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Research Article

Lead (Pb)-Resistant Bacteria Improve *Brassica chinensis* Biomass and Reduce Pb Concentration in Pb-Contaminated Soil

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ABSTRACT
Applications of inorganic fertilisers and pesticides frequently increase lead (Pb) content in the soil and food crops. This study aims to isolate Pb-resistant bacteria and test the isolated bacteria in reducing Pb concentration and increasing biomass production of *Brassica chinensis* on Pb-contaminated soil. Soil and plant samples were collected from agricultural land in Batu City, East Java, Indonesia. The isolated bacteria were tested for Pb resistance and then characterised according to 16S rRNA Sequence. A pot trial with a completely randomised block design consisting of 9 treatments and 3 replications was set to determine the effect of Pb-resistant bacteria inoculation on Pb residue, plant growth, and soil nutrients. The result showed that the isolated Pb-resistant bacteria were *Bacillus wiedmannii* and *Bacillus altitudinis*. The bacteria were resistant to Pb up to 10,000 mg/L $PbNO_3$. Inoculation of the bacteria increased *B. chinensis* growth and biomass production, namely increasing the number of leaves (12%) and dry weight (35%). Also, the bacteria reduced Pb residue in the soil by up to 88%. Moreover, soil essential nutrients such as total nitrogen, available phosphorus, and exchangeable potassium increased (12%, 73%, and 200%, respectively) after the application of Pb-resistant bacteria. The bacteria have the potential for bioremediation of Pb-contaminated soils on a large scale due to the bacteria prevent Pb uptake by food crops such as *B. chinensis* by reducing Pb content in the soil, which is good for food safety and environmental sustainability.

INTRODUCTION
Intensive farming is a type of agricultural system that usually uses large inputs of fertiliser and pesticides to increase plant production and prevent yield loss (Alexandratos & Bruinsma 2012; Scotti et al. 2015). Intensive farming is an agricultural system that farmers widely use, especially for horticultural crops (Mariyono 2019), such as in the highland of Batu City, East Java, Indonesia. Survey results showed that farmers in Sumberbrantas village, Batu City, apply ZA amount 300 kg/ha and NPK amount 200 kg/ha. While, application of various types of pesticides every two days. According to Statistics Indonesia (2018), the use of inorganic fertilisers and pesticides occupy almost 24.22% (potato) and 28.82% (cabbage) of total budget for these crops' cultivation. Using inorganic fer-

tilisers and pesticides continuously causes soil and water contamination (Sharma et al. 2019; Bisht & Chauhan 2020). Moreover, the residue remains in the crops and enters food chains, thus harmful to human health (Sharma et al. 2019). For ecosystem sustainability, pesticide use impacts biodiversity loss, e.g., loss of natural enemies and increased plant pests and disease resistance (Sánchez-Bayo 2021).

Intensive use of inorganic fertilisers and pesticides contributes to heavy metal contamination in agricultural land (Bisht & Chauhan 2020). Lead (Pb) is one of the heavy metals that contaminate agricultural soil, originally from agrochemical products such as fertilisers and pesticides containing Pb (Kumar et al. 2022). Pb content in fertiliser worldwide is around 1-300 mg/kg (phosphorus fertiliser), 1-15 mg/kg (nitrogen fertiliser), 2-125 mg/kg (lime fertiliser), 2-60 mg/kg (manure), respectively (Alengebawy et al. 2021). Several active ingredients of pesticides contain Pb above the permissible concentration (which is 10 ppb), such as glyphosate 58 ppb, isoproturon 30 ppb, and fluroxypyr 110 ppb, respectively (Defarge et al. 2018). The large amount of Pb content in fertilisers and pesticides applied during crop cultivation will increase Pb content in the soil and agricultural products (Kumar et al. 2022).

Pb contamination in soil is mainly from anthropogenic activities (Mallongi et al. 2022), such as agricultural activity. Pb concentration in agricultural soil ranges from 25.98 to 108.68 mg/kg (Astuti et al. 2021). The concentration exceeds the limit concentration of Pb in the soil for agricultural activities, which is less than 70 mg/kg (CCME - Canadian Council of Ministers of the Environment 1999). Moreover, Pb concentration in vegetable products, such as cabbage, is around 26.51 to 29.98 mg/kg. The concentration also exceeds the permissible level of Pb in vegetables according to the Regulation of Indonesia Food and Drug Agency Number 5 in 2018, which is less than 0.2 mg/kg. The results are alarming and remediation measures are crucially required to prevent further Pb contamination in the soil. The problem leads to disruption of soil function, affects plant growth, and is dangerous for human health as well as environmental sustainability (Bisht & Chauhan 2020).

There are many ways to remove Pb contamination in the soil. Yet, bioremediation is considered as cost-effective and environmentally friendly to remove metal contaminants from the soil (Dixit et al. 2015). Bioremediation uses microbes, either their biomass to absorb or their metabolism to detoxify contaminants in the soil (Ojuederie & Babalola 2017). Pb-resistant bacterium (e.g., *Rhodobacter sphaeroides*) is a promising alternative for Pb remediation in contaminated soil through the precipitation and formation of inert compounds such as Pb sulfate and Pb sulfide (Li et al. 2016). Also, phosphate (P) solubilising bacteria and biochar can immobilise Pb^{2+} and improve soil quality (Zhu et al. 2022). Compost and rhizobium addition are also a potential combination for removing Pb from contaminated soil (Rosariastuti et al. 2019). This study aims: 1) to isolate and to characterise Pb-resistant bacteria from Pb-contaminated soil due to intensive application of pesticides and fertilisers containing Pb; 2) to analyse the effect of Pb-resistant bacteria application in the soil and the growth of *Brassica chinensis* in Pb-contaminated soil. This study is crucial to support food safety and environmental sustainability.

MATERIALS AND METHODS

Soil and plants sampling site

Soil and plant samples were taken from agricultural land, specifically horticulture commodities such as Chinese Cabbage (*Brassica rapa* subsp. *pekinensis*) and potato (*Solanum tuberosum*), where farmers intensively ap-

plied pesticides and fertilisers containing Pb. The location lies on Sumber Brantas Village, Bumiaji Sub-Regency, Batu City, East Java, Indonesia (7°45'13" S and 112°31'04" E). The location is 953 m above sea level (m asl) with an average air temperature is 27 °C. Sampling points in a diagonal shape were determined using purposive random sampling (Figure 1). Soil samples were taken from rhizospheric areas and replicated three times at each sampling point. The samples (soil and plant) were kept in a polyethylene bag and stored in a cooling box. The samples were transported to the laboratory for further analysis.

Bacteria isolation and purification

Ten grams of Pb-contaminated soil were suspended in 90 mL of sodium chloride 0.85% in a 250 mL flask. Lead-resistant bacteria were isolated using a serial dilution method (10^{-1}-10^{-5}), 1 mL of aliquots were taken from serial dilutions 10^{-4} and 10^{-5} and then inoculated onto nutrient agar (NA) plates containing 50 mg/L $Pb(NO_3)_2$ according to a method proposed by Hafeez et al. (2018) with modification. To confirm that the isolated bacteria are Pb-resistant, the pure colonies were streaked three times onto NA agar plates containing 50 mg/kg $Pb(NO_3)_2$.

Lead minimum inhibitory concentration bioassay

Lead minimum inhibitory concentration test was done using paper discs 0.55 cm soaked with $Pb(NO_3)_2$ solution in different concentrations (0, 100, 1000, 5000, and 10000 mg/L). The tested bacteria were inoculated into Petri dishes containing NA medium using a pour plate method. The paper discs containing Pb were placed onto NA plates and then incubated at 28 °C for 48 h according to the method used by Ustiatik et al. (2022) with modification. The tested bacteria's ability in Pb detoxification was measured according to the inhibition zone on the medium (Equation 1).

$$LO = \pi r^2 \quad (1)$$

Where: LO = wide of inhibition zone

π = 3.14

r = inhibition zone (halo zone around the Paper Disc)

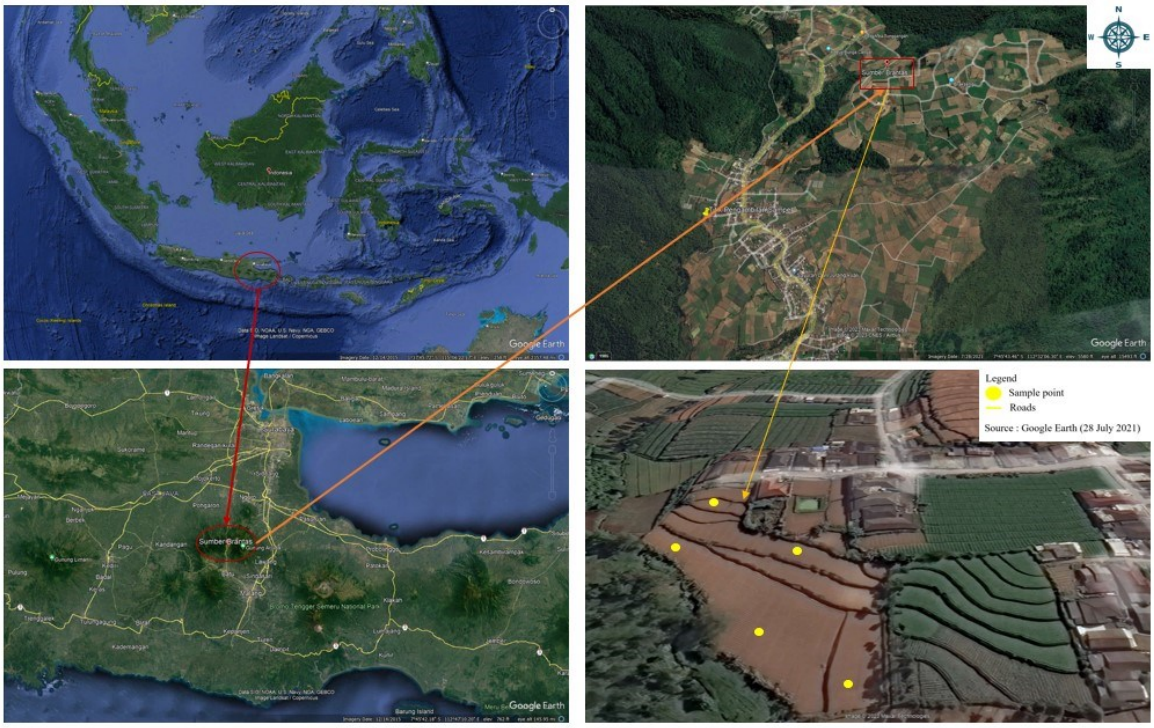

Figure 1. Soil and plant sampling site.

DNA isolation and 16S rRNA sequencing

Selected bacteria were chosen for genomic DNA extraction using Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). MyTaq Red Mix (Bioline, BIO-25048) was used for PCR amplification. For sample extraction, three loops of bacterial colonies (50-100 mg) were taken for bacterial lysis in ZR BashingBead™ Lysis Tubes (0.1 and 0.5 mm) containing BashingBead™ Buffer. Cell lysis was performed using mechanical lysis in a homogeniser with maximum speed for >5 min. After centrifugation, the supernatant was filtered and subsequently mixed with Genomic Lysis Buffer for DNA binding in a spin column. After DNA binding on the spin column, the column was then washed three times using DNA Pre-Wash Buffer and g-DNA Wash Buffer. The DNA was eluted using 35 µL DNA Elution Buffer. The DNA purity and concentration were measured using Nanodrop.

PCR master mix consisted of (1 x 25 µL) 9.5 dd H_2O, 12.5 MyTaq Red Mix (2x), 20 µM 27F Primer (AGAGTTTGATCMTGGCTCAG), 20 µM 1492R Primer (TACGGYTACCTTGTTACGACTT), and DNA Template, according to the product instruction (Ustiatik et al. 2022). For PCR condition, initial denaturation 95 °C (1 min), denaturation 96 °C (15 sec), annealing 52 °C (30 sec), extension 72 °C (45 sec). Subsequently, the PCR product was subjected to electrophoresis that was performed using 1% agarose gel run in 1x TBE Buffer. One microliter FloroSafe was added to 25 mL of 1% agarose for DNA staining, and then 2 µL PCR products of each sample were transferred to the well of agarose gel and run for 25 minutes at 135 V. For gel cutting, 10 µL FloroSafe was added to 25 mL of 1% agarose, and then the expected band was cut and proceed to gel purification before sequencing. Amplicons of 16S rRNA were purified and sequenced by Apical Scientific, Malaysia. The 16S rRNA sequences were compared with sequences in GenBank using BLAST program. The 16S rDNA sequences were aligned with reference sequences using MEGA V.6 program. Phylogeny trees were constructed and inferred with the neighbour-joining algorithm based on the Tamura-Nei model using 1000 replicates bootstraps.

Soil and plant analysis

Soil and plant samples were analysed for chemical properties that consisted of organic C (Walkey and Black), pH (Electrometry), available P (Bray I and HCl 25% extraction), total Pb of soil (acid mineralization) measured using Atomic Absorbance Spectrophotometry, Thermo-Fisher, USA.

Research design

Pot trial was designed as a completely randomised block design and consited of 9 treatments with 3 replications, i.e.: Pb-contaminated soil as control (KT); Pb-contaminated soil + fertiliser + pesticide application (TP); Pb-contaminated soil + fertiliser + pesticide application + *Bacillus altitudinis* (TA); Pb-contaminated soil + fertiliser + pesticide application + *B. wiedmannii* (TB); Pb-contaminated soil + fertiliser + pesticide application + *B. altitudinis* + *B. wiedmannii* (TAB); Pb-contaminated soil + fertiliser + pesticide application + *Brassica chinensis var. Parachinensis* (TS); Pb-contaminated soil + fertiliser + pesticide application + *B. chinensis* + *B. altitudinis* (TSA); Pb-contaminated soil + fertiliser + pesticide application + *B. chinensis* + *B. wiedmannii* (TSB); Pb-contaminated soil + fertiliser + pesticide application + *B. chinensis* + *B. altitudinis* + *B. wiedmannii* (TSAB). Polybags were filled with 3 kg of soil and base fertiliser was added (NPK 0.45 g/polybag). For pesticide and fertiliser application, ZA

0.3 g/polybag, pesticide abamectin 0.05 mL/polybag, sipermetrin 0.05 mL/polybag, and mancozeb 0.125 g/polybag. Bacteria starter in NB were prepared overnight and then applied 10 mL/polybag (1 x 10^8 CFU/mL).

Data analysis

Statistical analysis was conducted using GenStat 12th Edition. The obtained data were subjected to a data normality test using Shapiro Wilk's test. Abnormal distribution data were subsequently transformed using square root (Sqrt) or logarithm (Log10), and then statistically analysed using a one-way analysis of variance (ANOVA). The difference between treatment means was tested using Tukey Test at 5% significance level.

RESULTS AND DISCUSSION

Study site characteristics

Soil at the sampling site is fertile soil. According to Indonesia Soil Research Agency (2005) criteria soil organic C and available P were high, total N and exchangeable K were moderate. However, soil pH at the study site was acidic (Table 1). Intensive agriculture leads to soil acidification (decrease in pH) (Abure 2022) and is a sign of land degradation in a watershed (Felix et al. 2015). Total Pb in the soil was below the maximum Pb level, which is considered dangerous for agricultural activities (CCME - Canadian Council of Ministers of the Environment 1999). Pb content in the soil was below 140 mg/kg (111.81 mg/kg). However, Pb content in biomass exceeds the permissible level of Pb in vegetables according to Indonesia Food and Drug Agency Regulation Number 5 in 2018 (33.47 mg/kg). The concentration is harmful for human health as Pb is a non-bioessential and hazardous heavy metal (Naik & Dubey 2013).

Table 1. Soil and plant properties at the study site.

Parameter	Unit	Result
pH		5.01
Organic carbon	%	3.45
Total nitrogen	%	0.45
Available phosphorus	mg/kg	18.59
Exchangeable pottasium	me/100g	0.44
Lead content in Soil	mg/kg	111.81**
Lead content in biomass	mg/kg	33.47*
Total Pb-resistant bacteria	CFU/g	6.39 x 10^6

Remark: *Pb concentration exceeds Indonesia Food and Drug Agency Regulation Number 5 in 2018 (<0.2 mg/kg); **Pb concentration below the limit concentration of Pb in the soil for agricultural activities according to CCME - Canadian Council of Ministers of the Environment (1999).

Pb-resistant bacteria population

Pb-resistant indigenous bacteria have been successfully isolated from soil in a horticultural area that applies intensive farming and the total population was 6.39 x 10^6 CFU/g (Table 1). Previously Singh & Hiranmai (2021) reported that bacteria population in soil from different roadsides containing Pb >70 mg/kg is around 10^5-10^6 CFU/g. The isolated Pb-resistant are indigenous (local) bacteria that benefit the environment (Kumar & Gopal 2015). Indigenous bacteria have been reported to be beneficial for biodegradation, N fixation, P solubilisation, and other plant

growth-promoting (PGP) traits to increase soil fertility (Kumar & Gopal 2015; Bhat et al. 2022). However, this study did not test PGP traits of the isolated bacteria.

Pb minimum inhibitory concentration of the isolated Pb-resistant bacteria

Three potential Pb-resistant bacteria (isolate PT-3, PT-5, and PT-8) were tested for Pb resistant to reveal Pb minimum inhibitory concentration (MIC) of the tested bacteria. The result showed that MIC of the tested bacteria was 1000 mg/L. The concentration is higher compared to Manzoor et al. (2019) study, which is only 51 mg/L. The ability of the tested bacteria to survive under harsh environments (Pb presence in the medium) is significantly influenced by Pb concentration ($p<0.05$). High Pb concentrations inhibited bacteria growth, indicated by the width inhibition zone around paper discs containing Pb. There were significantly different among MIC of the tested bacteria ($p<0.05$). Two tested bacteria (isolate PT-3 and PT-5) survived up to 10,000 mg/L with an inhibition zone of less than 3 mm (Figure 2). Bacteria have several Pb-resistant mechanisms under high Pb concentration, i.e., bacterial cell wall adsorption, induction of Pb precipitation in the soil solution, and promoting bacterial community enrichment in producing plant growth-promoting substances (Qin et al. 2023).

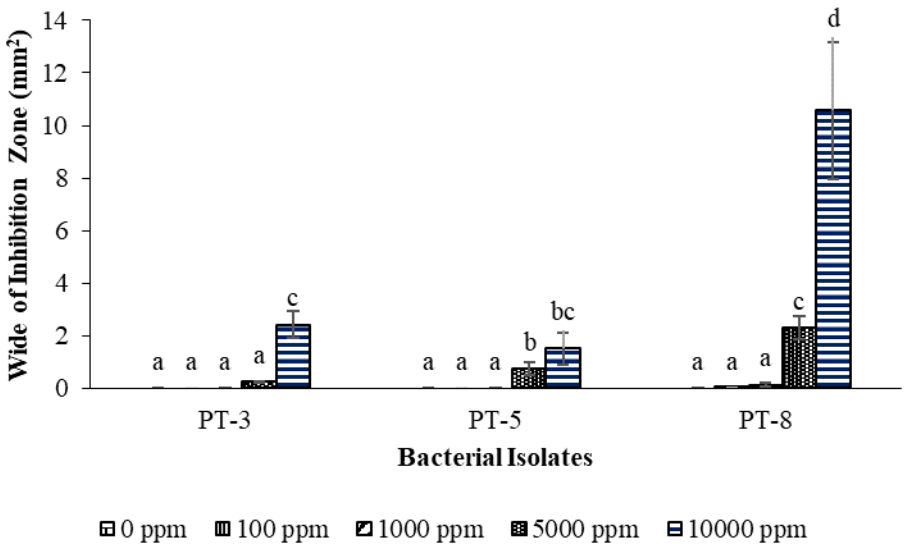

Figure 2. Pb minimum inhibitory concentration of the isolated Pb-resistant bacteria; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

Characteristics of the isolated Pb-resistant bacteria

16S rRNA sequencing result showed that isolate PT-3 is *Bacillus altitudinis* and PT-5 is *Bacillus wiedmannii* (Figure 3). *B. altitudinis* is a bacterium found in high-altitude places (Shivaji et al. 2006), such as the sampling site (intensive horticulture farming lands) at 953 m asl. The bacterium is also endophytic with plant growth-promoting traits (Zhang et al. 2021). The bacterium has also been reported to have the ability to lignocellulose degradation with many biotechnological applications, such as biofuels and biorefineries (Dar et al. 2021). Pb-resistance of *B. altitudinis* is related to the Stress-Alleviating Properties of the bacterium (Yue et al. 2019). The bacterium can detoxify Pb, or reduce the toxicity level of Pb by secreting enzymes or forming biofilms. A past study found that *B. altitudinis* can alleviate salinity and low phosphorus stress by producing en-

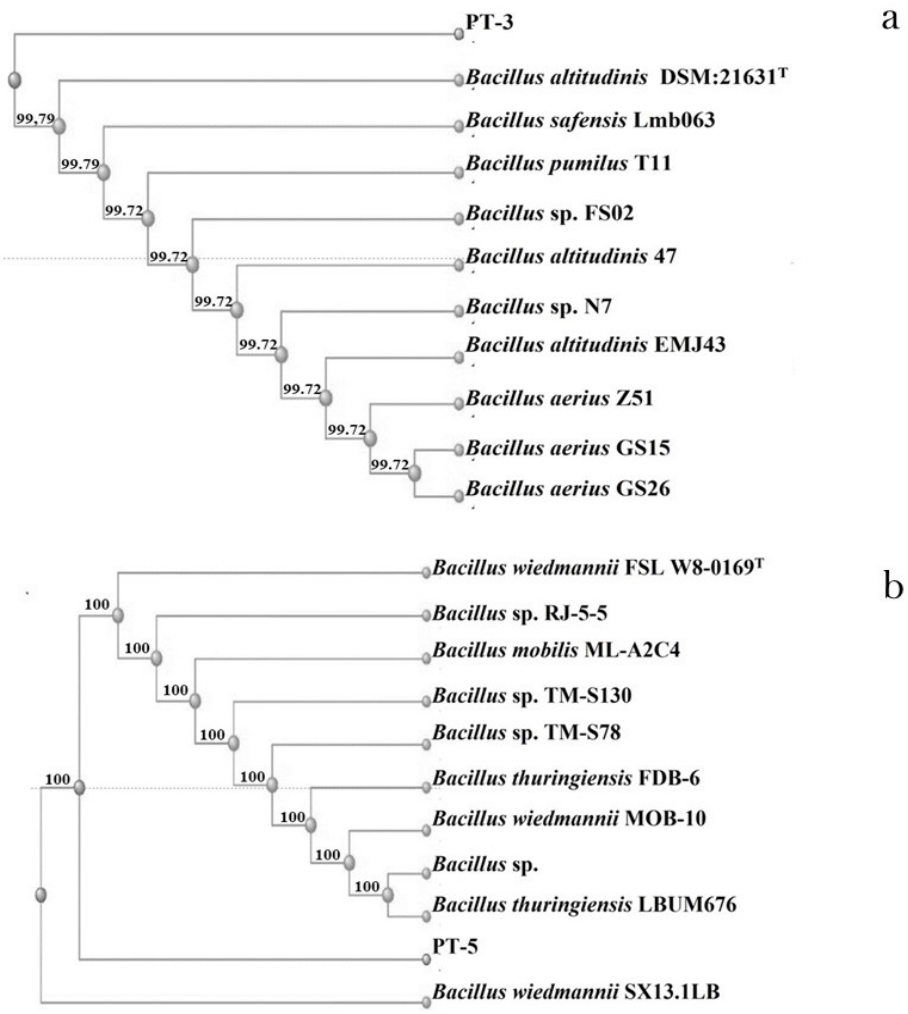

Figure 3. Phylogeny tree of the isolated Pb-resistant bacteria: a) *Bacillus altitudinis*, and; b) *Bacillus wiedmannii*.

zymes and biofilm (Yue et al. 2019). For isolate PT-5, *B. wiedmannii* is a psychrotolerant bacterium with a minimum growth temperature of up to 5 °C (Miller et al. 2016). The bacterium has agricultural importance as a biocontrol of root-knot nematode (Fallahzadeh-Mamaghani et al. 2023). Past studies revealed that the bacterium is tolerant to drought and heavy metals, also exhibits plant growth-promoting traits (Kalkan 2022; Fallahzadeh-Mamaghani et al. 2023). Plant growth-promoting traits are believed as tolerant mechanisms of bacteria under abiotic stress (Kumar et al. 2020), such as salinity, drought, and heavy metals. Pb resistance mechanism includes enhanced siderophore production, cell morphology alteration, extracellular sequestration, biosorption, precipitation, and intracellular bioaccumulation (Naik & Dubey 2013).

The effect of Pb-resistant bacteria inoculation on plant growth and Pb residue in the soil

The number of leaves

Pb-resistant bacteria consortium significantly increased the number of *B. chinensis* leaves ($p<0.05$; see Figure 4) by up to 12% compared to *B. chinensis* without Pb-resistant bacteria application. The increased number of leaves was found 3 and 4 weeks after planting. A similar result with the shoot length of *B. chinensis*, the highest number of leaves was recorded at TSAB treatment. A similar finding has been reported by Han et al. (2020) that Pb-resistant bacteria not only reduce Pb absorbed by plants but also increase plants' shoot length and the number of leaves.

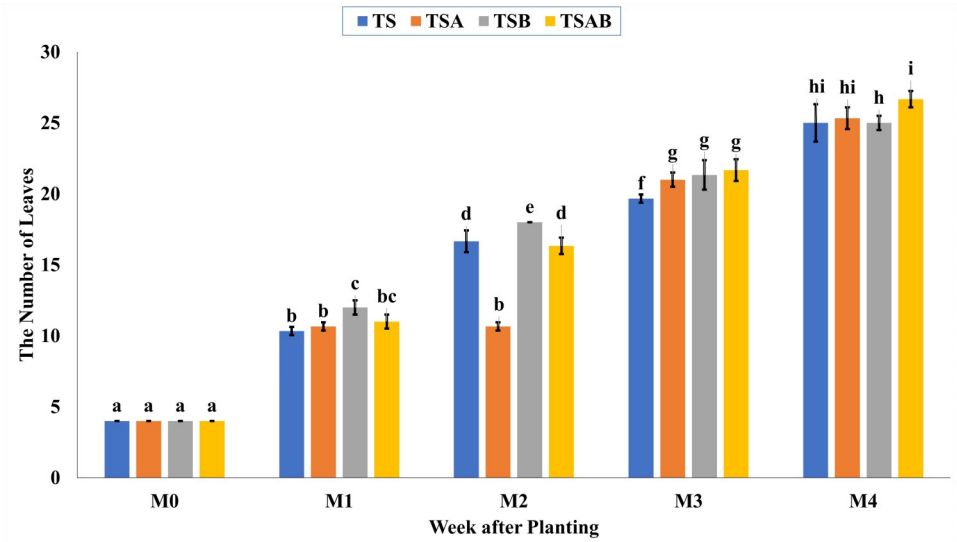

Figure 4. Effect of Pb-resistant bacteria inoculation on the number of *Brassica chinensis* leaves; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

Plant dry weight

Pb-resistant bacteria consortium significantly increased *B. chinensis* dry weight ($p<0.05$; see Figure 5) compared to *B. chinensis* without Pb-resistant bacteria application. *B. wiedmannii* had a similar effect as the Pb-resistant bacteria consortium (*B. altitudinis* + *B. wiedmannii)* on *B. chinensis* dry weight when applied as a single isolate ($p<0.05$). The highest *B. chinensis* dry weight was found at TSAB treatment. The dry weight increased by 30-35% after Pb-resistant bacteria inoculation. The results of this study agree with Najm-Ul-seher et al. (2021) that Pb-resistant bacteria can be used to improve the growth of *B. chinensis* where Pb pollution is a problem.

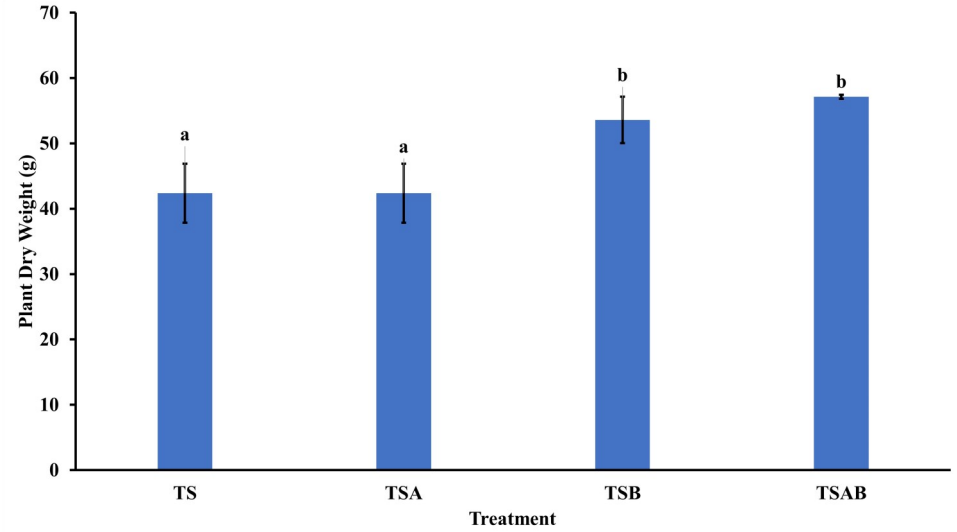

Figure 5. Effect of Pb-resistant bacteria inoculation on *Brassica chinensis* dry weight; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

Pb residue in the soil

This study revealed that intensive agriculture increases heavy metal concentrations in agricultural soil, especially from excessive fertiliser and pesticide application. Fertiliser and pesticide application on Pb-contaminated soil increase Pb residue in the soil by up to 0.9% (0.08 mg/kg) during the pot trial (Figure 6). Pb content will remain in the soil if

there are no further measures because Pb is an inert heavy metal (Alengebawy et al. 2021). Pb-resistant bacteria application significantly decreased Pb residue in the soil ($p<0.05$). The residues were 12 to 63% lower than untreated Pb-contaminated soil (control). Pb residues decreased around 55-88% in the treatment of Pb-resistant bacteria and *B. chinensis*, either applied as a single isolate or consortium. The lowest Pb residue was found on TSB (Pb-contaminated soil, *B. chinensis* and *B. wiedmannii*). The treatment reduced Pb residue by up to 88% compared to the control; the remaining Pb residue was only 10 mg/kg (Figure 6).

The low Pb residue in the soil on TSB treatment was due to *B. wiedmannii* adsorb Pb, as a previous study reported that *B. cereus*, which is closely related to *B. wiedmannii* can absorb Pb up to 96.58% through several mechanisms such as indole-3-acetic acid (IAA) secretion and inorganic phosphorus (P) dissolution (Miller et al. 2016; Li et al. 2023). Besides Pb adsorption ability, the bacterium is a biocontrol agent of the root-knot nematode (*Meloidogyne arenaria*) (Fallahzadeh-Mamaghani et al. 2023).

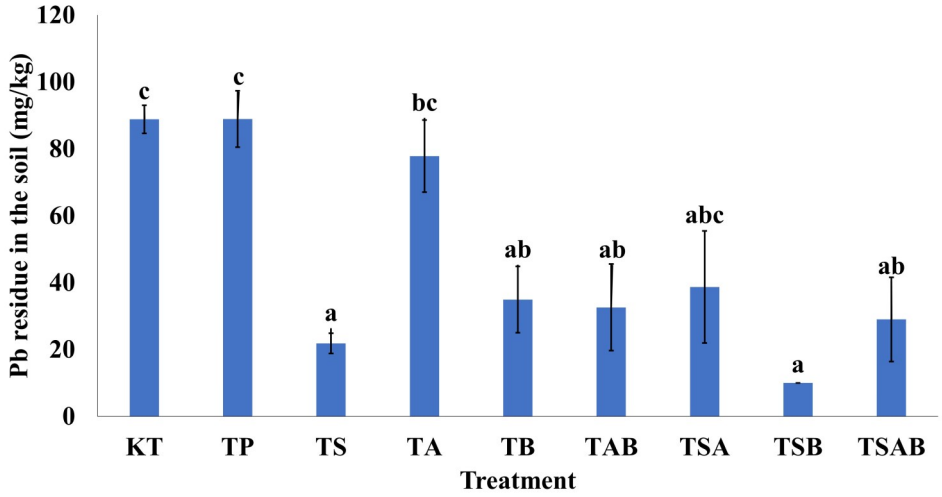

Figure 6. Effect of Pb-resistant bacteria inoculation on Pb residue in the soil; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

Effect of Pb-resistant bacteria inoculation on soil nutrients

Soil total-N

Pb-resistant bacteria inoculation significantly increased soil total N ($p<0.05$; Figure 7). Total N was significantly higher after inoculation of Pb-resistant bacteria consortium (TAB) compared to other treatments ($p<0.05$). This study noted that Pb-resistant bacteria inoculation increased total N from 4% to 36% compared to the control. Pb-resistant bacteria application on the soil without *B. chinensis* had significantly higher total N due to N in the soil absorbed by *B. chinensis*. The claim is proven by the low total N content on the soil planted with *B. chinensis*. Total N was 8-12% lower on the treatment with *B. chinensis* than control. The increase of total N content in the soil might be due to Pb-resistant bacteria consortium application leading to a symbiotic mutualism between these bacteria in fixing free N from the atmosphere. When these bacteria are applied to soil, they can form a symbiotic relationship with plants. The bacteria provide the plant with N, and the plant provides the bacteria with a safe place to live and nutrients. This can significantly improve crop yields (Kamaruzzaman et al. 2020; Roszak et al. 2021).

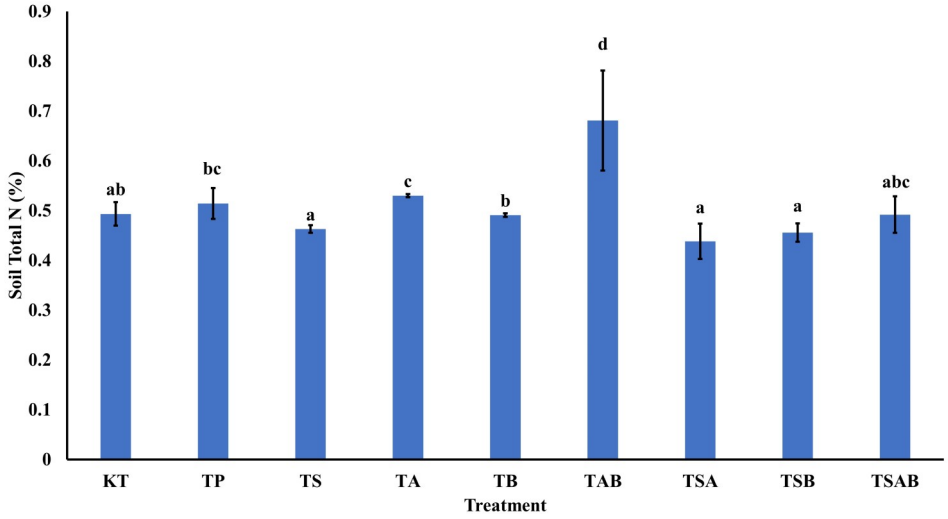

Figure 7. Effect of Pb-resistant bacteria inoculation on total N of Pb-contamiated soil; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

Soil available-P

The consortium of Pb-resistant bacteria application significantly increased available P on the Pb-contaminated soil ($p<0.05$; see Figure 8). The available P was increased up to 73% compared to the control. This finding indicates that Pb-resistant bacteria not only resistant to high concentrations of Pb in the soil but also exhibit P solubilising activity. A similar finding has been reported by Teng et al. (2019) that phosphate solubilising bacteria (PSB) were isolated from heavy metal-contaminated soils and had potentials for Pb immobilisation.

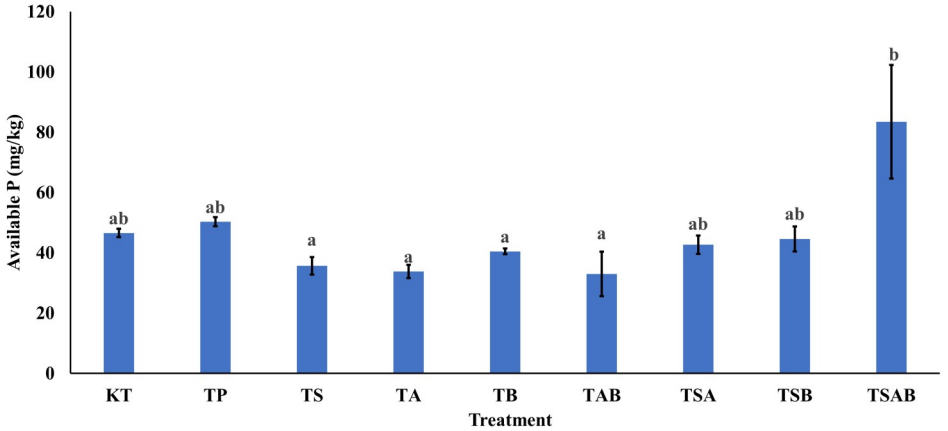

Figure 8. Effect of Pb-resistant bacteria inoculation on available P of Pb-contamiated soil; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

Soil exchangeable-K

Pb-resistant bacteria inoculation significantly influenced exchangeable K of Pb-contaminated soil ($p<0.05$, see Figure 9). Soil Exchangebale K increased from 85% to 200% after Pb-resistant bacteria inoculation, whether as a single isolate or consortium. However, on the treatment of Pb resistant bacteria and *B. chinensis* exchangeable K only increased 57% as K is an essential nutrient that is uptake by plants. This study agree with previous study that Pb-resistant bacteria change soluble-exchangeable fraction on soil nutrients in heavy metals-contaminated soil (Boechat et al. 2018).

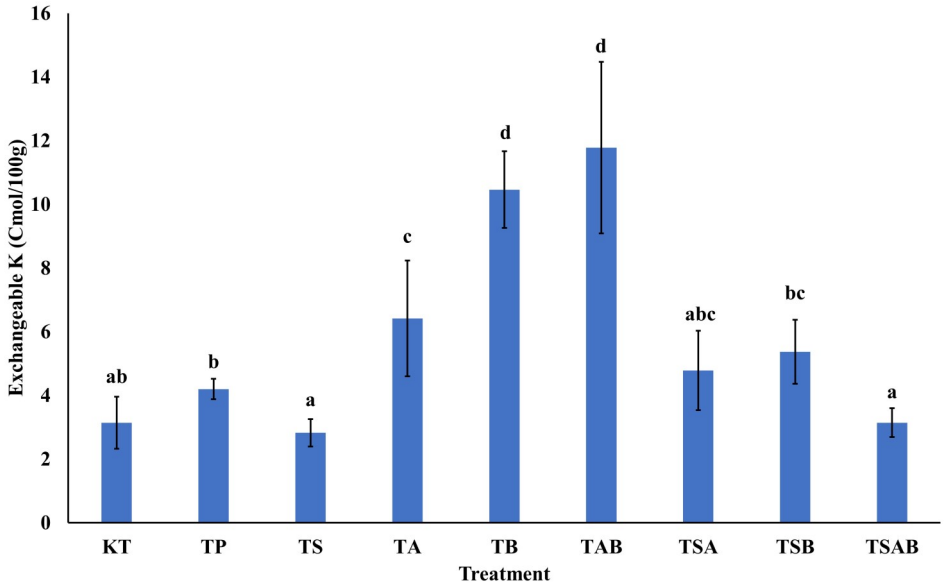

Figure 9. Effect of Pb-resistant bacteria inoculation on exchangeable K of Pb-contamiated soil; Means with different letters are significantly different ($p<0.05$) as determined by Tukey's test.

CONCLUSION

Intensive agriculture, frequent application of inorganic fertilisers and pesticides, lead to increase Pb content in the soil and food crops biomass. Bacteria isolated from Pb-contaminated soil exhibited Pb resistance (*Bacillus wiedmannii* and *Bacillus altitudinis*). The bacteria resistant to Pb up to 10,000 mg/L. Inoculation of the bacteria increased plant growth by increasing the number of leaves and dry weight of *Brassica chinensis* (12% and 35%, respectively). The bacteria reduced Pb residue in the soil by up to 88%. Moreover, the bacteria increased soil nutrients such as total N (12%), available P (73%), and exchangeable K (200%). The bacteria have the potential for bioremediation of Pb-contaminated soils in the field, as the bacteria can reduce Pb in the soil, thus preventing Pb uptake by food crops (such as *B. chinensis*), which is good for food safety and environmental sustainability.

AUTHORS CONTRIBUTION

B.L.S.P. designed the research, collected and analysed the data, wrote the initial draft of manuscript; R.U.: designed the research, analysed the data, project leader, supervised all the processes, wrote the manuscript; Y.N.: reviewed the manuscript;

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CONFLICT OF INTEREST

There is no conflict of interest regarding the research or the research funding.

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