ACTA SCIENTIARUM POLONORUM
Acta Sci. Pol. Hortorum Cultus, 19(5) 2020, 23–35

https://czasopisma.up.lublin.pl/index.php/asphc ISSN 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2020.5.3

ORIGINAL PAPER

Accepted: 3.09.2019

INOCULATION WITH N_2-FIXING PLANT GROWTH PROMOTING RHIZOBACTERIA TO REDUCE NITROGEN FERTILIZER REQUIREMENT OF LETTUCE

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ABSTRACT

This study was undertaken to determine the effect of three different mixtures of some N_2-fixing plant growth-promoting rhizobacteria (PGPR) on growth, yield, element content and nitrate accumulation as well as the effect on the reduction of nitrogen fertilization of lettuce (*Lactuca sativa* L.). The measurements were made in two separate experiments in 2015 between 6 June – 5 August (Experiment 1) and 2 July – 3 September (Experiment 2) under the field conditions. Butterhead form and heat tolerant summer cultivar 'Luna' was used as a plant material. *Agrobacterium rubi* RK-34, *Pantoea agglomerans* RK-79 and RK-92, *Pseudomonas putida* RK-142 and TV-42A, *Bacillus megaterium* TV-6D, TV-60D and TV-91C, *Pseudomonas flourescens* TV-11D and *Paenibacillus polymyxa* TV-12E were used as N_2-fixing plant growth promoting rhizobacteria. The treatments were 150 kg N/ha (available dose of AS) as ammonium sulphate (AS) [$(NH_4)_2SO_4$), (21% N)] and three different mixtures of PGPR. Further, combined uses of decreasing doses of AS (50%, 75 kg ha^{-1}AS and 75%, 112.5 kg ha^{-1} AS) and PGPR mixtures (M) such as M-1 + 75 AS, M-1 + 112.5 AS, M-2 + 75 AS, M-2 + 112.5 AS, M-3 + 75 AS and M-3 + 112.5 AS were used as additional treatments. All treatments increased the yield and the growth of lettuce according to the control. While inoculation with PGPR mixtures decreased the accumulation of heavy metals such as Cd, Ni, and Pb in lettuce, increased nutrient uptake of lettuce. It was determined that the nitrate accumulation of lettuce (cv. 'Luna') in PGPR mixtures were lower than the available dose of AS but higher than control. The yield in M-3 + 112.5 AS (48431 kg ha^{-1}) was similar and in the same statistical group with the available dose of AS (48225 kg ha^{-1}) in both experiments. Furthermore, according to the results of cost analyses, using 25% less of AS (112.5 kg ha^{-1}) with M-3 will supply the same income instead of using AS (150 kg ha^{-1}). It can be clearly said that the mixtures with some N_2-fixing plant growth promoting rhizobacteria (PGPR), especially M-3 (*P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C), have a great potential to decrease the nitrogen use (25%) for environmentally friendly crop production of lettuce.

Key words: biofertilizer, benefit/cost ratio, *Lactuca sativa* L., net profit, rhizobacteria, yield

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is a member of *Asteraceae* family, a major world salad crop [Jeffrey 2007] which has been cultivated since 4500 BC in the Mediterranean area and is a source of vitamins, nutrients which are highly required for human health [Chamangasht et al. 2012]. It thrives best in cool growing

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environments and can be grown in most temperate regions by selecting proper cultivars for the particular climate. Approximately 80% of lettuce growth occurs during the 3 to 4 weeks before harvest. The majority of the plant's nutritional requirements occurred during this period. Lettuce requires a well-watered and well-fertilized soil for high yield because it has a weak and shallow root system [Decoteau 2000].

Nitrogen is one of the essential key nutrients and is the world's largest used agricultural chemical. Nitrogen forms are widely used in vegetable production worldwide. However, farmers have increased the application of N fertilizers to their land year by year without considering the response of different species of N rate and forms. Whereas, a major drawback of fertilizer use, in particular excess use of N beyond the crops' needs, leads to a negative implication for the environment, especially groundwater pollution, health hazards and increases risk of chemical spills. Additionally, the production of chemical fertilizers is a highly energy-intensive process uses large amounts of fossil energy. High-input farming practices for achieving high yields have created environmental problems and degradation in natural resources [Şahin et al. 2004, Korkmaz et al. 2008, Abramovic et al. 2018]. Therefore, efforts are being made to replace chemical fertilizers with environmentally friendly and cost-effective resources such as plant growth promoting rhizobacteria (PGPR). It is known that alternative fertilization methods such as PGPR have gained importance for both healthy production of crops such as vegetables and healthy environment in the recent years.

The important limiting nutrient for lettuce growth and yield has been N, which has resulted in the increased use of fertilizer inputs. However, the principle of sustainable agriculture is low input and high output. Nitrogen fixation by microorganisms is a fascinating biological phenomenon which has been extensively studied in the last hundred years with the sole objective of harnessing its potential to provide low-cost nitrogen to increase crop productivity [Shantharam and Mattoo 1997]. Şahin et al. [2004] and Orhan et al. [2006] reported that bio-fertilizing with PGPR has gained importance in sustainable and environment friendly crop production, can overcome the adverse effects of chemical fertilization on the environment and can reduce the use of chemical fertilizer.

Long back, pioneer work of Kloepper and Schroth [1978] stated that microbial communities can be used to promote plant growth, yield and quality which have been called 'plant growth promoting rhizobacteria' (PGPR). PGPR plays an important role in plant growth by one or more mechanisms for direct plant growth promotion such as nitrogen fixation, phosphate solubilization, secreting of plant growth regulators [Kaymak 2010]. In other words, PGPR can be used as bioprotectant, biostimulant or biofertilizer to promote plant growth by increasing length and number of roots, as well as of chlorophyll production [Vessey 2003, Mohite 2013, Ruzzi and Aroca 2015].

Azospirillum is the first species suggested to promote the growth of plants by N_2-fixation [Bashan et al. 2004]. More examples can be given for N_2-fixing with PGPR such as *Pseudomonas putida* btyp B C3/101 and *Paenibacillus polymyxa* RC105 and RC14, *Bacillus cereus* RC18, *Bacillus licheniformis* RC08, *Bacillus megaterium* RC07, *Bacillus subtilis* RC11, *Bacillus* OSU-142, *Bacillus* M-13, *Pseudomonas putida* RC06 [Çakmakçı et al. 2007, Kaymak et al. 2013]. Also, *Pseudomonas* and *Bacillus* species [Kokalis-Burelle et al. 2002] and the other forms of PGPR species have gained attention in the recent years, because of their beneficial effects for sustainable and environment friendly production of vegetables and other crops. Furthermore, Vessey [2003] and Lai et al. [2008] declared that the uses of free-living PGPR as inoculants for a variety of crops are important and useful for crop productivity and environmental restoration.

Combined inoculations with both N_2-fixing and phosphate solubilizing bacteria were more effective than using single microorganisms, provides more balanced nutrition for plants [Şahin et al. 2004]. Therefore, this study was undertaken to determine the effect of three different mixtures of some N_2-fixing plant growth-promoting rhizobacteria (PGPR) on growth, yield, element content and nitrate accumulation as well as the effect on the reduction of nitrogen fertilization of lettuce (*Lactuca sativa* L.) under the field conditions.

MATERIALS AND METHODS

This study was conducted at Ataturk University, Agriculture Faculty, Erzurum, Turkey, in 2015 be-

tween 6 June – 5 August (Experiment 1) and 2 July – 3 September (Experiment 2). Butterhead form and heat tolerant summer cultivar 'Luna' (*Lactuca sativa* L.) was used as a plant material. Seedlings of cv. 'Luna' were supplied by ATLAS Seedling Co. Ltd. (Adana, Turkey).

The soil of the experiment area was clay loam texture (clay 36%, silt 35%, and sand 29%). Ustorthent great soil group with neutral pH (7.5) and EC 317 µmhos cm^{-1}. It had 1.90% organic matter, 23.30 cmol kg^{-1} Ca, 2.25 cmol kg^{-1} Mg, 1.27 cmol kg^{-1} K, 0.28 cmol kg^{-1} Na, 41.37 mg kg^{-1} P and 0.082% total N.

The bacterial strains used in this study were obtained from the culture collection unit of the Department of Plant Protection, Faculty of Agriculture at Atatürk University. The bacterial strains had been isolated from the rhizosphere of wild or cultivated plants growing in the eastern Anatolia region of Turkey. Hypersensitivity tests of all of the bacterial strains used in this work were made on tobacco plants according to the Klement et al. [1964]. The list of N_2-fixing and phosphate solubilizing plant growth promoting rhizobacteria strains used in this experiment and their some biochemical characteristics were shown in Table 1.

Before its use, the bacteria strains were hold in nutrient broth (NB) with 15% glycerol at –80ºC for long term storage and a colony from stock bacteria cultures were transferred to 500 ml flasks containing nutrient broth. Then, the bacteria strains were grown aerobically in the flasks on a shaker (rotating at 150 rpm) for 24 h at 27ºC. A UV-visible spectrophotometer (Shimadzu, Japan, UV 1201, SN A1080) were used to check the purity whether there is a contamination or not and the optical density of bacterial suspensions.

Table 1. Some biochemical characteristics of the bacterial strains used in this experiment

Bacterial strains	Isolated from	NF	PS	HR
Agrobacterium rubi RK-34	cherry	–	+	–
Pantoea agglomerans RK-79	apple	+	+	–
Pantoea agglomerans RK-92	pear	+	s+	–
Pseudomonas putida RK-142	pear	w+	w+	–
Bacillus megaterium TV-6D	wheat	+	+	–
Pseudomonas flourescens TV-11D	sugar beat	+	+	–
Paenibacillus polymyxa TV-12E	wheat	s +	+	–
Bacillus megaterium TV-60D	sugar cane	+	–	–
Bacillus megaterium TV-91C	wheat	+	w+	–
Pseudomonas putida TV-42A	wheat	w+	w+	–

+: positive reaction, –: negative reaction, s+: strong positive reaction, w+: weak positive reaction, NF: nitrogen fixation, PS: phosphate-solubilising activity, HR: hypersensitivity reaction

Table 2. PGPR mixtures used in this study

PGPR mixtures	Used bacterial isolates				
PGPR mixture 1	M-1	RK-79	RK-34	TV-6D	TV-42A
PGPR mixture 2	M-2	RK-92	TV-12E	TV-60D	
PGPR mixture 3	M-3	RK-142	TV-11D	TV-91C	

+: positive reaction, –: negative reaction, s+: strong positive reaction, w+: weak positive reaction, NF: nitrogen fixation, PS: phosphate-solubilising activity, HR: hypersensitivity reaction

Finally, obtained bacterial suspensions were diluted by using sterile distilled water. The final concentration of bacterial suspensions was 10^8 cfu/ml. The resulting suspensions (10^8 cfu/ml) of bacteria strains were used to prepare three PGPR mixtures (Tab. 2) and used to treat lettuce (cv. 'Luna') seedlings. Control seedlings were only treated with tap water before transplanting. All seedlings were held in each resulting suspension of PGPR mixtures and tap water for 45 min. There were no PGPR mixtures and N fertilizers applied in the control plots.

There were 15 lettuce seedlings that were transplanted on plots of 4 m^2 in the field, in rows of 160 cm long, at an inter-row spacing of 40 cm and intra-row spacing of 60 cm. The plants were irrigated on need basis (2 or 3 times a week) with furrow irrigation. All of the packages of growing practices such as irrigation, hoeing have been followed during the development phases.

All plots were received 100 kg P_2O_5/ha as triple superphosphate. Other treatments were 150 kg N/ha (available dose of AS) as ammonium sulphate (AS) [$(NH_4)_2SO_4$), (21% N)] and three different mixtures of PGPR. Further, the treatments were used in combination of decreasing doses of AS (50%, 75 kg ha^{-1}AS and 75%, 112.5 kg ha^{-1} AS) with PGPR mixtures. The combinations were PGPR mixture 1 + 75 kg ha^{-1} AS (M-1 + 75 AS), PGPR mixture 1 + 112.5 kg ha^{-1} AS (M-1 + 112.5 AS), PGPR mixture 2 + 75 kg ha^{-1} AS (M-2 + 75 AS), PGPR mixture 2 + 112.5 kg ha^{-1} AS (M-2 + 112.5 AS), mixture 3 + 75 kg ha^{-1} AS (M-3 + 75 AS) and PGPR mixture 3 + 112.5 kg ha^{-1} AS (M-3 + 112.5 AS). All of the P_2O_5 and half of N fertilizer were applied uniformly prior to planting and the remaining half of N was given 20 days after the planting onto the soil surface by hand and incorporated in all treatments [Güvenç et al. 2006].

In two of the experiments, the plants were harvested 60 days after transplanting. Their weights, diameters, length of their heads, dry weight of leaves and the yields were recorded. All the observations were made from randomly selected 12 plants out of 15 for each replication. In addition, the effect of the treatments for the element content and nitrate accumulation of the lettuce were evaluated.

The mineral contents of the lettuce samples were determined by using the methods of Mertens [2005a, 2005b]. Firstly, randomly selected lettuce leaf samples (250 to 300 g fresh weight) were dried at 68°C for 48 h in an oven and homogenized by using 1 mm sieve. The element content of lettuce leaves was determined by using a HNO_3–H_2O_2 acid mixture (2 : 3, v/v) in three steps (1st: 145°C, 75% RF, 5 min; 2nd: 180°C, 90% RF, 10 min; 3rd: 100°C, 40% RF, 10 min) in a microwave oven after wet digestion of dried and ground subsamples. Then, inductively Couple Plasma spectrophotometer was used to detect the contents of P, K, Ca, Mg, Na, Fe, Cu, Mn, Zn, B, Cd, Ni and Pb in the samples. Nitrogen content of the samples were determined by using the Kjeldahl method [Bremner 1996]. Finally, reflectoctoquant nitrate test (Merckoquant nitrate test 1.16995.0001) was used to determine NO_3-N in the lettuce samples.

At the end of the study, the economic analyses were made for all of the treatments including control treatment of the lettuce production for the field conditions. Gross production value, net profit and benefit/cost ratios were calculated and added into economic analyses. While the economic analyses were made, the data of Experiment 1 and Experiment 2 were combined and used.

The experimental design of these experiments was a completely randomized block design with 3 replications. ANOVA was applied to the data obtained in this study and Duncan's multiple range tests was used to compare the differences between the means. There were no statistical differences between the means of Experiment 1 and Experiment 2 for the elemental analyses; for this reason the data of the element contents and nitrate accumulation were combined and used in statistical analyses.

RESULTS AND DISCUSSION

The data for different treatments illustrating head diameter and length of cv. 'Luna' are presented in Table 3. The mean value of head diameter and length of cv. 'Luna' varied depending on the treatments. In both of the experiments the lowest value of head diameter and length were determined in control. When only bacteria mixtures are taken into consideration, the obtained results from both of the experiments were for head diameter range between 25.3 cm (M-2) and 26.7 cm (M-1) and, the results for head

Table 3. The effects of PGPR and nitrogen fertilization on head diameter and length of lettuce

PGPR mixtures		Experiment 1	Experiment 2	Mean
	treatments	head diameter (cm)		
	control	25.5 d	24.5 d	25.0 D
	ammonium sulphate (AS) 150 kg ha^{-1}	28.1 ab	28.7 a	28.4 A
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	26.7 c	26.0 bcd	26.3 BCD
	M-1 + 75 AS	27.4 bc	27.6 ab	27.5 AB
	M-1 + 112.5 AS	28.0 ab	28.6 a	28.3 A
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B. megaterium* TV-60D	M-2	26.2 cd	25.3 cd	25.7 CD
	M-2 + 75 AS	27.2 bc	27.3 abc	27.2 ABC
	M-2 + 112.5 AS	28.1 ab	28.0 ab	28.1 A
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	26.3 cd	26.6 abc	26.5 BCD
	M-3 + 75 AS	27.3 bc	27.9 ab	27.6 AB
	M-3 + 112.5 AS	28.8 a	28.9 a	28.8 A
		head length (cm)		
	control	30.5 f	30.7 f	30.6 G
	ammonium sulphate (AS) 150 kg ha^{-1}	35.3 ab	37.6 ab	36.5 A
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	31.8 ef	33.0 de	32.4 F
	M-1 + 75 AS	33.5 cd	34.6 cd	34.0 DE
	M-1 + 112.5 AS	34.9 abc	35.7 bc	35.3 BC
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B.megaterium* TV-60D	M-2	33.4 d	32.1 ef	32.8 F
	M-2 + 75 AS	34.0 bcd	34.3 cd	34.1 DE
	M-2 + 112.5 AS	35.5 a	35.7 bc	35.6 AB
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	32.6 de	33.8 cde	33.2 EF
	M-3 + 75 AS	33.7 cd	34.9 c	34.3 CD
	M-3 + 112.5 AS	34.9 abc	37.2 ab	36.1 AB

* Means with different letters on the column are significantly different at $P = 0.05$

length range between 31.8 cm (M-1) and 33.8 cm (M-2) – Table 3. On the other hand, the higher values of head diameter and length were determined in single usage of Ammonium Sulphate (AS) and bacteria mixtures with decreasing doses of AS. For example, the head diameter was changed to 28.1 cm in Experiment 1 and 28.7 cm in Experiment 2 from the available dose of AS, respectively. Similarly, the head diameter was changed to 27.2 cm in M-2+75 AS and 28.9 cm in M-3 + 112.5 AS. In other words, the values of head diameter and length obtained from the M-1 + 112.5 AS, M-2 + 112.5 AS and M-3 + 112.5 AS have similar results with using 150 kg ha^{-1} AS and also are in the same statistical group.

Results indicated that all of the treatments increased head diameter and length when compared with control. Furthermore, the results of the experiments indicated that the available dose (150 kg ha^{-1}) of nitrogen can be decreased by 25% with using PGPR mixtures for the highest plant diameter and length. A previous study declared that biofertilizers such as *Azospirillum* increased plant height of lettuce [Gasoni et al. 2001]. Similarly, Maroniche et al. [2016] reported that some *Pseudomonas* strains (LSR1, ZME4 and TAR5) promoted lettuce growth by inducing a significant increase in the length and fresh weight. Kaymak et al. [2013] declared that inoculation with *P. polymyxa* RC105 and *P. putida* C3/101 significantly increased the shoot length and diameter of mint. Kohler et al. [2006] also reported that the largest effect on the growth of lettuce was observed in the fertilization treatment, alone or in combination with *Pseudomonas mendocina*. Based on recent studies, it is known that PGPR can affect growth in a number of ways such as nitrogen fixation, phosphate solubilization and enhancement of vegetative growth. The effect of PGPR is documented for a range of crops such as vegetables. The plant growth promoting effects of bacteria used in this study for lettuce could be explained by similar reasons in the mentioned work.

There are significant differences between the treatments concerning the average leaf dry matter content in both experiments (Tab. 4). M-2+112.5AS provided the highest leaf dry matter content (5.09%) in Experiment 1. The lowest leaf dry matter content (4.19%) was determined in M-1 + 75AS. Flores-Félix et al. [2013] reported that the dry weight of lettuce was significantly increased for the plants inoculated with *Rhizobium leguminosarum* L. strain PEPV16, compared with uninoculated ones. Similarly, Chabot et al. [1996] inoculation of lettuce with *Serratia* sp. 22b or *R. leguminosarum* bv. *phaseoli* R1 increased the dry matter yield of lettuce. Plus, *Azosprillium* and *Azotobacter* increased leaf dry weight by about 43.95% compared with the control [Chamangasht et al. 2012].

Orhan et al. [2006] and Kaymak et al. [2013] declared that it was the best way to understand the importance of the effect of bacterial inoculations in plant nutrient element uptake was to determine the element content of plant leaves treated with plant growth promoting rhizobacteria (PGPR). Therefore, the effect of PGPR mixtures, ammonium sulphate and combined uses of PGPR mixtures and AS for the plant nutrient element uptake of cv. 'Luna' was given in Table 5. In this study, it was found that bacterial treatments and AS significantly increased the element content of the lettuce compared with control except for Na, Cu, Cd, Ni and Pb. When the Cd, Ni, and Pb were taken into consideration, it is known that these elements are heavy metals and hazardous for human health. Inoculation with PGPR mixtures decreased the accumulation of heavy metals in cv. 'Luna'. In addition, the highest P (2004 mg kg^{-1}), K (43665 mg kg^{-1}), Mg (7837 mg kg^{-1}), Fe (541 mg kg^{-1}), Cu (42.80 mg kg^{-1}), Ca (30298 mg kg^{-1}), Mn (57.67 mg kg^{-1}) and Zn (52.33 mg kg^{-1}) contents were obtained from M-1 + 112.5 AS, M-2 + 112.5 AS, M-2 + 75 AS, M-2 + 112.5 AS, M-1 + 75 AS, M-2 + 112.5 AS, M-3 + 112.5 AS, and M-3 + 75 AS, respectively, when compared with control and the available dose of AS. While the lowest percentage of N was determined in control (2.76%), and the highest N percentage was obtained from the available dose of AS (3.74%). The nitrate accumulation in lettuce showed a difference according to the treatments (Tab. 4). The effect of the treatments for the nitrate accumulation was found statistically significant. While the lowest nitrate accumulation was obtained in control (1132 mg kg^{-1}), and the highest nitrate accumulation was in the available dose of AS (1510 mg kg^{-1}). The nitrate accumulation of cv. 'Luna' in the combined treatments of decreasing doses of AS with all PGPR mixtures were higher according to the control, and it was lower than the available dose of AS (Tab. 4).

There are many studies on beneficial relationships between PGPR and nutrient uptake. For instance, PGPR

application (*Pseudomonas fluorescens* biotype G) significantly enhanced N, P and K uptakes [Naveed et al. 2008]. Similarly, inoculation with *Pseudomonas polymyxa* RC105 and *Paenibacillus putida* C3/101 significantly increased element contents of mint leaves compared with the control [Kaymak et al. 2013]. Kohler et al. [2006] reported that inoculation with *P. mendocina* had a significant effect on the dehydrogenase and phosphatase activities, 21 and 89%, respectively, compared with the control. In another report phosphate solubilizing microorganisms, *R. leguminosarum* bv. *phaseoli* strains P31 and R1, *Serratia* sp. strain 22b, *Pseudomonas* sp. strain 24 and *Rhizopus* sp. strain 68 were examined for their plant growth-promoting potential on lettuce and it was determined that 2 strains of *R. leguminosarum* bv. *phaseoli* solubilizing soil P can stimulate the growth of lettuce under the field conditions [Chabot et al. 1996]. It has also been indicated that mineral ions uptake of plants via stimulation of the proton pump ATPase can be increased by PGPR [Mantelin and Touraine 2004]. According to Orhan et al. [2006] report, this increase may also explained by organic acids production by plants and bacteria in the rhizosphere, which decreases soil pH and stimulate the availability of elements. The bacterial effect on plant growth can be attributed to an increase in nutrient availability in the rhizosphere [Kohler et al. 2006]. These findings in this study were supported by a number of previous studies [Chabot et al. 1996, Mantelin and Touraine 2004, Orhan et al. 2006, Kohler et al. 2006, Naveed et al. 2008, Kaymak et al. 2013].

Table 4. The effects of treatments on dry matter content (%) of leaves of lettuce

PGPR mixtures		Experiment 1	Experiment 2	Mean
	treatments	dry matter content (%)		
	control	4.90 ab	4.84 ab	4.87 AB
	ammonium sulphate (AS) 150 kg ha^{-1}	4.90 ab	4.78 abc	4.84 AB
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	4.41 ab	4.54 a-d	4.48 BCD
	M-1 + 75 AS	4.57 ab	4.19 d	4.38 CD
	M-1 + 112.5 AS	4.29 b	4.37 bcd	4.33 D
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B.megaterium* TV-60D	M-2	4.53 ab	4.30 cd	4.41 CD
	M-2 + 75 AS	5.05 ab	4.99 a	5.02 A
	M-2 + 112.5 AS	5.09 a	4.93 a	5.01 A
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	4.67 ab	4.92 a	4.79 ABC
	M-3 + 75 AS	5.04 ab	4.71 abc	4.87 AB
	M-3 + 112.5 AS	4.88 ab	5.05 a	4.96 A

* Means with different letters on the column are significantly different at $P = 0.05$

The experiments in this study showed that the treatments with some N_2-fixing PGPR mixtures, combinations of decreasing doses of ammonium sulphate (AS) and PGPR mixtures, and the available dose of AS affected the lettuce head weight and yield (Tab. 6). It was clear to say that all the treatments significantly increased head weight and yield of the lettuce compared with the control. The lowest head weight and yield were determined in control for both of the experiments. The head weight ranged between 869 g (control) and 1300 g (M-3 + 112.5 AS). Similarly, the lowest yield determined in control (32588 kg ha^{-1}) and the

Table 5. The effect of treatments on element contents of leaves of lettuce

PGPR mixtures		N*	NO_3	P	K	Ca
	control	2.76 g	1132 d	1578 d	35200 ef	22032 fg
	ammonium sulphate (AS) 150 kg ha^{-1}	3.74 a	1510 a	1636 d	36946 cde	27156 bcd
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	2.98 f	1312 a-d	1915 b	35710 de	28119 abc
	M-1 + 75 AS	3.40 d	1208 cd	1980 a	32296 f	29202 ab
	M-1 + 112.5 AS	3.56 bc	1273 bcd	2004 a	37054 cde	30298 a
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B. megaterium* TV-60D	M-2	3.18 e	1382 abc	1968 ab	40795 ab	29217 ab
	M-2 + 75 AS	3.66 ab	1477 ab	1837 c	43356 a	25499 cde
	M-2 + 112.5 AS	3.71 a	1476 ab	1822 c	43665 a	24735 def
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	3.10 ef	1307 a-d	1844 c	40300 abc	27060 bcd
	M-3 + 75 AS	3.43 cd	1350 abc	1907 b	39005 bcd	24156 efg
	M-3 + 112.5 AS	3.55 bc	1388 abc	1788 c	37991 b-e	21743 g
		Mg	Na	Fe	Cu	Mn
	control	6867 c	270 b	470 d	37.86 b	50.87 d
	ammonium sulphate (AS) 150 kg ha^{-1}	6324 d	300 ab	477 bcd	38.87 b	62.81 a
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	7128 bc	309 a	474 cd	39.69 ab	52.15 cd
	M-1 + 75 AS	7040 bc	265 b	515 abc	42.80 a	51.15 d
	M-1 + 112.5 AS	7279 bc	296 ab	524 a	42.32 a	50.93 d
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B. megaterium* TV-60D	M-2	6881 c	276 ab	517 ab	39.91 ab	52.46 cd
	M-2 + 75 AS	7837 a	298 ab	528 a	37.61 b	51.51 d
	M-2 + 112.5 AS	7413 ab	297 ab	541 a	37.27 b	54.70 c
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	7333 bc	289 ab	540 a	38.71 b	54.57 c
	M-3 + 75 AS	7045 bc	281 ab	530 a	38.61 b	52.92 cd
	M-3 + 112.5 AS	7196 bc	303 ab	523 a	38.23 b	57.67 b
		Zn	B	Cd	Ni	Pb
	control	46.89 b	32.33 b	0.170 a	2.68 a	25.31 a
	ammonium sulphate (AS) 150 kg ha^{-1}	50.81	37.13 a	0.157 ab	1.62 ef	20.67 bc
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	52.27 a	36.81 ab	0.122 de	1.43 f	18.35 bc
	M-1 + 75 AS	50.91 ab	34.43 ab	0.123 de	1.68 de	17.49 bc
	M-1 + 112.5 AS	49.81 ab	38.96 a	0.129 c-e	1.84 c-e	17.87 bc
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B. megaterium* TV-60D	M-2	39.54 c	34.99 ab	0.116 e	1.84 c-e	16.82 c
	M-2 + 75 AS	50.92 ab	37.48 a	0.133 c-e	1.91 bc	19.05 bc
	M-2 + 112.5 AS	49.02 ab	36.08 ab	0.137 cd	1.98 b	21.78 ab
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	52.25 a	35.10 ab	0.138 cd	1.87 bc	17.85 bc
	M-3 + 75 AS	52.33 a	36.63 ab	0.143 bc	1.84 c-e	17.93 bc
	M-3 + 112.5 AS	48.40 ab	37.54 a	0.143 bc	2.00 b	19.35 bc

* %, the other elements are mg kg^{-1}, ** means with different letters on the column are significantly different at $P = 0.05$

Table 6. The effects of treatments on head weight (g) and yield (kg ha^{-1}) of lettuce

PGPR mixtures		Experiment 1	Experiment 2	Mean
	treatments		head weight (g)	
	control	895 d	869 f	882 F
	Ammonium sulphate (AS) 150 kg ha^{-1}	1287 a	1285 a	1286 A
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	1057 cd	987 e	1022 E
	M-1 + 75 AS	1196 abc	1073 cde	1135 CD
	M-1 + 112.5 AS	1297 a	1180 abc	1239 AB
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B. megaterium* TV-60D	M-2	1046 cd	1004 de	1025 E
	M-2 + 75 AS	1191 abc	1108 bcd	1150 BCD
	M-2 + 112.5 AS	1237 ab	1207 ab	1222 ABC
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	1080 bc	1049 de	1064 DE
	M-3 + 75 AS	1169 abc	1181 abc	1175 BC
	M-3 + 112.5 AS	1300 a	1283 a	1292 A
			Yield (kg ha^{-1})	
	control	33550 d	32588 f	33069 F
	Ammonium sulphate (AS) 150 kg ha^{-1}	48275 a	48175 a	48225 A
M-1 = *A. rubi* RK-34 + *P. agglomerans* RK-79 + *B. megaterium* TV-6D + *P. putida* TV-42A	M-1	39638 cd	37013 e	38325 E
	M-1 + 75 AS	44838 abc	40250 cde	42544 CD
	M-1 + 112.5 AS	48650 a	44250 abc	46450 AB
M-2 = *P. agglomerans* RK-92 + *P. polymyxa* TV-12E + *B.megaterium* TV-60D	M-2	39213 cd	37650 de	38431 E
	M-2 + 75 AS	44650 abc	41563 bcd	43106 BCD
	M-2 + 112.5 AS	46388 ab	45263 ab	45825 ABC
M-3 = *P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C	M-3	40500 bc	39325 de	39913 DE
	M-3 + 75 AS	43825 abc	44300 abc	44063 BC
	M-3 + 112.5 AS	48750 a	48113 a	48431 A

* Means with different letters on the column are significantly different at $P = 0.05$

Table 7. Comparative cost and benefit analysis of some N_2-fixing plant growth promoting rhizobacteria use instead of fertilizer in lettuce production ($)

Application	Control	AS 150 kg ha^{-1}	M-1	M-1 + 75 AS	M-1 + 112.5 AS	M-2
1. Variable cost*	4063.4	4228.6	4080.7	4171.7	4208.6	4080.7
2. Capital interest (1 × 4%)	162.5	169.1	163.2	166.9	168.3	163.2
3.Total variable cost (1 + 2)	4226.0	4397.8	4243.9	4338.6	4377.0	4243.9
4. Soil rent	293.1	293.1	293.1	293.1	293.1	293.1
5. General management costs (3*3%)	126.8	131.9	127.3	130.2	131.3	127.3
6. Total fixed cost (4 + 5)	419.9	425.0	420.4	423.3	424.4	420.4
7. Total cost (3 + 6)	4645.9	4822.8	4664.3	4761.9	4801.4	4664.3
8. Yield (kg ha^{-1})	33069.0	48225.0	38332.0	42544.0	46450.0	38431.0
9. Selling price ($ da^{-1})	0.52	0.52	0.52	0.52	0.52	0.52
10. Gross production value (8*9)	17195.9	25077.0	19932.6	22122.9	24154.0	19984.1
11. Net profit ($ ha^{-1}) (10–7)	12550.0	20254.2	15268.3	17361.0	19352.6	15319.8
12. Benefit/cost ratio (10/7)	3.7	5.2	4.3	4.6	5.0	4.3

	M-2 + 75 AS	M-2 + 112.5 AS	M-3	M-3 + 75 AS	M-3 + 112.5 AS
1. Variable cost*	4171.7	4208.6	4080.7	4171.7	4208.6
2. Capital interest (1 × 4%)	166.9	168.3	163.2	166.9	168.3
3. Total variable cost (1 + 2)	4338.6	4377.0	4243.9	4338.6	4377.0
4. Soil rent	293.1	293.1	293.1	293.1	293.1
5. General management costs (3*3%)	130.2	131.3	127.3	130.2	131.3
6. Total fixed cost (4 + 5)	423.3	424.4	420.4	423.3	424.4
7. Total cost (3 + 6)	4761.9	4801.4	4664.3	4761.9	4801.4
8. Yield (kg ha^{-1})	43106.0	45825.0	39913.0	44063.0	48431.0
9. Selling price ($ da^{-1})	0.52	0.52	0.52	0.52	0.52
10. Gross production value (8*9)	22415.1	23829.0	20754.8	22912.8	25184.1
11. Net profit ($ ha^{-1}) (10–7)	17653.3	19027.6	16090.4	18150.9	20382.7
12. Benefit/cost ratio (10/7)	4.7	5.0	4.4	4.8	5.2

* Variable cost: seedling, plowing, irrigation, bacterium, fertilization, harvest and marketing

highest in M-3 + 112.5 AS (48750 kg ha^{-1}). The yield in the available dose of AS was 48275 kg ha^{-1}. When the mean values of Experiment 1 and 2 were taken into consideration, the percentage of yield increase from single PGPR mixtures according to the control were 16% in M-1 and M-2, and 21% in M-3. On the other hand, the percentage of yield decrease in single PGPR mixtures according to the available dose of AS were –21% in M-1, –20% in M-2 and –17% in M-3 (Tab. 6). However, the yield in combination of M-3 + 112.5 AS was similar and in the same statistical group with the available dose of AS in both of the experiments. It was clearly seen that the yield was 48 431 kg ha^{-1} in M-3 + 112.5 AS and 48225 kg ha^{-1} in the available dose of AS in the means of Experiment 1 and 2 (Tab. 6). Our results indicated that M-3 certainly reduce 25% of nitrogen requirement of lettuce.

Bashan et al. [2004] reported that *Azospirillum* was the first microorganism suggested to promote the growth of plants. Similarly, it was reported that *Azotobacter*, *Azoarcus*, *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Rhizobium*, etc. play an important role in plant growth by one or more mechanisms for direct plant growth promotion and yield such as nitrogen fixation, phosphate solubilisation, and secreting of plant growth regulators. On the other hand, previous reports have shown that the transfer of nitrogen fixed by PGPR to the plant is not enough and cannot fulfil all of the nitrogen requirements of the plants, nevertheless, contribute significant amounts of nitrogen. [Bashan et al. 2004]. For example, Kaymak et al. [2013] reported that yield of mint (*Mentha piperita* L.) obtained from bacteria inoculation (*P. polymyxa* RC105 and *P. putida* C3/101) was lower than urea treatment but more than control. Similarly, Gasoni et al. [2001] reported that *P. fluorescens* and *Bacillus pumilus* significantly promoted lettuce yield. Likewise, Chamangasht et al. [2012] declared that *Azotobacter*, *Azospirillum* and *Pseudomonas* strains increased lettuce yield compared with the control. Previous studies with PGPR were tested on lettuce and different species have been reported similar findings such as yield increase confirming results of this work. In addition to all of these previous reports on this subject, the most important outcome of our study, it can be clearly said that M-3 provides 25% of the nitrogen requirement of the lettuce (Tab. 6).

Unit cost of production land for lettuce, gross production value, net profit and benefit/cost ratios are given in Table 7. While the control has the lowest net profit (12550 $ ha^{-1}), and the highest net profit belongs to the M-3 + 112.5 AS with 20382.7 $ ha^{-1}. As the net profit of treatments are compared with the available dose of AS, M-3 + 112.5 AS makes the highest profit. When benefit/cost ratios are examined, similar results can be clearly seen from Table 7. According to benefit/cost ratio, for each dollar spent it is possible to make $5.2 income from both of the treatments M-3 + 112.5 AS and the available dose of AS. The results of cost analyses concluded that using M-3 + 112.5 AS will supply the same income with the available dose of AS. By using 25% less of AS, the lettuce producers will still make the same net profit instead of using the available dose of AS. This will let producers to make environment friendly and sustainable production.

CONCLUSION

Consequently, the general results clearly showed that single N_2-fixing PGPR mixtures, combinations with decreasing doses of ammonium sulphate (AS) and PGPR mixtures, and the available dose of AS were increased the yield and growth of lettuce according to the control. On the other hand, inoculation with PGPR mixtures decreased the accumulation of heavy metals such as Cd, Ni and Pb in lettuce, and nutrient uptake of lettuce were increased. It was determined that the nitrate accumulation of lettuce (cv. 'Luna') in PGPR mixtures was lower than the available dose AS but higher than the control. The yield in M-3+112.5 AS (48431 kg ha^{-1}) was similar and in the same statistical group with the available dose of AS (48225 kg ha^{-1}) in both experiments. Furthermore, according to the results of cost analyses, using M-3+112.5 AS will supply the same income instead of using the available dose of AS (150 kg ha^{-1}). As a result, the some N_2-fixing PGPR mixtures, especially PGPR mixture 3 (*P. putida* RK-142 + *P. flourescens* TV-11D + *B. megaterium* TV-91C), have a great potential to decrease the nitrogen requirement (25%) of lettuce for environmental friendly crop production.

SOURCE OF FUNDING

The study was funded by Atatürk University, Faculty of Agriculture, Department of Horticulture.

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