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Inoculation with *Bacillus licheniformis* MH48 to improve *Camellia japonica* seedling development in coastal lands

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Abstract: This study aimed to determine the promotion of the growth and nutrient uptake of *Camellia japonica* seedlings in coastal lands by bacterial inoculation. Soil salinity reduces plant growth and development in coastal areas due to the osmotic stress that perturbs nutrient uptake. The soil electrical conductivity at the sites of this study ranged from 1.02 to 1.89 dS m^{-1}. Application of chemical fertilizer resulted in the limited uptake of nutrients in seedlings under saline conditions as well as a low nutrient content in the soil caused by leaching, with no significant influence on the growth of the seedlings. However, *Bacillus licheniformis* MH48 increased the total nitrogen and total phosphorus in the soil due to atmospheric nitrogen fixation and the solubilization of phosphorus via organic acid exudation. In addition, *B. licheniformis* MH48 produces auxin, which stimulates root development and nutrient uptake. The bacterial inoculation could reduce the ethylene levels in seedlings by containing ACC deaminase, thus alleviating salt stress. Thus, bacterial inoculation significantly increased plant biomass to amounts of 15.67 g $plant^{-1}$ (the sum of the leaves and shoots) and 8.00 g $plant^{-1}$ in the roots of the seedlings. The nutrient uptake by seedlings also improved 2 to 3 times after the bacterial inoculation. The plant development effect appears to be direct, with the possible involvement of the bacterial inoculation as a plant development regulator. In view of environmental pollution due to excessive use of fertilizers and the high cost of producing fertilizers, the bacterial inoculation tested in our study has the potential to be used for environmentally benign plant production. However, the survival rate between the groups decreased due to salt stress. In particular, the survival rate of the seedlings that received bacterial inoculation was not significantly different from that of uninoculated seedlings. *C. japonica* seedlings are considered moderately sensitive to salinity.

Key words: Auxin, fertilizer, nutrition, plant growth-promoting rhizobacteria, reclaimed land, salt stress

1. Introduction

Camellia japonica is a broad-leaf evergreen tree that is naturally distributed in coastal areas of the southern parts of Korea (Park et al., 2017). This tree constitutes an important type of coastal vegetation in Korea because the tree is relatively tolerant to sea breeze and salinity. However, most *C. japonica* forests in the coastal areas of Korea have been degraded due to human activities such as road construction, urban development, and seedling collections for potted planting. The Korean government has made efforts to restore *C. japonica* forests in coastal areas, but restoration is very difficult (Park et al., 2017). In coastal environments, plant growth and survival are affected by the salinity of the soil and by the low availability of nutrients (Williams et al., 1998; Weber and D'Antonio, 1999). In particular, excess salt in the soil can decrease plant growth by inhibiting the osmotic uptake of water by roots or by causing nutritional imbalance through competitive ion absorption (Koyro, 2006).

Plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms including phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, phytohormone production, and interference with pathogen toxin production. The potentiality of PGPR in agriculture and forestry is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides, and other supplements (Ercisli et al., 2003; Koç, 2015; Tomic et al., 2015; Aslam et al., 2016).

PGPR can protect plants against salt stress by exogenously producing secondary metabolites (Suzuki et al., 2003; Yao et al., 2010). PGPR can lower ethylene levels in plants by synthesizing 1-aminocyclopropane-1-carboxylated (ACC) deaminase, thereby reducing environmental stress on plants (Glick et al., 1998; Saleem et al., 2007; Çakmakçı, 2016). *Bacillus* species are important

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PGPR microorganisms that can produce phytohormones such as auxin and cytokinin, which promote root development (Ercisli et al., 2003; Erturk et al., 2010; Park et al., 2017). In addition, *Bacillus* organisms contain ACC deaminase and alleviate the adverse effects of salt stress, resulting in an increase in plant tolerance to salt (Suzuki et al., 2003). Nutrients such as nitrogen and phosphorus are necessary for plant growth and yield (Orhan et al., 2006). *Bacillus* can increase nitrogen and phosphorus in the soil by fixing atmospheric nitrogen and solubilizing inorganic phosphate (Park et al., 2017). Numerous studies have focused on the effects on agricultural crops in saline conditions to demonstrate the benefits of PGPR on plants and yield (Paul and Lade, 2014). However, research on the effects of PGPR on the growth and nutrient uptake of trees in coastal areas is very limited (Park et al., 2017).

Saemangeum is the largest land reclamation project in Korea. A 33-km sea dike was completed in April 2006 to create an 11,800-ha freshwater lake and 28,300 ha of reclaimed land (Lie et al., 2008), and this reclaimed land was not provided with landfills for soil improvement. The Saemangeum reclaimed land has been slated for plant growth. To construct *C. japonica* forests in coastal reclaimed land, it is very important to understand the effects of PGPR on the growth and nutrient uptake of *C. japonica* seedlings under salt stress and low nutrient conditions. Therefore, the objective of this study was to determine the effects of inoculation with *B. licheniformis* MH48 on the growth and nutrient uptake of *C. japonica* seedlings in the Saemangeum coastal reclaimed land in Korea.

2. Materials and methods

2.1. Site description

A field experiment was conducted in the Saemangeum coastal reclaimed land in Gunsan, Korea (Figure 1) from July 2014 to April 2015. Two rivers (the Mangyung and Dongjin Rivers) flow into the estuary and then into the Yellow Sea (Figure 1). The construction of a 33-km sea dike in 2006 created an 11,800-ha freshwater lake that has a storage capacity of approximately 530×10^6 t. The wetlands were transformed into 28,300 ha of arable land, which is the largest reclaimed land area in Korea. Many researchers and nongovernmental organizations have concerns regarding the water pollution and eutrophication caused by nutrient leaching from large-scale agricultural lands. Therefore, the government has enacted a law prohibiting the use of chemical fertilizers in the Saemangeum reclaimed land.

The Saemangeum reclaimed land was not provided with landfills to improve the soil. The soil at the reclaimed

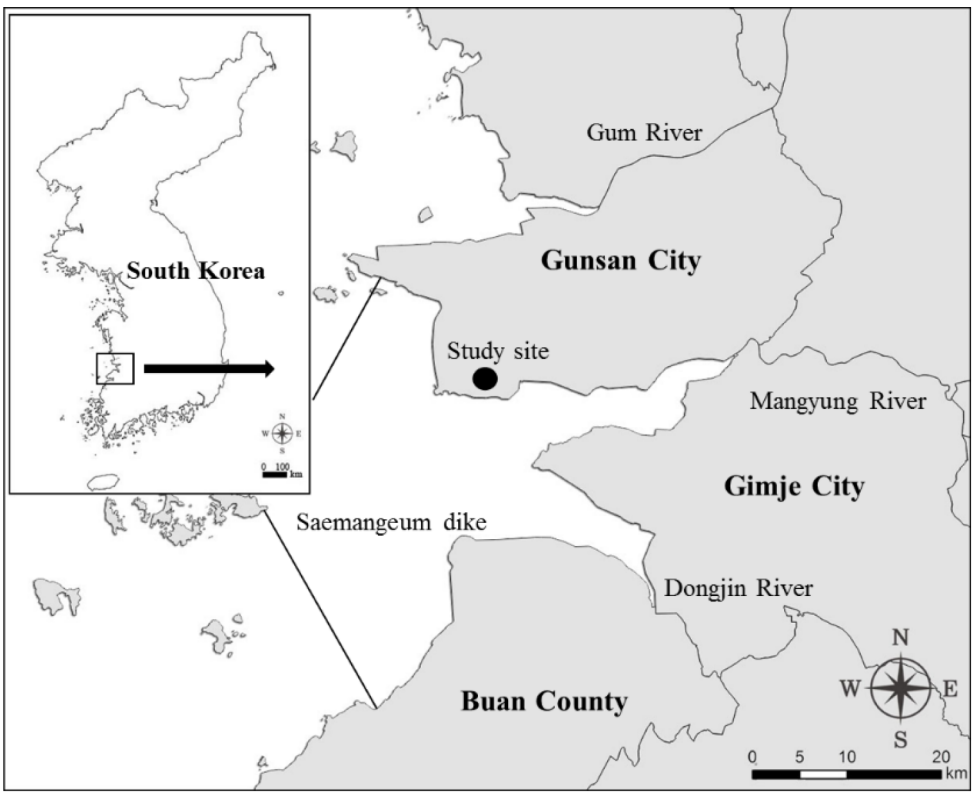

Figure 1. Location of the study sites in the Saemangeum coastal reclaimed land.

land site consists of a silt loam with a slope of 0%–2% (Park et al., 2017). The rainfall data recorded in the study area from July 2014 to June 2015 show a total annual rainfall of 1189 mm (Korea Meteorological Administration, 2015), and the soil in the study area contains moisture, except for the surface soil. The average annual temperature at the site during the study period was 12 °C.

2.2. Bacterial strain, culture conditions, and microbial counts

The *B. licheniformis* MH48 bacterial strain was obtained from the Laboratory of Soil Microbiology, Chonnam National University, Gwangju, Korea. This bacterial strain was isolated from rhizosphere soil collected from the Saemangeum coastal reclaimed land (Park et al., 2017). To prepare an inoculum, *B. licheniformis* MH48 was cultured in broth medium [1.36% urea ($(NH_2)_2CO$), 0.41% potassium phosphate monobasic (KH_2PO_4), 0.30% potassium chloride (KCl), 0.10% organic compost, and 2.06% sugar] and incubated at 30 °C for 5 days. To analyze the actual concentration of *B. licheniformis* MH48, colony-forming units (CFUs) were counted in diluted inocula (0.1%, 0.3%, 0.5%, 0.7%, and 1%) and incubated at 30 °C for 7 days on tryptone soya agar.

2.3. Analysis of auxin production

Auxin phytohormone production by *B. licheniformis* MH48 was tested in tryptophan-containing culture media (0.1% L-tryptophan, 1.36% urea, 0.41% potassium phosphate monobasic, 0.30% potassium chloride, 0.10% organic compost, and 2.06% sugar). The culture media were inoculated with *B. licheniformis* MH48 and incubated at 30 °C in a shaking (140 rpm) incubator for 5 days. The culture broth was then centrifuged at 8000 rpm for 20 min. The supernatant was collected and acidified to pH 2.8 using H_3PO_4. The organic fraction was extracted using the same volume of ethyl acetate and concentrated under a vacuum using a rotary evaporator (Buchi, Flawil, Switzerland). The dry matter was dissolved in methanol and analyzed using high-performance liquid chromatography (HPLC; Shimadzu, Japan) equipped with a C18 reversed-phase column. For the mobile phase of HPLC, acetonitrile and 50 mM KH_2PO_4 (pH 3, 30/70) were used at a flow rate of 1 mL min^{-1} (Patten and Glick, 2002). The production of auxin was determined by comparing the retention time and the standard peak size displayed at 7000 ppm for an auxin standard (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Experimental conditions

Experimental treatments at the coastal reclaimed land site were arranged in a randomized complete block design. The treatment area was divided by cutting large furrows with dimensions of 5 m (length) × 5 m (width) × 1 m (height). Each treatment was performed in three replicates. The following three treatments were used: 1) control (without fertilizer or bacteria); 2) chemical fertilizer; and 3) *B. licheniformis* MH48 inoculation. *C. japonica* seedlings (4 years old at a height of 50–60 cm) were planted at 70 cm × 70 cm spacing in July 2014, with a total of 54 seedlings planted per treatment (3 replicates per treatment, 18 seedlings per replicate). Regarding coastal tree planting, the Korea Forest Service recommends high amounts of seedlings of 30–50 cm in height that are 4–5 years old to withstand adverse environments, such as dryness and salinity in soils. The planted seedlings were similar in size in terms of height and roots and were produced by cuttings from the same tree (Park, 2016). One month after planting, bacteria (20 L of *B. licheniformis* MH48 culture, as mentioned above) and chemical fertilizer (0.15% urea, 0.04% potassium phosphate monobasic, and 0.03% potassium chloride) were applied through 20 L of water. Control seedlings were not treated with fertilizer or bacteria. The fertilizer application rate was determined based on the recommended basal chemical fertilizer application rate for *Camellia sinensis* (N : P : K = 60 : 20 : 30 kg $year^{-1}$) (Park, 2016). Each treatment was applied approximately once per month.

2.5. Soil chemical properties and plant nutrient analysis

Soil samples were taken from soils at a depth of 0 to 30 cm three times (July 2014, September 2014, and March 2015) to analyze the electrical conductivity (EC), total nitrogen, and total phosphorus content. The soil samples were oven-dried at 105 °C for 24 hours followed by sifting through a 2-mm sieve.

To determine the total nitrogen and total phosphorus uptake by *C. japonica* seedlings, the dry weights and nutrient contents of the seedling samples were measured. All surviving seedlings were sampled in April 2015. The seedlings were excavated using shovels because they did not grow many roots due to the salinity. In the seedling samples, aerial portions (leaves and shoots together) and roots were separated and rinsed with deionized water. The aerial portions and roots were separated at the boundary of the uppermost part of the root. Dry weights in separated aerial portions and roots were recorded after oven-drying at 105 °C for 16 h. These samples were then pulverized and filtered through a 30-mesh screen followed by total nitrogen and total phosphorus content analyses. Nutrient uptake by *C. japonica* seedlings was calculated using the following formula: nutrient uptake (mg $plant^{-1}$) = [dry weight (g $plant^{-1}$) × nutrient content (%)] × 10 (Park et al., 2017).

The soil EC of a 1 : 5 soil/water suspension was measured using an EC meter (CM31-EC meter, Monta, Japan) to determine the salinity of the soil. The total nitrogen content of the soils was determined using the Kjeldahl method (Mulvaney, 1996) following wet digestion with H_2SO_4. The total nitrogen content of the seedlings

was analyzed using an elemental analyzer (Variomax CN Analyzer, Elementar Analysensysteme GmbH, Germany) and a thermal conductivity detector after combustion at a high temperature (1200 °C) with nitrogen and helium gases. The total phosphorus content of the soil and seedlings was determined using an inductively coupled plasma optical emission spectrometer (ICP-OES Optima 8300, PerkinElmer, USA) after digestion in concentrated nitric acid in a microwave (MARS Xpress, CEM Corporation, USA).

2.6. Analysis of the survival rate of C. Japonica seedlings

The survival rate of *C. japonica* seedlings was measured after inoculation with *B. licheniformis* MH48. The survival rate was measured from July 2014 to April 2015, and the seedlings were considered dead when they had no leaves. The survival rate of *C. japonica* seedlings was calculated using the following formula: survival rate (%) = (surviving seedlings / total seedlings) × 100.

2.7. Statistical analysis

The data were statistically analyzed using the Kruskal–Wallis test followed by the Mann–Whitney U test for pairwise comparisons, except for EC, total nitrogen, and total phosphorus in the soils. An adjusted P-value of <0.017 after Bonferroni correction was taken to indicate significance. The data of EC, total nitrogen, and total phosphorus in the soils showed a normal distribution; therefore, they were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.01$). Statistical analyses were performed using IBM SPSS Statistics version 21 (Armonk, NY, USA).

3. Results and discussion

The efficiency of PGPR in promoting plant growth has been demonstrated in numerous greenhouse and field studies using various types of agricultural crops and herbs (Esitken et al., 2003; Orhan et al., 2006; Aslantaş et al., 2007; Yao et al., 2010; Chakraborty et al., 2012; Çakmakçı, 2016). However, the use of PGPR to promote tree development and tolerance to salt stress in coastal areas has been poorly studied. We discuss the beneficial effects of inoculation with *B. licheniformis* MH48 on the growth and nutrient uptake of *C. japonica* seedlings in coastal reclaimed land.

3.1. Colony number and auxin production in *B. licheniformis* MH48

The bacteria were able to form colonies in different diluted inocula (Figure 2). The colony numbers increased with increasing concentration of bacterial inoculation. In particular, the CFU count for bacteria showed 8.7×10^7 CFU mL^{-1} of 1.0% concentration of inoculation sample used for this experiment (Figure 2).

Auxin production in the *B. licheniformis* MH48 cultures was confirmed via HPLC analysis (Figure 3). The ethyl acetate-extracted fraction of the culture supernatant of *B. licheniformis* MH48 in the media amended with 0.1% L-tryptophan produced a sharp HPLC peak at the same retention time (6.74 min) as that observed for the auxin standard.

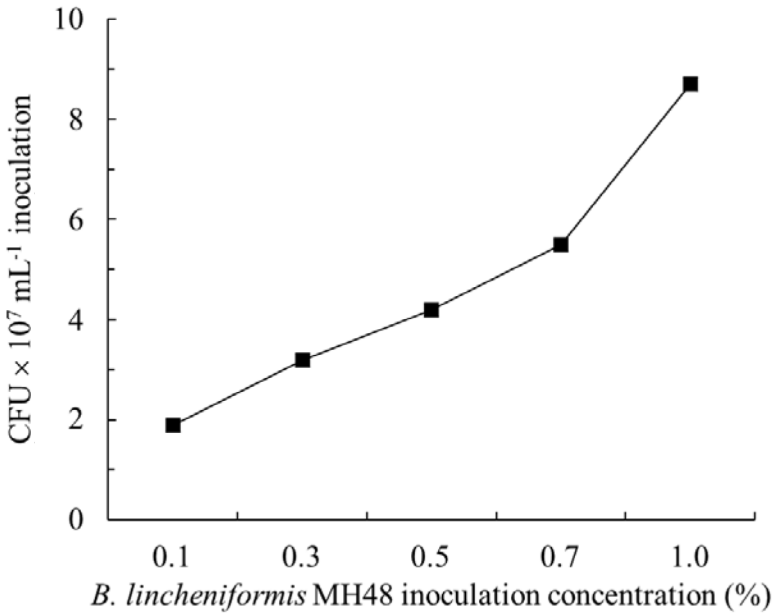

Figure 2. Colony numbers in different diluted inocula (0.1%, 0.3%, 0.5%, 0.7%, and 1%) of *B. licheniformis* MH48.

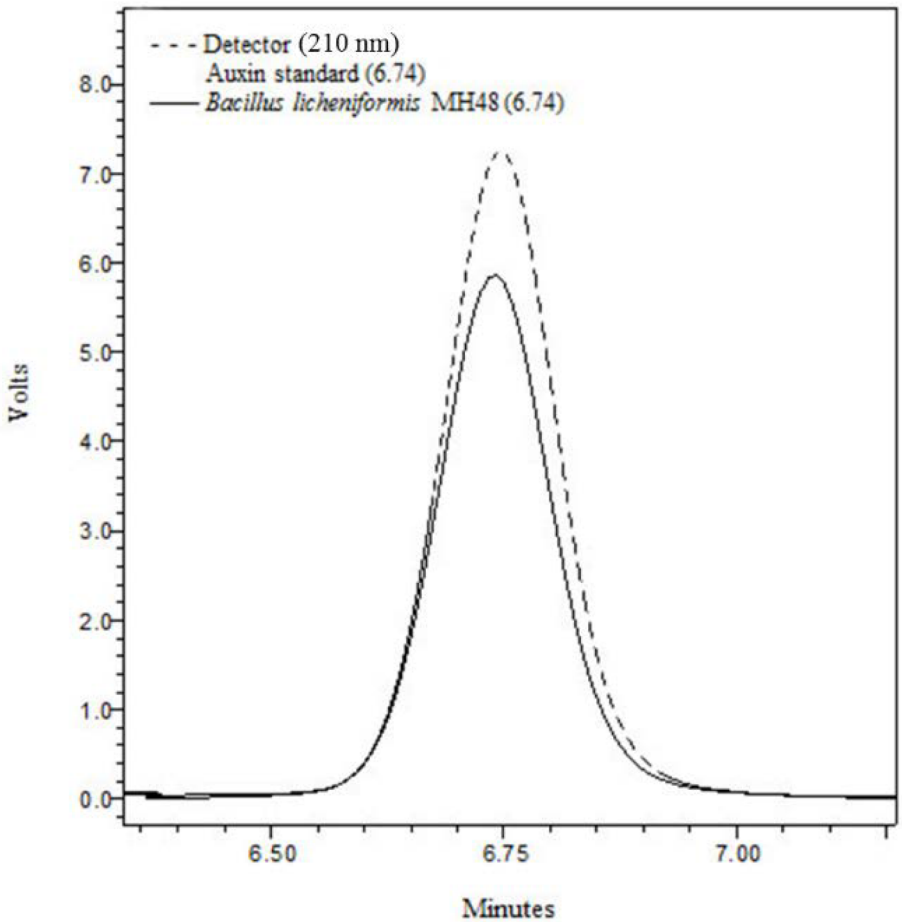

Figure 3. HPLC chromatograms of the auxin standard (dotted line) and the ethyl acetate-extracted organic fraction of the *B. licheniformis* MH48 culture supernatant (solid line).

3.2. Nutrients in soils and growth and nutrient uptake of seedlings

Total nitrogen and total phosphorus contents of the soils with chemical fertilizer treatment were significantly lower than in those that received bacterial inoculation (Table 1). Nitrogen and phosphorus from chemical fertilizers are easily leached (Adesemoye and Kloepper, 2009). In the soils inoculated with *B. licheniformis* MH48, the total nitrogen and total phosphorus contents were significantly higher than those in the chemical fertilizer and control treatments (Table 1). *B. licheniformis* MH48 can increase soil nutrients through mechanisms such as atmospheric nitrogen fixation and the solubilization of phosphorus by organic acids (Park et al., 2017).

Chakraborty et al. (2012) and Çakmakçı (2016) reported that PGPR stimulate *C. sinensis* growth, including shoot development, leaf yield, and nutrient uptake. The aerial (leaves and shoots) and root yields of *C. japonica* seedlings treated with bacterial inoculate increased 2.0–2.6 times and 2.0–2.2 times, respectively, compared with those of control seedlings (without chemical fertilizer or bacterial inoculation) and those of seedlings treated with chemical fertilizer (Table 2). Nitrogen and phosphorus are essential nutrients for plant growth and yield (Orhan et al., 2006). However, chemical fertilizer application had no significant influence on the yields of aerial portions or roots compared to control treatment (Table 2). The nutrient leaching of chemical fertilizers decreased the nutrient content of the soils (Table 1).

B. licheniformis MH48 produces auxin (Figure 3), which promotes root development (Table 2), resulting in increased nutrient uptake. The highest total nitrogen uptake observed in the seedlings was 428.06 mg plant^{-1}, which occurred after treatment with the *B. licheniformis* MH48 inoculate (Table 2). The lowest total nitrogen uptake of seedlings was 134.41 mg plant^{-1}, which was exhibited in the control group. The total amounts of phosphorus uptake of the seedlings following inoculation with *B. licheniformis* MH48, following treatment with chemical fertilizer, and in the control treatment were 50.00, 26.13, and 17.84 mg plant^{-1}, respectively (Table 2). Thus, bacterial inoculation significantly influenced nutrient uptake by *C. japonica* seedlings.

Table 1. Electrical conductivity, total nitrogen, and total phosphorus in soils among the control, chemical fertilizer, and *B. licheniformis* MH48 inoculation treatments.

Treatments	EC (ds m^{-1})	Total nitrogen (g kg^{-1})	Total phosphorous (g kg^{-1})
Control	1.02 ± 0.19 a*	0.91 ± 0.19 a	0.44 ± 0.07 a
Chemical fertilizer	1.05 ± 0.21 a	0.90 ± 0.14 a	0.46 ± 0.13 a
B. licheniformis MH48	1.89 ± 0.43 b	1.61 ± 0.25 b	2.25 ± 0.59 b

*Different letters indicate significant differences among treatment groups according to Duncan's multiple range test ($P < 0.01$).

Table 2. Average dry weight, nutrient content, and nutrient uptake of *C. japonica* seedlings in coastal reclaimed land following control, chemical fertilizer, and *B. licheniformis* MH48 inoculate treatments.

Treatments	Dry weight (g $plant^{-1}$)		Nutrient content (%)		Nutrient uptake (mg $plant^{-1}$)	
	Ariel part	Root	Total nitrogen	Total phosphorus	Total nitrogen	Total phosphorus
Control (n = 18)	6.09 ± 1.81 a*	3.62 ± 0.58 a	1.33 ± 0.28 a	0.15 ± 0.03	134.41 ± 47.19 a	17.84 ± 7.63 a
Chemical fertilizer (n = 15)	7.82 ± 2.20 a	3.91 ± 0.79 a	1.73 ± 0.12 b	0.26 ± 0.04	224.74 ± 68.32 b	26.13 ± 10.18 b
B. licheniformis MH48 (n = 24)	15.67 ± 4.04 b	8.00 ± 2.07 b	1.70 ± 0.14 b	0.20 ± 0.04	428.06 ± 123.44 c	50.00 ± 16.08 c

*Different letters indicate significant differences among treatment groups at $P < 0.017$ (Kruskal–Wallis test followed by the Mann–Whitney U test after Bonferroni correction).

The growth and nutrient uptake of *C. japonica* seedlings was higher after treatment with *B. licheniformis* MH48 inoculate compared with that of the other two treatments tested (chemical fertilizer and control) (Table 2). *B. licheniformis* MH48 can produce auxin (Figure 3), and auxin can stimulate the formation of lateral roots and absorbent root hairs (Liu et al., 2013), resulting in better uptake of nutrients from the soil and better plant growth (Suzuki et al., 2003; Orhan et al., 2006; Aslantaş et al., 2007; Liu et al., 2013). In addition, the bacterial inoculate contains ACC deaminase, which is the immediate precursor of the phytohormone ethylene, thereby inhibiting ethylene levels and reducing salt stress in *C. japonica* seedlings (Glick et al., 1998; Patten and Glick, 2002; Çakmakçı, 2016). Our results indicated that the inoculation of *B. licheniformis* MH48 is able to promote tree growth and nutrient uptake and reduce the need for chemical fertilizers for tree seedlings in coastal reclaimed lands.

3.3. Survival rate of *C. japonica* seedlings

Salinization in coastal reclaimed lands involves the process of salt accumulation near the soil surface. It is often caused by the capillary rise of salt from water tables due to an increased potential for evapotranspiration (Salama et al., 1999; Rengasamy, 2006). Salt stress can restrict plant survival because the uptake of water and nutrients from high-salinity soil is limited (Koyro, 2006; Park et al., 2017). The survival rate in *C. japonica* seedlings decreases when the EC is greater than 1.3 dS m^{-1} (Park, 2016). In this study, the EC values among the three treatments ranged from 1.02 to 1.89 dS m^{-1} (Table 1), which were 3- to 6-fold higher than those of the forest soil (Park, 2016). The survival rates of *C. japonica* seedlings in the *B. licheniformis* MH48-inoculated, control, and chemical fertilizer-treated groups were 38.88%–50.00%, 27.77%–38.89%, and 18.16%–37.40%, respectively (Table 3). The values of survival rates among the groups were highly variable, and bacterial inoculation treatment had no significant effect compared to the control and chemical fertilizer treatments (Table 3). Inoculation with *B. licheniformis* MH48 induced the growth and development of *C. japonica* seedlings under salinity (Table 2), but inoculation limited the improvement of the survival rate to resist salt stress (Table 3). This result

Table 3. Average survival rate of *C. japonica* seedlings in the control, chemical fertilizer, and *B. licheniformis* MH48 inoculate treatment groups in coastal reclaimed land.

Treatments	Survival rate (%)
Control	33.33 ± 5.56
Chemical fertilizer	27.78 ± 9.62
B. licheniformis MH48	44.44 ± 5.56

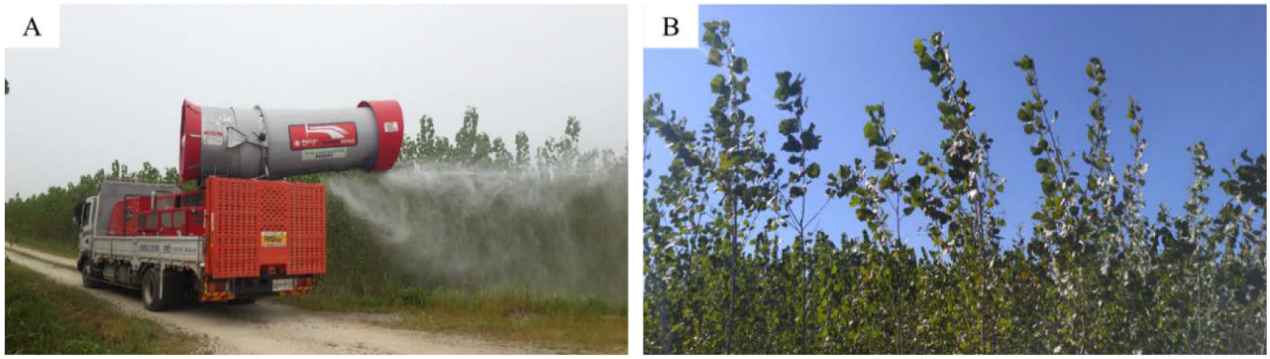

Figure 4. (A) Foliar application of *B. licheniformis* MH48 inoculation in *P. euramericana* forests of Saemangeum coastal areas in June 2016 and (B) the growth improvement of poplar forests in October 2016.

may be due to a capillary rise in salt from underground water sources because the dry surface of the soil continued during the fall season in 2014 and spring season in 2015 (Korea Meteorological Administration, 2015). In addition, *C. japonica* seedlings are considered moderately sensitive to salinity.

3.4. Conclusions

PGPR are important microorganisms that can increase the growth and yield of plants in coastal areas. However, there is little information on the beneficial effects of PGPR inoculation on the growth of coastal forest seedlings. The application of PGPR inoculation is an effective method to improve the growth and nutrient uptake of *C. japonica* seedlings in coastal reclaimed land due to the combined actions of nutrient-enhancement systems and root development. In addition, *B. licheniformis* MH48 containing ACC deaminase inhibited the levels of ethylene and reduced salt stress. PGPR are crucial to the effectiveness of this technique to manage coastal forests. However, the survival rate of seedlings that received bacterial inoculation was not significantly different from that of uninoculated seedlings.

From 2012 to 2016, the Korea Forest Service established 180 ha of *Populus euramericana* forests in Saemangeum reclaimed land to produce wood pellets. However, the growth of poplar trees was recently reduced due to salt stress and limited nutrients in the soils. To examine the effects of inoculation with *B. licheniformis* MH48 on the growth of poplar forests (2 ha) under salt stress conditions, we performed 5 foliar applications of bacterial inoculation at 2-week intervals from June to August in 2016 (Figure 4A). Foliar fertilization was carried out after diluting the bacterial inoculation (1000 L) with water (1000 L) in 2-ha poplar forests. In October 2016, the growth of the poplar trees that received the bacterial inoculation treatment increased (Figure 4B). A calculation of the cost of producing the bacterial inoculation including the mineral nutrients shows that *B. licheniformis* MH48 inoculation cost approximately \$80 ha^{-1}, which is inexpensive. This strategy is a new method to improve the management of applied fertilizers in coastal forests.

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