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Application of Pb-Resistant Bacteria to Reduce Pb-Accumulation in *Brassica* sp. on Pb-Contaminated Soil

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ABSTRACT

Brassica sp. is a horticultural crop with high demand for consumption. To meet the demand, farmers apply intensive farming to increase yields and prevent plant pests and diseases that cause yield loss. Agrochemical applications in the form of inorganic fertilizers and pesticides contribute to lead (Pb) contamination in agricultural soils and increase lead (Pb) content in the cultivated plants. Nowadays, using bacteria for remediation (bioremediation) is environmentally friendly and effective in cleaning pollutants by converting organic Pb into inorganic Pb which is less toxic. This study aims to explore Pb-resistant bacteria that can reduce Pb-accumulation on food crops such as Brassica sp. grown in Pb-contaminated soil. This study isolated 15 isolates that survived on nutrient agar containing 1,000 mg/L Pb(NO3)2. The study showed 2 potential Pb-resistant bacteria that reduced Pb accumulation in Brassica sp. up to 30.5%. The bacteria are gram-positive bacilli and non-human pathogens (PT-3 and PT-5). DNA barcode identification results showed the isolates identified as Bacillus altitudinis (PT-3) and Bacillus wiedmannii (PT-5). Aplication of the bacteria increases the shoot length and fresh weight of Brassica sp. Application of the bacteria improves food crops quality by reducing heavy metals accumulation, such as Pb. Thus, the bacteria are potential as biofertilizers for reducing agrochemicals use in intensive agriculture areas and preventing environmental destruction and food contamination.

1. INTRODUCTION

Brassica sp. is a horticultural crop with high demand for consumption. Data from the Center of Data and Information (PUSDATIN, 2020) in 2019 reported that *Brassica* sp. consumption reached up to 601,000 tons. To meet the large demand, farmers apply intensive farming to increase yields and prevent pests and diseases causing yield loss. Agrochemical applications in the form of inorganic fertilizers and pesticides contribute to lead (Pb) contamination in agricultural soils and increase Pb accumulation in cultivated plants. Pb content in inorganic fertilizers and pesticides such as urea is around 4.45 mg/kg, nitro-ponska 2.16 mg/kg, superphosphate 21 mg/kg, foliar fertilizers 16 mg/kg (Rahayu *et al.,* 2019), and TSP 7-225 mg/kg (Alloway 1995; Rasman & Hasmayani, 2018). Rahayu *et al.* (2019) stated that Pb content in fungicides is around 8 mg/L, insecticides 99 mg/L, acaricides 3.77 mg/L, herbicides 2 mg/L, and adhesives pesticides 0.71 mg/L.

The application of inorganic fertilizers and pesticides increases Pb content in horticultural crops by 30.65 mg/kg and also increases Pb content in the soil by around 11.35-11.62 mg/kg (Rahayu *et al.,* 2019). High Pb accumulation in cultivated plants has negative impacts on human health due to carcinogens. Pb can accumulate in the human body, causing health problems, including kidney illness, hypertension, and reproductive disease, and moreover, inhibit fetus growth and development (Maghfirah, 2020).

According to The Indonesian National Standard SNI 7387:2009 (BSN, 2009), Pb content in vegetables have to be less than 0.5 mg/kg. Due to the harmful effect of Pb on human and environmental health, it is necessary to prevent Pb uptake by food crops. Bioremediation is a method that utilizes microorganisms to remedy contaminated environments. Bioremediation is an effective alternative method for cleaning up pollutants that have been rapidly developed in America and Europe (Hamzah *et al.,* 2019). Bacteria are one of the remedial agents that are effective in degrading heavy metals through the detoxification of heavy metal and organic pollutants by extraction and/or absorbing the heavy metals in the cell surface. The initial mechanism is heavy metals mobilization and then immobilization, which causes intracellular accumulation and enzyme transformation (Ahmed, 2018). Therefore, this study aims to explore Pb-resistant bacteria that can reduce Pb-accumulation on food crops, namely *Brassica* sp. grown in Pb-contaminated soil. In this study, exploration and a pot trial were carried out to analyze Pb-resistant bacteria' effectiveness in reducing Pb accumulation in plant biomass.

2. MATERIALS AND METHODS

2.1. Sampling Location

This study was conducted in Soil Biology Laboratory and Green House, Faculty of Agriculture, University of Brawijaya, from June 2022 to December 2022. Pb-contaminated soil was collected from intensive agricultural lands in Sumber Brantas Village, Bumiaji District, Batu City, East Java (7^{o}45'13'' S and 112^{o}31'04'' E).

2.2. Pb-resistant Bacteria Isolation and Characterization

2.2.1. Pb-resistant Bacteria Isolation

The Pb-resistant bacteria were isolated using nutrient agar containing 50 mg/L Pb (NA+Pb(NO_3)$_2$) (Al-Hazmi, 2019). The bacteria were tested for Pb resistance in different concentrations of 0, 100, 500, and 1,000 mg/L (Kurnia *et al.,* 2015).

2.2..2 Pb-resistant Bacteria Characterization

Bacteria characterization included gram staining, pathogenicity tests on blood agar, and hypersensitivity tests using tobacco leaves (*Nicotiana tabacum*). A blood agar test was done by streaking the bacterial isolate onto the blood agar and then incubating it for 24 hours. If the incubation results are clear zones (lysis), the bacteria are pathogenic for humans (Faruq, 2018). Hypersensitivity test was carried out by injecting the tested bacteria that had been prepared and grown in NB (Nutrient Broth) into the tobacco leaves' stomata and then incubating for 3 days. If necrosis forms on tobacco leaves, the bacteria are pathogenic to plants (Rahmayuni *et al*., 2018).

Morphological characteristics of the isolated bacteria grown on NA+$Pb(NO_3)_2$ medium with 50 mg/L $Pb(NO_3)_2$ were further characterized according to the morphology of the bacterial colonies as explained in Bergey's Manual of Systemic Bacteriology. These characteristics include size, pigmentation, shape, margin, and elevation. The potential bacteria were subjected to DNA extraction for species identification.

2.3. Experimental Design

This study used a completely randomized design with 4 treatments. The treatment code was presented in Table 1.

Table 1. Application of Pb-resistant Bacteria on *Brassica* sp.

Code	Treatments
TS	Pb-contaminated soil + *Brassica* sp. (Control)
TSA	Pb-contaminated soil + *Brassica* sp. + *Bacillus altitudinis*
TSB	Pb-contaminated soil + *Brassica* sp. + *Bacillus wiedmannii*
TSAB	Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* + *B. wiedmannii*

The research procedure for pot trial in Green House consisted of 1) growth medium preparation using 3 kg mixture of Pb-contaminated soil, manure, and the addition of Pb $(NO_3)_2$ with a concentration of 100 mg/kg; 2) the application of isolated Pb-resistant bacteria of 20 mL/polybag ($1x10^8$ CFU/mL) (Hindersah & Matheus, 2015); 3) fertilizers application at 7 and 20 days after planting (DAP). Pesticide application every two days; 4) daily irrigation with 500 mL/polybag. Fertilizer application consists of ZA 200 kg/ha (0.3 g/polybag) and NPK 300 kg/ha (0.45 g/polybag). Pesticide application consists of abamectin (0.05 mL/polybag), cypermethrin (0.05 mL/polybag), and mancozeb (0.125 g/polybag).

2.4. Data Analysis

The obtained data were analyzed using Analysis of Variance (ANOVA) followed by Tukey at 5% significant level. Data analysis were carried out using Genstat software.

3. RESULT AND DISCUSSION

3.1. Soil Chemical Properties

The result showed that soil pH on the horticultural land of Sumberbrantas Village, Bumiaji District, Batu City, East Java, was acidic (Table 2). The low pH is mainly due to the application of ZA fertilizer, which exceeds the recommended dose by Ministry of Agriculture. The application of ZA fertilizer ($(NH_4)_2SO_4$) in the soil will dissolve according to the reaction $(NH_4)_2SO_4 + H_2O \rightarrow NH_4OH + H_2SO_4$ where H_2SO_4 is a strong acid which can decrease soil pH (Atmaja *et al*., 2021). In addition, low soil pH will also increase the solubility of heavy metals that easily taken up and accumulated in plant biomass (Binh *et al.*, 2021).

High Pb accumulation in *Brassica* sp. is due to high Pb solubility in the soil due to the low soil pH. Wijayanti *et al.* (2020) stated that *Brassica* sp. is a hyperaccumulator plant that can accumulate large amounts of heavy metals without exhibiting toxicity symptoms. *Brassica* sp. uptakes Pb from both soil and air as farmers apply inorganic fertilizers and pesticide intensively. Rahayu *et al.* (2019) stated that Pb content in insecticides is 99 ppm, and in fungicides is 8 ppm. Thus, the habit of farmers applying pesticides every two days contributes to the increasing of Pb content in cultivated plants.

Table 2. Soil chemical properties and lead (Pb) concentrations in horticultural land of Sumberbrantas Village, Bumiaji District, Batu City, East Java

Soil properties	**Unit**	**Value**
pH H_2O	--	4.12 (±0.08) $^{(SM)}$
Organic C	%	4.03(±0.11) $^{(T)}$
Total N	%	0.46(±0.018) $^{(S)}$
Available P	mg/kg	110.82(±2.26) $^{(ST)}$
Exchangeable K	me/100g	0.53(±0.038) $^{(S)}$
Pb in Soil	mg/kg	17.6(±0.78)
Pb in Biomass	mg/kg	32.48(±1.63)*

Note: *Pb concentration in plants exceeds the maximum limit of SNI; SM = Very Acidic; T = High; S = Moderate; ST = Very High (Balai Penelitian Tanah, 2009).

3.2. Identification of Pb-Resistant Bacteria

The study obtained 15 isolates of Pb-resistant bacteria, which had different colony morphology, as explained in Table 3.

Table 3. Morphological characteristics and Pb-resistant bacteria

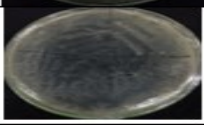
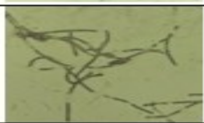
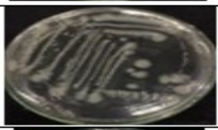
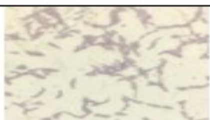
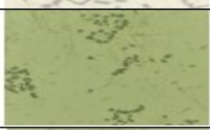
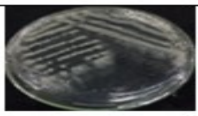
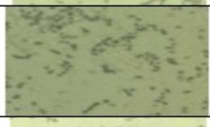
Isolate	Morphology of Colony	Gram Reaction	Description
PT-1			Shape of colony: filaments Color: Cream Margin: Entire Elevation: flat Shape of cell: bacilli Gram reaction: (+)
PT-2			Shape of Colony: rhizoid Color: white Margin: filamentous Elevation: flat Shape of cell: bacilli Gram reaction: (+)
PT-3			Shape of Colony: filaments Color: white Margin: lobate Elevation: flat Shape of cell: bacilli Gram reaction: (-)
PT-4			Shape of Colony: circullar Color: cream Margin: entire Elevation: flat Shape of cell: bacilli Gram reaction: (+)
PT-5			Shape of Colony: circullar Color: yellow Margin: entire Elevation: flat Shape of cell: bacilli Gram reaction: (+)
PT-6			Shape of Colony: circullar Color: cream Margin: lobate Elevation: convex Shape of cell: cocci Gram reaction: (+)
PT-7			Shape of Colony: irregular Color: white, Margin: undulate Elevation: flat Shape of cell: cocci Gram reaction: (-)

Table 3. Continued

Isolate	Morphology of Colony	Gram Reaction	Description
PT-8			Shape of Colony: circular Color: yellow Margin: entire Elevation: convex Shape of cell: cocci Gram reaction: (+)
PT-9			Shape of colony: filaments Color: Cream Margin: Entire Elevation: flat Shape of cell: bacilli Gram reaction: (+)
PT-10			Shape of Colony: circular Color: white, Margin: rhizoid Elevation: convex Shape of cell: cocci Gram reaction: (+)
PT-11			Shape of Colony: circular Color: yellow Margin: filamentous Elevation: rhizoid Shape of cell: cocci Gram reaction: (-)
PT-12			Shape of Colony: irregular Color: white Margin: lobate Elevation: flat Shape of cell: cocci Gram reaction: (+)
PT-13			Shape of Colony: circular Color: white, Margin: rhizoid Elevation: flat Shape of cell: cocci Gram reaction: (+)
PT-14			Shape of Colony: rhizoid Color: white Margin: filamentous Elevation: flat Shape of cell: bacilli Gram reaction: (+)
PT-15			Shape of Colony: filaments Color: white Margin: rhizoid Elevation: flat Shape of cell: bacilli Gram reaction: (-)

3.3. Pb-resistant Test

The tested bacterial isolates survived on media containing 1,000 mg/L $Pb(NO_3)_2$ (Figure 1). The result proved that the isolated indigenous bacteria have high Pb resistance as Pb is a toxic metal to bacteria. The study highlighted that bacterial resistance in Pb-containing media means these bacteria can detoxify Pb through Pb binding mechanism in bacterial cells due to protein or mineral granules such as polyphosphate. Thus, it can be concluded that these bacteria are potential bacteria as bioremediation agents for Pb -contaminated soil (Zhang *et al.,* 2011; Fahruddin *et al.,* 2020).

Figure 1. Lead resistance test of the isolated Bacterial Isolates

3.4. Gram Staining

The 15 isolates of Pb-resistant bacteria were bacilli (53.3%) and cocci (46.7%). Bacilli bacteria can absorb (biosorbents) and detoxify heavy metals toxicity. The bacterial cells consisted of gram-positive (80%) and gram-negative (20%) bacteria (Table 3). Gram-positive bacteria are more tolerant to Pb toxicity than gram-negative bacteria due to gram-positive bacteria have a cell wall surface with a high capacity in ions metal binding than gram-negative bacteria. The cell wall of gram-positive bacteria consists of a carboxyl group, a heavy metal absorbing agent. The source of the carboxyl group is teichoic acid, which is associated with peptidoglycan in the bacterial cell wall (Sa'diyah *et al.,* 2016).

3.5. Pathogenicity Test of the Isolated Pb-resistant Bacteria

The hemolysis test using blood agar (Figure 2) showed that there were 10 isolates (66.67%) formed beta-hemolysis, one isolate formed alpha-hemolysis (6.67%), and 4 isolates formed gamma-hemolysis (26.67%). Bacterial isolates included in beta-hemolysis and alpha-hemolysis are pathogenic to humans and animals. However, bacterial isolates that form gamma-hemolysis are non-pathogenic bacteria (Sukmadewi *et al.,* 2017).

The hypersensitivity test (Figure 3) found 8 isolates of non-pathogenic (53.33%) and 7 pathogenic bacteria to plants (46.67%). The positive result of pathogenic bacteria on plants is indicated by the presence of brownish spots, by the day, the spot on the leaf surface became necrosis, which can tear the leaves. Discoloration occurred on tobacco leaves as plant adaptation (defense reaction) due to pathogenic bacterial inoculation. The hypersensitivity response occurs through rapid cell death in the tissue around the inoculated area (Hastuti *et al.,* 2014). This is in line with Hanif & Susanti (2017) statement that hypersensitivity reactions occur due to rapid and localized cell death due to bacterial infection to inhibit the pathogens.

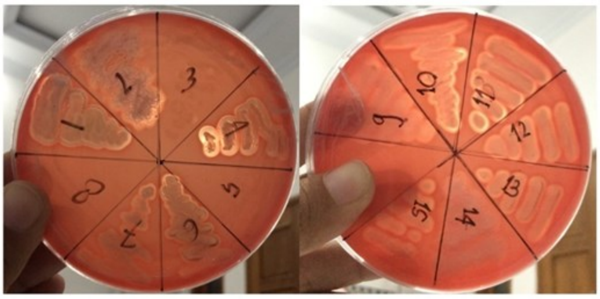


Figure 2. Hemolysis test on blood agar medium

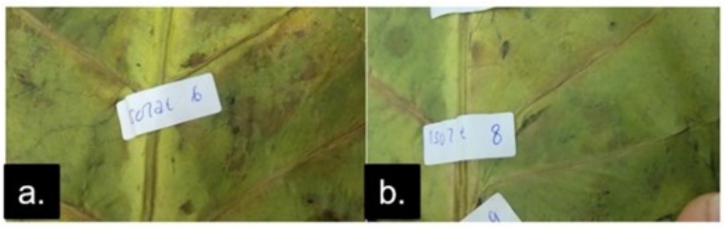


Figure 3. Hypersensitivity test on tobacco Leaves: a) Forming Necrosis; b) Not forming Necrosis.

3.6. Identification of Pb-resistant Bacteria

The bacterial identification consisted of DNA extraction, amplification, and sequencing. The result of DNA amplification is visualized on 1.5% agarose gel and showed a 1500 bp (Figure 4). The sequences were used to determine bacteria species according to the database from GenBank of NCBI (Puspitasari *et al.,* 2014). According to the analysis, PT-3 is *Bacillus altitudinis,* and PT-5 is *Bacillus wiedmannii.*

Pb-resistant bacteria that have been identified are potential bacteria with non-pathogenic for humans and plants. Furthermore, Pb-resistant bacteria will be applied to lead-contaminated soil with *Brassica* sp. to determine the effect of Pb-resistant bacteria on the growth of *Brassica* sp. and lead uptake by *Brassica* sp.

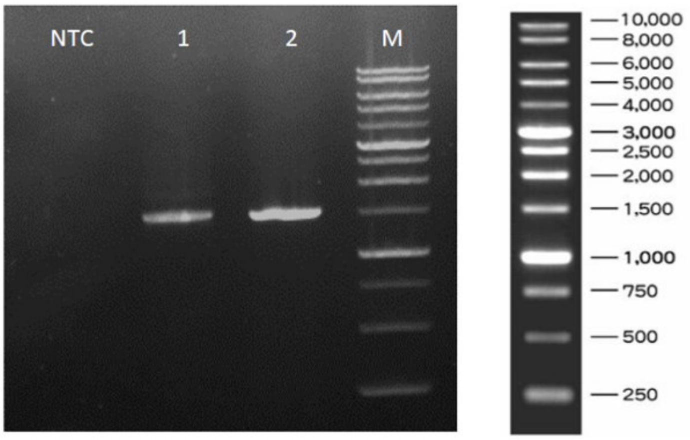


Figure 4. DNA bands from primer amplification ladder 1 kb

3.7. Shoot Length of *Brassica* sp.

The results showed that the application of Pb-resistant bacteria had no significant effect on *Brassica* sp. shoot length ($p>0.05$) (Figure 5). Four weeks after planting, Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* + *B. wiedmannii* (TSAB) had the highest shoot length compared to the control, 28.17 cm (Figure 5).

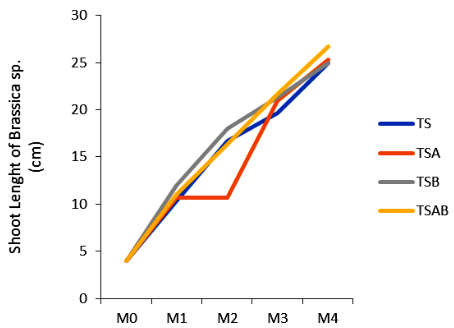


Figure 5. Shoot Lenght of *Brassica* sp.; Pb-contaminated soil + *Brassica* sp. (TS); Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* (TSA); Pb-contaminated soil + *Brassica* sp. + *B. wiedmannii* (TSB); Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* and *B. wiedmannii* (TSAB).

The result might be due to the bacterial consortia exhibiting plant growth-promoting traits such as nitrogen (N) fixation, phosphate (P), and potassium (K) solubilization for plant absorption. Finney *et al*. (2017) and Ustiatik *et al*. (2022) reported that heavy metals-resistant bacteria exhibit plant growth-promoting traits and support plant growth due to the provision of plant nutrients such as P, iron (Fe), and N. The beneficial traits are important for sustainable agroecosystems, including (i) N_2 fixation, phosphate solubilization, indole acetic acid, and siderophore production and (ii) biocontrol of plant pathogens (Martin & Isaac, 2018).

3.8 Lead Uptake by *Brassica* sp.

The application of Pb-resistant bacteria affected Pb uptake by *Brassica* sp. ($p<0.05$). However, compared to the control, Pb-resistant bacteria application had no significant difference in Pb uptake by *Brassica* sp. Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* (TSA) had the lowest Pb uptake, namely 0.01 mg/kg, but not statistically significant (Figure 6). The low Pb uptake is due to the application of *B. altitudinis* that decreased Pb content in Pb-contaminated soil due to Pb-resistant bacteria absorbing Pb from the soil. Thus, it results in a decrease in Pb uptake by plants. Thus, we assumed that the application of *B. altitudinis* is beneficial for reducing Pb accumulation in horticulture or primarily agricultural plants. However, further studies are required to elucidate the effects of the bacterium in reducing Pb accumulation on various crops.

The mechanism of Pb-resistant bacteria in absorbing Pb is initiated by the metal transport of PIB-type ATPase, which is a group of proteins involved in the transport of heavy metals outside the cell membrane and regulates the heavy metal resistance of bacteria. PIB-type ATPases can regulate the efflux of toxic heavy metals outside the cell membrane and prevent excessive accumulation of highly reactive and toxic heavy metals. Furthermore, bioaccumulation occurs, and one of the common mechanisms is the induction of specific metal-binding proteins that facilitate the uptake/ bioaccumulation of toxic metals in cells. These well-studied metal-binding proteins are referred to as metallothioneins (MTs). Metallothionein plays an important role in the immobilization of toxic heavy metals, thereby protecting the bacterial metabolic processes catalyzed by enzymes. The following mechanism is extracellular sequestration, a metal immobilization strategy applied by microbes to counter the toxic effects of heavy metals. Bacterial exopolysaccharide (EPS) plays a vital role in the initial attachment of cells to different substrates, cell-to-cell aggregation, protection against desiccation, and resistance to harmful exogenous materials. Surface biosorption is also a mechanism for the extracellular sequestration of toxic heavy metals to prevent their entry into the bacterial cell, thereby maintaining metal homeostasis.

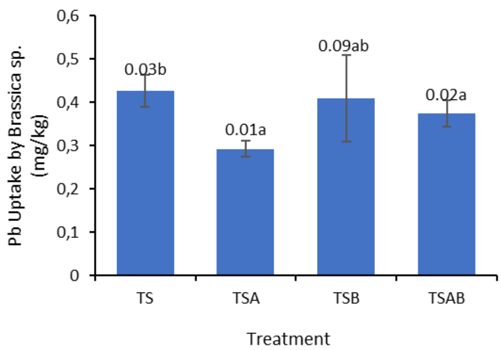


Figure 6. Pb Uptake by *Brassica* sp.; Pb-contaminated soil + *Brassica* sp. (TS); Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* (TSA); Pb-contaminated soil + *Brassica* sp. + *B. wiedmannii* (TSB); Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* and *B. wiedmannii* (TSAB).

The last process is bio-precipitation and biotransformation. Bio-precipitation is the precipitation of toxic metals into insoluble complexes, reducing their bioavailability and toxicity. Organo-Pb biotransformation, natural microorganisms can also degrade organo-Pb using a biotransformation mechanism. Microbial consortia have been reported to degrade tetra-ethyl Pb in soil. In nature, tetra-alkyl lead compounds, such as tetra-ethyl lead and tetra-methyl Pb, can undergo photolysis and evaporation. The degradation progresses from tri-alkyl species to di-alkyl species and finally to inorganic Pb (Naik & Dubey, 2013).

3.9. *Brassica* sp. Fresh Weight

The application of Pb-resistant bacterial isolates increased *Brassica* sp. fresh weight ($p<0.05$). However, there was no significant difference in the increasing fresh weight compared to the control ($p>0.05$). Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* + *B. wiedmannii* (TSAB) treatment had the highest fresh weight compared to other treatments, namely 433.3 g and statistically different (Figure 7). The increasing fresh weight of *Brassica* sp. after bacterial consortium application occurred due to a symbiotic mutualism of two bacterial isolates that acted as Plant Growth Promoting Bacteria. Thus, we assumed that besides reducing Pb uptake, the application of Pb-resistant bacteria as a consortium increases plant fresh biomass production. The ability of bacteria with plant growth-promoting traits to fix N from the atmosphere results in increased N availability for plants (Saban *et al.,* 2018). In line with the statement of Sondang *et al.* (2020) that increasing N, P, and K availability will stimulate plant growth. The application of growth-promoting bacteria increases the number and size of leaves, enlarges the diameter of the stem, and increases the length of plant roots so that the weight of plant shoots will also increase. Moreover, the bacteria act as a growth promoter that regulates essential nutrients for plant growth.

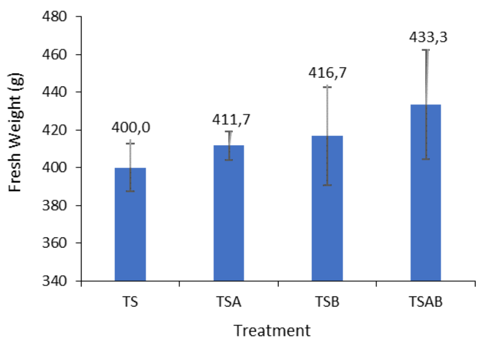


Figure 7. Fresh Weight of *Brassica* sp.; Pb-contaminated soil + *Brassica* sp. (TS); Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* (TSA); Pb-contaminated soil + *Brassica* sp. + *B. wiedmannii* (TSB); Pb-contaminated soil + *Brassica* sp. + *B. altitudinis* and *B. wiedmannii* (TSAB).

4. CONCLUSION

Exploration of Pb-resistant bacteria in intensive agriculture lands obtained two potential bacterial isolates, *Bacillus altitudinis* and *Bacillus wiedmannii*. The bacteria reduce Pb uptake in *Brassica* sp. up to 30.5%. Aplication of the bacteria increases the shoot length and fresh weight of *Brassica* sp. Thus, the bacteria are potential as biofertilizers for reducing agrochemicals use in intensive agriculture areas and preventing environmental destruction and food contamination.

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