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Plant Growth-Promoting Rhizobacteria Improved Salinity Tolerance of *Lactuca sativa* and *Raphanus sativus*

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Salinity stress is an important environmental problem that adversely affects crop production by reducing plant growth. The impacts of rhizobacterial strains to alleviate salinity stress on the germination of *Lactuca sativa* and *Raphanus sativus* seeds were assessed using different concentrations of NaCl. Plant growth-promoting rhizobacteria (PGPR) strains were also examined to improve the early germination of Chinese cabbage seeds under normal conditions. *Lactobacillus* sp. and *P. putida* inoculation showed higher radicle lengths compared with non-inoculated radish (*Raphanus sativus*) seeds. LAP mix inoculation increased the radicle length of lettuce (*Lactuca sativa*) seedlings by 2.0 and 0.5 cm at salinity stress of 50 and 100 mM NaCl concentration, respectively. Inoculation by *Azotobacter chroococcum* significantly increased the plumule and radicle lengths of germinated seeds compared with non-inoculated control. *A. chroococcum* increased the radicle length relative to the uninoculated seeds by 4.0, 1.0, and 1.5 cm at 50, 100, and 150 mM NaCl concentration, respectively. LAP mix inoculation significantly improved the radicle length in germinated radish seeds by 7.5, 1.3, 1.2, and 0.6 cm under salinity stress of 50, 100, 150, and 200 mM NaCl concentration, respectively. These results of this study showed that PGPR could be helpful to mitigate the salinity stress of different plants at the time of germination.

Keywords: PGPR, stress mitigation, microbial inoculation, early seed germination

Introduction

The severity of abiotic stresses, particularly salinity, disturbs perspectives of crop production. Ecological stresses are the most restricting aspects to seed germination [1, 2]. Salinity is an abiotic stress common in arid and semiarid regions that substantially declines the yield of crops by more than 50% [3]. Recently, high soil salinity became a very serious factor limiting the agricultural production in a wide proportion over the world [4]. Failure of germination on saline soils is often due to a high salt level in the planting zone as well as upward movement of the soil solution and subsequent evaporation at the soil surface [5]. Moreover, ethylene stress occurs, in which ethylene is biosynthesized by the plant as a response to these stresses [3]. This ethylene stress induces the symptoms of senescence in the plant, such as abscission and chlorosis, which affect plant growth and survival [6]. Some rhizobia strains can produce the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCD), which hydrolyzes the ethylene precursor (ACC) in plants [7]. Consequently, ACCD-producing bacteria may improve plant growth by declining the malicious impacts of ethylene stress. Glick [8] demonstrated that ACCD can be produced by numerous rhizobacterial strains. Moreover, plant growth-promoting rhizobacteria (PGPR) can enhance plant growth by different direct and indirect mechanisms [9]. One of these mechanisms is elevating the plant tolerance to dry condition, salinity, and metal toxicity. Therefore, the rhizobacterial population is the main source for effective microorganisms that can improve plant survival under different abiotic stresses, such as drought and salinity, through several mechanisms [10]. Increasing plant hormone synthesis, such as of indole acetic acid (IAA) and gibberellins, is the main trigger of the

activity of specific enzymes that promote seed germination and growth [11]. Thus, PGPR were found to endure salinity stress, which are frequently harmful to the growth of essential crop plants [12]. The aim of the present work was to demonstrate the adverse effect of salinity on plant growth and to report the potentiality of rhizobacterial strains to mitigate this effect on the early seed germination of *Lactuca sativa* and *Raphanus sativus*.

Materials and Methods

Microorganisms and Inocula

Microorganisms that strongly produce IAA were previously isolated from the rhizosphere of *Panax schinseng* (ginseng plant) South Korea [13]. The bacterial strain cultures were activated in Tryptic soy agar (TSA) medium, pH 7.2. Optical density was used to control the inoculum size in liquid media. Specifically, 1 ml of the newly growing spores (1×10^6 CFU/ml) were transferred to 250 ml of fresh TSB liquid medium supplemented with (5%) NaCl and incubated at 30°C for 48 h.

Effect of NaCl Stress on Seed Germination

Plant seeds were surface disinfected by immersing in 70% ethanol for 1 min, and then washed three times in sterile distilled water. Ten seeds were set for every Petri dish lined with filter paper (Whatman No. 1) wetted by 5 ml of 0, 50, 100, and 150 mM of sterile NaCl solution. The experiments were run intriplicate. All Petri dishes of various treatments were incubated at 28°C under darkness in a plant growth chamber (DASOL; Scientific Co., Ltd., Korea). Germination development was checked for 4 days. The number of germinated seeds was recorded after every 24 h.

Seed Inoculation

The bacterial spore suspension was adjusted into 1×10^7 CFU/ml for all actively growing culture using a UV-VIS Spectrophotometer (Hitachi U-2900) and OD at 600 nm. The inocula levels were calibrated to reach a turbidity of 0.4 McFarland Standard (matches approximately 5×10^7 CFU/ml) [14]. Seeds were agitated at 150 rpm with their spore suspension for 2 h at room temperature. The seeds were separated from the spore suspension using sterile tea mesh sieves and set as 10 seeds/dish [15]. The seeds were placed on sterile filter paper (Whatman No. 1) that had been moistened by 5 ml of sterile distilled water. The trial was conducted in triplicate.

Table 1. Seed germination percentages of *Brassica rapa*, *Lactuca sativa*, and *Raphanus sativus* under different salinity levels after 96 h.

NaCl treatment (mM)	Germination rate (%)		
	Chinese cabbage	Lettuce	Radish
Control	100 ± 0.707a	100 ± 0a	100 ± 1.41a
50	100 ± 0.353a	100 ± 0a	80 ± 1.41a
100	95 ± 0.353a	75 ± 0.71ab	80 ± 1.41a
150	90 ± 0.707a	0.00 ± 0c	80 ± 1.41a
200	90 ± 0.707a	0.00 ± 0c	50 ± 1.41b

Different letters indicate significant difference ($p < 0.05$) between control and NaCl treatments.

Seeds Inoculation under Salinity Conditions

The seed germination test under different salinity levels of NaCl (50, 100, and 150 mM) was conducted under single- and multi-inoculated conditions. The seed surface was disinfected and washed as stated above. Seeds were inoculated by dipping for 2 h in 30 ml of their bacterial spore suspension (1×10^7 CFU/ml) under shaking condition of 150 rpm. In the multi-inoculated treatment, 10 ml of every culture were added to make the spore suspension. Sterile distilled water was used instead of spore suspension to serve as a control. Using the sterile mesh sieve, 10 seeds were separated and each placed in a Petri dish lined by sterile filter paper (Whatman No. 1) [15]. Salinity and/or moisture conditions were provided by adding 5 ml of the sterile 0, 50, 100, and 150 mM NaCl solution. The germination percentage (%) was calculated according to kader's [16] equation (Germination % = (Number of germinated seeds/Total number of seeds) × 100).

Statistical Analysis

Data were subjected to the analysis of variance. Significance at the 5% level was verified by least significant difference by means of the Statistical Analysis Software (SAS) ver. 9.1 program [17].

Results

Stress tolerance due to microbial inoculants has become of great interest to plant and soil scientists. In the present study, the germination of *Lactuca sativa* and *Raphanus sativus*

Table 2. Effect of PGPR on seed germination of lettuce *Lactuca sativa* under different salinity levels.

NaCl (mM)	Germination rate (%)				
	Control	*A. chroococcum*	*Lactobacillus* sp.	*P. putida*	LAP mix
50	100 ± 0a	100 ± 0a	100 ± 0a	100 ± 0a	100 ± 0a
100	75 ± 0.71ab	100 ± 0a	100 ± 0a	75 ± 0.71ab	100 ± 0a
150	0.00 ± 0c	95 ± 0.71a	15 ± 0.71c	0.00 ± 0c	50 ± 2.83b
200	0.00 ± 0c	90 ± 0a	0.00 ± 0c	0.00 ± 0c	0.00 ± 0c

Table 3. Effect of PGPR on the root length of lettuce *Lactuca sativa* under different salinity levels.

NaCl (mM)	Radicle length (cm/plant)				
	Control	*A. chroococcum*	*Lactobacillus* sp.	*P. putida*	LAP mix
50	0.43 ± 0.11ef	2.31 ± 0.24b	1.03 ± 0.05c	0.70 ± 0.12de	2.56 ± 0.16a
100	0.37 ± 0.21f	1.00 ± 0cd	0.50 ± 0ef	0.50 ± 0ef	0.88 ± 0.15cd
150	0.00 ± 0g	0.00 ± 0g	0.00 ± 0g	0.00 ± 0g	0.00 ± 0g
200	0.00 ± 0g	0.00 ± 0g	0.00 ± 0g	0.00 ± 0g	0.00 ± 0g

seeds under different levels of salinity were checked. Consequently, the effects of some selected PGPR strains to mitigate salinity stress at the early germination process of *L. sativa* and *R. sativus* plants were studied in laboratory environment. Treatment of 50 mM of NaCl did not affect the *Brassica rapa* and *L. sativa* seed germination; however, the *R. sativus* seed germination rate was reduced to 80%. The salinity of 100 mM of NaCl also caused 80% germination of *R. sativus* seeds and decreased the *B. rapa* seed germination to 95%, and 75% for *L. sativa*. The salinity of 200 mM of NaCl reduced the germination of *R. sativus* by 50% and only 10% for *B. rapa* seeds (Table 1).

PGPR Impact on Seed Germination at Different Salinity Stress Levels

Application of *A. chroococcum* significantly increased the germination rate of *L. sativa* seeds by 25%, 95%, and 90% at salinity stress of 100, 150, and 200 mM of NaCl, respectively, as shown in Table 2. However, the other inoculants showed no significant seed germination at 200 mM of NaCl. LAP mix increased the seed germination by 25% and 50% at salinity levels of 100 and 150 mM NaCl, respectively. *P. putida* inoculation showed no increase higher than control; however, *Lactobacillus* sp. enhanced the seed germination by 25% and 15% at NaCl stress of 100 and 150 mM, respectively (Table 2). LAPmix inoculation increased the radicle length of *L. sativa* seedling significantly by 2.0 and 0.5 cm at salinity stress of 50 and 100 mM NaCl, respectively. Similarly, inoculation of *A. chroococcum* increased the radicle length significantly at salinity stress of 50 and 100 mM NaCl by 1.9 and 0.5 cm, respectively. However, *Lactobacillus* sp. and *P. putida* did not show a significant increase in the radicle length at the same salinity levels as compared with the control. All inoculants could not enhance the radicle length at salinity stress of 150 and 200 mM NaCl (Table 3). Inoculation of *A. chroococcum* and LAPmix increased the plumule length of *L. sativa* germinated seed by 2.0 and 2.3 cm at 50 mM NaCl, respectively (Fig. 1). *Lactobacillus* sp. inoculation increased the length of plumule by 2.0 cm at 50 mM NaCl, and by 0.3 cm at 100 mM NaCl, higher than the control. None of the inoculants increased the *L. sativa* plumule growth at salinity stresses of 150 and 200 mM NaCl. *P. putida* inoculation showed the lowest activity to enhance the *L. sativa* growth (Table 4). *R. sativus* seed germination

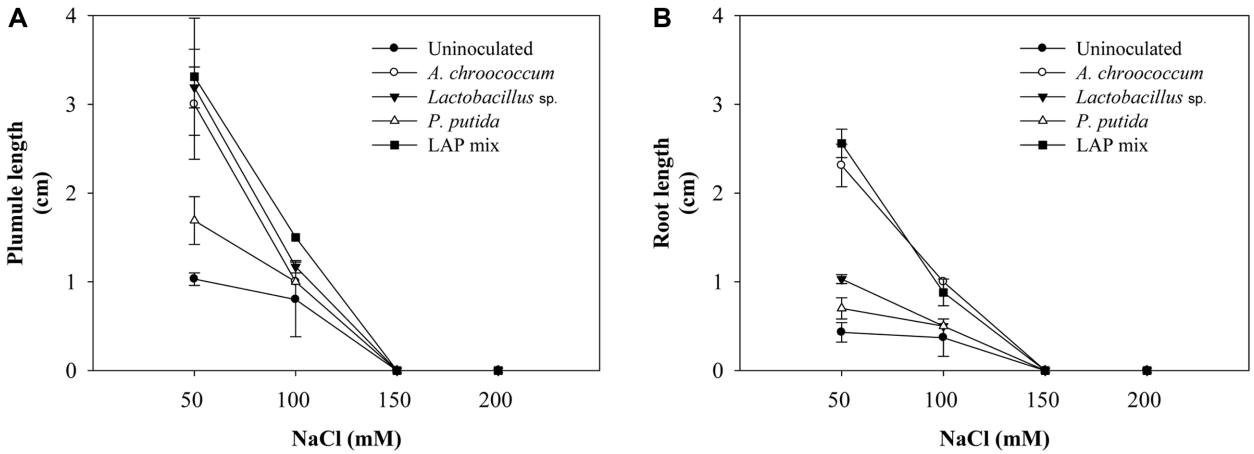

Fig. 1. Bacterial inoculation enhances the salinity tolerance of the stressed *Lactuca sativa* plant.
(**A**) Plumule length and (**B**) root length of lettuce after 96 h of different salinity stress and bacterial applications.

Table 4. Effect of PGPR on the plumule length of lettuce *Lactuca sativa* under different salinity levels.

NaCl (mM)	Plumule length (cm/plant)				
	Control	*A. chroococcum*	*Lactobacillus* sp.	*P. putida*	LAP mix
50	1.03 ± 0.07d	3.00 ± 0.62a	3.19 ± 0.23a	1.69 ± 0.27b	3.31 ± 0.66a
100	0.80 ± 0.42d	1.00 ± 0d	1.17 ± 0.07cd	1.00 ± 0d	1.50 ± 0c
150	0.00 ± 0e	0.00 ± 0e	0.00 ± 0e	0.00 ± 0e	0.00 ± 0e
200	0.00 ± 0e	0.00 ± 0e	0.00 ± 0e	0.00 ± 0e	0.00 ± 0e

was enhanced under salinity stress by *Lactobacillus* sp. only at 100 mM NaCl, and by LAP mix inoculation at 200 mM NaCl. Interestingly, all bacterial inoculants could not improve the *R. sativus* seed germination rate significantly (Table 5). The *R. sativus* radicle was greatly enhanced by the help of *A. chroococcum* inoculation, and it further increased the radicle length higher than the uninoculated seeds, by 4.0, 1.0, and 1.5 cm at NaCl concentrations of 50, 100, and 150 mM, respectively (Fig. 2). LAP mix inoculation significantly increased the radicle length in germinated *R. sativus* seeds by 7.5, 1.3, 1.2, and 0.6 cm under salinity stress of 50, 100, 150, and 200 mM NaCl, respectively. *Lactobacillus* sp. and *P. putida* inoculation showed higher radicle lengths compared with non-inoculated *R. sativus* seeds but lower than *A. chroococcum* (Table 6). Inoculation by *A. chroococcum* significantly increased the plumule length of the germinated *R. sativus* seeds compared with the non-inoculated control. *A. chroococcum* enhanced the plumule length by 3.0, 0.8, and 0.6 cm under salinity stress of 50, 100, and 150 mM NaCl, respectively. However, at 200 mM NaCl, the bacterial inoculation did not show any change or length as compared with the control. This result was similar to that shown by LAP mix inoculants, which increased the plumule length by 2.9, 0.8, and 0.6 cm at NaCl treatments of 50, 100, and 150 mM, respectively (Fig. 3). *Lactobacillus* sp. increased the plumule length by 2.0 cm at 50 mM NaCl, and *P. putida* increased the plumule length by 1.0 cm at the same salinity level. However, all inoculants did not enhance the plumule growth at 200 mM NaCl (Table 7).

Discussion

Chinese cabbage (*Brassica rapa*), lettuce (*Lactuca sativa*), and radish (*Raphanus sativus*) are known as main economic plant crops cultivated and used in Asia. Most of the plant species are sensitive to high concentrations of salts in the soil, and that affects the cultivated area steadily. Therefore, salinity is one major severe stress factor affecting crop productivity. In recent years, it has been revealed that bacteria belonging to different genera, such as *Rhizobium*, *Bacillus*, *Pseudomonas*, *Burkholderia*, *Achromobacter*, *Methylobacterium*, and *Variovorax*, can provide tolerance to host plants against different abiotic stresses [18]. These bacteria can attenuate the environmental stresses in agriculture and can be helpful for mitigating soil salinity. Sadeghi *et al.* [19] reported increases in the growth of a wheat plant when they inoculated the wheat seedlings with an auxin- and siderophore-producing strain of *Streptomyces* under saline soil conditions. In this study, *L. sativa* and *R. sativus* were used to check the mitigation potential of PGPB upon salinity stress. The influence of the selected PGPR strains on plant growth was investigated under normal (Fig. 4) and stress conditions. The ability of rhizobacteria to enhance plant tolerance and promote growth has been recorded. Salinity level affects essential biochemical processes in plants, such as protein synthesis, photosynthesis, and lipid metabolism [20]. However, most plants have adaptation mechanisms to overcome the deteriorations of salinity, such as regulation and immobilization of metals, stimulation of plant hormones, and changes in photosynthetic pathways. The isolation of

Table 5. Effect of PGPR on seed germination of radish *Raphanus sativus* under different salinity levels after 96 h.

NaCl (mM)	Germination rate (%)				
	Control	*A. chroococcum*	*Lactobacillus* sp.	*P. putida*	LAP mix
50	80 ± 1.41ab	80 ± 1.41ab	80 ± 1.41ab	80 ± 1.41ab	80 ± 1.41ab
100	80 ± 1.41ab	80 ± 0.71ab	95 ± 0.71a	80 ± 0.35ab	80 ± 0.57ab
150	80 ± 1.41ab	75 ± 0.71ab	55 ± 0.71b	80 ± 0.71ab	75 ± 0.71ab
200	50 ± 1.41b	50 ± 1.41b	50 ± 0.71b	50 ± 1.41b	60 ± 1.41ab

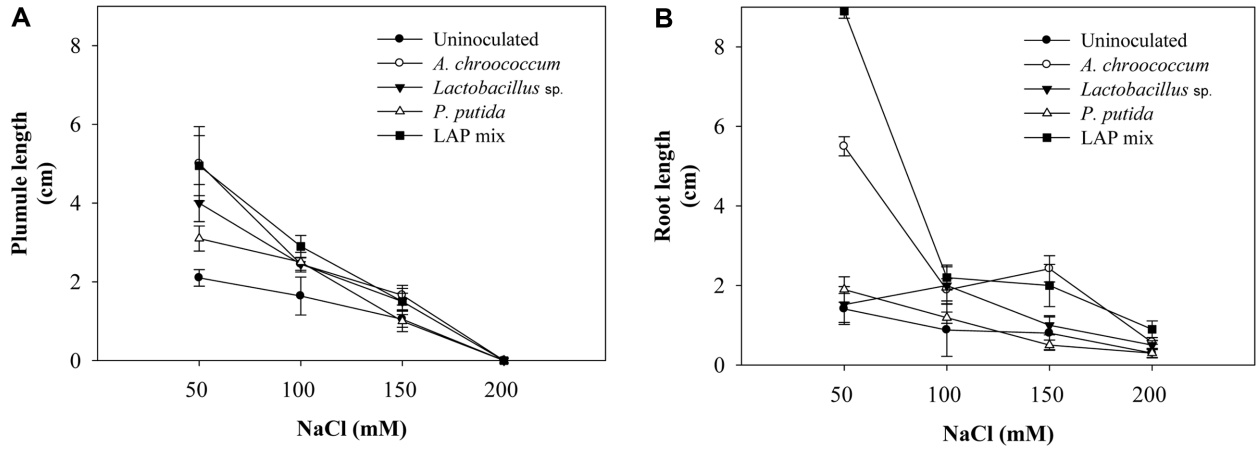

Fig. 2. Inoculation alleviates salinity stress in the early seed germination of radish *Raphanus sativus*.
(**A**) Plumule length and (**B**) root length after 96 h of different salinity stress and bacterial applications.

indigenous strains from stress-influenced soils and screening on the premise of their stress resistance and PGP qualities might be helpful in the quick determination of effective strains that could be used as bioinoculants for stressed plant crops. According to the results of this study, inoculation of *A. chroococcum* significantly increased the germination rate of *L. sativa* seeds by 25%, 95%, and 90% higher than uninoculated seeds at salinity stress of 100, 150, and 200 mM NaCl, respectively. Moreover, *Lactobacillus* sp. enhanced the seed germination by 25% and 15% at NaCl stress of 100 and 150 mM, respectively (Table 5). Rhizobacteria able to conduct positive interactions with plant roots, well-known as plant growth-promoting rhizobacteria, are prospective for their beneficial use in sustainable agriculture. In this study, LAPmix inoculation increased the radicle length of *L. sativa* seedlings significantly by 2.0 and 0.5 cm at salinity stress of 50 and 100 mM NaCl, respectively. However, all inoculants could not enhance the radicle length at salinity stresses over 100 mM NaCl (Table 6). Plant-associated microorganisms can play a significant role in acquiring resistance to abiotic stresses. The rhizosphere, endophytic, and symbiotic bacteria and fungi may act via different mechanisms, such as phytohormone supply, inducing an osmotic response, biosynthesis of siderophores, nutrients uptake, and protection. Inoculation of *A. chroococcum* and LAP mix increased the plumule length of *L. sativa* germinated seed by 2.0 and 2.3 cm at 50 mM NaCl, respectively. *Lactobacillus* sp. inoculation increased the length of plumule by 2.0 cm at 50 mM of NaCl, and by 0.3 at 100 mM NaCl higher than the control (Table 7). The *R. sativus* radicle was greatly enhanced by *A. chroococcum* inoculation, which further increased the radicle length higher than the uninoculated seeds by 4.0, 1.0, and 1.5 cm at 50, 100, and 150 mM NaCl, respectively. LAP mix inoculation significantly improved the radicle length in germinated *R. sativus* seeds by 7.5, 1.3, 1.2, and 0.6 cm under salinity stress of 50, 100, 150, and 200 mM NaCl, respectively (Table 6). The rhizo-microorganisms secrets certain compounds on the root tip mucilage, providing a defensive zone and elongating root cells [21]. Dimkpa *et al.* [22] reported that inoculation of the plant by PGPR enhanced lateral root development owing to higher nitric acid and IAA production, and decreased ethylene-induced stress in plants. In the present results, *A. chroococcum* enhanced the plumule length by 3.0, 0.8, and 0.6 cm under salinity stress of 50, 100, and 150 mM NaCl, respectively.

Table 6. Effect of PGPR on the root length of radish *Raphanus sativus* under different salinity levels.

NaCl (mM)	Radicle length (cm/plant)				
	Control	*A. chroococcum*	*Lactobacillus* sp.	*P. putida*	LAP mix
50	1.41 ± 0.39efghi	5.5 ± 0.24b	1.52 ± 0.45efgh	1.90 ± 0.32def	8.90 ± 2.18a
100	0.88 ± 0.66hij	1.89 ± 0.28defg	2.0 ± 0.47def	1.19 ± 0.14fghij	2.20 ± 0.31de
150	0.80 ± 0.41hij	2.42 ± 0.33d	1.0 ± 0.24ghij	0.50 ± 0.13j	2.00 ± 0.53def
200	0.30 ± 0.12j	0.57 ± 0.05ij	0.50 ± 0.11j	0.30 ± 0.11j	0.90 ± 0.21hij

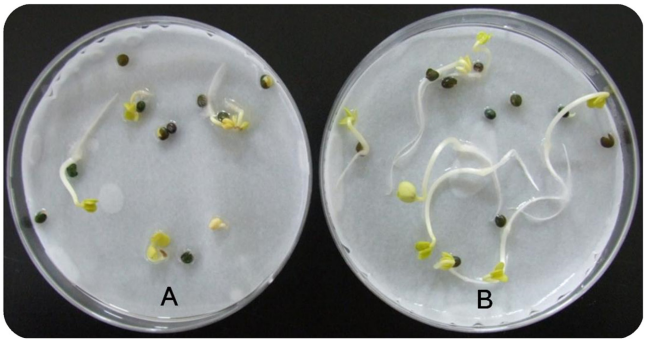

Fig. 3. PGPR effect to mitigate salinity stress of 100 mM NaCl either for percentage of seed germination or plumule and root lengths of radish (*Raphanus sativus*) after 96 h of treatment. (**A**) Uninoculated seeds. (**B**) Inoculated seeds with LAP mix.

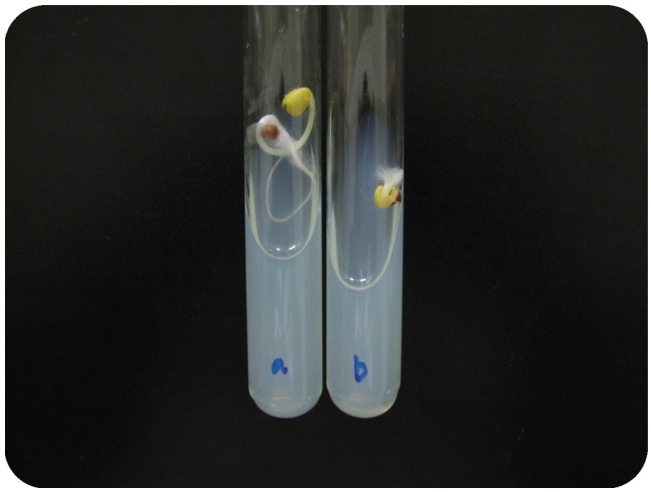

Fig. 4. Effect of PGPR on early seed germination of Chinese cabbage under normal conditions (after 96 h).
a: Seeds were inoculated with *Lactobacillus* sp.; b: Uninoculated seeds.

LAP mix increased the plumule length of *R. sativus* by 2.9, 0.8, and 0.6 cm at NaCl treatments of 50, 100, and 150 mM, respectively (Table 7). Ethylene plays an important physiological role in plants, either in normal plant growth or in the stressed plant. Ethylene synthesis is highly affected by slight changes in the environmental factors, including light, temperature, and other phytohormones, and its production increases as a response to several stresses, both abiotic and biotic, suggesting that it acts as a bridge between a changing environment and developmental adaptation. The abiotic stress conditions that trigger ethylene synthesis include submergence, heat, shade, exposure to heavy metals and high salt, low nutrient availability, and water deficiency [23]. The ethylene biosynthesis pathway consists of a simple three-step process: methionine is converted into *S*-adenosyl methionine, which is further converted by ACC-synthases to ACC, the direct precursor of ethylene. Recycling of methylthioadenosine enables rapid ethylene biosynthesis when necessary [24]. Some bacterial species possess the ability to synthesize ACC deaminase that breaks this ACC [25]. *Burkholderia* spp. can degrade excess amounts of ACC and produce nitrogen and energy as a by-product, consequently reducing the stress response and enhancing plant growth [26]. PGPR may improve plant growth, either by enhancing plant tolerance or producing plant growth hormones. In this study, the effects of the PGPR to mitigate the salinity stress on the early germination of plant seeds were tested. The significant differences found in both radicle and plumule lengths of *L. sativa* and *R. sativus* may be due to the bacterial inoculation. When plant growth suppression is the result of ethylene stress, PGPR with ACCD can be exploited [27]. ACCD metabolizes ACC, a precursor of ethylene in the biosynthesis pathway, thus limiting the amount of ethylene stress that can be produced [28, 29]. Polyamines secreted by PGPR have also been exposed to mitigate stress ethylene levels and alleviate osmotic stress [30, 31]. PGPR have been demonstrated to activate the synthesis of antioxidants and indole acetic acid, which can stimulate root growth [28, 32, 33]. Recently, PGPR were shown to alter mineral uptake, which results in a favorable increase in the cellular ratio of K+/Na+; and elevated generation of quorum-sensing molecules, which can lead to modifications

Table 7. Effect of PGPR on the plumule length of radish *Raphanus sativus* under different salinity levels.

NaCl (mM)	Plumule length (cm/plant)				
	Control	*A. chroococcum*	*Lactobacillus* sp.	*P. putida*	LAP mix
50	2.1 ± 0.21ef	5.00 ± 0.94a	4.00 ± 0.47b	3.1 ± 0.32c	4.95 ± 0.76a
100	1.64 ± 0.48fg	2.45 ± 0.16de	2.45 ± 0.16de	2.5 ± 0.25de	2.9 ± 0.28cd
150	1.05 ± 0.20h	1.66 ± 0.25fg	1.5 ± 0.21hg	1.0 ± 0.27h	1.5 ± 0.33hg
200	0.00l ± 0h	0.00l ± 0h	0.00l ± 0h	0.00l ± 0h	0.00l ± 0h

in the rhizosphere [34–36]. *Lactobacillus* sp. and *A. chroococcum* showed remarkable activation either in normal or under stress conditions. Co-inoculation with the three rhizobacterial strains (LAP mix) showed a synergistic impact that improved the early germination of the seeds. The consequences of this investigation are promising for use of rhizobacterial strains that have plant growth-elevating traits to upgrade the plant flexibility against salinity.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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