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[1]School of Applied Biosciences, Kyungpook National University, Daegu, Korea
[2]Department of Botany, Abdul Wali Khan University Mardan, Pakistan
[3]Department of Agriculture, Abdul Wali Khan University Mardan, Pakistan

Bacillus subtilis JW1 enhances plant growth and nutrient uptake of Chinese cabbage through gibberellins secretion

Sang-Mo Kang[1], Muhammad Hamayun[2], Muhammad Aaqil Khan[1], Amjad Iqbal[3], In-Jung Lee[1*]

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Summary

In the present study, we have isolated rhizospheric bacteria JW1 from rice paddy in Andong, South Korea. The culture filtrate (CF) analysis of JW1 showed higher contents of gibberellins GA_1, GA_4, GA_7, organic acids, fatty acids and tricalcium phosphates. The 16S rDNA gene sequencing and phylogentic analysis revealed that the strain JW1 has a 99% homology with *Bacillus subtilis* sequences from BLAST search.The growth promotion capability of the strain JW1 was initially assessed on Waito-C and Whayoung-beyo rice cultivars, which improved the growth attributes of the rice cultivars. Similarly, a significant increase in plant height, biomass, chlorophyll contents and nutrient uptake have been noticed, when the Chinese cabbage was treated with JW1 strain. From the results, it is concluded that the integrative use of *B. subtilis* JW1 can promote plant growth by secreting bioactive compounds. Therefore, *B. subtilis* JW1 may be utilized as an eco-friendly bio-fertilizer in the agricultural fields after successful field trials.

Keywords: *Bacillus subtilis* JW1; Bioactive GAs; Phosphate-solubilization; Nutrient uptake; Growth promotion

Introduction

Over the last few years, the multifaceted interactions of plant growth promoting microrganisms have been extensively explored as a productive source of novel bioactive natural products (Bilal et al., 2018; Hamayun et al., 2017; Hussain et al., 2018; Ikram et al., 2018; Mehmood et al., 2019). In fact, the growth and development of the host plant under stress conditions can be improved by the production of microbial secondary metabolites (Ismail et al., 2019; Ismail et al., 2018; Jan et al., 2019; Joo et al., 2009; Mehmood et al., 2018; Nusrat et al., 2019). Moreover, beneficial rhizospheric bacteria or plant growth promoting rhizospheric bacteria (PGPR) can also promote the growth of host plant under normal as well as stress conditions through the release of bioactive secondary metabolites (Souza et al., 2015). These bioactive compounds, include indole acetic acid (IAA), cytokines (CK), jasmonic acid (JA), gibberellins (GA) and abscisic acid (ABA). In other words, these bioactive compounds are known as plant hormones or growth regulators (Marques et al., 2010). Similarly, PGPR can fix biological nitrogen, solubilize the insoluble/non-available phosphorus (Jeon et al., 2003), and alleviate stress through changes in ACC deaminase expression (Souza et al., 2015). Furthermore, various PGPR have shownhostile activity against phyto-pathogenic microorganisms by releasing anti-pathogenic compounds (Lucy et al., 2004).

During the last few decades, researchers have concentrated on the role of soil microorganisms that convert insoluble phosphate to soluble forms (Ordoñez et al., 2016; Ruangsanka, 2014). Most of rhizospheric bacteria that solubilize inorganic phosphate belongs to *Pseudomonas*, *Enterobacter*, *Bacillus* and some soil fungi, such as *Aspergillus* (Osorio Vega, 2007; Patgiri and Bezbaruah, 1990; Rao, 1982; Whitelaw, 1999). PGPR are also reported to produce organic acids, including gluconic, acetic, malic, citric, lactic, oxalic, formic, and 2-keto-gluconicacids. These microrganisms also release protons into the soil (Osorio Vega, 2007), thus converting the non-available form of phosphorus into available forms. Phosphate solubilizing and siderophore producing microbes, such as *Acinetobacter rhizosphere*, *Burkholderia cepacia*, *Streptomyces tendae* and *Serratia marcescens*are considered as important PGPR because of their proven activity inenhancingcrop production (Ben Farhat et al., 2009; Dimkpa et al., 2009; Gulati et al., 2010; Song et al., 2008; Vejan et al., 2016). Bacillus species have also shown support towards the optimal growth of the host plants by protecting them against the enimies and/or by secreting phytohormones and organic acids (Tahir et al., 2017; Yi et al., 2018). Previously, various bacterial strains have been reported for cabbage growth promotion under stress conditions (Hussein et al., 2016; Liu et al., 2016).

Chinese cabbage (*Brassica rapa* L.) is grown for vegetable purpose and for their active nutritional value. Due to its nutritional value, high yield varieties were introduced, which increases its production, although it requires large quantities of fertilizers and chemicals. For sustainable cultivation of Chinese cabbage in an eco-friendly manner, and less dependence on synthetic fertilizers and agro-chemicals, utilizing native microflora in the form of PGPR can be an ideal strategy. The current study was designed to identify the novel rhizospheric bacteria from the paddy fields in Andong, South Korea; to screen the isolated bacteria for plant growth promotion abilities (like phosphate solubilizing, siderophore formation and phytohormones production) in Chinese cabbage.

Material and methods

Isolation of rhizospheric bacteria

Rhizospheric soil collected from the rice paddies in Andong was screened for bacterial isolates capable for plant growth promotion. Plant samples with rhizospheric soil were packed in sterilized polyethylene zip-bags. The bags were then stored in an ice box and the samples were transported to the laboratory. From the roots of each plant 1 g of soil was dissolved in saline water (0.85%) and marked as a stock solution. A serial dilution (10^{-1} to 10^{-9}) was initially setup and finally 10^{-4} dilution was selected for further analysis on the basis of results (Kang et al., 2009). LB agar media were used for bacterial isolation by direct plating method and the plates were incubated at 28 °C till the emergence of bacterial colonies. Pure colonies were obtained by picking a single colony and re-plated on LB agar media. Pure colonies were identified by observing the size, color, shape and growth pattern (Barillot et al., 2013; García-Salamanca et al., 2013).

* Corresponding author

Phosphate solubilization

Bacterial isolate JW1 was inoculated on 0.5% ofNational Botanical Research Institute's phosphate (NBRIP) media plates (generously provided by National Botanical Research Institute) (NAUTIYAL, 1999). The plates were then incubated 30 °C for 3-days. The formation of halos by the inoculated bacteria indicates phosphate solubilization. The process of phosphate solubilization was monitored by checking the pH of liquid NBRIP medium after every 12 h till the end of the experiment.

Organic acids production

Millipore filter (0.22 µm) was used to obtain bacterial culture filtrate. The filtered cultures (10 µl) were then injected into a high performance liquid chromatography (HPLC; Model: Waters 600E) for further analysis. The HPLC was equipped with a Refractive Index Detector (Model: Waters 410) and RSpak KC-811 (8.0 × 300 mm) column. The conditions for the analysis were: Eluent = 0.1% H_3PO_4/ H_2O, Flow rate = 1.0 ml/min, Temperature = 40 °C. The concentration of each organic acid was determined through comparison with their respective standards (retention times and peak areas). Succinic acid (Sigma-Aldrich, USA), lactic acid, malic acid, and butyric acid (Supelco, USA) were used as standards (KANG et al., 2012).

Screening of isolate JW1 on rice

To assess the growth activity of the bacterial isolate JW1, Waito-C (dwarf and GAs mutant rice cultivar) and Whayoung-beyo (normal GAs producing rice cultivar) were used as test plants. Initially, seeds of the tested cultivars were surface sterilized with sodium hypochlorite and then thoroughly washed with sterilized distilled water. The clean seeds were spread in a plate containing sterilized distilled H_2O for germination. Germinated seedlings were transplanted in magenta box, containing 90 ml of water agar media (0.8%). The box wastransferred to thepresetgrowth chamber (light intensity = 1,000 µmol m^{-2} s^{-1} + temperature = 35 °C for 16 h as a day and 20 °C for 8 h as a night). When the tested rice cultivars reached to two-leaved stage, a 10 µl of lyophilized bacterial filtrate suspension was applied to the tip of apical meristem.The growth attributes of both rice cultivars were analyzed after 10 days of treatment.

Quantification of bacterial secreted gibberellins

The bacterial isolate JW1 was inoculated into nutrient broth (120 ml) for 7 days at 30 °C (shaking incubator-120 rpm) as described earlier (KANG et al., 2009). The culture medium and bacterial cells were separated through centrifugation (2500 × g at 4 °C for 15 min). The culture medium (50 ml) was used to extract and purify the GAs as described by KANG et al. (2009). Before purification, deuterated GA internal standards (20 ng; [17, 17-2 H2] GA_1, GA_4, GA_7), obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia, were added to the CF. The CF was subjected to chromatographic and mass spectroscopy techniques for identification and quantification of GAs. The data were calculated in ng/ml and the analysis was repeated three times.

Molecular identification of isolate JW1

The genomic DNA of isolate JW1 was isolated following the standard protocol of SAMBROOK (2001). The 16S rRNA gene was amplified and sequenced using the 27F (5′- AGAGTTTGATC (C/A) TGGCTCAG-3′) and 1492R (5′-CGG (T/C) TACCTTGTTACGACTT-3′) universal primers (KHAN et al., 2014). The BLAST search program of NCBI GenBank database/ eztaxon was used to determine the nucleotide sequence homology of bacterial isolate JW1. To conduct phylogenetic analysis, neighbour joining method was used with the help of MEGA v. 6.1 (TAMURA et al., 2013). The closely related sequences with highest homology, query coverage, and lowest E-values were used for alignment with ClustalW. The isolate JW116S rRNA gene sequence was submitted to GenBank.

Isolate JW1 bioassay on Chinese cabbage

Healthy and disease free seeds of Chinese cabbage were obtained from Seminis Korea Co. (Korea) with a 95% germination rate. These seeds were surface sterilized using 2.5% sodium hypochlorite solution for 20 minutes and washed twice with autoclave distilled water. The seeds were then subjected to germination assay in plastic trays under greenhouse conditions with the day/night cycle: 14 h at 28 °C/10 h at 25 °C; relative humidity 60-70%. Uniform size seedling were transplanted into pots (15 seedlings per treatment) filled with autoclave horticulture soil. The composition of horticultural soil was as follows: peat moss (10-15%), perlite (35-40%), coco peat (45-50%), zeolite (6-8%), and NH^{+4}~ 0.09 mg/g, NO^{-3}~ 0.205 mg/g, P_2O_5 ~ 0.35 mg/g, and K_2O ~ 0.1 mg/g to prepare the microbe-free condition. The experimental treatments comprised, (1) control (2) isolate JW1 treated plants and (3) inoculation of Chinese cabbage with bacterial free CF (KANG et al., 2009). A 25 mL of bacterial cells and cell free extracts were applied to plants, twice consecutively, in a week. After 2 weeks of such treatment, the experiment was harvested and analyzed for shoot length, root length, fresh and dry biomass, chlorophyll contents (SPAD-502 Minolta, Tokyo, Japan). For dry weight data, respective plants were randomly collected and dried in oven at 70 °C for 72 h.

Nutrient uptake by Chinese cabbage

Minerals like calcium, potassium, magnesium, phosphate and sulfate were extracted from dried plant samples and determined by Inductively Coupled Plasma Mass Spectroscopy (VG Elemental, Plasma Quad 3, Perkin Elmer, United States). The quantity of minerals was estimated using known standard values.

Statistical analysis

The data were subjected to analysis of variance using SAS-9.1 software (SAS Institute, Cary, NC). Mean values among treatments were compared by Duncan's Multiple Range Test (DMRT) test at $P \leq 0.05$ significance level.

Results

Phosphate solubilization by *Bacillus subtilis* JW1

The halos formation shows the tricalcium phosphate solubilization capacity of bacterial isolate JW1, which reached to its maximum after 60 h. The phosphate solubilization potential of the isolate JW1 was cross checked by monitoring the pH of the media after every 12 h, which showed a steady decline up to 48 h (Fig. 1). The pH of the broth was decreased in response to phosphate solubilizing activity of the isolate JW1, accompanied by a significant drop from an initial pH of 7.0 to pH 4.1 after 72 h.

Organic acid production by isolate JW1 in LB media

Current study shows that the CF of *B. subtilis* JW1 contained citric acid, butyric acid, lactic acid, malic acid, and succinic acid. The organic acid analysis showed that JW1 strains have the ability to produce significantly higher quantities of lactic acid (28.7 µg/ml) in LB medium, followed by succinic acid (10.7 µg/ml), butyric acid and malic acid (1.85 µg/ml) respectively (Fig. 2).

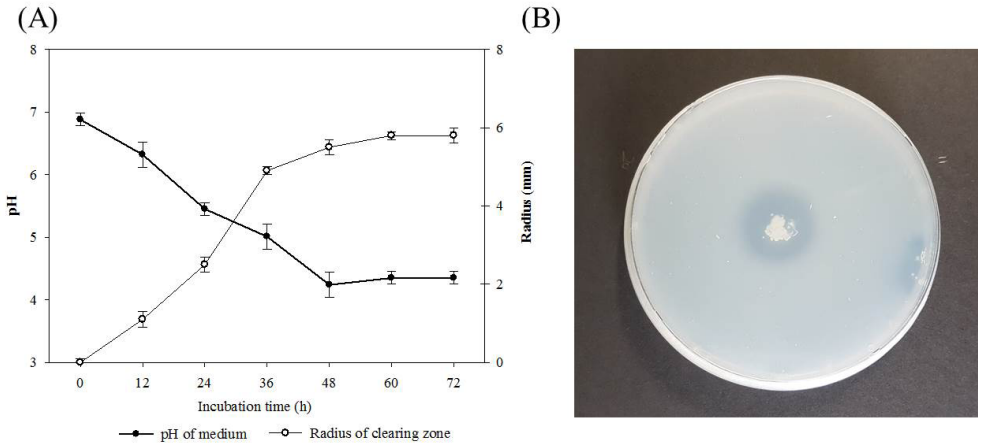

Fig. 1: (A) The rate of halos formation of *B. subtilis* JW1 in liquid NBRIP medium and pH of the medium. Values given are means of three replicates and error bars indicate standard deviation (B) the corresponding *B. subtilis* JW1on NBRIP growth media plates after 72 hours of incubation at 30 °C.

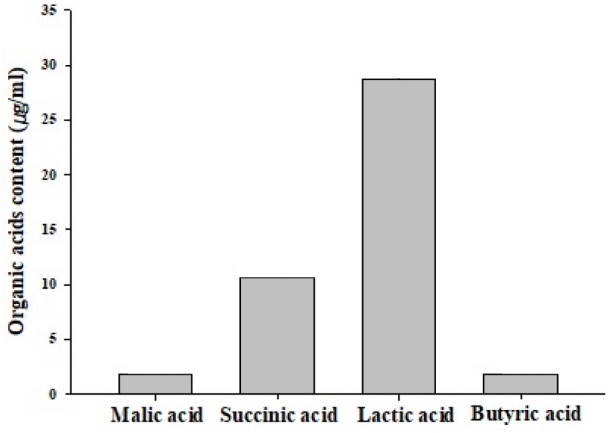

Fig. 2: Organic acids production of *Bacillus subtilis* JW1 after five days of incubation at 28 °C.

Bioactive GAs detected in the culture filtrate of isolate JW-1

The CF of isolate JW-1 was evaluated for the existence of bioactive gibberellins. Our results confirmed the existence of bioactive GAs, i.e. GA_4, GA_1 and GA_7. The quantities of bioactive GAs were, GA_4 (2.26 ng/ml), GA_1 (0.12 ng/ml) and GA_7 (0.08 ng/ml) as given in Fig. 3.

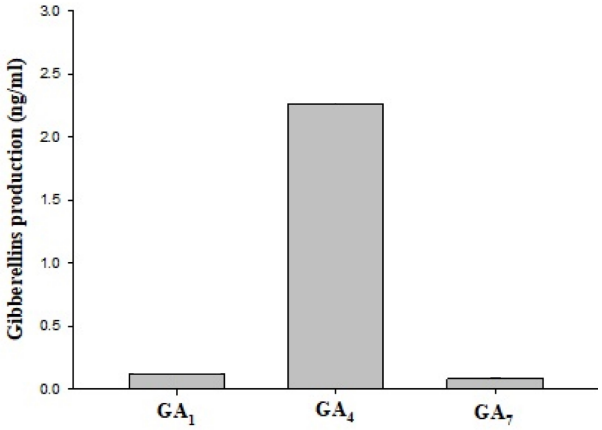

Fig. 3: Bioactive GAs detected in the CF of *Bacillus subtilis* JW1.

Screening of isolate JW1 on rice

We assessed the effect of CF of isolate JW1 on shoot length and plant fresh biomass of Waito-C and Whayoung-beyorice seedlings after seven days of incubation. It was observed that application of isolate JW1 on Waito-C significantly promoted the shoot length (20.86%) and fresh plant weight (31.75%) as compared to control. A similar growth promotion was also observed in Whayoung-beyo (Tab. 1; Fig. 4).

Tab. 1: Effects of *Bacillus subtilis* JW1 on growth attributes of Waito-C and Whayoung-beyo

Treatment	Waito-C		Whayoung-beyo	
	Shoot length (cm)	Fresh biomass (g/plant)	Shoot length (cm)	Fresh biomass (g/plant)
Control	12.94±0.38[b]	0.19±0.01[b]	12.10±0.44[b]	0.13±0.01[b]
Isolate JW1	15.64±0.39[a]	0.25±0.01[a]	15.06±0.36[a]	0.18±0.01[a]

Each value in the table are mean ± SE of three replicates. Values in columns followed by different letters significantly different at P > 0.05 based on DMRT analysis.

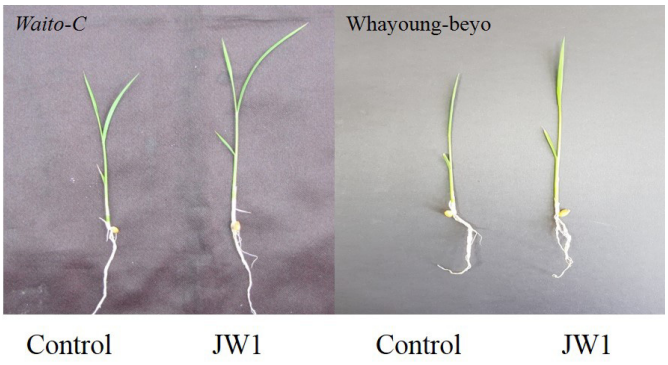

Fig. 4: Influence of *B. subtilis* JW1 culture filtrates (10 µl) on growth attributes of Waito-C and Whayoung-beyoseedlings after seven days of incubation.

Molecular identification and phylogenetic analysis

The isolate JW1 was identified by relating the amplified sequences of 16S rRNA region using universal primers, with the associated

sequences existing in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The closely related sequences were recovered from GenBank and subjected to phylogenetic analysis (Neighbor joining method) by using MEGA 6.1. The closely related sequences with highest homology, query coverage, and lowest E-values were used for alignment with ClustalW. The JW1 isolate was identified as *Bacillus subtilis* (Fig. 5). The newly identified isolate *B. subtilis* JW1 16S rRNA gene sequence was submitted to GenBank and was allotted accession no. KM 264401.

Isolate JW1 promotes growth of Chinese cabbage

The current study revealed that application of isolate JW1 and cell free CF significantly promoted plant growth attributes including, shoot and root length, fresh/dry weight, and chlorophyll contents as compared to control (Tab. 2; Fig. 6). The shoot length of Chinese cabbage was significantly increased by cell free CF (12.65%) followed by isolate JW1 (3.16%), as compared to control. The same trend was noted for the plant fresh and dry biomass (Tab. 2). Higher chlorophyll contents were detected in Chinese cabbage when treated with cell free CF and isolate JW.

***B. subtilis* JW1 promotes nutrients uptake of Chinese cabbage**

Chinese cabbage inoculated with cell free CF and with isolate JW1 significantly enhanced P, K, Mg and S contents of the treated plants as compared to the control (Tab. 3). Inoculation of plants with isolate JW1 significantly increases the nutrient content of K (6.48%) and S (45.91%) as compared to cell free CF (S-2.85%; K-32.29%) and control treatments. However, no significant differences in Mg contents were observed in different treatments. However, Ca and P contents were significantly higher in plants treated with bacterial cell free CF i.e. Ca (11.53%) and P (73.30%), and JW1 i.e. Ca (4.17%) and P (50.54%) as compared to control (Tab. 3).

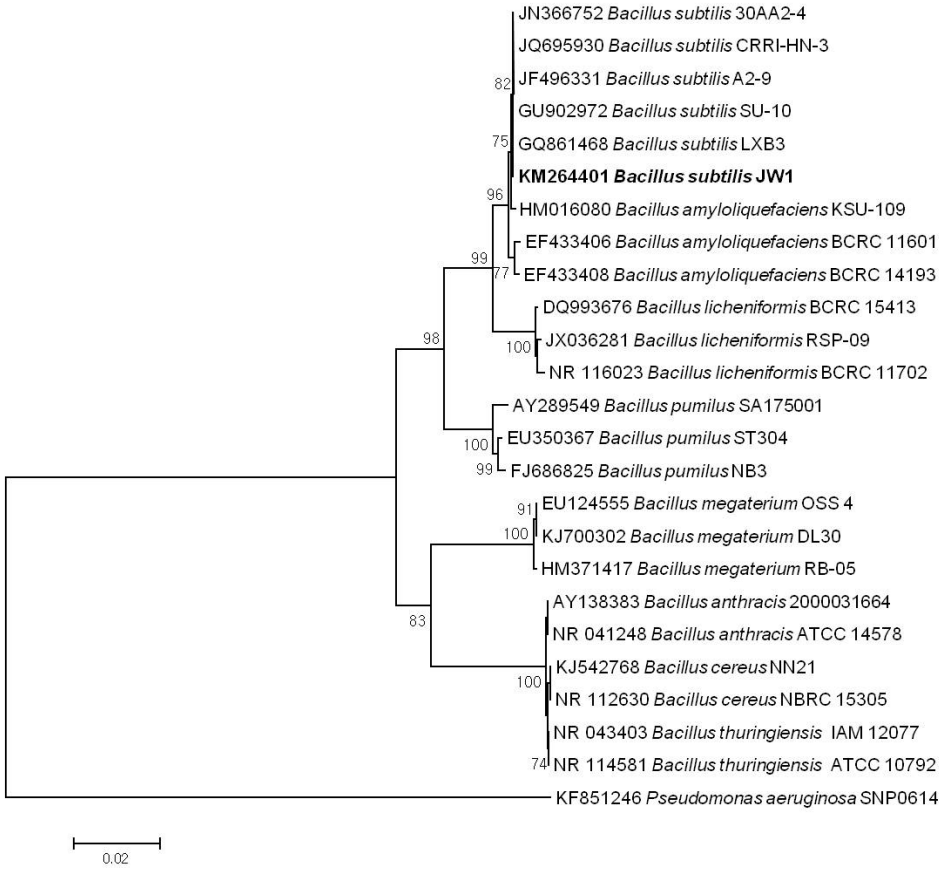

Fig. 5: The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Evolutionary analysis were conducted in MEGA v. 6.1. The phylogenetic analysis was performed by constructing Neighbour Joining (NJ) tree using 16S rDNA gene sequences from isolate JW1and related bacterial strains.

Tab. 2: Effect of *B. subtilis* JW1 and cell free CF of *B. subtilis* JW1 on growth attributes and chlorophyll contents of Chinese cabbage

Treatments	Shoot length (cm/plant)	Root length (cm/plant)	Fresh biomass (g/plant)	Dry biomass (g/plant)	Chlorophyll (SPAD)
Control	11.38±0.33^{b}	6.36±0.07^{c}	2.14±0.08^{c}	0.21±0.01^{c}	29.32±0.35^{c}
***B. subtilis* JW1**	11.74±0.22^{ab}	7.22±0.13^{b}	2.40±0.04^{b}	0.24±0.01^{b}	31.48±0.42^{b}
Cells-free extract	12.82±0.21^{a}	7.84±0.13^{a}	2.82±0.10^{a}	0.31±0.01^{a}	33.92±0.50^{a}

Each value in the table are mean ± SE of three replicatesand subsequent comparisons were conducted using Duncan's multiple range tests at $P<0.05$.

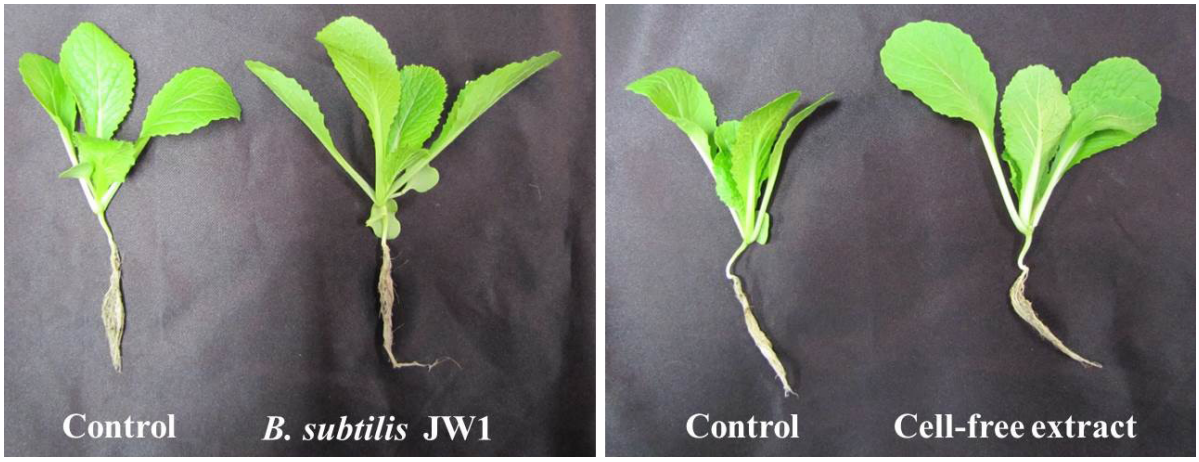

Fig. 6: Influence of *B. subtilis* JW1 and cell free CF on growth attributes of Chinese cabbage.

Tab. 3: Effect of isolate JW1 and bacterial cell free CF on nutrient uptake of Chinese cabbage

Treatments	K	Ca	Mg	P	S
Control	10.49±0.01^{c}	31.12±0.01^{c}	5.35±0.02^{b}	4.57±0.01^{c}	2.57±0.03^{c}
***B. subtilis* JW1**	11.17±0.02^{a}	32.42±0.03^{b}	5.38±0.02^{b}	6.88±0.01^{b}	3.75±0.01^{a}
Cell-free extract	10.79±0.02^{b}	34.71±0.03^{a}	5.69±0.02^{a}	7.92±0.06^{a}	3.40±0.02^{b}

Each value in the table are mean ± SE of three replicatesand subsequent comparisons were conducted using Duncan's multiple range tests at $P<0.05$.

Discussion

The area that surrounds the plant roots (rhizosphere) is a multifarious system in which PGPRs interact with the host plants for development and higher production. The exact mechanism of PGPR on plant growth promotion is still unknown. Many researchers have previously reported a significant improvement in crops yield in response to microbial inoculations under normal as well as stress conditions. In the current study, we have also observed an increase in plant height and dry biomass in the JW1 associated plants that might be due to the availability of soluble P in the presence of phosphate solubilizing rhizospheric bacteria. Higher halos formation with respect to the time indicated towards low pH and thus the transformation of phosphate from non-available in an available form. The pH of the media has been monitored for every 12 h, a decline in pH reflected the release of organic acids (most probably gluconic acid and 2-ketogluconic acid) that indicates the conversion of insoluble phosphate into soluble phosphate. Current results confirmed the findings of Nautiyal (1999), who has demonstrated the efficiency of bacterial strains NBRI0603, NBRI2601, NBRI3246 and NBRI4003 to solubilize phosphorous and make it available for the plants to achieve normal growth. Similarly, Li et al. (2018) has demonstrated that *Bacillus subtilis* strain SEM-9 has the ability to release P from inorganic phosphate sources, including calcium hydrogen phosphate, aluminum phosphate, calcium phosphate and ferric phosphate. Also, the strain *B. subtilis* JW1 has produced optimum amounts of organic acids (malic acid, succinic acid, lactic acid and butyric acid) after five days of incubation at 28 °C. The production of these organic acids by our isolate *B. subtilis* JW1 might be associated with the digestion of soil organic matter, i.e. through the oxidation of carbonaceous matter in the rhizosphere. The production of organic acids by the *Bacillus sp.* makes the pH of the soil low that make the P soluble and available to the plants (Li et al., 2018).

Furthermore, the optimum availability of minerals could enhance soil fertility and prolong micro-organisms survival in the soil (Vejan et al., 2016; Yang et al., 2011). In the current study, the higher nutrient uptake in plant inoculated with *B. subtilis* JW1 suggests an increased availability of soluble nutrients in the rhizosphere of Chinese cabbage. *Bacillus megaterium* strain TV-91C and *Bacillus subtilis* strain TV-17C has been reported to improve the macro and micronutrients uptake by cabbage seedlings (Turan et al., 2014). Indeed, plant can take up the minerals from the rhizosphere, where the PGPR can interact with host roots and help them to absorb optimum amounts of mineral nutrients from the soil (Turan et al., 2012).

Moreover, we have also observed that the CF of *B. subtilis* JW1 promoted the growth attributes (such as shoot and root lengths, biomass and chlorophyll contents) of Chinese cabbage, which can be attributed to its potential to release bioactive GA in the culture medium. GA is well known phytohormone that can promote plant growth even under stressful conditions (Khan et al., 2018; Kim et al., 2009). It has been demonstrated previously that some of the plant growth promoting microorganisms, *Asprgillus fumigatus* TS1 and *Fusarium proliferatum* BRL1can be utilized as a plant growth promoter as it secretes GAs. In addition, the isolates have intensively colonized host roots and significantly enhance endogenous GA, thus promoted host plant growth (Bilal et al., 2018). Similarly, higher root and shoot growth and GA contents have been recorded in cabbage seedlings inoculated with *Bacillus megaterium* strain TV-91C, *Pantoeaagglomerans* strain RK-92, and *Bacillus subtilis* strain TV-17C (Turan et al., 2014). In the light of these findings, it is obvious that using such useful GAs producing PGPR as eco-friendly biofertilizer might enhance the growth of economically important crop plants to sustain agriculture.

Conclusion

From the growth promoting capabilities of our isolate *B. subtilis* JW1, it is concluded that the current strain secreted higher amounts of exogenous gibberellins and have the ability to metabolize phos-

phate into a form that is readily available to the plant. Further studies are needed to isolate the genes coding for gibberellin production in *B. subtilis* JW1 and for determining how to use the new knowledge under field conditions.

Author contributions

Formal analysis, Muhammad Hamayun and AmjadIqbal; Funding acquisition, In-Jung Lee; Investigation, Sang-Mo Kang, Muhammad Hamayun and Muhammad Aaqil Khan; Project administration, In-Jung Lee; Resources, In-Jung Lee; Supervision, In-Jung Lee; Writing – original draft, Sang-Mo Kang; Writing – review & editing, Muhammad Hamayun, AmjadIqbal and In-Jung Lee.

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ORCID
Amjad Iqbal https://orcid.org/0000-0001-6141-8337

Address of the authors:
Prof. In-Jung Lee (Corresponding author): E-mail: ijlee@knu.ac.kr
Dr. Amjad Iqbal: E-mail: amjadiqbal@awkum.edu.pk