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Inoculation of *Capsicum annuum* L. and *Lactuca sativa* L. Plants with Local Bacillus Species for Evaluating the Protein Amount

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Abstract

Bacteria with plant growth promoting activity can produce metabolites for facilitating nutrient availability and uptake in plants. *Bacillus* species can colonize in root regions and form symbiotic relationships. They provide benefits as binding free nitrogen, dissolving phosphorus, and producing hormones for growth promotion. Local isolates of *Bacillus thuringiensis*, *B. subtilis*, *B. niratireducens* and *B. paramycoides* were coated to seeds of pepper and lettuce plants in greenhouse conditions according to randomized complete block experimental design with five replications in pots with five kg soil. Bacteria were incubated overnight in Luria Bertani medium at 37° C at 180 rpm until reaching $1x10^8$ CFU/ml. Subsequently, coated to surface sterilized seeds through incubating at 100 rpm for 30-45 min. The plants were harvested and kept at 65-70 °C until drying. Samples were pulverized for determining the amount of N using the Kjeldahl method. The amount of total protein content was also estimated by proportioning the amount of N. The N% in the roots of pepper was found to be 5.00 in control group, while the respective values were 6.14 and 6.04 in *Bt* SY49.1 and *Bs* PSY1 treated groups with considerable increase. Likewise, while the N % in shoot samples of pepper was 5.34% in control, it was 6.26 % in *Bt* SY49.1, 6.29 % in *Bn* PSY1, and 8.26 % in *Bp PSY1* treated groups. For root samples of lettuce, the amount was 6.64% in control, however, in *Bt* PSY1 and *Bs* PSY1 treated groups the results were around 8.00%. In lettuce shoot samples, N% was 12.42 in control, and 13.17, 12.87, and 13.12 % in groups treated with *Bn* PSY1, *Bt* PSY1, *Bs* PSY1, respectively. It is known that, nearly all N incorporate into protein structure in food, and can be deduced that the applied bacterial species cause considerable increase in protein amounts in plants.

Keywords: *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus paramycoides*, *B. nitratireducens*, Nitrogen

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1. INTRODUCTION

In recent years, the interest in sustainable agriculture has increased thanks to the realization that all kinds of resources, including soil, used in nourishing living things, are not endless and inexhaustible, and thus, residue-free production is aimed. PGPR bacteria, which is the abbreviation of 'Plant Growth Promoting Rhizobacteria', is important for sustainable agriculture in terms of promoting plant growth or being used as a biocontrol agent [1].

Bacteria that colonize the roots of plants and increase plant growth after inoculation into the seed are called 'rhizobacteria promoting plant growth' (PGPR) [2]. These bacteria include the species in *Bacillus, Lactobacillus, Paenibacillus, Arthobacter, Streptomyces, Pseudomonas, Burkholderia, Comamonas, Hydrogenophaga, Agrobacterium, Alcaligenes, Enterobacter, Pantoae, Enterobacter, Xanthomonas, Serratia, Rhizotobium, Bradyrbium bacterium, Azizobium* genera [3]. Among these, the most important bacterial groups are included in Pseudomonas, Bacillus, Enterobacter and Erwinia [2].

Plant pathogens originating from fungi or bacteria exist in the rhizosphere and can cause various plant diseases by obtaining nitrogen, which is essential for their survival, from the plant. PGPR bacteria mostly colonize in plant roots [3]. These bacteria live on the root surface and in the rhizosphere, stimulating plant growth directly or indirectly. They benefit the plants directly with their properties like nitrogen fixation, plant hormone production (Gibberellin and Cytokinin), reducing the effect of ethylene hormone, increasing phosphate solubility, increasing iron intake by binding iron to the plant root and indirectly benefit the plants through reducing the effect of harmful microorganisms [4]. In addition, Indoleacetic Acid (IAA) produced by bacteria can increase the effects of plant auxin and directly affect root growth by stimulating the plant's cell division and elongation [2].

It is known that Bacillus species constitute the majority of bacteria in the rhizosphere. İmriz et al. [5] reported in a study that the first commercial preparation was produced from the strain of *Bacillus subtilis* A-13 in the USA in 1985 as a biological control agent. Bacillus type of bacteria show antagonistic effect against plant pathogens as well as stimulating plant growth [6].

One of the crucial nutritional elements that plants need is nitrogen, as it is included in the building units of proteins. It is also found in enzymes, chlorophyll and vitamins. Although nitrogen is the main component of the atmosphere, plants can directly use very few of it. In order for living beings to use nitrogen; it must reduce the triple bond in the nitrogen molecule to the double bond and the nitrogen must combine with oxygen and hydrogen. This phenomenon is called nitrogen fixation. Only bacteria, blue-green algae and some fungi can directly use nitrogen in nature. Bacillus type of bacteria are among such kind of organisms [7].

Within the scope of this study, it was aimed to examine the effect of local Bacillus species on the uptake and utilization efficiency of N in root and shoot samples of Lettuce and Pepper plants. Also correspondingly estimating the protein contents in those samples.

2. MATERIALS AND METHODS

Pepper (*Capsicum annuum* L.) (BATEM Özge Hibrit Sivri Biber) and lettuce (*Lactuca sativa* L.) (Yedikule M5701 variety) seeds were used as experimental material. Local isolates of *Bacillus thuringiensis, Bacillus subtilis* and *Bacillus paramycoides* species were used to determine their effects on these plants.

2.1. Activation of bacteria from stock culture

B. nitratireducens PSY1, *B. thurungiensis* PSY1, *B. subtilis* PSY1 and *B. paramycoides* PSY1 isolates were activated from the stock culture by incubating them in LB agar medium for overnight at 37 ° C at 180 rpm. Then they were transferred to LB medium and incubated

overnight at the same conditions till obtaining $1x10^8$ CFU / ml.

2.2. Surface sterilization and inoculation of seeds

For coating trials, Pepper and Lettuce seeds were surface sterilized with 70% ethanol and 1% sodium hypochlorite. After drying, the seeds were kept at 100 rpm in a shaking incubator for 30-45 minutes in a bacterial solution containing 0.1% sucrose. Seeds were dried on a blotter paper in laminar flow for 1 hour and planted in pots.

2.3. Trial pattern

The trials were carried out under greenhouse conditions in 5 replicates for *B. thuringiensis* SY49.1, *B. nitratireducens* PSY1, *B. thuringiensis* PSY1, B. *subtilis* PSY1, and *B. paramycoides* PSY1 and control group. The experimental soil was composed of 1/2 garden soil, ¼ peat and ¼ pumice (sandy-loam; pH 7.83, 1: 2.5v / v; organic matter 1.86; lime 1.59%; P2O5 kgP2O5 / da EC 0.104). The experiments were carried out under greenhouse conditions. Plants were watered periodically as needed. To the plants in each pot 6 g of 15-15-15 composite and 4 g of ammonium nitrate were applied. Lettuce and pepper samples were harvested when they reached a certain volume. The roots and stems of the harvested samples were separated and dried in the oven at 65-70 °C. The dried samples were ground and powdered for protein determination. Photos of the trials are given in figure 1 below.

Figure 1 Growing plants in greenhouse conditions

C: Transplanting seeds into pots

D: Pepper in pots

F: Lettuce in pots

2.4. Total protein determination with Kjeldahl method

Three basic steps are applied in the Kjeldahl method [8].

1. Incineration
2. Distillation
3. Titration

1g of sample was weighed into the incinerator tubes of the Kjeldahl device. 20 ml of sulfuric acid was added to kjeldahl tablet and burned in the device until there was no turbidity and particles for 1 hour. The incinerator tubes were then removed and cooled. After adding 50 ml of distilled water on it, the distillation phase was started. Nearly 70 ml NaOH solution is taken into incinerator tubes during distillation stage. Then, for the titration stage, 60 ml of 3% boric acid with (pH, 4.65) was added into 100 ml beakers. Subsequently the whole amount of distilled solution was added onto the boric acid in the beaker. In the titration stage, the mixture in the beaker was titrated with 0.1 M sulfuric acid till the pH comes to 4.65. During this process the amount of sulfuric acid used for titration stage was recorded. The amount of nitrogen percentage was calculated according to the following equation, and subsequently multiplied by the pre-determined factor 6.25 for estimating the percentage of protein amount in samples [3].

$$\% N = \frac{(V1 - V0) \times N \times 0.014}{m} \times 100$$

$$\% \boldsymbol{Protein} = \% \boldsymbol{N} \times \boldsymbol{F}$$

V1: The amount of sulfuric acid consumed in titration
V0: The amount of sulfuric acid spent in blank trial titration
N: The normality of sulfuric acid solution used in titration
m:The amount of food sample taken (g)
F: General protein coefficient of foods (6.25)

2.5. Statistical analysis

Analysis of variance (ANOVA) for the data were conducted using SPSS 13.0 (SPSS inc, 2001). Post-hoc analyses were performed with Duncan and Dunnett's T3 tests. P<0.05 was considered as significant [9].

3. RESULTS AND DISCUSSION

The Kjeldahl method is a well-known procedure for estimating the amount of nitrogen in the samples. Since almost all of the nitrogen in foods is added to the protein structure, the percentage of nitrogen is considered to be equivalent to the protein amount [8]. The protein amounts of the dried and ground pepper and lettuce samples estimated by the Kjeldahl method in the root and stem parts are given in Table 1.

Table 1 Percentage of protein content in root and shoot samples of pepper and lettuce

Bacillus spp. isolates	Pepper (protein%)		Lettuce (protein%)	
	Root	Stem	Root	Stem
Control	5±0,06^{a*}	5±0,34^{a}	6±0,64^{b}	12±0,42^{bc}
B. thuringiensis SY49.1	6±0,14^{b}	6±0,26^{b}	4±0,59^{a}	7±1,33^{a}
B. nitratireducens PSY1	5±0,58^{a}	6±0,29^{b}	5±0,36^{ab}	13±0,17^{c}
B. thuringiensis PSY1	3±0,45^{-}	6±0,34^{b}	8±0,32^{c}	12±0,87^{bc}
B. subtilis PSY1	6±0,04^{ab}	5±0,14^{a}	8±0,23^{c}	13±0,12^{c}
B. paramycoides PSY1	5±0,49^{a}	8±0,26^{c}	6±0,95^{b}	9±0,45^{ab}

*Values indicated by different letters in the same column are statistically different.

As seen in Table 1, the protein content of root samples of pepper treated with *B. thuringiensis* SY49.1 (P=0.038) and *B. subtilis* PSY1 (P=0.043) was considerably high compared to the control group. In the samples treated with *B. nitratireducens* PSY1, and *B. paramycoides* PSY1, there was no difference compared to the control. *B. thuringiensis* SY49.1 (P=0.031), *B. nitratireducens* PSY1 (P=0.030), *B. thuringiensis* PSY1 (P=0.031), *B. paramycoides* PSY1 (P=0.00) caused remarkable increase in N amount in stem samples compared with control. There was no significant difference in *B. subtilis* PSY1 treated samples compared to the control group.

In root samples of lettuce, it was clear that, *B. thuringiensis* PSY1 (P=0.029), and *B. subtilis* PSY1 (P=0029) isolates caused an increase in percentage of N and consequently the protein amount compared to control group.

In stem samples of the same plant *B. nitratireducens* PSY1 (P=0.26), and *B. subtilis* PSY1 (P=0.26) isolates resulted in higher N content compared to control. PGPR is known to increase enzyme and hormone levels in plants. Thus, the increase in protein amount is related with enzyme and hormone production. In present study, it was clear that the corresponding increases in N and protein amounts were directly related with the PGPR activity of bacterial species. The differences in nitrogen content in root and stem samples is the indication of high protein synthesis in vegetative parts and thus growth promotion.

The protein contents of sources from vegetables were specified and indicated that green leafy vegetables contain high amount of protein [10]. Li et all. [11] stated that the protein content of lettuce samples was between 18-26% of dry weigh. In another study Yardım [12] reported that the amount of nitrogen in soil and plant samples were around 3.06% in tomato leaf and 2.92% in peach leaf. In a study carried out with *Tribulus terestris L.*, the total amount of protein was estimated as 14.48% using the Kjeldhal method. For producing bacterial cellulose from the peels of various fruits and vegetables, it was reported that cucumber, melon, kiwi, tomato, apple, quince, and pomegranate contained 21.25, 4.93, 10.14, 13.46, 2.43, 3.12, 4.18% protein,

determined with Kjeldhal method, respectively [13].

Breedt et al. [14] examined the yield improvement efficiency of five different bacterial isolates in corn plant in different soil types and reported that nitrogen fixation and consequently the yield was improved [14]. When the corn plants under salinity stress and normal conditions were treated with *Serratia liquefaciens* KM4, total free amino acids, soluble proteins and proline amounts were significantly increased [15]. In another study, cyanobacteria and PGPR were applied to rice in field conditions and caused 73% increase in N content. In the same study, *Providencia sp.* PW5 caused 25% increase in N content in wheat [16].

In conclusion, the local bacterial isolates harboring the genes for plant growth promoting activity can be used for developing new formulations after testing in field conditions and on other plant species.

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Author contribution

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This study does not require ethics committee approval or any special permission.

The Declaration of Ethics Committee Approval

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The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science."

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