. (6): Nodule characters of *Pisum sativum* plants contaminated with lead metal at a concentration of 6.3 mM as $Pb(No_3)_2$ and inoculated with wild-type strain and mutants 6.3 mM $Pb(No_3)_2$. (a) Nodule fresh weight (b) Nodule dry weight (c) Nodule numbers. Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

4.5 Nitrogen content

The ability to fix nitrogen in symbiosis with wild-type strain and mutants was studied. The performance of *Rhizobium* strains in recording nitrogen content in pea plants was statistically different (Figure 7). It is noticed that the

Rhizobium inoculant significantly prevented the negative effect of lead stress on nitrogen content in pea plants. The highest nitrogen content in the plants was obtained with the RLM9 inoculation under stress conditions. The lowest value among mutants was RLM4.

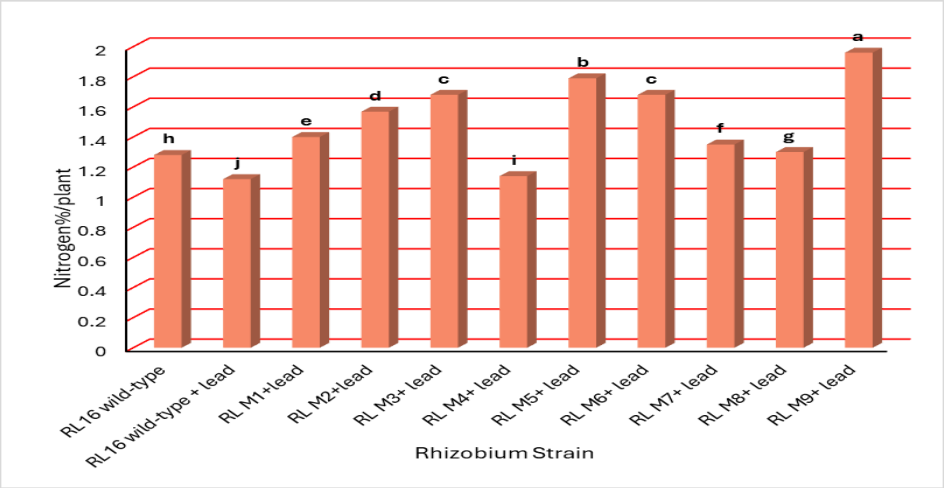

Fig. (7): Nitrogen percent in *Pisum sativum* plants contaminated with lead metal at a concentration of 6.3 mM as Pb(No3)2 and inoculated with wild-type strain and mutants 6.3 mM $Pb(No_3)_2$. Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

5. Proline content in plants

Proline in plants plays a significant role under stressful conditions. In the first observation, the proline estimation in pea plants showed a significant increase in all strain inoculations under stress compared to the control without stress (Figure 8). In the second observation, the mutant inoculations showed significantly higher proline concentrations in pea plants than in control except RLM2 and RLM9. The highest significant value was RLM7.

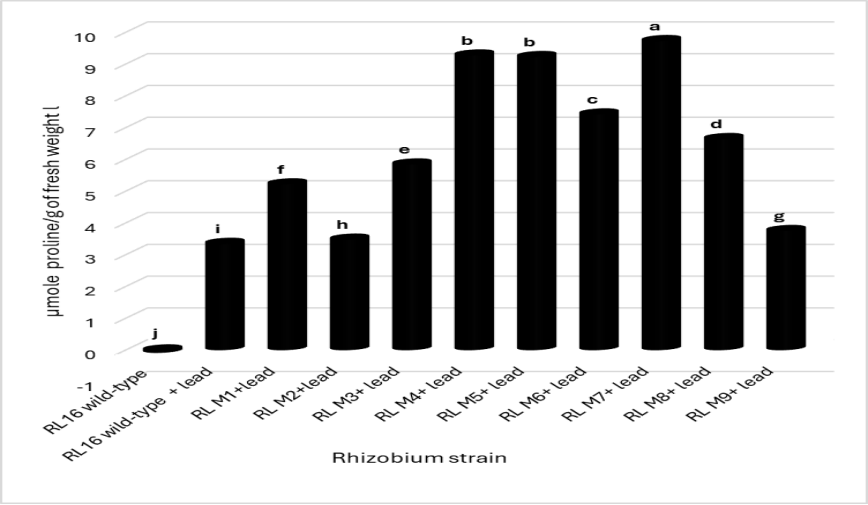

Fig. (8): Proline concentration in *Pisum sativum* plants contaminated with lead metal at a concentration of 6.3 mM as Pb $(No_3)_2$ and inoculated with wild-type strain and mutants 6.3 mM $Pb(No_3)_2$. Per each column, means with the same letter are not significantly different according to Duncan's multiple tests, $p \leq 0.05$.

6. Molecular characterization of Pb resistant gene (pbrA).

PCR amplification using a specific primer revealed the presence of the resistance gene *pbrA* for Pb in *Rhizobium* (Borremans *et al.*, 2001). The primer used to amplify the Pb-resistant gene yielded a band of approximately 450 bp (Fig. 9) with wild-type strains and mutants. This result demonstrated that in *R. leguminosarum* the tolerance of lead metal was accompanied by the presence of *pbr*A gene.

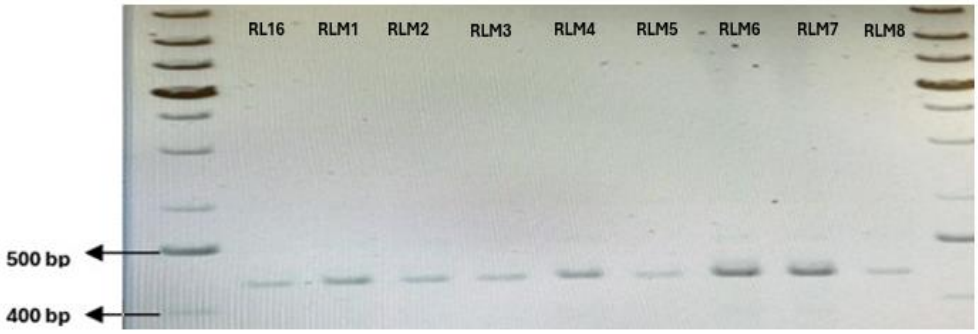

Fig (9): PCR amplicon of resistance gene *pbr*A in wild-type strain and mutants. M. Gene ruler 1kb plus Thermo Scientific.

Discussion

In this study, a wild-type *Rhizobium* strain (Hegazy WN *et al*., 2024) tolerant to lead heavy metal was subjected to chemical mutagenesis EMS to induce an improved mutant more tolerant to lead heavy metal. After chemical mutation nine strains were identified as survivors. The EMS random mutagenesis can induce gene mutations and chromosomal breaks. The frequency of these aberrations is directly proportional to the applied dose. In the present study, only one dose was used at different times. It was found that with increased exposure time, the bacterial colonies' survival percentage went down. Thus, it can be concluded that the effect of EMS was accumulative disruptive. The developed mutants exhibited improved growth compared to their native isolates. The similar result was obtained by Gnanachitra and Sridar (2019), who used EMS to create a better Rhizobium mutant. In addition, Shah *et al.*, (2024) developed mutants of *Rhizobium* by gamma (γ)-irradiation random mutagenesis. Moreover, Zelalem *et al.*, (2014), and Mekonnen *et al*., (2022) have derived efficient *Rhizobia* mutants using other chemical mutagens.

Before evaluating the efficacy of developed mutants in the greenhouse, the nine superior-growing mutants were tested for their production of proline and IAA compared to the corresponding native strain RL16. Proline provides protective benefits against abiotic and biotic stresses in a broad range of organisms (Sharma *et al*., 2011; Liang *et al*., 2013). The result of this study demonstrated clearly that the mutants were able to produce more extracellular proline than the wild-type under lead stress. Therefore, it provides compelling evidence that proline confers protection and increases the ability of *Rhizobium* to survive in lead heavy metal stress conditions. Related results were proved by Abou-Aly *et al*. (2019) in *Bacillus cereus* and *Alcaligenes faecalis*, and Goswami *et al*. (2022) in *Bacillus megaterium*. Also, other researchers established that proline is a multifunctional amino acid associated with several essential functions including carbon and nitrogen metabolism (Goldmann *et al.*, 1991) protein synthesis, and protection against various environmental factors such as metal toxicity (Chen and Dickman, 2005; Tripath *et al*., 2006). Although it is proved that proline provides heavy metal tolerance, the actual mechanism of its action warrants thorough investigation (Goswami *et al*., 2022). As for IAA, the data indicated that all strains including wild-type had excess

amounts under Pb stress conditions than nonstresses. Many researchers showed that Pb treatment increased IAA synthesis in plant-growth-promoting rhizobacteria i.e. Asadullah *et al.* (2022) with *Pseudomonas putida*, Wang *et al*. (2020) with *Enterobacter bugandensi*s, and *Bacillus megaterium*, Abdelkrim *et al.* (2018) with *Luteibacter* sp. and *Variovorax* sp., Mitra *et al.* (2018) with *Klebsiella michiganensis*, Carlos *et al.* (2016) with *Escherichia*, *Enterobacter*, *Enterobacter*, and *Serratia*. Also, data revealed that the presence of L-tryptophan increased IAA amounts. This aligns with Shoukry *et al.* (2018) and Bharucha *et al.* (2013), who reported comparable results with L-tryptophan. This increase is due to L-tryptophan as an essential amino acid and is crucial for various biological processes (Bhutani *et al*., 2018).

Following confirmation of enhanced IAA and proline production, the symbiotic infection behavior of each mutant was evaluated on pea plants under controlled greenhouse conditions. The effectiveness of *Rhizobia* -legume symbiosis is directly related to the symbiotic nitrogen fixation process carried out by the *Rhizobia* inside the nodules (Lindström and Mousavi 2019). Therefore, the nodulation ability is also associated with the effectiveness of *Rhizobia* strains, since nodule formation and growth are regulated in response to the nitrogen extant in the legume phloem and/or in the soil (Murray *et al*., 2017). Moreover, as previously indicated, symbiotic nitrogen fixation was the first studied mechanism of plant growth promotion; therefore, the parameters analyzed to determine the effectiveness of *Rhizobia* strains have been chlorophyll content, root characters, shoot characters, nodule characters, and the nitrogen content of pea plants. Ur Rahman *et al*. (2024) indicated that Pb-affected plants show significant retardation in physiological attributes such as chlorophyll content, root characters, shoot characters, nodule characters, and nitrogen content. However, the nodule bacteria *R. leguminosarum* bv. *viciae* commonly plays a key role in pea plants' tolerance to toxic heavy metals. Therefore, efficient integration of symbiotic partners and the success of plants and bacteria under stress requires that both symbionts be tolerant to both stress factors (Belimov *et al*., 2019). This fact was true in this experiment where chlorophyll content, root characters, shoot characters, nodule characters, and nitrogen content of pea plants showed a significant reduction as expected due to Pb heavy metal (Aponte *et al*., 2020; Chowdhury and Rasid 2021). However, *Rhizobium* application improves the pea characters and *Rhizobium* mutants indicated better recovery because the mutants were more tolerant to Pb than the wild-type strain.

Proline estimation was performed to elucidate the role of proline metabolism in pea plants under lead stress circumstances. All treatments demonstrated a greater buildup of proline in the presence of lead heavy metal. This indicates more proline accumulation when the plant suffers from severe environmental stress. Thus, the extreme sensitivity of the metabolic processes of proline synthesis and degradation themselves may be of benefit by regulating metabolic processes adversely affected by stress (Hare and Cress, 1997). This research's result supports the idea that Rhizobium mutants showed more proline production, which agrees with their ability to recover the negative effect of Pb contamination in pea characters.

PCR amplification of the *pbrA* gene showed all wild-type strain and mutants contained the Pb-resistant gene. Pb-resistant gene presence in varied species and genus suggested that there was a possible transfer of Pb-resistant gene (Zhang *et al*., 2008). It has also been reported that many species were highly abundant in *pbrA* gene in heavy metal-contaminated environments and remained rare in uncontaminated areas (Hemme *et al.*, 2016; Abdel-lateif 2017). Therefore, it may be claimed that Rhizobia has been improving its heavy metal resistance mechanisms with the lateral gene transfer through the evolutionary process to quickly adapt to abrupt harsh environmental stresses (Barcellos *et al.*, 2007).

Conclusions

This study was conducted to investigate the ability of pea plants to grow in Pb-contaminated soil. Results reported that efficient and heavy metal-tolerant *Rhizobium* bacteria increase the pea plants' biomass production. Furthermore, combining bacteria and legume plants could be a novel bioremediation technique that allows a more efficient clean-up of heavy metal-polluted soils by accelerating phytoremediation processes or by regenerating and enriching moderately contaminated soil.

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تطوير وتوصيف طفرات الريزوبيا المقاومة للرصاص لتعزيز نمو البازلاء

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الملخص بالعربى

التركيزات المتزايدة من الرصاص وجد أنها عالية السمية للكائنات الحية الدقيقة الموجودة بالتربة وممكن ان تؤثر على تباينها التركيبى لذلك فان الدراسة الحالية استهدفت استحداث طفرات فائقة من بكتريا الريزوبيوم باستخدام مادة ايثيل ميثان سلفونات ومعاملة السلالة البرية واختبار مدى تاثير الطفرات على محصول البسلة. وجد ان تسع طفرات فائقة تتحمل التركيز العالى من نترات الرصاص (٦,٣ ملليمول) بالمقارنة بالسلالة البريه

هذه الدراسة أوضحت ان الطفرات المتحصل عليها انتجت برولين اكثر من السلالة البرية والذى يزيد من قدرتها على البقاء وتحمل ضغط الرصاص .

استخدام الريزوبيوم يحسن من الصفات المحصولية لنبات البسلة وقد أعطت طفرات الريزوبيوم اعلى تحمل للرصاص بالمقارنة بالسلالة البرية وقد أظهرت النتائج ان تلقيح نباتات البسلة بطفرات الريزوبيوم اعطى اكثر انتاجا للبرولين والذى يتوافق مع قدرتها على تحمل التاثير السلبى للتلوث بالرصاص. وقد اظهرتفاعل البلمرة المتسلسل وجود جين تحمل الرصاص في كلا من السلالة البرية والطفرات .

الكلمات المفتاحية: طفرات الريزوبيا، البرولين، حمض الإندول الأسيتيك(IAA) ، المعالجة الحيوية، البازلاء، جينpbrA