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Plant growth-promoting rhizobacteria improved growth, nutrient, and hormone content of cabbage (*Brassica oleracea*) seedlings

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Abstract: A greenhouse experiment was conducted to observe the effects of *Bacillus megaterium* strain TV-91C, *Pantoea agglomerans* strain RK-92, and *B. subtilis* strain TV-17C inoculation on the growth, nutrient, and hormone content of cabbage seedlings. The seeds of cabbage were incubated in flasks by shaking at 80 rpm for 2 h at 28 °C to coat the seeds with the rhizobacteria. Plant growth-promoting rhizobacteria (PGPR) treatments increased fresh and dry shoot and root weight, stem diameter, seedling height, chlorophyll reading values, and leaf area of cabbage seedlings compared with the control. Among the strains, *B. megaterium* TV-91C gave the greatest seedling nutrient content and growth parameters, although the maximum values for leaf area, gibberellic acid, salicylic acid, and indole acetic acid (IAA) contents of seedlings were obtained with the *P. agglomerans* RK-92 treatment. Seed inoculation with *B. megaterium* TV-91C increased fresh and dry shoot and root weight by 32.9%, 22.6%, 16.0%, and 35.69%, respectively. Inoculations also increased the stem diameter, seedling height, and SPAD chlorophyll values by 47.5%, 27.2%, and 5.8%, respectively. Furthermore, compared to the control, *P. agglomerans* RK-92 increased gibberellic acid, salicylic acid, IAA, and leaf area by 13.9%, 70.9%, 38.5%, and 27.3%, respectively. PGPR treatments may improve seedling growth and quality in cabbages.

Key words: Seedling quality, plant growth promoting rhizobacteria, hormone, plant nutrition element

1. Introduction

The seedling is one of the most important inputs for high-yield production of vegetable crops. Seedling quality is a prerequisite for successful stand establishment and uniform plant growth and development. Plant growth-promoting rhizobacteria (PGPR) have been used for plant production but little is known about the mechanism(s) involved in their effect on seedling performance (Yildirim et al., 2011a).

There has been growing evidence that extensive use of chemical fertilizers can be costly and can create serious environmental problems. Large amounts of chemical fertilizers are used to replace soil nitrogen and phosphorus, but they can be expensive and also contaminate the environment. The efficiency of applied fertilizer is estimated to be about or lower than 50% for N, less than 10% for P, and about 40% for potassium in chemical fertilizer, and it is lower for manure (Baligar et al., 2001). Despite the deleterious environmental effects, the total amount of inorganic fertilizers used worldwide is expected to increase to produce more food via intensive agriculture for the increasing world population (Adesemoye et al., 2009). Current efforts have been focused on the decreased use of chemical pesticides and inorganic fertilizers in agriculture, prompting the search for alternative ways to enhance soil fertility and crop production. Because the use of PGPR with the aim of improving nutrient availability for plants can be important for agriculture (Freitas et al., 2007), PGPRs have been recently used increasingly worldwide in sustainable agriculture as biological fertilizer (Yildirim et al., 2011a).

The growth-promoting activities of PGPR on plants can be explained in various ways, including through biocontrol and induction of disease resistance in the inoculated plant, biological N_2 fixation, phosphorus solubilization, and/or production of phytohormone (Mia et al., 2012). Research should not ignore the potential to improve plant production through PGPR inoculation via mechanisms that do not involve biological nitrogen fixation. Many soil- and plant-associated rhizobacteria are able to synthesize phytohormones (Bastian et al., 1998). *Rhizobium*, *Azotobacter*, *Acetobacter*, and *Herbaspirillum*

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isolates can excrete and synthesize gibberellins, auxin, and cytokinins (Atzorn et al. 1988; Bastian et al., 1998). Earlier studies showed that PGPR could stimulate the growth and yield of some vegetable crops such as tomato, lettuce, and broccoli (Turan et al., 2007; Yildirim et al., 2008, 2011b; Gunes et al., 2009).

Commercial vegetable seedling production in Turkey has increased significantly in recent years and annual production has reached 2.5 billion. A large number of *Brassica* seedlings, including cabbage, are produced commercially. Liquid fertilizers including N are used during seedling production. Soilless plant growth media mixes have been reported to be ideal for delivery of PGPR for transplanted crops (Kokalis-Burelle et al., 2002). PGPR treatments significantly increased the shoot and root weight and improved the seedling quality of melon and watermelon (Kokalis-Burelle et al., 2003). Most studies with PGPR have been conducted to determine the direct beneficial effects on plant growth and yield, but there is a lack of information about the effect of PGPR on the seedling quality of cabbage. Therefore, this study was carried out to investigate the effects of PGPR seed inoculation on seedling growth and plant nutrient and hormone content of cabbage under greenhouse conditions.

2. Materials and methods

2.1. Growth conditions and plant materials

This study was conducted under greenhouse conditions at Atatürk University, Turkey, in 2012. Cabbage (*Brassica oleracea* var. *capitata* 'Yalova1') seedlings were maintained under natural light conditions, approximate day/night temperatures of 25/14 °C, and 75% relative humidity during the experiment. Cabbage seeds were sown into 45-celled trays (30 cm^3) filled with peat. There was no nutrition application during the experiments.

2.2. Bacterial strains

The bacterial strains (*Bacillus megaterium* TV-91C, *Pantoea agglomerans* RK-92, and *Bacillus subtilis* TV-17C) were obtained from the culture collection unit of the Department of Plant Protection, Faculty of Agriculture, Atatürk University, Turkey. They had been isolated from plant rhizospheres and phyllospheres in the East Anatolia region of Turkey (Kotan et al., 2005). The bacterial cultures were grown on nutrient agar for routine use and were maintained in Luria Broth with 15% glycerol at –80 °C for long-term storage.

2.3. Identification of the bacterial strains by microbial identification system

Identification of the bacterial strains tested was confirmed by using a microbial identification system (MIS). Preparation and analysis of fatty acid methyl esters (FAMEs) from whole-cell fatty acids of bacterial strains was performed according to the method described in the manufacturer's manual (Sherlock Microbial Identification System version 4.0, MIDI, Inc., Newark, DE, USA). FAMEs were separated by gas chromatography (HP-6890, Hewlett-Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m × 0.2 mm, with cross-linked 5% phenyl methyl silicone). Each bacterial strain was identified by comparing its FAME profile with those in commercial databases (TSBA 40) using the MIS software package.

2.4. Characteristics of the bacterial strains

The bacteria were tested for N_2-fixing ability as described by Dobereiner et al. (1988). Ability of rhizobacterial isolates to grow on Dobereiner N-free culture medium indicated their nonsymbiotic N_2-fixation ability. Phosphate solubilization capacity was tested on the National Botanical Research Institute's phosphate growth medium (NBRIP-BPB) according to Metha and Nautiyal (2001). The bacterial colonies, selected and purified, were inoculated (50 µL inoculum with approximately 1 or 2 × 10^9 cfu mL^{-1}) into 5 mL of NBRIP-BPB medium. Autoclaved, uninoculated media served as controls. Furthermore, we determined the hormone content [gibberellic acid, indole acetic acid (IAA), and salicylic acid] of the PGPR used in the study. PGPR strains have been shown to produce gibberellic acid, IAA, and salicylic acid (data not shown here).

2.5. Media and growth condition for bacteria

Tryptic soy agar (TSA, Oxoid) and tryptic soy broth (TSB, Oxoid) were used in the experiments. All bacterial isolates were incubated in TSA at 27 °C for 24 h. After incubation, single colonies were transferred to 500-mL flasks containing TSB and grown aerobically on a rotating shaker (150 rpm) for 48 h at 27 °C (Merck KGaA, Germany). The bacterial suspension was then diluted in sterile distilled water (sdH_2O) to a final concentration of 1 × 10^8 cfu mL^{-1} as measured with a turbidimeter.

2.6. Coating procedure of bacteria on the seeds

The seeds were surface-disinfected to avoid the presence of any saprophytic and/or pathogenic microorganisms on the seed surface by dipping the seeds for 3 min in 3% sodium hypochlorite and washing 4 times in sterilized water. Seeds were left to dry on sterile Whatman filter papers overnight in a laminar flow hood.

Bacteria were grown in 50-mL flasks containing 20 mL of TSB medium on a rotary shaker at 27 °C for 24 h. Absorbance of the bacterial suspensions was measured spectrophotometrically at 600 nm and they were diluted to 1 × 10^8 cfu mL^{-1} in sdH_2O. Approximately 0.2 g of sucrose (10 mg mL^{-1}) was added to each Erlenmeyer flask, and 90 g of the surface-sterilized seeds were soaked in this suspension. The seeds were incubated in the flasks by shaking at 80 rpm for 2 h at 28 °C to coat the seeds with the bacteria. After shaking, the seeds were air-dried on sterile Whatman filter papers overnight in the laminar flow hood. Seeds soaked in TSB medium amended with sucrose served as the control.

2.7. Chlorophyll reading values

A portable chlorophyll meter (SPAD-502; Konica Minolta Sensing, Inc., Japan) was used to measure leaf greenness. This estimates total chlorophyll in leaves in a nondestructive method (Neufeld et al., 2006). For each plant, measurements were taken at 4 locations on each leaf, 2 on each side of the midrib on all fully expanded leaves (Khan et al. 2003), and the same leaves were used for chemical analyses.

2.8. Growth parameters

Forty days after sowing, 20 plants from each replicate were harvested, and shoot and root fresh and dry weights, stem diameter, seedling height, and leaf number were determined. The plant material for dry weight was dried at 70 °C for 48 h. The area of the green leaves was quantified with a leaf area meter (LI-3100, LI-COR).

2.9. Mineral analysis

Leaf tissue samples were taken during harvest, then oven-dried at 68 °C for 48 h, ground, and passed through a 1-mm sieve. The Kjeldahl method and a Vapodest 10 Rapid Kjeldahl Distillation Unit (Gerhardt, Germany) were used to determine total N (Bremner, 1996). Macroelements (P, K, Ca, Mg, and Na) and microelements (Fe, Mn, and Cu) were determined after wet digestion of dried and ground subsamples using a HNO_3-H_2O_2 acid mixture (2:3, v/v) with 3 steps [first step: 145 °C, 75% radio-frequency power (RF), 5 min; second step: 180 °C, 90% RF, 10 min; and third step: 100 °C, 40% RF, 10 min] in a microwave digester (Bergof Speedwave Microwave Digestion Equipment MWS-2; Berghof Products and Instruments, Germany) (Mertens, 2005a). Tissue P, K, Ca, Mg, Na, Fe, Mn, and Cu were determined using an inductively coupled plasma spectrophotometer (Optima 2100 DV, ICP/OES; PerkinElmer, USA) (Mertens, 2005b).

2.10. Hormone analysis

Extraction and purification processes were executed as described by Kuraishi et al. (1991) and Battal and Tileklioğlu (2001). Methanol 80% at –40 °C was added to fresh leaf samples (Davies, 1995). After the material was homogenized for 10 min with Ultra Turrax, it was incubated for 24 h in the dark. The samples were filtered through Whatman No. 1 filter paper and the supernatants were filtered again through a 0.45-µm pore filter (Cutting, 1991). Supernatants were dried at 35 °C using an evaporator pump. Dried supernatants were dissolved in 0.1 M KH_2PO_4 (pH 8.0). Extracts were centrifuged at 5000 rpm for 1 h at 4 °C to separate fatty acids (Palni et al., 1983). Polyvinylpyrrolidone (PVPP), 1 g, was added to the supernatant to separate phenolic and colored materials (Chen, 1991; Hernandez-Miana, 1991; Qamaruddin, 1996). The supernatant was then filtered through Whatman No. 1 paper to remove the PVPP (Cheikh and Jones, 1994). For further specific separation, a Sep-Pak C-18 (Waters) cartridge was used. Hormones absorbed by the cartridge were transferred to vials using 80% methanol. The hormones were analyzed by high-performance liquid chromatography (HPLC) using a Zorbax Eclipse-AAA C-18 column (Agilent 1200 HPLC) and by absorbance at 265 nm in a UV detector. Flow speed was set to 1.2 mL min^{-1} at a column temperature of 25 °C. Gibberellic acid, salicylic acid, IAA, and abscisic acid levels were determined using 13% acetonitrile (pH 4.98) as the mobile phase.

2.11. Statistical analysis

Experiments were repeated twice. Each experiment consisted of a completely randomized design with 4 replicates per treatment and 45 plants per replicate. Data were subjected to analysis of variance using SPSS 18 (PASW Statistics 18) (SPSS Inc., 2010). Means were separated by Duncan's multiple range test (DMRT). There were no significant interactions by experiments; therefore, the data were pooled.

3. Results

3.1. Nitrogen fixation and phosphate solubilization activity

The bacterial strains were confirmed as *Bacillus megaterium* TV-91C, *Pantoea agglomerans* RK-92, and *Bacillus subtilis* TV-17C (Table 1). All strains showed capacity to grow in N-free conditions and to solubilize phosphate.

3.2. Seedling growth parameters

PGPR treatments improved the growth parameters of cabbage seedlings. All parameters investigated, with the exception of true leaf numbers, were significantly affected by PGPR inoculations (Table 2). PGPR treatments increased fresh and dry shoot and root weight, stem diameter, seedling height, chlorophyll reading values, and leaf area of cabbage seedlings compared with the control. Highest fresh and dry shoot and root dry weight, stem diameter, seedling height, and chlorophyll reading values of cabbage seedlings were obtained from *Bacillus megaterium* TV-91C and following *P. agglomerans* RK-92 and *B. subtilis* TV-17C treatment. However, the leaf area was the greatest in *P. agglomerans* RK-92 (Table 2).

On average, seed inoculation with *B. megaterium* TV-91C increased fresh and dry shoot and root weight by 32.9%, 22.6%, 16.0%, and 35.6%, respectively, and increased stem diameter, seedling height, and SPAD chlorophyll by 47.5%, 27.2%, and 5.8%, respectively, compared with the control, while the leaf area increase rate was 27.3% with *P. agglomerans* RK-92.

3.3. Nutrient content

The concentrations of macro- and micronutrients in cabbage seedlings in response to PGPR treatments

Table 1. Nitrogen fixation and phosphate-solubilizing activity of the tested bacterial strains.

Bacterial strains	Isolated from	Nitrogen fixation	Phosphate solubilization
Bacillus megaterium TV-91C	Sugar beet	+	w+
Pantoea agglomerans RK-92	Pear	+	s+
Bacillus subtilis TV-17C	Rye	+	w+

+: Positive reaction; s+: strong positive reaction; w+: weak positive reaction.

Table 2. Seedling growth parameters and chlorophyll reading values of cabbage seedlings in response to PGPR treatments.

Parameters	Control	TV-91C	RK-92	TV-17C
Fresh shoot weight (g)	32.0 b**	42.5 a	40.8 a	33.6 b
Dry shoot weight (g)	3.31 b*	3.84 a	3.82 a	3.69 a
Fresh root weight (g)	9.85 b*	12.08 a	11.28 ab	10.86 ab
Dry root weight (g)	0.59 c**	0.80 ab	0.83 a	0.71 b
Seedling height (cm)	11.3 b*	14.3 a	13.9 a	13.8 a
Stem diameter (mm)	2.76 c**	4.07 a	3.63 ab	3.33 b
True leaf number	3.67 ns	4.00	4.03	4.00
Chlorophyll reading value	40.1 b*	42.4 a	39.5 b	41.4 ab
Leaf area	21.54 c*	25.17 ab	27.41 a	22.83 bc

*P < 0.05; ** P < 0.01; ns: P > 0.05. Means within rows not followed by the same letter differ significantly at P < 0.05 by DMRT.

are shown in Table 3. The nutrient content of cabbage seedlings was significantly affected by PGPR treatments. PGPR inoculations increased the plant nutrient element content, with the exception of Na and Cu. The highest concentrations for N and P were recorded in *B. megaterium* TV-91C, while in *B. subtilis* TV-17C for Ca, Na, and Fe and in *P. agglomerans* RK-92 for K, Mg, and Mn.

Seed inoculation with *B. megaterium* TV-91C increased N and P by 18.0% and 10.2%; *P. agglomerans* RK-92 increased K, Mg, and Mn concentrations of plants by 5.0%, 25.3%, and 21.7%; and *B. subtilis* TV-17C increased Na, Ca, and Fe concentrations by 4.5%, 10.9%, and 36.6%, respectively, compared with the control.

3.4. Hormone content

The hormone content of cabbage seedlings was significantly affected by PGPR treatments. PGPR inoculations increased gibberellic acid, salicylic acid, and IAA concentrations. The greatest values for gibberellic acid, salicylic acid, and IAA content were recorded in *P. agglomerans* RK-92, while abscisic acid was highest in the control treatment. *B. subtilis* TV-17C decreased the abscisic acid content compared to the other treatments. The increase rates of gibberellic acid, salicylic acid, and IAA were 13.88%, 70.93%, and 38.51%, respectively, in comparison to the control (Table 4).

4. Discussion

The improved root and shoot growth in response to all inoculants compared with the control indicates the beneficial role of these rhizobacteria. The improving effect of seed inoculation with rhizobacteria on shoot dry weight and yield of plant were reported earlier by Yildirim et al. (2008, 2011b), Gunes et al. (2009), Karlidag et al. (2011), and Turan et al. (2012). Such an improvement might be attributed to the N_2-fixing and phosphate-solubilizing capacities of bacteria, as well as the ability of these microorganisms to produce growth-promoting substances such as IAA (Salantur et al., 2006).

PGPRs have been reported to stimulate nutrient content in tomato, radish, lettuce, and strawberry (Turan et al., 2007; Yildirim et al., 2008, 2011b; Gunes et al., 2009; Karlidag et al., 2011). PGPR applications such as *Bacillus* M3 OSU-142 increased N, P, Ca, Fe, and Zn concentrations of plant leaves, consistent with the present results. PGPRs promote the growth of the plant and increase the root surface area or the general root architecture (Bhattacharyya and Jha, 2012). Auxins excreted by rhizobacteria can improve root growth, resulting in an increased uptake of essential nutrients (Vikram, 2007). Plant developmental processes are also controlled by internal signals that depend on

Table 3. Macro- and micronutrient element content of cabbage seedlings in response to PGPR treatments

Element	Control	TV-91C	RK-92	TV-17C
N (%)	0.78 c **	0.92 a	0.79 bc	0.86 ab
Na (μg g^{-1})	142 ab*	130 b	139 ab	149 a
K (μg g^{-1})	4599 b***	4829 a	4830 a	4810 a
Ca (μg g^{-1})	3826 c***	4074 b	3919 c	4242 a
Mg (μg g^{-1})	189 c***	218 b	236 a	197 c
P (μg g^{-1})	671 c***	740 a	699 b	713 b
Fe (μg g^{-1})	28.6 c**	32.8 b	33.7 ab	36.6 a
Cu (μg g^{-1})	3.21 ns	3.15	3.25	3.19
Mn (μg g^{-1})	3.87 d***	4.45 b	4.71 a	4.29 c

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: $P > 0.05$. Means within rows not followed by the same letter differ significantly at $P < 0.05$ by DMRT.

Table 4. Hormone content of cabbage seedlings in response to PGPR treatments.

	Control	TV-91C	RK-92	TV-17C
Gibberellic acid (ng μL^{-1})	190 b*	215 a	216 a	214 a
Salicylic acid (ng μL^{-1})	44.7 b**	50.6 b	76.4 a	53.8 a
Abscisic acid (ng μL^{-1})	0.23 a*	0.17 b	0.21 a	0.22 a
Indole acetic acid (ng μL^{-1})	6.31 c**	7.67 b	8.74 a	7.82 b

* $P < 0.05$; ** $P < 0.01$. Means within rows not followed by the same letter differ significantly at $P < 0.05$ by DMRT.

the adequate supply of mineral nutrients by soil to roots. Thus, the availability of nutrient elements can be a major constraint to plant growth in many environments of the world, especially in the tropics where soils are extremely low in nutrients. Plants take up most of their mineral nutrients through the rhizosphere, where microorganisms interact with plant products in root exudates. Plant root exudates consist of a complex mixture of organic acid anions, phytosiderophores, sugars, vitamins, amino acids, purines, nucleosides, inorganic ions (e.g., $HCO3^-$, OH^-, H^+), gaseous molecules (CO2, H2), enzymes, and root border cells, which have major direct or indirect effects on the acquisition of mineral nutrients required for plant growth (Bottini et al., 2004; Turan et al., 2012).

The present experiment revealed that seed inoculation with *B. megaterium* TV-91C, *P. agglomerans* RK-92, and *B. subtilis* TV-17C resulted in increased root and shoot weight, seedling height, leaf area, and chlorophyll content in the greenhouse. The most effective bacteria in terms of fresh and dry shoot and root weight, stem diameter, seedling height, and chlorophyll reading values of cabbage seedlings was *B. megaterium* TV-91C, but *P. agglomerans* RK-92 was the most effective on leaf area of the seedlings. In a previous study, it was reported that application of *P. agglomerans* RK-92, also used in the present study, increased growth and yield parameters of dry bean (Tozlu et al., 2012). The positive effects of PGPR on the yield and growth of crops such as wheat (Ozturk et al., 2003; Salantur et al., 2006; Bulut, 2013), maize (Egamberdiyeva, 2007), soy bean (Cattelan et al., 1999), and sugar beet (Cakmakci et al., 2006) were explained by N_2-fixation ability, phosphate-solubilizing capacity, and phytohormone production. Similar increases in plant root and shoot weight, stem diameter, and leaf area were observed in different crops inoculated with *Pseudomonas*, *Azospirillum*, and *Azotobacter* strains (Martinez-Toledo et al., 1998; Siddiqui and Shaukat, 2002; Shaukat et al., 2006).

It is suggested that the tested PGPR strains influenced root hormone levels by producing IAA and/or other plant hormones in the rhizosphere, which were then absorbed by the root. Abbass and Okon (1993) suggested that IAA and other plant hormones could be responsible for improved growth of canola, tomato, and wheat inoculated with *Azotobacter paspali*.

Researchers have recently identified cytokinin, gibberellin, auxin, and 1-aminocyclopropane-1-carboxylate deaminase production by PGPR (Timmusk et al., 1999; Gutierrez Mañero et al., 2001). Many PGPRs have the ability to produce plant growth regulators, and these regulators may play an important role in plant growth

promotion (Bent et al., 2001; Patten and Glick, 2002). Application of IAA to P-deficient plants improved root surface area, carbohydrate release, and acid phosphatase activity (Wittenmayer and Merbach, 2005). In this study, the N-fixation ability of PGPRs could have been the main factor affecting seedling growth. Solubilization of P and production of hormones such as IAA may also have positive effects on seedling growth on cucumber.

The present study indicates that P-solubilizing, N_2-fixing, and phytohormone-producing PGPR strains stimulated seedling nutrient content and root and shoot growth. Microbial fertilization could be an alternative to mineral N and P fertilizer sources for seed germination and seedling performance in greenhouse conditions. Because of the environmental pollution associated with the excessive use of N and P fertilizers, PGPR can be a promising alternative for vegetable seedling production. This study showed that inoculation of seeds with PGPR under greenhouse conditions may help to reduce or replace the total amount of starter mineral fertilizers necessary to obtain maximum seedling performance for sustainable agriculture. Further studies may be important to investigate the effect of PGPR on seedling performance of different cultivated plants.

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