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A versatile Bacillus spp. from garlic plant as a promising eco-friendly agent: Multiple abiotic stress tolerance, exopolysaccharides production, antioxidant and antifungal activities

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Abstract

The selection of plant-associated bacteria tolerant to various stressors offers an eco-efficient strategy for sustainable agriculture. This study evaluates the resilience of four *Bacillus* spp. endophytes isolated from garlic (*Allium sativum*) roots against key abiotic and biotic challenges, including drought, salinity, herbicide (florasulam/2,4-D ester), heavy metals (Pb and Cd), and the phytopathogen *Botrytis cinerea*. Among the isolates, AsEB4 exhibited the highest stress tolerance, achieving maximal growth (0.985±0.27) under drought conditions (−1.0 MPa) and maintaining a survival rate of 92.62±1.32 under high salinity (400 mM NaCl). All strains tolerated herbicide concentrations up to 1200 µL mL^{-1}, and maintained robust growth under heavy metal stress, with a maximum tolerated concentration of 5 mM for Pb and Cd. EPS production and antioxidant activity increased dose-dependently with metal concentration, tending to a saturation point at higher levels (Tukey's test; $p<0.05$). Additionally, the isolates displayed strong antagonistic activity against *Botrytis cinerea* (>96%). These findings highlight the potential of garlic-associated *Bacillus* endophytes as sustainable microbial resources for stress mitigation, supporting the development of bio-based agents to reduce chemical inputs and enhance environmental resilience.

Keywords: Endophytic *Bacillus*, garlic roots, drought, salinity, herbicide, heavy metals, biological activity.

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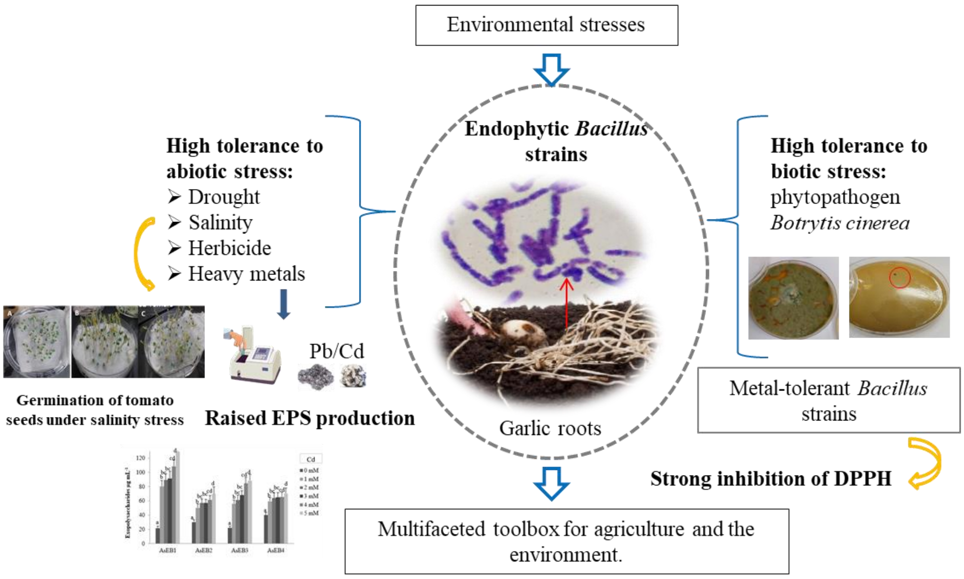
Graphical abstract



1. Introduction

In light of climate change and population pressure, numerous abiotic and biotic stresses have toxic effects on ecosystems and can cause serious human, plant, and animal health risks. Currently, the most significant factors that affect agricultural productivity are salinity, drought and toxic chemicals such as pesticides and heavy metals, which can disturb nutritional balance, photosynthesis, physiological functions, and development of plants (Kamran *et al.*, 2020; Sharath *et al.*, 2021; Anand *et al.*, 2023). Exposure time, concentrations, and formulations of herbicides can seriously alter the metabolism and the growth of soil microbial biodiversity, leading to negative consequences such as the unavailability of nutrients, low soil fertility, a slowdown in plant development, and increased vulnerability to pathogens (Vera *et al.*, 2025). Furthermore, toxic metals like cadmium and lead, which are released in ecosystems via anthropogenic activities (industrial waste, electronics, mining, and agriculture), can lead to several damages including water and soil pollution, cell deformation in plants and animals, and contamination of the human food chain (Kumar *et al.*, 2025; Zhang *et al.*, 2024). Many plants are impacted by these hazardous metals, which cause alterations in nutrient uptake and water absorption, leading to reduced plant development and production, and even death (Shahid *et al.*, 2024). In addition to abiotic stresses, biotic stressors, including pathogens, can cause significant economic losses. *Botrytis cinerea* is one of phytopathogens that can infect more than 200 plant species (*e.g.,* tomato) and cause severe loss in crop development (Toral *et al.*, 2020).

Increased mass concerns about environmental stresses, food safety, chemicals in food, crop losses, and pollution of ecosystems have led to encourage the investigation of biological approaches. Currently, plant growth-promoting bacteria (PGPB), known for their beneficial abilities, represents a crucial solution for minimizing serious agricultural impacts while promoting yields and food security. Among these bacteria, endophytic *Bacillus* species play a key role in the protection of plants against harmful stresses (Rafanomezantsoa *et al.,* 2025). The endospore forming ability allows the members of *Bacillus* genus to cope with harsh environmental conditions and climate-induced vulnerabilities. Furthermore, their capability to solubilize nutrients and produce phytohormones, siderophores, organic acids, hydrolytic enzymes, and several antimicrobial substances make them valuable candidates for mitigating both abiotic and biotic stresses in diverse cultivated plants and enhancing productivity (Nandana and Anith, 2025). Another noteworthy characteristic of PGPB *Bacillus* is the production of EPS, which are macromolecular constituents, synthesized by planktonic cells within metabolism and growth. EPS supply energy for cells, retain water, generate biofilm, and adsorb ions like toxic heavy metals (Reddy et *al*., 2024). Recently, numerous research highlighted the importance of EPS-producing bacteria (more than 70 species have been characterized) in heavy metal bioremediation which contribute to environmental detoxification and plant protection (Zhang *et al*., 2024; Bhardwaj, 2025). Microbial EPS are currently used in several sectors due to their unique qualities and the simplicity of their extraction. Therefore, the selection of potential EPS-producers remains one of the most crucial approaches. Under various stresses, including toxic metals, reactive oxygen species, highly reactive molecules, can be generated and damage cells (Abou-Aly *et al.,* 2019). To counteract this oxidative damage, endophytes can produce potent antioxidant compounds that inhibit the activation or multiplication of oxidative chain reactions (Muhtari *et al*., 2024). Accordingly, endophytes can also be a key source of novel pharmacological products.

In recent years, the attention of researchers has been attracted by endophytes from medicinal and aromatic plants, which can have similar abilities to their hosts in producing bioactive compounds such as antioxidants and antimicrobials, playing a role in the protection and the development of plants (Sarjono *et al*., 2019; Muhtari *et al*., 2024). To date, Garlic (*Allium sativum* L.) is known for its culinary and medicinal characteristics (*e.g*., antimicrobial, antioxidant, anti-inflammatory, and anti-cancer effects) thanks to its bioactive molecules, among which saponins, polysaccharides, and organosulfur compounds (Poplawska *et al*., 2024). So, garlic plant can also constitute a rich source of endophytic bacteria with biotechnological and medical properties. Our previous study (unpublished data) demonstrated that garlic roots of Guelma district (Northeastern Algeria) represents an exceptional source of endophytes that exhibited PGP features, which supported the findings of Srivastava *et al.* (2024), who recovered several endophytes from garlic plant with antibacterial activity. However, few studies have investigated the tolerance of garlic-associated bacteria to abiotic stresses, although *Allium sativum,* rich in reactive organosulfur compounds (e.g., allicin) (Poplawska *et al*., 2024), represents a chemically selective niche that may select for adapted and functionally valuable endophytes with potential as a multifaceted microbial toolbox for sustainable agriculture and environmental resilience. In the agricultural areas of Guelma, there is no information on the tolerance and the compatibility of endophytes with the common pollutants. In order to discover interesting functional properties of garlic-root endophytic bacteria in this zone, with a view to future environmental applications, the current study aimed to assess the ability of endophytic *Bacillus* spp. from garlic-roots in Guelma district to tolerate various abiotic stresses (drought, salinity, herbicide, and heavy metals), determine their antioxidant activity and EPS production under lead and cadmium stressors, and evaluate their ability to control the phytopathogen *Botrytis cinerea* in vitro.

2. Materials and Methods

2.1. *Selection of Bacterial strains from garlic roots*

Four *Bacillus* spp. were selected among 13 endophytic bacteria isolated from the garlic plant roots *Allium sativum* L. The plant material (n = 5) was sampled during the spring of 2024, from Ben Djerrah region (36° 25′ 56″ N, 7° 22′ 7″ E), situated in Guelma district, northeastern Algeria. The samples were transported in aseptic conditions to the laboratory within 1 hour of harvest. Healthy roots were initially washed in tap water to remove soil attached, and then surfaces were sterilized twice with 70% ethanol for 30 seconds, separated by a wash with 5% sodium hypochlorite for 3 min. In order to select only the endophytic bacteria, the roots were also soaked in a 10% sodium bicarbonate solution for 15 min and then washed at least five times with distilled water. One hundred ml of the final wash were streaked into nutrient agar (NA) (Merck, Germany) and incubated for a day at 28 °C to confirm the sterilization procedure. The sterilized roots were macerated in 90 ml of sterile physiological saline at180 rpm for one hour at ambient temperature. Aliquots of 50 µl were streaked on the NA and the Luria Bertani Agar (LBA) (Merck, Germany), and incubated at 30 °C for 24 hours (Nagah *et al*., 2024). After phenotypic identification according to microbiological tests described in Bergey’s Manual of determinative Bacteriology (Holt, 1994), and the determination of PGP characteristics, the four best promising *Bacillus* spp. strains were selected for this study to assess their tolerance to the most significant abiotic and biotic stresses. Isolates were denominated AsEBx (AsEB1, AsEB2, AsEB3, and AsEB4). To obtain a standard inoculum, each strain was adjusted to 0.5 McFarland scale after incubation in the Luria-Bertani broth (LBB) (Merck, Germany) at 150 rpm and 30 °C for 18 hours.

2.2. *Tolerance to drought stress*

In order to estimate the growth of endophytic bacteria under osmotic potentials of 0 MPa, −0.25 MPa, −0.5 MPa, −0.75 MPa, and −1.0 MPa, screening of drought tolerant AsEBx strains was performed on LBB medium enriched with 0, 2.4, 4.7, 6.5 and 7.95 g/100 ml polyethylene glycol 6000 (PEG), respectively. As PEG can alter the physicochemical properties of the medium, the pH was carefully adjusted to 7.0±0.2 following its addition in all treatments. After 24 h of incubation at 37 °C with shaking at 180 rpm, the optical density (OD) of 10 ml of each isolate was estimated spectroscopically (600nm). The stains were classified into: Highly sensitive (OD<0.3), sensitive-to-tolerant (0.3<OD<0.39), tolerant (0.4<OD<0.5), and highly tolerant (OD>0.5) (Nader et al., 2024).

2.3. *Tolerance to salt stress*

The standard bacterial inoculums were inoculated into the Tryptone Soy Broth (TSB) contained escalating concentrations of NaCl (0, 50, 100, 200, and 400 mM) (Merck, Germany), and incubated in a shaker at 140 rpm and 30 °C for 5 days. The growth rate of tolerant-bacteria was calculated every day after measurement of the OD at 600 nm and comparison with the control (Soto-Varela *et al*., 2024). The bacterial strain exhibiting the highest tolerance was selected for subsequent germination assays on tomato seeds under salt stress as described by Akbaba and Özden (2023). Briefly, surface-sterilized seeds (1% NaClO, 1 min) were soaked for 30 min in bacterial suspensions (OD_{600} = 0.1). After drying under sterile conditions, the seeds were incubated on moist filter paper in Petri dishes containing either distilled water or 400 mM NaCl. Germination percentage and seedling growth were assessed after 5 days at 25 °C in the dark.

2.4. *Bacterial growth in response to florasulam/2,4-D stress*

The exploration of the bacterial growth under herbicide stress was assessed in LBB supplemented with different concentrations of florasulam/2,4-D. This herbicide is widely used in Algeria especially in high production cereal fields. It contains two active compounds, 17.4 mM of florasulam and 900 mM of 2,4-D ester. According to the doses recommended by the producer to use in cereal areas, five concentrations (60, 150, 300, 600, and 1200 µl/ml) were tested. The quantitative tolerance of the four strains was determined using a spectrophotometer (OD600 nm) after 1, 2, 3, 4, 7, 8, 9 and 10 days of incubation at 180 rpm and 30 °C. The dry biomass weight (g L^{-1}) was estimated after cell bacteria recuperation, washing with PBS (Phosphate-Buffered Saline) (Merck, Germany), and drying at 60°C. A calibration curve DO_{600} *vs* dry biomass weight was used (Briceño *et al.*, 2020).

2.5. *Bacterial growth in response to lead and cadmium stress*

The tolerance screening of the endophyte isolates was firstly confirmed qualitatively by inoculation of the four AsEBx strains on LBA supplemented with different concentrations (ranging from 0.1 to 0.9 mM) of lead ($Pb(NO_3)_2$) and cadmium ($CdSO_4$) (Sigma-Aldrich, Germany). After incubation at 30 °C for 48 hours, the tolerant heavy metal strains were selected based on their ability to grow rapidly even at the highest concentration, and the creamy aspect of their colonies which facilitate their handling (Alvarado-Campo *et al*., 2023). Quantitative heavy metal tolerance was performed using broth diffusion method. Briefly, concentrations of 1, 2, 3, 4 and 5 mM of heavy metals salts were prepared in LB. A volume of 1 ml of each standard inoculum was added to 1 ml of each concentration and incubated at 30 °C and 140 rpm for 10 days. Bacterial growth was measured spectroscopically (OD600nm) at 1, 2, 3, 4, 7, 8, 9 and 10 days. Negative and positive controls (non-inoculated LB and LB without heavy metal salts, respectively) were prepared in the same condition. The experiment was conducted in triplicate. Maximum tolerance concentration (MTC) was determined by spotting of 10 µl of each inoculum on LBA. MTC corresponds to the highest concentration that shows visible bacterial colonies (Shylla *et al.,* 2021).

2.6. *Estimation of exopolysaccharide production for tolerating heavy metal stress*

The concentrations of EPS produced by heavy metal-tolerant strains were deduced using sulfuric acid anthrone colorimetry method (Zhang *et al*., 2024). For this, broth cultures were centrifuged at 7 000 rpm for 10 min at 4°C. The cell pellet was homogenized with three volumes of absolute ethanol and kept overnight at 4 °C to precipitate EPS and remove residual medium sugars. After drying at 37 °C, the pellet was resuspended in 1 mL distilled water and treated with 0.5 mL of Sevage reagent for protein removal, followed by centrifugation after an hour at 37 °C. The purified EPS fraction was then mixed with 5 mL sulfuric acid anthrone reagent, heated at 100 °C for 10 min and OD was determined spectrophotometrically (620 nm). EPS concentrations were expressed as µg mL^{-1} using glucose standard curve.

2.7. *Antioxidant activities of metal-tolerant strains*

The non-enzymatic antioxidant activities of the four metal-tolerant AsEBx strains were carried out using the experimental protocol described by Abou-Aly *et al.* (2019). One percent of each bacterial strain was inoculated in Mueller-Hinton broth (Merck, Germany) supplemented with the different concentrations of lead and cadmium previously cited, and incubated for a day (150 rpm/30 °C). The obtained cultures were centrifuged at 10,000×*g* for 5 min at 4 °C in order to obtain Cell Free Extracts (CFE). A mixture of 500 µL of CFE and 3000 µL of 2-DiPhenyl-2-Picryl hydrazyl hydrate ([DPPH] = 5 mg 100 mL^{-1} ethanol) (Sigma-Aldrich, Germany) was incubated in the dark for 30 min. The ethanol is used to prepare the negative control. The ODs were determined at 517 nm using acid ascorbic as a standard. The rate of antioxidant activities was calculated as:

$$\text{DPPH residue}(\%)=\frac{OD_{(517\,control)}-OD_{(517\,sample)}}{OD_{(517\,control)}}\times 100 \quad (1)$$

$$\text{Inhibition DPPH}(\%)=100-\text{DPPH residue}(\%) \quad (2)$$

2.8. *Assessment of antifungal ability of AsEBx strains*

The antifungal activity of AsEBx strains against the phytopathogen *Botrytis cinerea* (isolated from unhealthy tomato and supplied by the Annaba University, Algeria) was carried out on potato dextrose agar (PDA) (Merck, Germany). In the same plate, a 5 mm fragment of a fresh *B. cinerea* and 5 µL of a cultured *Bacillus* sp. strain in TSB were spotted facing each other. After 7 days of incubation at 28°C, the growth inhibition rate of the fungal pathogen was calculated in comparison with the control, which was PDA inoculated with *B. cinerea* and TSB without bacteria (Weinand *et al*., 2023):

$$\text{Inhibition rate}(\%)=\frac{\text{Diameter control fungal colony}-\text{Diameter fungal colony in presence of bacteria}}{\text{Diameter control fungal colony}} \quad (3)$$

2.9. *Statistical analysis*

The results were reported as mean values with standard deviation represented by bars. The analysis of variance (ANOVA) and Tukey's test were performed to estimate statistically significant differences ($p<0.05$) using SPSS software (IBM, USA).

3. Results

3.1. *Attributes of selected Bacterial strains from garlic roots*

From 13 endophyte bacteria isolated from garlic roots *Allium sativum* L., 4 *Bacillus* spp. were selected for the current study. In our previous research, it has been shown that these endophytes exhibited several functional traits such as high enzymatic activity, photohormone production, phosphate solubilization, and promoting development of wheat and tomato seedlings (see **Supplementary Table S1**). Phenotypic profiles demonstrated that our strains belong to the *Bacillus* spp.

species. All the interesting characteristics of the AsEBx strains were mentioned in **Table 1**.

Table 1. Phenotypic profiles of isolated *Bacillus* spp. from garlic roots.

Isolate	AsEB1	AsEB2	AsEB3	AsEB4
Morphological characterization	**Gram (+); Endospore forming rods; Mobile; Aerobic.**	**Gram (+); Endospore forming rods; Mobile; Aerobic.**	**Gram (+); Endospore forming rods; Mobile; Aerobic.**	**Gram (+); Endospore forming rods; Mobile; Aerobic.**
Biochemistry characterization				
Catalase	+	+	+	+
Oxidase	-	-	-	-
Citrate	+	+	+	+
Amylase	+	+	+	+
Esterase	+	+	+	+
Gelatinase	+	+	+	+
Lipase	+	+	+	+
Pectinase	+	+	+	+
Protease	+	+	+	+
Urease	+	+	+	+
Lactose	+	+	+	+
Glucose	+	+	+	+
Mannitol	-	+	+	+
Rhamnose	-	+	-	-
Saccharose	+	+	+	-
Methyl red test	+	-	-	-
VP test	+	-	-	+
Indole	-	-	-	-
PGP traits				
Phosphate solubilization	+	+	+	+
Hydrogen cyanide production	+	-	-	+
Indole-3-acetic acid production	> 114 µg/ml	> 97 µg/ml	> 96 µg/ml	> 108 µg/ml
Germination rate of seeds	> 94 %	> 82 %	> 80 %	> 93 %

PGP: Plant Growth Promoting, (+): positive reaction, (-): negative reaction

3.2. *Tolerance to drought and salinity stresses*

The results in **Table 2** demonstrate that all strains are highly tolerant to drought stress. Regarding the OD values under unstressed conditions, there was a decrease in strains' growth under different drought intensities ($p<0.05$). Nevertheless, the isolates grew well and their OD values remained above 0.5. At high osmotic pressure, the strains AsEB1 and AsEB3 showed the lowest OD values. However, the highest growth level was recorded for AsEB4 followed by AsEB2, indicating the best drought tolerance. With the increase in NaCl concentration, a significant progressive reduction in the growth rate of AsEB1, AsEB2, and AsEB3 strains was observed ($p<0.05$). The AsEB4 isolate revealed the highest tolerance to salinity stress (**Table 2**). Compared to the control, the reduction in AsEB4 growth was very slight at 200 mM (1.45%) and remained low even at 400 mM (7.38%). In contrast, the AsEB2 strain was the most affected by NaCl, with growth reductions of 22.17% and 22.68% at 200 mM and 400 mM NaCl, respectively. Nonetheless, it is important to emphasize that the growth rate of all tested strains remained above 70%.

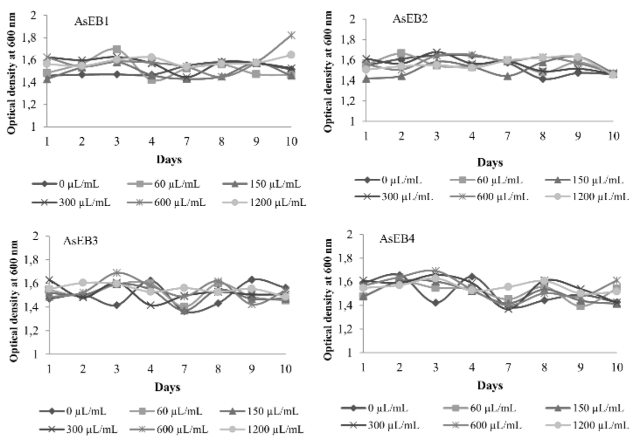


Figure 1. Growth of endophytic *Bacillus* spp. isolated from garlic roots under florasulam/2,4-D herbicide stress.

The AsEB4 strain positively influenced the germination of tomato seeds under salinity condition (400 mM NaCl) (**Table 3**) (Tukey's test; p<0.01). Although salinity reduced seedling growth compared to the standard condition (seeds inoculated with AsEB4), the recovered values remained substantial, suggesting that AsEB4 can play a role in mitigating salt stress during germination (see **Supplementary Figure S1**).

Table 2. Drought and salinity tolerance of endophytic *Bacillus* spp. isolated from garlic roots.

Isolate	AsEB1	AsEB2	AsEB3	AsEB4
Drought stress*				
0 Mpa	1.887±0.01^{a}	1.721±0.3^{ab}	1.717±0.24^{ab}	1.907±0.03^{a}
−0.25 Mpa	1.652±0.21^{ab}	1.551±0.02^{abc}	1.641±0.05^{ab}	1.703±0.1^{ab}
−0.5 Mpa	0.972±0.03^{cd}	0.911±0.01^{cd}	0.981±0.03^{cd}	1.314±0.2^{bc}
−0.75 Mpa	0.725±0.32^{de}	0.845±0.23^{cd}	0.872±0.1^{cd}	1.016±0.1^{cd}
−1.0 Mpa	0.693±0.18^{e}	0.832±0.27^{cd}	0.632±0.13^{e}	0.985±0.27^{cd}
Salinity stress**				
0 mM	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}
50 mM	99.82±15.29^{a}	97.16±5.5^{ab}	99.44±6.35^{a}	100±0.00^{a}
100 mM	96.56±7.96^{ab}	88.70±12.11^{bc}	92.13±7.36^{ab}	100±0.00^{a}
200 mM	96.43±7.99^{ab}	77.83±15.04^{c}	87.37±6.52^{bc}	98.55±8.45^{a}
400 mM	89.75±11.84^{b}	77.32±16.20^{c}	81.82±15.29^{c}	92.62±1.32^{a}

**: Results are expressed in OD_{600} /24 h, **: Results are expressed in survival rate (%)/5 days, results are mean±SD, $^{a-e}$: indicate significant differences (Tukey's test; p<0.05).*

Table 3. Effect of AsEB4 strain on germination and seedling growth of tomato under saline condition after 5 days.

Parameters	Tomato seeds		
	Control*	Standard condition**	Saline condition***
Germination (%)	50.7±2.1^{a}	83.4±1.0^{c}	71.7±1.7^{b}
Shoot length (cm)	1.4±0.3^{a}	7.6±0.3^{c}	6.1±0.6^{b}
Root length (cm)	2.03±0.5^{a}	9.8±0.2^{c}	7.9±0.4^{b}
Shoot fresh weight (mg)	3.3±0.1^{a}	19±0.1^{c}	14.7±0.2^{b}
Root fresh weight (mg)	5.1±0.2^{a}	47.3±0.2^{c}	39±0.2^{b}
Shoot dry weight (mg)	0.5±0.2^{a}	2.7±0.3^{c}	1.9±0.1^{b}
Root dry weight (mg)	2.7±0.3^{a}	5.9±0.2^{c}	3.3±0.2^{b}

: seeds without bacterial strain, **: seeds inoculated with AsEB4, *: seeds inoculated with AsEB4 in 400 mM NaCl, results are mean±SD, $^{a-c}$: indicate significant differences between values within the same line (Tukey's test; p<0.05).*

Table 4. Non-enzymatic antioxidant activities of endophytic *Bacillus* spp. isolated from garlic roots.

	AsEB1		AsEB2		AsEB3		AsEB4	
Heavy metal Concentration	DPPH residue (%)	Inhibited DPPH (%)	DPPH residue (%)	Inhibited DPPH (%)	DPPH residue (%)	Inhibited DPPH (%)	DPPH residue (%)	Inhibited DPPH (%)
0 mM (Control)	38.5^{a}	61.5	42.7^{a}	57.3	40.3^{a}	59.7	39.7^{a}	60.3
Pb								
1 mM	20.3^{b}	79.7	29.5^{b}	70.5	25.5^{b}	74.5	18.7^{b}	81.3
2 mM	18.7^{c}	81.3	21.8^{c}	78.2	20.2^{c}	79.8	15.6^{c}	84.4
3 mM	10.6^{d}	89.4	18.2^{cd}	81.2	19.7^{cd}	80.3	12.3^{d}	87.7
4 mM	9.6^{d}	90.4	12.8^{d}	87.2	13.4^{d}	86.6	10.7^{d}	89.3
5 mM	9.1^{d}	90.9	10.4^{d}	89.6	9.5^{d}	90.5	9.3^{d}	90.7
Cd								
1 mM	17.4^{b}	82.6		71.6	24.7^{b}	75.3	20.3^{b}	79.7
2 mM	15.3^{c}	84.7	28.4^{b}	74.3	19.8^{c}	80.2	17.8^{c}	82.2
3 mM	10.2^{cd}	89.8	25.7^{c}	79.7	17.3^{cd}	82.7	15.4^{cd}	84.6
4 mM	8.4^{d}	91.6	20.3^{cd}	86.4	9.7^{d}	90.3	11.6^{d}	88.4
5 mM	6.8^{d}	93.2	13.6^{d}	90.4	8.1^{d}	91.9	7.9^{d}	92.1

$^{a-d}$: indicate significant differences between metal concentrations; Residual DPPH % were used for statistical tests (Tukey's test; p<0.05).

3.3. *Bacterial growth in response to florasulam/2,4-D stress*

As illustrated in **Figure 1**, all isolates showed high tolerance to florasulam/2,4-D concentrations up to 1200 µL mL-1. Compared to the control, strain AsEB2 exhibited non-significant growth reductions (<10%) after 10 days (p=0.72). Furthermore, relative increases in growth of AsEB1, AsEB3, and AsEB4 were noted at high herbicide concentrations. For the isolate AsEB1, the most of OD values at 1200 µL mL-1 were higher than those measured in the control, indicating a better adaptive ability and a prolonged tolerance to the herbicide stress. The dry biomass determination revealed that the strain AsEB1 showed the highest biomass weight (1.87 – 2.41 g L-1), followed by AsEB4 (1.81 – 2.23 g L-1), AsEB3 (1.8 – 2.22 g

L-1), and AsEB2 (1.73– 2.19 g L-1). Detailed significance groupings at each time point are presented in **Supplementary Table S2** (Tukey's test; $p<0.05$).

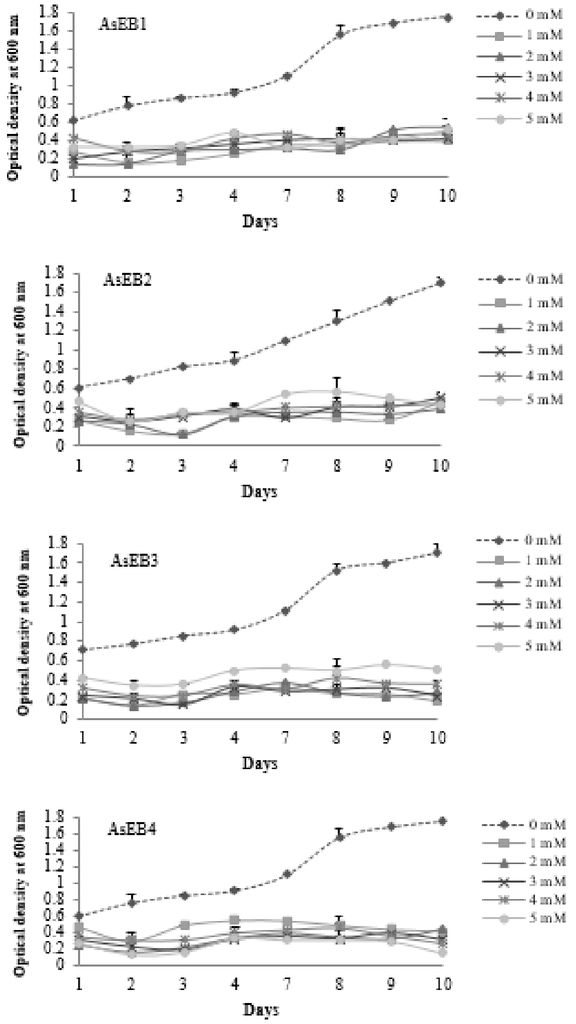


Figure 2. Growth of endophytic *Bacillus* spp. isolated from garlic roots under lead stress.

3.4. *Bacterial growth in response to lead and cadmium stress*

The first screening of heavy metal-tolerant bacteria showed that all the AsEBx strains exhibited appropriate growth with creamy colonies at the maximum concentration 0.9 mM of Pb and Cd. The quantitative tolerance screening at increased toxic concentrations revealed that the four endophytic *Bacillus* spp. remained tolerant to tested heavy metals. Overall, all strains showed comparatively ($p>0.05$) a decrease in their OD values, especially between the first and the third day. Starting from the fourth day, the strains demonstrated a slight increase in growth and maintained stability under a potential metal stress until 10 days (**Figures 2 and 3**). In the case of lead, the isolate AsEB2 reached a higher growth level (OD=0.572) at 5 mM after 8 days. On the other hand, the isolate AsEB3 exhibited a robust growth at 5 mM of Pb, compared to lower concentrations, recording maximum OD values over the entire period of incubation (**Figure 2**). In the presence of cadmium, the growth of AsEB1 in toxic concentrations showed the highest OD mean values. In the other strains, OD_{600} mean values fluctuate between 0.3 and 0.5 were noted (**Figure 3**). Detailed significance groupings for bacterial growth under Pb and Cd stresses at each time point, as determined by Tukey's test, are presented in **Supplementary Table S3** and S4. Based on the culture on LBA, results showed important colony development at all tested concentrations with a MTC of 5 mM of each heavy metal (**Figure 4**), confirming a greater tolerance of the AsEBx strains to the top level of Pb and Cd.

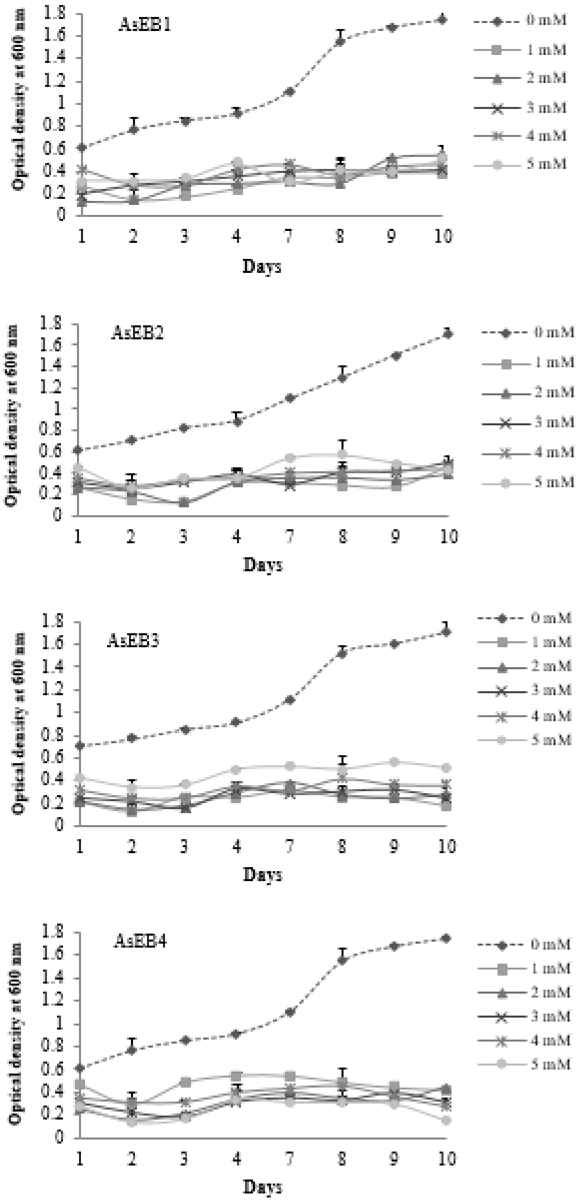


Figure 3. Growth of endophytic *Bacillus* spp. isolated from garlic roots under cadmium stress.

3.5. *Exopolysaccharide production for tolerating heavy metal stress*

All the strains exhibited the same trends of EPS production (**Figure 5**). The lowest EPS quantities were noted in control (without any metal). However, EPS production was raised with the increasing of heavy metals concentrations, with partial saturation at higher levels (Tukey's test; $p<0.05$), indicating probably an adaptive response in the AsEBx strains. Among the four heavy metal-tolerant strains, AsEB1 isolate was the most important producer of EPS. The highest EPS amounts were noted in presence of Cd at 4 and 5 mM (108.61 and 129.1 µg mL^{-1}, respectively). Under lead exposure, the maximum ability for EPS secretion was recorded in AsEB3 isolate, which reached 79.25 µg mL^{-1} EPS at 5 mM of Pb. Furthermore, isolates AsEB2 and AsEB4 showed an enhanced production of EPS up to >65 µg mL^{-1} under each tested heavy metal stress.

4. Antioxidant activities of metal-tolerant strains

Results present in **Table 4** revealed that the four heavy metal-tolerant *Bacillus* spp. strains were able to inhibit DPPH and exhibited effective antioxidant activities. Important rates (> 90 %) of inhibited DPPH were observed under the highest concentrations of the two heavy metals. Minimum rates of residual DPPH under Pb and Cd were recorded by AsEB1 (9.1-6.8%, respectively), followed by AsEB4 (9.3-7.9%, respectively), AsEB3 (9.5-8.1%, respectively), and AsEB2 (10.4-9.6%, respectively). Whereas, the bottom level of antioxidant activities occurred at the control (without stress), demonstrating that DPPH inhibition effectiveness was gradually increased under stress conditions.

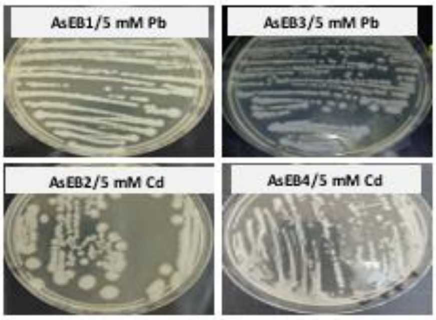


Figure 4. Colony development of heavy metal-tolerant *Bacillus* spp. isolated from garlic roots at the maximum tolerance concentration of lead and cadmium.

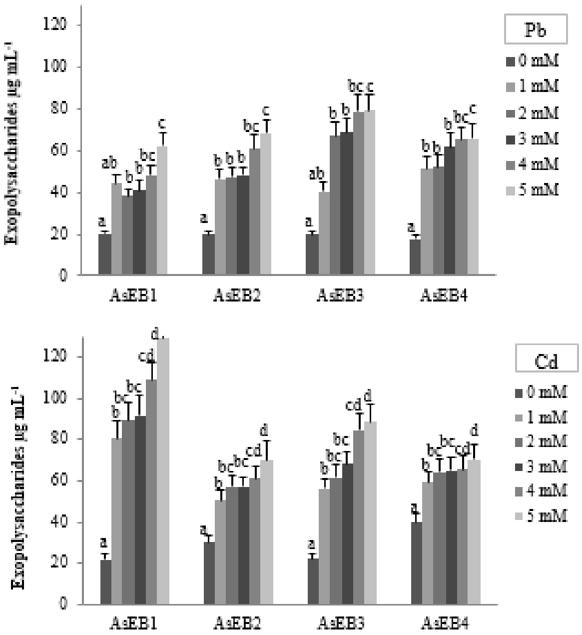


Figure 5. Exopolysaccharides production by endophytic *Bacillus* spp. strains under lead and cadmium stresses. [a-d]: indicate significant differences (Tukey's test; p<0.05).

4.1. *Antifungal ability of AsEBx strains*

The *in vitro* assays indicated that AsEBx strains exhibit antifungal activity against the gray mold *Botrytis cinerea.* The co-cultivation experiments revealed a strong inhibition of mycelial growth at an average rate of 97.78±0.21% by AsEB1, 96.97±0.1% by AsEB2, 96.11±0.24% by AsEB3, and 97.22±0.11% by AsEB4. A representative image of the dual-culture assay is provided in **Figure 6**.

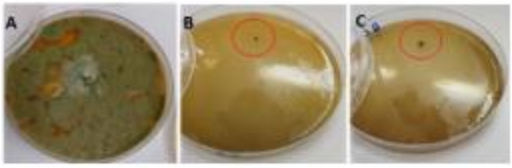


Figure 6. Effect of endophytic *Bacillus* spp. strains on the mycelial growth of *Botrytis cinerea* after 7 days at 28 ± 0.2 °C. (A) Colony growth of *B. cinerea* on the PDA medium as control; (B) Inhibition of *B. cinerea* growth by AsEB1; (C) Inhibition of *B. cinerea* growth by AsEB4.

5. Discussion

5.1. *Bacillus strains from garlic roots*

Endophytic *Bacillus* species are well documented for their plant growth-promoting functions and stress mitigation capacity (Abuhena *et al.*, 2024). Our investigation indicates that garlic (*Allium sativum* L.) roots constitute a selective ecological niche that favors metabolically versatile and stress-adapted bacterial populations. While previous studies have mainly focused on classical PGP traits, the present work advances current knowledge by evaluating isolates under both abiotic and biotic pressures. To the best of our knowledge, such a diverse multifunctional profile has rarely been reported for garlic root-associated *Bacillus* endophytes. The robust tolerance observed in the AsEBx strains, combined with their EPS-production, antioxidant activity, and biocontrol capacity, reinforces their relevance as biological inputs for resilient, low-input cropping systems (Nagah *et al*., 2024). Although the AsEBx were assigned to the genus *Bacillus* based on phenotypic and biochemical characteristics, molecular identification using 16S rRNA gene sequencing would provide a more precise taxonomic resolution and will be considered in future studies.

5.2. *Tolerance to drought and salinity stresses*

Water deficit and soil salinity are major constraints that reduce crop productivity and threaten global food security. The exploration of endophytic bacteria capable of tolerating such stresses is critical for developing strategies that enhance plant resilience under adverse environmental conditions. In the present investigation, the ability of *Bacillus* spp. strains to maintain functional activity under severe osmotic stress (−1.0 MPa), highlights its potential for sustaining crop germination and nutrient availability under low water conditions. By facilitating nutrient acquisition when water is scarce, these bacteria may act as biological buffers, mitigating the economic risks associated with drought-induced yield losses (Sharath *et al.*, 2021). Regarding salinity, *Bacillus* spp. isolates displayed a broad tolerance range (50 to 400 mM NaCl). These findings corroborate previous research that indicate the ability of *Bacillus* species to alleviate salinity stress (Kaur and Karnwal, 2023). The inoculation with AsEB4 strain supported robust germination of tomato seeds under salinity (400 mM NaCl. These data support the potential role of *Bacillus* spp. in mitigating salt stress, possibly through mechanisms such as osmolyte synthesis and antioxidant activity. The observed stress resilience may be attributed to enhanced production of exopolysaccharides and proline, which act as osmoprotectants and free radical scavengers (Abuhena *et al.*, 2024; Nadar *et al*., 2024).

5.3. *Tolerance to florasulam/2,4-D stress*

The screening of herbicide-tolerant bacteria represents a strategic approach to reduce the toxic effects of herbicides and sustain crop productivity in intensive agricultural systems. The present study advances current knowledge by documenting the adaptive capacity of garlic-associated *Bacillus* spp. isolates to florasulam/2,4-D stress. These isolates maintained high cell densities, and biomass accumulation progressed over time despite herbicide pressure. Similar to our data, previous studies reported the tolerance of certain *Bacillus* strains exposed to different active molecules, such as atrazine and paraquat, where growth remained unaffected or was only moderately influenced (Farias *et al*., 2021; Inthama *et al.*, 2021). While other studies have reported total growth inhibition of all *Bacillus* strains at 500 ppm of paraquat (Pradhan and Jena, 2023), our data suggest a higher level of metabolic resilience. The pronounced tolerance observed in our garlic root-associated isolates may be related to their endospore forming capability, which ensures persistence under chemical stress. While the clarification of the culture medium after incubation could suggest a potential transformation of the herbicide, this hypothesis requires further analytical confirmation. Notably, previous investigations have shown that certain *Bacillus* species can degrade herbicides via specific metabolic pathways, which may provide a plausible explanation for this observation (Ichor *et al*., 2024; Zameer *et al.*, 2023). The deployment of such herbicide-tolerant endophytes could help preserve beneficial microbiota in treated soil, reduce the need for repeated chemical inputs, and potentially contribute to the in-situ biodegradation of residual compounds.

5.4. *Tolerance to heavy metals*

Heavy metal pollution constitutes one of the foremost concerns for the environment and living organisms' health. Currently, irrigation water and agricultural soils in Guelma district are under increasing heavy metal pressure (Benhalima *et al*., 2020; Sassane and Touati, 2024). Consequently, resistance development in plant-associated bacteria to cope with heavy metal stressors is possible. In this context, our isolates from Guelma agricultural region demonstrate remarkably high tolerance to Pb and Cd. These levels align with the robust defense mechanisms identified in other specialized endophytes (Biswas *et al.*, 2024; Kumar *et al.*, 2025). In addition to the detected PGP traits (direct processes), our data suggest that AsEBx isolates can also improve plant growth by increasing plant tolerance to high heavy metal levels (indirect process). The high metal tolerance observed in our isolates likely reflects their prior exposure to metal-contaminated soils, as persistent heavy metals significantly influence resistance development within endophyte communities (Liu J. *et al*., 2024). However, the initial decrease in growth observed during the first days, followed by a recovery phase, may correspond to a metal-induced lag phase, indicating that these isolates undergo necessary physiological adjustments to cope with metal toxicity. This behavior is commonly associated with adaptive metabolic responses, including intracellular redox regulation, metal sequestration, and the expression of chelating proteins, before resuming active proliferation (Liu L. *et al*., 2024). Such resilient microbial resources could support the development of cost-effective and environmentally sustainable strategies for managing metal-affected soils (Liang *et al*., 2025).

5.5. *Exopolysaccharide production for tolerating heavy metal stress*

The production of EPS by the studied *Bacillus* spp., which followed a dose-response relationship with respect to the concentration of tested heavy metals, highlights its role as a key tolerance mechanism against lead and cadmium stresses. EPS are known to act as extracellular biosorbents thanks to their anionic functional groups (e.g., carboxyl and phosphate), which can bind cationic metals through electrostatic interactions and complexation. This physiological plasticity allows bacterial strains to construct a physical barrier, effectively immobilizing and sequestering toxic ions before they reach the intracellular environment. The higher EPS production observed under Cd stress compared to Pb may reflect variations in metal behavior and interaction with EPS matrix. Cd^{2+} can exert strong intracellular stress due to its mobility and bioavailability, inducing a more pronounced EPS synthesis. In contrast, Pb^{2+} is more readily immobilized at the cell surface and forms stable precipitates, which may reduce its intracellular impact and consequently the need for elevated EPS production (Zadeh *et al.*, 2023; Zhang *et al.*, 2024). Such differences suggest that EPS may contribute to distinct sequestration dynamics for Cd and Pb in our strains, although these interactions require further investigation to be confirmed. These findings highlight these strains as efficient, eco-friendly agents capable of transforming toxic stress into a manageable biological response. In this context, integrating such metal-tolerant bacteria into contaminated soils could help reduce the bioavailability of Pb and Cd through a self-sustaining biological process. As noted in similar observation, the sequestration of metals by EPS not only ensures bacterial survival but also contributes to the establishment of a protected rhizospheric niche (Bhardwaj, 2025).

5.6. *Antioxidant activities of metal-tolerant strains*

The significant activation of antioxidant systems in AsEBx strains under Pb and Cd stress reflects a dynamic metabolic adaptation to metal-induced oxidative stress. Our findings suggest that these strains can reprogram environmental stress into a physiological trigger for the synthesis of high-value secondary metabolites. Such metabolic plasticity opens avenues for the biotechnological production of natural antioxidants and additives. The strong antioxidant potential of endophytic *Bacillus* is driven by the secretion of specialized compounds, notably polyphenols and flavonoids, which effectively neutralize free radicals (Tran *et al*., 2025). Given the therapeutic richness of garlic, its associated endophytes appear to recapitulate the host's chemical complexity to enhance plant defenses. This symbiotic legacy positions our isolates not only as defensive partners for the host plant but also as versatile biological agents capable of bridging the gap between microbial resilience and the bioeconomy (Muhtari *et al*., 2024).

5.7. *Antifungal ability of AsEBx strains*

The selection of beneficial bacterial strains as biocontrol agents represents a feasible and sustainable approach controlling post-harvest deterioration of several crops and reducing the overuse of chemicals (Herpich *et al*., 2025). In this study, all tested *Bacillus* sp. strains exhibited remarkably strong antifungal activity against *B. cinerea*, with inhibition rates exceeding 96%. This inhibition level surpasses most recent reports and highlights the exceptional antagonistic capacity of garlic-associated endophytes (Abuhena *et al.*, 2024). The strong inhibitory activity observed in AsEBx strains may be associated with the production of hydrolytic enzymes, antimicrobial metabolites, siderophore, as well as competition for nutrients, which contribute to pathogen suppression by limiting fungal growth and interfering with cell integrity (Nagrale *et al.* 2023). The ability of our isolates to almost completely inhibit a major post-harvest pathogen suggests their potential as high-value bio-fungicides.

6. Conclusion

This study reveals the potential of garlic root-associated *Bacillus* endophytes as multifunctional microbial resources capable of enhancing plant tolerance to multiple stresses, with promising relevance for sustainable agricultural practices and bioeconomic strategies. The combination of plant growth-promoting traits, tolerance to abiotic stressors, and strong antifungal activity underscores their capacity to simultaneously mitigate biotic and abiotic constraints. In addition, the production of EPS and antioxidant activity further strengthens their biotechnological relevance, as these traits may contribute to soil stabilization, stress mitigation, and the development of more robust bioinoculant formulations. Further research should focus on validating their performance under field conditions, clarifying the molecular mechanisms underlying their stress-adaptive responses, and developing scalable formulations to support environmentally responsible agricultural practices.

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Supplementary Material

Table S1. Effect of endophytic *Bacillus* spp. isolated from garlic roots on germination and seedling growth of wheat and tomato seeds after 14 days.

Parameters	Wheat seeds					Tomato seeds				
	Control	Treatment				Control	Treatment			
		AsEB1	AsEB2	AsEB3	AsEB4		AsEB1	AsEB2	AsEB3	AsEB4
Germination (%)	49.6±4.1ᵃ	90.4±2.6ᵇ	85.2±1.2ᵇ	88.3±4.6ᵇ	95.3±1.2ᶜ	51.3±2.1ᵃ	94.6±1.8ᶜ	82.5±1.4ᵇ	81.7±1.2ᵇ	93.4±2.0ᶜ
Shoot length (cm)	9.1±1.5ᵃ	19.4±1.1ᶜ	19.8±0.7ᶜ	20.2±1.2ᶜ	21.3±0.5ᵈ	9.8±0.6ᵃ	16.2±0.3ᶜ	17.3±0.2ᶜ	20.4±0.6ᵈ	20.8±0.9ᵈ
Root length (cm)	13.15±0.5ᵃ	21.2±1.5ᵈ	22.±0.5ᶜ	22.8±0.4ᶜ	23.7±0.1ᵈ	7.2±0.2ᵃ	15.8±0.2ᶜ	18±0.9ᶜ	19.9±0.5ᵈ	20.2±0.6ᵈ
Shoot fresh weight (mg)	10±0.1ᵃ	150±0.1ᵉ	203±0.2ᶜ	367±0.1ᵈ	369±0.1ᵈ	8±0.2ᵃ	48±0.2ᶜ	39±0.3ᵇ	68±0.4ᵈ	70±0.2ᵈ
Root fresh weight (mg)	7±0.03ᵃ	220±0.1ᵉ	214±0.1ᶜ	299±0.2ᵈ	301±0.1ᵈ	7±0.1ᵃ	89±0.2ᶜ	79±0.2ᵇ	91±0.3ᶜ	96±0.1ᵈ
Shoot dry weight (mg)	1.9±0.02 ᵃ	23.6±0.02ᵇ	29.2±0.03ᵇ	57.1±0.1ᵈ	58.3±0.2ᵈ	1.7±0.1ᵃ	8.1±0.03ᶜ	4.9±0.02ᵇ	11.8±0.1ᵈ	12.1±0.1ᵈ
Root dry weight (mg)	1.1±0.1ᵃ	32.3±0.2bᶜ	32.2±0.02ᶜ	43.8±0.04ᵈ	44.4±0.3ᵈ	1.08±0.1ᵃ	13.2±0.2ᶜ	12.6±0.2ᶜ	14.1±0.1ᶜ	14.9±0.2ᵈ

[a-d]: Significant differences between values within the same line (Tukey's test; $p \leq 0.05$).

Table S2. Tukey's post hoc test letters indicating significance of bacterial growth under herbicide stress at each time point.

Strain	Florasulam/2,4-D (µL/mL)	Days							
		1	2	3	4	7	8	9	10
AsEB1	0	b	a	b	b	a	a	a	b
	60	b	a	a	c	a	a	a	b
	150	b	a	b	b	b	b	a	b
	300	a	a	b	a	b	a	a	b
	600	a	a	b	a	a	b	a	a
	1200	a	a	b	a	a	a	a	a
	p-value	0.0002	0.1542	0.0000	0.0000	0.0001	0.0000	0.1248	0.0001
AsEB2	0	ab	a	ab	a	ab	c	b	a
	60	ab	a	b	b	a	c	b	a
	150	b	c	b	b	b	b	a	a
	300	a	b	a	ab	ab	c	b	a
	600	ab	b	ab	a	ab	a	b	a
	1200	ab	b	b	b	ab	a	a	a

	p-value	0.0031	0.0000	0.0001	0.0012	0.0002	0.0001	0.0005	0.7245
AsEB3	0	b	b	c	a	c	c	a	ab
	60	b	b	b	a	c	a	c	c
	150	b	b	b	b	c	ab	c	b
	300	a	b	b	c	b	b	b	ab
	600	b	ba	a	a	b	a	c	a
	1200	a		b	b	a	b	b	b
	p-value	0.0001	0.0004	0.0000	0.0000	0.0000	0.0000	0.0000	0.0015
AsEB4	0	b	a	c	a	b	b	a	b
	60	b	b	b	b	b	ab	a	b
	150	b	a	b	b	b	b	a	b
	300	a	b	a	b	b	a	a	b
	600	a	a	a	b	b	b	a	a
	1200	a	b	b	b	a	a	a	b
	p-value	0.0004	0.0001	0.0000	0.0001	0.0001	0.0001	0.1142	0.0001

Different letters within the same column indicate significant differences between treatments at the same time point (Tukey's test; p<0.05).

Table S3. Tukey's post hoc test letters indicating significance of bacterial growth under lead stress at each time point.

Strain	Pb (mM)	Days							
		1	**2**	**3**	**4**	**7**	**8**	**9**	**10**
AsEB1	0	a	a	a	a	a	a	a	a
	1	b	d	c	d	c	c	c	c
	2	c	d	b	cd	c	c	b	b
	3	bc	bc	b	bc	bc	b	bc	bc
	4	b	b	b	b	b	c	bc	bc
	5	b	c	b	b	c	b	bc	b
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AsEB2	0	a	a	a	a	a	a	a	a
	1	c	c	c	a	a	a	a	a
	2	c	c	c	a	a	a	a	a
	3	bc	bc	bc	a	a	a	a	a
	4	b	ab	b	a	a	a	a	a
	5	ab	ab	ab	a	a	a	a	a
	p-value	0.0004	2.33e-05	6.35e-05	0.384	0.234	0.605	0.096	0.197
AsEB3	0	a	a	a	a	a	a	a	a
	1	c	c	bc	c	a	c	bc	c
	2	c	c	c	c	a	c	c	c
	3	bc	bc	c	bc	a	bc	bc	c
	4	b	b	bc	bc	a	bc	c	bc
	5	ab	ab	b	b	a	b	b	b
	p-value	0.0003	0.000	0.000	0.008	0.21	0.0016	0.012	0.000
AsEB4	0	a	a	a	a	a	a	a	a
	1	ab	b	b	ab	a	a	a	b
	2	bc	b	c	b	a	a	a	b
	3	bc	b	c	b	a	a	a	b
	4	ab	b	b	ab	a	a	a	b
	5	c	b	c	b	a	a	a	b
	p-value	0.0003	0.0000	0.0000	0.0382	0.1245	0.1872	0.0912	0.0415

Different letters within the same column indicate significant differences between treatments at the same time point (Tukey's test; p<0.05).

Table S4. Tukey's post hoc test letters indicating significance of bacterial growth under cadmium stress at each time point.

Strain	Cd (mM)	Days							
		1	**2**	**3**	**4**	**7**	**8**	**9**	**10**
AsEB1	0	a	a	a	a	a	a	a	a
	1	b	b	a	b	a	a	a	b
	2	b	b	a	b	a	a	a	b

	3	ab	b	a	b	a	a	a	b
	4	b	ab	a	b	a	a	a	b
	5	ab	b	a	b	a	a	a	ab
	p-value	0.0421	0.0156	0.0812	0.0485	0.4125	0.2841	0.0954	0.0388
AsEB2	0	a	a	a	a	a	a	a	a
	1	b	b	b	c	b	b	b	b
	2	ab	b	b	c	b	b	b	b
	3	b	b	b	b	b	b	b	b
	4	ab	b	b	b	b	b	b	b
	5	ab	b	c	c	b	b	b	b
	p-value	0.0042	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AsEB3	0	a	a	a	a	a	a	a	a
	1	b	b	b	b	b	b	b	b
	2	b	b	b	b	b	b	b	b
	3	b	b	b	b	b	b	b	b
	4	b	b	b	b	b	b	b	b
	5	b	b	b	b	b	b	b	b
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AsEB4	0	a	a	a	a	a	a	a	a
	1	c	b	b	b	b	b	b	b
	2	b	c	c	b	b	b	b	b
	3	bc	b	b	b	b	b	b	b
	4	bc	b	b	b	b	b	b	b
	5	bc	b	c	c	b	b	b	b
	p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Different letters within the same column indicate significant differences between treatments at the same time point (Tukey's test; $p<0.05$).

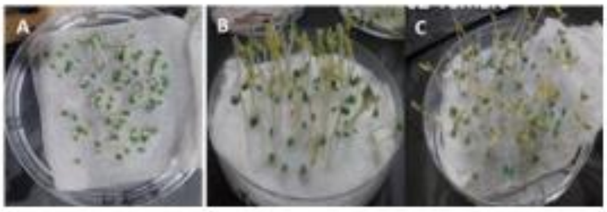

Figure S1. Effect of AsEB4 strain on germination of tomato seeds after 5 days. (A) Control, (B) Seeds inoculated with AsEB4 under standard condition, (C) Seeds inoculated with AsEB4 under salinity stress (400 mM NaCl).