

Mitigation of N and P leaching from irrigated wheat area as influence plant growth promoting rhizobacteria (PGPR)

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Abstract

Uses of bio-fertilizers containing beneficial micro organisms instead of synthetic chemicals are known to improve plant growth through the supply of plant nutrients and may help to sustain environmental health and soil productivity. This study was conducted to determine effects of PGPB and chemical fertilizer application on the growth of wheat and the leaching loss of nitrate, ammonium and phosphate. For this aim, a pot experiment with nine treatments: control, *Bacillus OSU-142* (BA-142), *Bacillus M-13* (M-13), BA-142 + M-13, NH_4NO_3 (AN) fertilizer, $(\text{NH}_4)_2\text{SO}_4$ (AS) fertilizer, Triple superphosphate (TSP) fertilizer, AN + TSP, AS + TSP were carried out. Results showed that BA-142 can work on this high initial available nitrogen soil and increase the nitrogen loss, ranged 124 to 193%, but reduce the nitrogen loss compared to chemical nitrogen fertilizers application by 53.8 to 70.2%. The application of phosphate solubilizing bacteria, M-13, significantly increased the concentration of soil available P and the P concentration and dry matter production of wheat shoot. This application also increased the leaching loss of P by 37.4 to 57.3 %, compared to treatments with no chemical phosphate fertilizer and this bacteria application. However, this application system reduces the leaching loss P by 52.0 to 53.1 %, compared to the treatments with chemical phosphate fertilizer applied.

Keywords: Entisol, bio-fertilizer, nitrogen, leaching, phosphorus, plant uptake, wheat

Introduction

An appropriate application of fertilizers guarantees the high yield and quality of agricultural products. Nitrogen and phosphorus fertilizers are the two most often used fertilizers in quantity. The energy consuming in the production of their chemical fertilizers are very high, especially the energy consuming of using nitrogen fertilizers occupied more than 50 % of whole agricultural energy consuming in European [1]. While N and P fertilizers have positive contribution in agricultural production, they have been concerned in water pollution. Since the point-source of N and P have been reduced by wastewater treatment, the contribution of N and P pollutants from the agricultural sectors become the major sources [2]

The pollution of NO_3^- in groundwater is an international problem Ünlü et al., [3] reviewed NO_3^- pollution in agricultural land and groundwater in Turkey. Their data showed that there was a rising trend in NO_3^- concentrations over time. Some of the agricultural lands in Turkey, NO_3^- concentration is already higher than the WHO [4] recommended level which is set at 50 mg l⁻¹ for drinking water.

Nitrate pollution of groundwater in Erzurum and Hasankale plain, in eastern part of Turkey, is of particular concern because of the proximity to environmentally sensitive areas and a large number of people in city and rural areas relying on using groundwater as drinking water. Over use of N- P fertilizer and poor irrigation managements may lead to NO_3^- and P leaching and polluting the groundwater. Several studies documented adverse effects of high NO_3^- levels,

most notably methemoglobinemia, stomach cancer and non-Hodgkin's Lymphoma [5]. So far a considerable number of bacterial species mostly associated with the plant rhizosphere, have been tested and found to be beneficial for plant growth, yield and crop quality. They have been called "plant growth promoting rhizobacteria" including the strains in the genera *Cinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* [6]. In previous studies, it was found that PGPR could stimulate growth and increase yield in mulberry, apricot, sweet cherry and raspberry [7, 8, 9, 10,]. N_2 -fixing and P-solubilizing bacteria are the two most important function modes of bio-fertilizers because they have been proved to decrease the use of chemical fertilizers.

Our objectives were to determine the impact of bio- and chemical fertilizer application on nitrate, ammonium and phosphate leaching, yield and nutrient content of wheat grown in the common Entisol Erzurum province in Turkey.

Materials and methods

Initial soil sampling and characterization

Soil was collected from the Ap horizon (0-20 cm) of an Entisol with parent materials mostly consisting of volcanic, marn and lacustrin residual and transported material in Erzurum province (39° 55' N, 41° 61' E), Turkey. Mean annual temperature, precipitation, evaporation and relative humidity in the study area are 6.3°C, 498 mm, 1060 mm and 64%, respectively. Soil was air-dried indoors, and then crumbled to pass through a 4 mm sieve for the pot experiment and a 2 mm sieve for chemical and physical analyses. Particle size distribution, cation exchange capacity (CEC), Kjeldahl-N, plant-available P, pH and calcium carbonate, organic matter (OM), exchangeable K, Ca, Mg and Na, and DTPA extracted micro-nutrients, and water contents of field capacity and wilting point [11] were shown in Table I.

Table 1. Chemical and physical properties of soil used (mean \pm standard deviation).

pH (1/2.5)	NH ₄ -N	NO ₃ -N	Organic Matter	CaCO ₃	Clay	Silt
	-----mg kg ⁻¹ -----		----- % -----			
7.60 \pm 0.6	24.6 \pm 4.3	43.2 \pm 3.6	1.80 \pm 0.3	3.20 \pm 3.9	36 \pm 5.7	39 \pm 6.1
FC and WP	Na ⁺ †	K ⁺	Ca ⁺⁺	Mg ⁺⁺	CEC‡	Olsen -P¶
%	-----cmol _c kg ⁻¹ -----					mg kg ⁻¹
19.2 and 3.70	0.15 \pm 0.03	1.95 \pm 0.15	18.8 \pm 1.3	6.3 \pm 1.4	27.4 \pm 3.1	11.8 \pm 1.8

†Extractable nutrient determined using ammonium acetate buffered at pH 7

‡CEC: Cation Exchange Capacity, ¶EC: Electrical Conductivity, FC: Field capacity, WP: Wilting point

Bacterial strains: Bacterial strains (OSU-142 and M-13) source and preparation methods employed in this study were permitted by Dr. Sahin (Yeditepe University, Department of Genetics and Bioengineering, Istanbul, Turkey). Both bacteria have been proved having positive effects as biofertilizers and bio-control agents (12 Sahin et al. 2004.). Bacteria were grown on Nutrient Agar (NA) for routine use, and maintained in Nutrient Broth (NB) with 15% glycerol at -80°C for long-term storage.

Seed inoculation: Before pot experiment proceeded, a single colony of preserved bacteria was transferred to a 500 mL flask containing NB, and grown aerobically in a flask rotating shaker (150 rpm) for 48 h at 27 °C. The bacterial suspension was then diluted with sterilized distilled water containing 0.025% Tween 20 to the bacteria population of around 10⁹CFU mL⁻¹. Wheat seeds were sterilized in 70% ethanol for 2 min, then soaked in 1.2%

sodium hypochlorite for 10 min and rinsed ten times with sterilized tap water. Seeds were then suspended in bacterial solutions for 30 min under sterilized chamber for inoculation treatment.

Pot experiment: Each polyethylene pot (20 cm diameter with 15 cm depth) was sterilized with 20% sodium hypochlorite solution before soil (2 kg soil pot⁻¹) was filled. Nine fertilization treatments were designed as follows: (1) control (with no bacteria inoculation and no chemical fertilizer), (2) Bacillus OSU-142 (BA-142), (3) Bacillus M-13 (M-13), (4) Bacillus OSU-142 + Bacillus M-13 (BA-142 + M-13), (5) NH₄NO₃ (AN) fertilizer (35 mg N kg⁻¹ soil), (6) (NH₄)₂SO₄ (AS) fertilizer (35 mg N kg⁻¹ soil), (7) Triple superphosphate (TSP) fertilizer (20 mg P kg⁻¹ soil), (8) NH₄NO₃ + Triple superphosphate fertilizers (AN + TSP) (35 mg N kg⁻¹ soil + 20 mg P kg⁻¹ soil), (9) (NH₄)₂SO₄ + Triple superphosphate (AS + TSP) fertilizers (35 mg N kg⁻¹ soil + 20 mg P kg⁻¹ soil). Pots were put on a bench in a greenhouse and arranged by a randomized complete block design with three blocks. Wheat seeds were placed at approximately 2.5 cm below the soil surface. The greenhouse was controlled under a day/night cycle of 15/9 h natural light (ranged from 900 to 1,200 μmol/m⁻² s⁻¹), 25/16°C and 55% relative humidity. After seven days of sowing (7 DAS), seedlings were thinned to remaining five healthy seedlings. Soil water content was maintained at 70% of field capacity throughout the experiment period by addition of deionized water weekly, except the leaching treating periods. For simulating the province irrigated amount, 360 ml pot⁻¹ of deionized water was added, made soil water status to around 150% of water holding capacity and maintained for three days, then collected the drained water, called leaching water in this manuscript. Leaching treatments were conducted at 10, 20, 40 and 80 DAS, the collected water were around 250 ml pot⁻¹, and diluted to 300 ml before analysis. The concentration of ammonium, nitrate and phosphorus in leaching water was determined within three days after collected and kept at 4 °C. The wheat plants were pulled out and cut to separate shoot and root at 120 DAS. Plants samples were oven-dried at 68 °C for 48 h and then ground. The Kjeldahl method and a Vapodest 10 Rapid Kjeldahl Distillation Unit were used to determine total N. Phosphorus content was determined spectrophotometrically by using the indophenol-blue and ascorbic acid method with a UV/VIS Aquamat Spectrophotometer and K, Ca, Mg, Fe, Mn, Zn, and Cu were determined with a Perkin–Elmer 360 Atomic Absorption Spectrophotometer after wet digestion with HNO₃-HClO₄ acid mixture (4:1 v/v) [11].

Statistical analyses: All data were subjected to a two way analysis of variance (ANOVA) and their significant level presented by LSD and calculated by using SPSS computer program (SPSS).

Results

Effect of Fertilizer Application on Dry Matter Yield and Nutrient Content of Wheat

The BA-142 and M-13 were initially selected and investigated for their antifungal and antibacterial properties. The BA-142 also has N₂-fixing function in field experiments with sugar beet and barley, although it was screened from tomato root. The P solubilizing ability of M-13 was also proved in laboratory and field experiments [12]. The significantly positive effect of the application of PGPR and chemical fertilizers was found in production of total and shoot dry matter (DM) of wheat (Table II), though no significant effect observed in the DM of root. The highest group of total DM was obtained of the AS + TSP (6.60 g pot⁻¹), AS (6.45 g pot⁻¹) and AN + TSP (6.44 g pot⁻¹) treatments, which had chemical nitrogen fertilizers applied with no matter of having chemical phosphate fertilizers. The superior effect of the application of ammonium sulphate to ammonium nitrate in alkaline soil is well known for its higher acidifying effect on decreasing soil pH. The DM production of treatments of the

application of combination of N₂-fixing with P solubilising bacteria (BA-142 + M-13) and ammonium nitrate (AN) treatments was 6.21 g pot⁻¹ and 6.20 g pot⁻¹, respectively. They were the second highest group but having no significant differences among treatments of these two groups. This showed that the application of bio-fertilizer with the formula having N₂-fixation and P solubilising potential bacteria together will have enhanced effect. The superior effect of the application of AS to AN in alkaline soil is well known for its higher acidifying effect on decreasing soil pH. And the higher rate of nitrate loss through leaching after soil water status raised to 150 % of field water capacity simulating of irrigation amount of province, which will be discussed latter. The application of only TSP or BA-142 or M-13 was the third high group in DM production, although they are significantly lower than the highest group but significantly higher than the control. This result showed that the BA-142 bacteria can work on such high available nitrogen soil. The summation of initial NH₄⁺-N (24.6 mg kg⁻¹) and NO₃⁻-N (43.2 mg kg⁻¹) was 67.8 mg kg⁻¹, this is high enough for no nitrogen fertilizer recommended for wheat cultivation, according to [13]. However, over irrigation will cause a high loss of nitrate nitrogen and made a low final crop production if no other PGPR and chemical fertilizer applied. The available P of this soil is at a medium level and needs phosphate fertilizer applied to secure a high production of wheat [14]. The results showed that there were no significant differences in DM production between M-13 and TSP treatments. This clearly proves that the phosphate solubilizing bacteria M-13 really can replace part of the application of TSP in wheat plantation (Table II).

Table 2. Effect of fertilizer types on the dry weight of shoot and root and nutrients' concentration of shoot of wheat

	Fertilizer Type								
	Control	OSU-142	M-13	OSU-142 + M-13	AS	AN	TSP	AS + TSP	AN + TSP
	g pot ⁻¹								
Root ^{ns}	0.93	1	0.99	1.02	1.04	1.02	1	1.1	1.06
Shoot ^{**}	3.65c ¹	4.83b	4.76b	5.19a	5.41a	5.18a	4.87b	5.50a	5.38a
Total DM ^{**}	4.58d	5.82bc	5.75c	6.21ab	6.45a	6.20ab	5.87bc	6.60a	6.44a
	%								
N ^{**}	1.05c	1.26b	1.07c	1.3ab	1.43a	1.36ab	1.06c	1.40a	1.38ab
P ^{**}	0.20bcd	0.20cde	0.22a	0.22ab	0.19de	0.18e	0.23a	0.22a	0.21abc
K ^{ns}	3.36	3.38	3.32	3.31	3.25	3.25	3.29	3.29	3.28
Ca ^{ns}	0.56	0.55	0.55	0.55	0.54	0.53	0.54	0.55	0.54
Mg ^{ns}	0.23	0.24	0.23	0.24	0.24	0.24	0.25	0.24	0.23
	mg kg ⁻¹ dw								
Fe ^{ns}	94.1	96.3	94.6	94.2	93.7	91	96.5	94	95.4
Mn ^{ns}	71.2	71.2	71.7	71.5	71.6	70.6	70.1	71.6	72.6
Zn ^{ns}	63.9	67.7	61.3	62.4	63.2	62.1	61.1	62.4	61.9
Cu ^{ns}	15.7	16.1	16.6	15.7	15.8	16.2	15.4	16.1	16

Nutrients concentration of wheat shoot at 120 DAS showed that fertilizer types had no significant effects on K, Ca, Mg, Fe, Mn, Zn and Cu content of plant (Table II). It is reasonable because none of these nutrients applied in this experiment and their concentrations are also high enough, although some workers reported that the use of beneficial bacteria may improve the availability of micronutrients [15]. However, highly significant differences ($p < 0.01$) were observed in N and P concentrations. As expected, the highest N concentration was found in treatments AS, AS + TSP, AN and AN + TSP fertilizer due to the application of nitrogen fertilizers. The N concentration of BA-142 + M-13 treatment with the application of combination of N₂-fixing and phosphate solubilizing bacteria was a little lower than the chemical nitrogen fertilizer applied treatments but did not reach significant level. Even only

the application of BA-142, its N concentration was significant higher than those of treatments of control, TSP and M-13. The DM (dry weight of wheat shoot) was highly significantly linearly correlated to the N_{shoot} (nitrogen concentration of wheat shoot) for whole treatments (n=9) as: $DM=2.03 + 3.15 N_{\text{shoot}}$, $R^2 = 0.651^{**}$. While the data of TSP and M-13 treatments were excluded (n=7) the relationship reached highly highly significant with: $DM=-0.83 + 5.24 N_{\text{shoot}}$, $R^2 = 0.956^{***}$. The highest P concentration was observed in treatments applied with chemical phosphate fertilizer and phosphate solubilizing bacteria: TSP, AS + TSP, AN+TSP, M-13, and BA-142 + M-13. The lowest shoot P concentration was reasonably found in treatments (AS and AN) applying chemical nitrogen fertilizer with no phosphate fertilizer and no phosphate solubilizing bacteria application. The concentration of P of treatments Control and BA-142, however, in the median was due to their DM were not high though there were neither inorganic phosphate and nor phosphate solubilizing bacteria applied. A phenomenon found from this experiment result that the initial soil available nitrogen (ammonium and nitrate) $135.6 \text{ mg pot}^{-1}$ was much higher than the amount of nitrogen of wheat uptake in control treatment, 38.3 mg pot^{-1} , and even still much higher than the highest nitrogen uptake treatment AS, 77.4 mg pot^{-1} , but the plant growth still responses to the application of inorganic nitrogen fertilizer and the N_2 -fixation bacterial application. This is due to the nutrient loss, especially the high loss of nitrogen during the leaching treatment with extra water applied, simulating heavy raining. The following data are direct evidence to explain this phenomenon.

Discussion

The changes of concentration of ammonium and nitrate in soil and in leaching water and their relationship: There were two levels of total available concentration of nitrogen ($\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$), with the initial concentration at 67.8 mg kg^{-1} for control, BA-142, M-13, BA-142+ M-13. and TSP treatments, and 102.8 mg kg^{-1} for AS, AN, AS + TSP, and AN + TSP treatments, respectively, for 35 mg kg^{-1} chemical nitrogen fertilizer applied. Because two kinds of chemical nitrogen fertilizers used, AS and AN, the initial concentration of the $\text{NH}_4\text{-N}$ of soil was in three levels, with control, BA-142, M-13, BA-142 + M-13 and TSP treatment at 24.6 mg kg^{-1} , AN and AN + TSP at 42.1 mg kg^{-1} , and AS and AS + TSP at 59.6 mg kg^{-1} , respectively. While the initial concentration of $\text{NO}_3\text{-N}$ was in two levels with control, BA-142, M-13, BA-142 + M-13, AS and AS + TSP treatments at 43.2 mg kg^{-1} , and AN and AN + TSP at 60.7 mg kg^{-1} , respectively. Therefore, inevitably the available concentrations of $\text{NH}_4\text{-N}$, and $\text{NO}_3\text{-N}$ in soil and in leaching water through the cultivation period were statistically significantly different among groups of treatments (Table III and IV).

Table 3. Effect of fertilizer types on $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, and P concentration (mg kg^{-1}) in soil after leaching treatment

Day	$\text{NH}_4\text{-N}$ (mg kg^{-1})								
	Control	OSU-142	M-13	OSU-142 + M-13	AS	AN	TSP	AS + TSP	AN + TSP
Initial	24.6 c ¹	24.6c	24.6c	24.6c	59.6a	42.1b	24.6c	59.6a	42.1b
10	22.5c	34.0b	22.8c	34.2b	43.2a	33.8b	23.7c	42.3a	35.9b
20	17.7d	23.0bc	17.6d	22.3c	37.3a	26.7bc	17.2d	39.7a	27.3b
40	18.3d	24.4bc	15.6d	23.6bc	33.9a	27.5b	16.1d	31.7a	22.7c
80	13.7de	18.4cd	14.9d	20.1c	21.6b	26.1ab	13.7de	28.8ab	23.3bc
					$\text{NO}_3\text{-N}$ (mg kg^{-1})				
Initial	43.2b	43.2b	43.2b	43.2b	43.2b	60.7 a	43.2b	43.2b	60.7 a
10	6.83c	21.9b	6.53c	19.5b	33.3a	37.5a	7.40c	31.8a	36.1a
20	6.20e	10.5d	6.80e	10.1d	17.9c	24.5a	6.20e	15.5c	21.4b
40	6.67ef	9.17de	6.10f	9.43de	13.4c	21.7a	6.93ef	11.1cd	16.4b
80	5.03c	7.80b	6.40bc	7.77bc	12.4a	7.37bc	5.50c	12.8a	14.9a
					P (mg kg^{-1})				
Initial	12.8b	12.8b	12.8b	12.8b	12.8b	12.8b	32.8a	32.8a	32.8a
10	12.2c	12.7c	17.9b	16.1b	12.5c	12.7c	27.7a	25.7a	27.1a
20	10.7cd	12.4c	16.9b	16.3b	8.90d	10.4cd	26.8a	25.3a	25.3a
40	9.47c	10.1c	13.6b	14.2b	8.53c	8.70c	22.4a	21.0a	21.4a

DAS	Control	OSU-142	M-13	OSU-142 + M-13	AS	AN	TSP	AS + TSP	AN + TSP
80	9.40c	9.70c	13.7b	13.4b	8.27c	8.67c	11.0b	18.9a	20.6a
Table IV. Effect of fertilizer types on concentration of NH ₄ -N, NO ₃ -N, and P concentration (mg kg ⁻¹) in leaching water									
NH ₄ -N (mg kg ⁻¹)									
10	0.80c ¹	1.03c	0.63c	1.50c	6.27a	3.47b	0.93c	6.53a	2.57bc
20	0.80d	1.70d	0.93d	1.77d	6.53a	3.73c	1.87d	5.93ab	5.13b
40	0.83c	0.73c	1.03c	1.17bc	3.03a	1.30bc	1.00c	2.80a	1.77b
80	0.63c	0.67c	0.50c	0.77c	1.67a	1.33ab	0.50c	1.67a	1.20b
Total	3.06	4.13	3.1	5.2	17.5	9.83	4.3	16.9	10.7
NO ₃ -N (mg kg ⁻¹)									
10	2.10c	13.9b	1.87c	13.47b	13.3a	29.7b	2.33c	15.5a	28.7b
20	1.60c	2.90c	1.57c	2.63c	8.43b	12.5a	1.93c	9.60b	13.5a
40	1.30d	1.17d	0.83d	0.90d	7.20c	15.0a	1.10d	6.27c	13.5b
80	0.60d	0.83d	0.50d	0.90d	3.50c	10.4a	0.67d	3.13c	8.63b
Total	5.6	18.8	4.77	17.9	32.5	67.6	6.03	34.5	64.4
T-AN ²	8.66	22.93	7.87	23.1	50	77.43	10.33	51.4	75.1
LN ³ , %	-167	-0.74	-193	0	53.8	70.2	-124	55	69.2
P (mg kg ⁻¹)									
10	0.73c	0.70c	1.20b	1.13b	0.53c	0.33c	2.53a	2.57a	2.33a
20	0.63c	0.63c	1.03b	0.70c	0.53c	0.63c	1.37a	1.43a	1.60a
40	0.83c	0.90c	1.17b	1.13b	0.90c	0.87c	1.83a	1.80a	1.90a
80	0.37b	0.30b	0.63b	0.57b	0.27b	0.40b	1.80a	1.57a	1.63a
Total	2.57	2.53	4.03	3.53	2.22	2.23	7.53	7.36	7.46
LP ⁴ , %	-37.4	-39.5	12.4	0	-59.0	-58.3	53.1	52.0	52.7

1. T-AN: the summation of total loss of NH₄-N and NO₃-N of the four leaching treatments.
2. LN, the ratio of the total nitrogen loss of other treatments (LN_{other}) compared to that of OSU+M-13 treatment (LN_{OSU-142+M-13}) as: $100 * (LN_{other} - LN_{OSU-142+M-13}) / LN_{OSU-142+M-13}$
3. LP, the ratio of the total phosphorus loss of other treatments (LP_{other}) compared to that of OSU+M-13 treatment (LP_{OSU-142+M-13}) as: $100 * (LP_{other} - LP_{OSU-142+M-13}) / LP_{OSU-142+M-13}$

The changes of concentration of available nitrogen through cultivation period in this experiment can be divided into four groups, according to the effect of forms of bio- and chemical nitrogen fertilizers as follows:

Group N-1 (control, M-13, and TSP): There were no chemical nitrogen or N₂-fixing bacterial fertilizer added for the treatments of this group. The concentrations of ammonium and nitrate nitrogen both of soils and leaching waters were significantly lower than those of other groups from the beginning through the whole experimental period, but no significant difference among them (Table III, Table IV). The concentration of NH₄-N in soils (NH₄-N_S) of this group was linearly decreased with DAS (days after sowing), NH₄-N_S = 22.9 – 0.125 DAS, R² = 0.776 (p < 0.0001). The magnitude of the decrease of NH₄-N_S is only about 10 mg kg⁻¹, from the initial 24.6 mg kg⁻¹ decreased to around 14 mg kg⁻¹ at 80 DAS. The concentration of NH₄-N in leaching water (NH₄-N_L) of this group was also very low, lower than 1 mg L⁻¹ of the control, and lower than 2 mg L⁻¹ of M-13 and TSP treatments. The concentration of NO₃-N in soils (NO₃-N_S) of this group was sharply decreased after the first leaching treatment (10 DAS), then remained nearly at a constant level, ranged from 5.03 mg kg⁻¹ to 7.40 mg kg⁻¹, through the rest of cultivation period. The concentration of NO₃-N in leaching water (NO₃-N_L) of this group ranged from 0.50 to 2.33 mg-NO₃-N L⁻¹ was much lower than the limit concentration of tap water of USA and European Union, 44 mg-NO₃ L⁻¹ (9.9 mg-NO₃-N L⁻¹) and 50 mg-NO₃ L⁻¹ (11.3 mg-NO₃-N L⁻¹), respectively, cited from Addiscottls and Benjam [16]. . The magnitude of the decrease of soil nitrate nitrogen after the

first leaching treatment was dramatically high, about 84.9 ~ 82.9 %, from 43.2 mg kg⁻¹ decreased to lower than 7.40 mg kg⁻¹. However, the loss of nitrogen from the uptake of wheat at this stage should be very low, the loss of nitrogen accounted from the leaching water is low (0.42 mg pot⁻¹). The main loss of nitrate (the uncounted nitrate) should be due to the denitrification for the soil status remained at 150% of water holding capacity for three days for each leaching treatment. This result strongly suggests that the nitrate concentration of soil should not remain at high level during rainy season.

Group N-2 (AS, and AS + TSP): In this group the initial concentration of NH₄-N_S in soil was 59.6 mg kg⁻¹ with 35 mg kg⁻¹ higher than that of the first group. The increase of 35 mg-NH₄-N kg⁻¹ was due to the application of ammonium sulfate (AS). The change of concentration of NH₄-N_S in soil with DAS was also described by linear relationship as it is shown in group one: NH₄-N_S = 50.6 – 0.360 DAS, R² = 0.762 (p<0.001). The higher decrease rate comparing to group one, around triple times higher, was due to its higher initial concentration caused a higher nitrification and loss of ammonium nitrogen through leaching. But its NH₄-N_S still remained the highest level, higher than 42 mg kg⁻¹, compared to other treatments (Table III). The change of concentration of NO₃-N_S of this group was also decreased sharply at the first two leaching periods then remained at around 12 mg-N kg⁻¹. Although their initial concentration of NO₃-N_S of this group was the same as that of the first group, its nitrate concentration during the whole cultivation period was significantly higher than those of group one (Table III) mainly due to the applied ammonium fertilizer which in turn increasing soil nitrate concentration through the nitrification. Accordingly, the concentration of NO₃-N_L in leaching water, ranged from 3.13 to 15.5 NO₃-N L⁻¹, was much higher than those of group one with their concentration at the first leaching water higher than the tap water limit, 11.3 mg-NO₃-N L⁻¹ (Table IV).

Group N-3 (AN, and AN + TSP): The initial concentration of NH₄-N_S in soils of this group was 42.1 mg kg⁻¹ with 17.5 mg kg⁻¹ higher than that of the first group and 17.5 mg kg⁻¹ lower than that of the second group for the application of 35 mg-N kg⁻¹ in form of ammonium nitrate (AN), half of NH₄-N and half of NO₃-N. The change of concentration of NH₄-N_S in soil with DAS had no linear relationship because that its concentration was sharply decreased in the first two leaching treatments then remained at around 25 mg kg⁻¹. The concentration of NO₃-N_S of this group was also decreased more sharp at the first two leaching periods then decreased slowly, and could be present in two linear lines divided at 20 DAS. The two lines were NO₃-N_S = 59.0 – 1.89 DAS, R² = 0.973 (p<0.0003) and NO₃-N_S = 26.9 – 0.197 DAS, R² = 0.754 (p<0.025), presented the periods 0 to 20 DAS and 20 to 80 DAS periods, respectively. The concentration of NO₃-N_S of this group was significantly higher than those of treatments of other three groups through the whole cultivation period (Table III) mainly due to the application of 17.5 mg-NO₃-N kg⁻¹ at the beginning. Undoubtedly, their concentration of NO₃-N_L in leaching water, ranged from 8.63 to 29.7 mg-NO₃-N L⁻¹, was much higher than those of other groups, with their concentration higher than the tap water limit for the first three leaching periods (Table IV).

Group N-4 (BA-142 and BA-142 + M-13): The concentration of NH₄-N_S and NO₃-N_S in soils of both treatments of this group was all higher than those of treatments of group 1, and in most periods reached to statistically significant levels, although the initial NH₄-N_S and NO₃-N_S of both groups was the same. The extra available nitrogen source was due to the inoculation of BA-142 which has been evidenced to have N₂-fixation function [12] (Table III). The high fixation potential of BA-142 made the concentration of NH₄-N_S in treatments applied with the PGPR, even increased 10 mg-NH₄-N kg⁻¹ higher than the initial concentration after leaching treatment at 10 DAS. The concentration of NO₃-N_S of this group was also decreased sharp at the first two leaching periods then decreased slowly, similar to

group 3, and also could be present in two linear lines divided at 20 DAS. The first line was: $\text{NO}_3\text{-N}_S = 41.2 - 1.64 \text{ DAS}$, $R^2 = 0.952$ ($p < 0.0008$), and the second line was: $\text{NO}_3\text{-N}_S = 11.0 - 0.413 \text{ DAS}$, $R^2 = 0.997$ ($p < 0.0002$). The concentration of $\text{NO}_3\text{-N}_L$ in leaching water of this group had no significant difference to group 1, except the concentration at 10 DAS was as high as those of group 2 which were higher than the allowable limit of tap water. While the concentration of $\text{NH}_4\text{-N}_L$ in leaching water of this group had no significant difference to group 1 through the whole cultivation period. Taking the whole data as a pool, the concentration of $\text{NH}_4\text{-N}_L$ and $\text{NO}_3\text{-N}_L$ in leaching water was highly significantly linearly related to their concentration of $\text{NH}_4\text{-N}_S$ and $\text{NO}_3\text{-N}_S$ in soil determined in previous period: $\text{NH}_4\text{-N}_L = -2.27 + 0.148 \text{ NH}_4\text{-N}_S$, $R^2 = 0.775$ ($P < 0.0001$); $\text{NO}_3\text{-N}_L = -1.25 + 0.351 \text{ NO}_3\text{-N}_S$, $R^2 = 0.606$ ($P < 0.0001$), respectively. As excluding the outliers of data of first leaching (10 DAS) of treatments control, M-13 and TSP, the relationship of concentration of nitrate between in leaching water and in soil was highly improved as $\text{NO}_3\text{-N}_L = -2.12 + 0.442 \text{ NO}_3\text{-N}_S$, $R^2 = 0.867$ ($P < 0.0001$). It showed that the leaching loss rate of nitrate nitrogen was about 3 times higher than ammonium nitrogen.

The effect of fertilization practices on the loss of nitrogen during heavy rain: The above data showed that the loss of available nitrogen either ammonium or nitrate through leaching of both forms are highly linearly related to their concentration in soil, no matter of what fertilization practices which include chemical and bio-fertilizers. Comparing the loss of available nitrogen in the leaching water of all other treatments to that of treatment BA-142 + M-13, it is found that the application of N_2 -fixation bacteria (BA-142) increased nitrogen loss by 124 to 193 % to treatments (control, M-13, and TSP) with no N_2 -fixation bacteria and no chemical phosphate fertilizers applied, but reduced nitrogen loss 53.8 to 70.2 % to treatments (AS, AN, AS + TSP, and AN + TSP) with chemical nitrogen fertilizers applied (Table IV). In this study there was some loss of the available nitrogen uncounted, especially during the first leaching period. It is well known that crop generally has exponential growth of dry mass and uptake of nutrient characters [17], therefore, the growth of wheat during the first 20 days will be much lower than the final stage at normal situation. Thus the amount of uptake of nitrogen will be ignored comparing to the content of available nitrogen ($\text{Avai-N} = \text{NH}_4\text{-N} + \text{NO}_3\text{-N}$) existed in soil of this study. In this study system the concentration of available nitrogen at any stage $(\text{Avai-N})_t$ was affected by initial soil available concentration $(\text{Avai-N})_0$, chemical fertilizer added $(\text{Avai-N})_{\text{CF}0}$, N_2 -fixed by bio-fertilizer $(\text{Avai-N})_{\text{BF}t}$, removed by plant uptake $(\text{Avai-N})_{\text{PU}t}$, leaching loss $(\text{Avai-N})_{\text{LL}t}$, and uncounted loss $(\text{Avai-N})_{\text{UL}t}$, such as ammonium volatilization or denitrification, and related as: $(\text{Avai-N})_t = (\text{Avai-N})_{\text{S}0} + (\text{Avai-N})_{\text{CF}0} + (\text{Avai-N})_{\text{BF}t} - (\text{Avai-N})_{\text{PU}t} - (\text{Avai-N})_{\text{LL}t} - (\text{Avai-N})_{\text{UL}t}$. The parameters of $(\text{Avai-N})_{\text{BF}t}$ and $(\text{Avai-N})_{\text{UL}t}$ were not measured and being estimated by other measured parameters. Assuming the amount of $(\text{Avai-N})_{\text{BF}t}$ of control was zero and $(\text{Avai-N})_{\text{PU}t}$ was negligible, the amount of uncounted loss of $(\text{Avai-N})_{\text{UL}10}$ was 76.3 mg kg^{-1} , which is calculated by: $(\text{Avai-N})_{\text{UL}10} = (\text{Avai-N})_{\text{S}0} + (\text{Avai-N})_{\text{CF}0} - (\text{Avai-N})_{\text{S}10} - (\text{Avai-N})_{\text{LL}10}$. The amount of $(\text{Avai-N})_{\text{UL}10}$ of M-13 and TSP treatments, 72.9 and 71.1 mg kg^{-1} (Table IV), respectively, was nearly equal to that of control treatment. This is consistency for them belong to the same group N-1. The uncounted loss may be due to the denitrification because soil water status was remained to 150 % of water holding capacity for three days. The amount of $\text{Avai-N}_{\text{UL}10}$ of BA-142 was 20.4 mg pot^{-1} , which is 55.9 mg pot^{-1} lower than the control. This may be explained by: 1. the loss of available nitrogen compensated by bio-fixation of the BA-142 bio-fertilizer, or 2. the BA-142 can immobilize $\text{NO}_3\text{-N}_S$. The former effect has been demonstrated by previous workers [12] but most of them showed its fixation through plant uptake only with no detail analysis of soil available nitrogen, however the latter concept needs further research work to answer. The magnitude of $\text{Avai-N}_{\text{UL}10}$ of BA-142 + M-13 treatment, 24.8 mg pot^{-1} , was similar to that of BA-142 treatment. The magnitude of $\text{Avai-N}_{\text{UL}10}$ of AS, AN, AS + TSP and

AN + TSP (groups N-2 and N-3) were similar, ranged from 48.1 ~ 55.4 mg pot⁻¹, having more than 20 mg pot⁻¹ lower than that of control. There is no good explanation on this difference without further study. The uncounted loss of available nitrogen at 20 DAS (Avai-N_{UL20}) was very different to that of 10 DAS (Avai-N_{UL10}) among different treatments. The quantity of Avai-N_{UL20} of group N-1, ranged from 9.3 to 14.7 mg pot⁻¹ became the lowest because their low soil available nitrogen after the first leaching. The Avai-N_{UL20} of the rest of all other six treatments (i.e. groups N-2, N-3 and N4), ranged from 32.8 to 41.4 mg pot⁻¹, were two to three times of those of group N-1. For groups N-2 and N-3 the uncounted loss was due to the high available nitrogen fertilizer added, however the high Avai-N_{UL20} of group-4 shall be contributed by the inoculated BA-142 (N₂ fixation bacteria).

The changes of concentration of phosphate in soil and in leaching water and their relationship: The initial concentration of available P of soil used in this study was in two levels with treatments of TSP, AS + TSP, and AN + TSP at 32.8 mg kg⁻¹ which had 20 mg-P kg⁻¹ added in form of triple superphosphate, and other six treatments at 12.8 mg kg⁻¹, respectively. As it has been discussed that the application of bio-fertilizer will affect the phosphorus status in soils, definitely the application of phosphate solubilizing bacteria of M-13 in treatments of M-13 and BA + M-13, significantly increased the available P concentrations, comparing to control, BA-142, AS and AN treatments, though most of their values were still significantly lower than those of triple superphosphate application treatments. Apparently the concentration of available P (P_S) in soils and dissolved P (P_L) in leaching water during cultivation period showed in three groups, P1 (treatment control, BA-142, AS and AN), P2 (M-13, BA-142 + M-13) and P3 (TSP, AS + TSP and AN + TSP), and being statistically significantly different among different groups (Table III).

Group P1 (control, BA-142, AS and AN): Statistically, the concentration of available P among treatments in this group had no difference, but still having some different characteristic among them (Table III). The concentration of available P (P_S) in treatments AS and AN was a little lower than those of control. This was mainly due to the plant uptake of AS and AN treatments was higher with significantly higher dry matter growth (Table II). The concentration of available P of BA-142 treatment was a little higher than those of control, even its dry matter growth was significantly higher than that of control. This may be due to the acidifying effect from the release of acid during N₂ fixation [18], and the nitrification from its fixed ammonium. The concentration of P_L of leaching water in this group was lowest among different groups and never higher than 1 mg-P L⁻¹, ranged 0.37 to 0.90 mg-P L⁻¹.

Group P2 (M-13, BA-142 + M-13): The concentration of available P of this group was increased shortly after the application of phosphate solubilizing bacteria M-13 then slowly decreased, but remained higher than the initial concentration even at 80 DAS, although no chemical phosphate fertilizer added (Table III). This is not a new discovery for the P solubilizing ability of M-13 was previously demonstrated in other works [12]. Some of concentration of P_L of leaching water in group was higher than 1 mg L⁻¹, ranged 0.57 to 1.20 mg-P L⁻¹ (Table IV).

Group P3 (TSP, AS + TSP and AN + TSP): The application of TSP made the available P of soil in this group was significantly higher than those of other groups especially at the early stage. The magnitude of the higher P was decreased with the going of the cultivation for a higher decrease rate of P_S with DAS in soils of this group. The change of concentration of available P with DAS for the control treatment was described by a linear relationship: P_S = 13.0 – 0.128 DAS, R² = 0.978 (n=5, P<0.0218). A linear relationship was also presented for group P3 treatments: P_S = 31.9 – 0.258 DAS, R² = 0.984 (n=15, P<0.0008). The decrease rate of concentration of available P of group P3 is doubled to control treatment. The concentration of P_L in leaching water of this group all higher than 1 mg-P L⁻¹, even higher than 2 mg-P L⁻¹,

ranged from 1.37 to 2.57 mg-P L⁻¹ (Table IV). Comparing the loss of available P in the leaching water of all other treatments to that of treatment BA-142 + M-13, it is found that the application of phosphate solubilizing bacteria (M-13) increased phosphorus loss by 37.4 to 59.0 % to treatments (control, BA-142, AS, and AN) with no phosphate solubilizing bacteria and no chemical phosphate fertilizers applied, but reduced phosphorus loss by 52.0 to 53.1 % to treatments (TSP, AN + TSP, and AS + TSP) with chemical phosphate fertilizers applied (Table IV).

This study has showed that fertilizer type greatly influences plant nutrient uptake and nutrient leaching out of the soil. The inoculation of N₂-fixation bacteria and phosphate solubilizing bacteria can replace part of chemical nitrogen and phosphate fertilizers and also alters the status of soil available nitrogen and phosphate. The use of bio-fertilizers with a good management can decrease the leaching loss of nitrate and phosphate from the agricultural land and improve the ground water quality. The high energy dependent of production of chemical fertilizers and the limited phosphate sources will make a harsher impact on food production. Therefore, a proper use of bio-fertilizers should be a good policy in future fertilization system.

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