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Research article

Gibberellins producing *Bacillus methylotrophicus* KE2 supports plant growth and enhances nutritional metabolites and food values of lettuce

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ABSTRACT

The nutritional quality of green leafy vegetables can be enhanced by application of plant beneficial micro-organisms. The present study was aimed to increase the food values of lettuce leaves by bacterial treatment. We isolated bacterial strain KE2 from Kimchi food and identified as *Bacillus methylotrophicus* by phylogenetic analysis. The beneficial effect of *B. methylotrophicus* KE2 on plants was confirmed by increasing the percentage of seed germination of *Lactuca sativa* L., *Cucumis melo* L., *Glycine max* L. and *Brassica juncea* L. It might be the secretion of array of gibberellins (GA_1, GA_3, GA_7, GA_8, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, GA_{34} and GA_{53}) and indole-acetic acid from *B. methylotrophicus* KE2. The mechanism of plant growth promotion via their secreted metabolites was confirmed by a significant increase of GA deficient mutant rice plant growth. Moreover, the bacterial association was favor to enhance shoot length, shoot fresh weight and leaf width of lettuce. The higher concentration of protein, amino acids (Asp, Thr, Ser, Glu, Gly, Ala, Leu, Tyr and His), gama-aminobutric acid and fructose was found in bacterial culture (KE2) applied plants. The macro and micro minerals such as K, Mg, Na, P, Fe, Zn and N were also detected as significantly higher quantities in bacteria treated plants than untreated control plants. In addition, the carotenoids and chlorophyll *a* were also increased in lettuce at bacterial inoculation. The results of this study suggest that *B. methylotrophicus* KE2 application to soil helps to increase the plant growth and food values of lettuce.

1. Introduction

The growth and nutritional quality of plants were affected by soil pH, available nutrients, water, temperature and other biotic and abiotic factors. The chemical fertilizers and pesticides are being used by farmers to improve the crop yield during unfavorable conditions. Those agricultural chemicals deposits in soil and mixed with water to cause hazardous effects to human and other living organism (Flores-Felix et al., 2013). For example, mancozeb is a fungicide used to protect the plants from wide range of fungal diseases (Paro et al., 2012), and it causes detriment effects on plant metabolism (Pereira et al., 2014). Therefore, the application of plant beneficial microbes in the rhizosphere enhances food production and helps plants by reducing the stress effects against environment stresses (Morrissey et al., 2004). Plant growth promoting rhizobacteria (PGPR) exhibit several mechanisms to increase the plant growth by nitrogen fixation, phosphates solubilization, increase the availability of mineral nutrients, plant growth promoting metabolites synthesis and prevent the phytopathogens growth and infection (Kang et al., 2014a,b; Tank and Saraf, 2003). Soil living and endophytic bacteria and fungi can act as biofertilizers to plants and improve the physic-chemical properties of soil. The identification and application of plant hormones producing bacteria and fungi on crop plants to promote the plant growth have been major interest in sustainable agriculture. Recently, the numbers of gibberellin (GA) and indole-acetic acid (IAA) and other plant growth promoting metabolites were identified and reported in several bacterial species, which helped to improve the plant growth under unfavorable environmental stress conditions (Ferrara et al., 2012; Kang et al., 2014b). GA plays a vital role in seed germination, plant growth, flowering and fruiting. The role of IAA in root development is well

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determined in plants. The micro-organisms assist to absorb the nutrients and water through root cells for their growth and development.

Lettuce is a green leafy vegetable and cultivated worldwide. The global production of lettuce is increasing every year due to their nutritional values. It is eaten raw because it is rich in nutrients such as K, Na, Ca, Mg, Fe, Mn, Cu, Zn and Se and other phytochemicals (Pinto et al., 2014). The yield of lettuce has been affected by chemical fertilizers, particularly nitrogen (Kamata, 1969). Several studies suggest that application of plant beneficial micro-organisms is environmental friendly to increase the crop yield (Dutt et al., 2013; Flores-Felix et al., 2013; Garcia-Fraile et al., 2012). The numbers of plant growth promoting bacteria (PGPB) have been identified in *Acetobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Pseudomonas*, and *Serratia* genera (Glick, 1995; Jones et al., 2007). Several researchers reported that PGPB were able to produce the GA and support the plant growth (Gutierrez-Manero et al., 2001; Kang et al., 2014b). A very few studies demonstrated the plant growth promoting effects of *B. methylotrophicus* in crop plants, but no detailed study was conducted on their effects on plant metabolisms. Moreover, the plant hormones including IAA and GA production from *B. methylotrophicus* are not reported. In this investigation, we examined the interaction of *B. methylotrophicus* with crop plants by their bioactive metabolites induced nutritional changes in lettuce plants. The objective of this study was to determine the effect of newly isolated bacterial strain, *B. methylotrophicus* KE2 on seed germination, plant growth and food values including protein, amino acids, sugars, minerals, elements, carotenoids and chlorophylls of lettuce.

2. Materials and methods

2.1. Isolation and identification Bacillus methylotrophicus

The bacterial strain (KE2) was isolated from Kimchi, a fermented Korean traditional food. Kimchi was added to sterile saline solution and inoculated on plates containing tryptic soy/agar (TSA; Merck Co., Germany) medium and incubated for 48 h at 30 °C. The bacterial colonies were differentiated by their morphology, pigmentation and growth rate. The bacterial isolate, KE2 was identified on the basis of partial 16S ribosomal DNA (rDNA) sequence. The 27F primer (5′-AGAGTTTGATC(AC)TGGCTCAG-3′) and 1492R primer (5′-CGG(CT)TACCTTGTTACGACTT-3′) were used for PCR amplification of 16S rDNAs. The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to find the nucleotide sequence homology of this bacterial isolate. The relatively similar nucleotide sequences were aligned by ClustalW and MEGA (version 5.0) software, and the neighbor-joining tree was generated. Bootstrap replication (1000 replications) was used to a statistical support for the nodes in the phylogenetic tree.

2.2. Plant materials and bacterial inoculation

Seeds of *Lactuca sativa* L., *Cucumis melo* L., *Glycine max* L. and *Brassica juncea* L. were soaked in water for 5 h and surface sterilized with 0.5% sodium hyphochloride and 70% ethanol, and then rinsed with sterile distilled water three times to remove the sterilization chemicals. The seeds were inoculated in *Bacillus methylotrophicus* KE2 (7.5×10^{-8} CFU/ml of tryptic soy broth) bacterial culture for 30 min. The culture medium was drained from the tubes and seeds were transferred to three layered petri-plates and incubated at 25 °C in a dark incubator. The rate of seed germination was measured at 3 days.

2.3. Bioactive metabolites in KE2 culture

Plant growth promoting metabolites, gibberellins (GAs) were extracted from 3-days -old *B. methylotrophicus* KE2 culture filtrates, according to the method followed by Joo et al. (2009). The extracted samples were fractionated by reverse-phase C18-HPLC and were chromatographed on a 3.9 × 300 m Bondapak, C18 column (Waters Corp., USA) and eluted at 1.5 ml/min with the following gradient: 0–5 min, isocratic 28% methanol (MeOH) in 1% aqueous acetic acid; 5–35 min, linear gradient from 28 to 86% MeOH; 35–36 min, 86–100% MeOH; 36–40 min, isocratic 100% MeOH. The forty eight fractions (each fraction contains 1.5 ml) were collected and prepared for injection to gas chromatography/mass spectrometer (GC/MS) with selected ion monitoring mode (SIM) (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, USA). For each GA type, 1 ml of sample was injected to a 30 m × 0.25 mm (i.d.), 0.25 mm film thickness DB-1 capillary column (J & W Scientific Co., USA). The GC oven temperature was programmed for a 1 min hold at 60 °C, then to rise at 15 °C/min to 200 °C followed by 5 °C/min to 285 °C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector with an interface and source temperature of 280 °C, an ionizing voltage of 70 eV and a dwell time of 100 ms. Full scan mode (the first trial) and three major ions of the supplemented [$^2H_2$] GAs internal standards and the bacterial gibberellins were monitored simultaneously. The retention time was determined using hydrocarbon standards to calculate the KRI (Kovats Retention Index) value, while the GAs quantification was based on peak area ratios of non deuterated (extracted) GAs to deuterated GAs.

For indole acetic acid (IAA) analysis, *B. methylotrophicus* KE2 was cultured in medium with 0.5 g/L D-tryptophan and incubated for seven days at 28 ± 2 °C. The microbes were separated from the culture. The filtrate pH was adjusted to 2.8–3.0, and ethyl acetate was added. The obtained organic layer was vacuum evaporated. The extracts were re-suspended in 0.1 M acetic acid and transferred to a reverse-phase C18 column. The extract was eluted using stepwise elution with 30% methanol (MeOH), 50% MeOH, and 100% MeOH. All the eluted samples were combined and dried. Methyl esters of the samples were prepared by dissolving the residues in MeOH and adding ethereal diazomethane; the methyl esters were then re-dissolved in ethyl acetate and analyzed using GC-MS with selected ion monitoring (SIM). The IAA concentration in culture filtrate was measured by a known standard peak area.

2.4. Bioassay on gibberellins (GAs) deficient mutant Waito-C rice

The mechanism of plant growth promotion during *B. methylotrophicus* KE2 interaction was analyzed by performing screening bioassay on gibberellins (GAs) deficient mutant *Waito-C* rice. Its seeds were surface sterilized with 2.5% sodium hypochlorite for 30 min, rinsed with autoclaved distilled water, and incubated for 24 h with 20-ppm uniconazol. The germinated seeds were grown on agar medium (0.8%) in a growth chamber for 10 days under controlled conditions. After attaining two leaves stage, 20 μl culture filtrate of *B. methylotrophicus* KE2 was applied at the apex of *Waito-C* rice seedlings. The length, fresh and dry weight of shoot were recorded in rice plants after 7 days. The shoots of rice plants were kept at 65 °C for 48 h in an oven and measured the dry weight of shoots.

2.5. Lettuce plant growth and bacterial isolate KE2 treatment

The surface sterilized lettuce seeds (50 seeds per treatment) were sown in autoclaved horticulture soil mixture containing peat