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Effects of *Bacillus megaterium* Inoculation on Metabolic Profiles of Hungarian Vetch (*Vicia pannonica Roth*) at Different P Levels from Poultry Manure and Chemical Fertilizer

Tavuk Gübresi ve Kimyevi Gübre Kaynaklı Farklı P Seviyelerinde *Bacillus megaterium* Aşılamasının Macar Fiği (*Vicia pannonica Roth*) Metabolik Profilleri Üzerine Etkileri

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ABSTRACT

This study was carried out to evaluate the metabolic effects of two different doses of poultry manure (0, 3 t ha^{-1}), and three different doses of phosphorus fertilizer (0, 50, 100 kg P_2O_5 ha^{-1}) with or without *Bacillus megaterium* M-3 inoculation on the biochemical parameters such as total protein, glucose, triglyceride of Hungarian vetch (*Vicia pannonica Roth*). The results indicated that 10^{-8} CFU ml^{-1} *Bacillus megaterium* inoculation with ~80 kg P/ ha^{-1} affected all biochemical parameters of *Hungarian vetch* positively.

ÖZET

Bu çalışma iki farklı doz tavuk gübresi (0, 3 t ha^{-1}) ile üç farklı doz (0, 50, 100 kg P_2O_5 ha^{-1}) fosforlu gübrenin *Bacillus megaterium* M-3 varlığında ve yokluğunda Macar fiğinde toplam protein, glikoz, trigliserit gibi biyokimyasal parametrelere metabolik etkilerini değerlendirmek amacıyla yapılmıştır. Sonuç olarak Macar fiğinde 10^{-8} CFU ml^{-1} *Bacillus megaterium* ve ~80 kg P/ ha^{-1} uygulaması tüm biyokimyasal parametreleri olumlu etkilemiştir.

INTRODUCTION

Phosphorus (P) is a growth limiting macronutrient required by crops in large amounts (Silber et al., 2002; Manitoba 2013). P influences plant metabolism by contributing directly to metabolic pathways (carbohydrate metabolism), by creating intermediate substance required in metabolic pathways (protein, lipid, and nucleic acid metabolism) (Marschner, 1995; Armstrong, 1999; Silber et al., 2002; Manitoba, 2013), or by controlling the many enzymatic reactions which regulate different metabolic processes (Theodorou and Plaxton, 1993). Consequently, P is vital in cell division - development of new plant tissues, and thereby essential for plant growth (Armstrong, 1999; Manitoba, 2013).

The unique source of the phosphorus for crops is soil, and plants require sufficient P for optimal crop yield from the very early stages of growth (Grant et al., 2001). However, the concentration of soil Pi that required for crops commonly lower than 10 mM (Bieleski, 1973), and is not adequate for growing crop tissues where the essential concentration is 5 - 20 mM (Raghothama, 1999). Because of the low P level in soils, applications of mineral or animal P fertilizers are the most common and fastest way to ensure P availability for crops (Manitoba, 2013). Addition of the mineral P fertilizers or animal manures results increase in P level in soils regardless of the native soil P concentration (Mullins, 2009). Further, applying

phosphorus fertilizer increases crop growth, and yields (Mullins, 2009). On the other hand, phosphorus may become toxic when accumulated by plants in high concentrations (Silber et al., 2002), and as in deficiency, inhibit plant growth (Armstrong, 1999). On the other hand, as a consequence of characteristic of phosphorus - in acidic soils Pi chelates with Fe + Al, and in alkali soils chelates with Ca, additional Pi precipitates (Manitoba, 2013). As a result, ≥80% of the P becomes "unavailable" for crops (Holford, 1997). Therefore, efficacy of P fertilizer is about 10-25% (Isherword, 1998), and concentration of P increase 1.0 mg kg^{-1} in soil (Goldstein, 1994) that insufficient for an optimal crop growth (Goldstein, 2000). Also, repeated applications of P fertilizer that exceed crop requirements may promote the eutrophication of surface waters (Mullins, 2009; Manitoba, 2013).

The establishing a new effective fertilization approach based on supplying adequate P to crops while reducing the harmful effects of fertilization on environment is critical. At this point, use of bio-fertilizer such as soil microorganisms gain importance for sustaining crop production with optimized P fertilization because of their role in mineralization of P (Richardson, 2001). Phosphate solubilizing bacteria (PSB) are being used as bio-fertilizer since 1950s (Kudashev, 1956). Use of phosphate solubilizing bacteria with or without other P fertilizers increases plant available Pi concentration and reduce P fertilizer application up to 50% without a significant decrease in crop yield (Sundara et al., 2002; Jilani et al., 2007). This study assessed a wide-range analysis of the metabolic effects of PSB inoculations with/without mineral, and animal P fertilizers on the protein, carbohydrate, lipid, and phenolics metabolism of Hungarian vetch.

MATERIAL and METHODS

Crop management and experimental design

Research was carried out in fields of Agricultural research and extension center of Ataturk University, Faculty of Agriculture during year 2010. The experimental area was located 39^0 55^1 N and 41^0 61^1 E at an altitude of 1850 m. The soil texture of experimental fields was loam with 27.5 kg ha^{-1} available P_2O_5, 118 kg ha^{-1} corresponding available K_2O, 0.82% lime content, 1.40% organic matter content, pH of 7.45 according to laboratory analysis results.

Experiments were conducted in a randomized complete block design with three replications. Hungarian vetch seeds were sown in 5 x 2.8 m (L x W) rows with 30 cm row spacing, and with 0.5 m inner - 2 m outer buffer zones. The sowing rate was 8 kg da^{-1}, and sowing was performed with a hand drill. Experimental treatment was arranged as 0, 50 and 100 kg ha^{-1} P_2O_5 triple super phosphate (P) as chemical fertilizer; 0, and 3000 kg ha^{-1} poultry manure (M)(contains ~30 kg (29.8) Pi in 3000 kg manure); 10^{-8} CFU ml^{-1} *Bacillus megaterium* (B) as phosphorus solubilizing bacteria (PSB) inoculation (Table 1). Harvest was performed excluding one row from each side, and 0.5 m from both edges, and plant samples were grinded and sieved through a 2 mm sieve and prepared for chemical analyses.

Table 1. Experimental groups, phosphorus sources, and the applied P levels with or without *Bacillus megaterium*

Group	Manure P	Fertilizer P	Applied Total P	B
Ctrl	0	0	0	0
B	0	0	0	10^{-8} CFU ml^{-1}
M	30 kg ha^{-1}	0	30 kg ha^{-1}	0
BM	30 kg ha^{-1}	0	30 kg ha^{-1}	10^{-8} CFU ml^{-1}
P_1	0	50 kg ha^{-1}	50 kg ha^{-1}	0
BP_1	0	50 kg ha^{-1}	50 kg ha^{-1}	10^{-8} CFU ml^{-1}
P_1M	30 kg ha^{-1}	50 kg ha^{-1}	80 kg ha^{-1}	0
BP_1M	30 kg ha^{-1}	50 kg ha^{-1}	80 kg ha^{-1}	10^{-8} CFU ml^{-1}
P_2	0	100 kg ha^{-1}	100 kg ha^{-1}	0
BP_2	0	100 kg ha^{-1}	100 kg ha^{-1}	10^{-8} CFU ml^{-1}
P_2M	30 kg ha^{-1}	100 kg ha^{-1}	130 kg ha^{-1}	0
BP_2M	30 kg ha^{-1}	100 kg ha^{-1}	130 kg ha^{-1}	10^{-8} CFU ml^{-1}

Ctrl: Control, B: *Bacillus megaterium*, M: Poultry manure, P; Phosphorus fertilizer, Total P: Total phosphorus.

Homogenization of plant samples: One gram of plant samples were mixed with solubilization buffer (0.5 M Tris-HCl, pH 8.8, 5.0% SDS, 1.0% SDC, 1.0% Tween 20, 1.0% Triton x100), and then homogenized at 5.000 rpm for 2' using a tissue homogenizer. During homogenization all samples were kept in ice bath.

Determination of phosphorus concentration: To 100 µl homogenized sample, 3 ml solution of ammonium molybdate (0.48 mM) in sulfuric acid (220 mM) was added. After vortexing, samples were incubated at 37^{0}C for 5 min, and the absorbances of the samples were measured at 340 nm (Amador and Urban, 1972). Results were calculated by using a standard phosphorus solution (5 mg/dL), and expressed as micrograms of P per gram of dry weight (µg P/g DW).

High Performance Thin Layer Chromatography (HPTLC) of plant lipids: Five hundred microliters of n-hexane-isopropanol 3:2 (v/v) mixtures was added to 1000 µL of homogenized plant samples in an eppendorf tube. After vortexing vigorously, the tubes were centrifuged at 5.000 x g, +4^{0}C for 5 min, and the upper phase was used for chromatographic analysis of plant lipids. HPTLC silica gel 60 plates (20 x 10 cm) were used for separation, and identification of lipids. Standard lipid mixture (cholesterol, palmitate, glycerol di-palmitate, L-α-phosphatidyl choline), and plant lipid extracts were spotted on the HPTLC plates. The lipids were developed with n-hexane: diethyl ether: formic acid; 80:20:2 (v/v) to 5 cm above the application point. After developing, the entire plate was dipped in charring solution (10% $CuSO_4$ (w/v) in 8% H_3PO_4 (v/v)), and lipid classes were visualized by incubating the plates at 180°C (Kaynar et al., 2013). Vetch lipids were separated into the following classes: Free fatty acids (FFA), sterols (STE), diacylglycerols (DAG), and phospholipids (PL). HPTL chromatograms were analysed with TL 120 software, and results were obtained as percentage of individual lipid class in total lipid composition of plant samples (Kaynar et al., 2013).

Determination of triglyceride concentration: To 100 µl homogenized sample, 1 ml triglyceride (GPO) reagent (4-Chlorophenol 3.5 mM, ATP >0.5mM, magnesium salt 10 mM, 4-Aminophenazone 0.3 mM, microbial glycerol kinase >250 U/L, microbial glycerol phosphate oxidase >4500U/L, horseradish peroxidase >2000 U/L, microbial lipase >200.000 U/L, buffer (pH 7.3), sodium azide (0.01%)) was added. After 30 min incubation, absorbance of sample was measured at 505 nm (Fossati and Prencipe, 1982). Results were calculated by using a standard triglyceride (TG) solution (50 mg/dL) and expressed as milligrams of TG per gram of dry weight (mg TG/g dw).

Determination of total protein concentration: The plant homogenates mixed with 0.5 ml sodium deoxy cholate (10%), and 0.5 ml TCA (10%). The mixture was incubated in 37^{0}C for 30 min, and centrifuged at 5.000 x g, +4°C for 5 min. The precipitate was dissolved in 5.0 ml 0.1 N NaOH. Further, 5.0 ml alkaline copper reagent was added in to the same tube and, after 10 min, 0.5 ml folin reagent was added and incubated at room temperature for 30 min. Finally absorbance values of samples and protein standards were recorded at 660 nm at spectrophotometer (µ-Quant, BioTek) against the blank solution (Lowry et al., 1951). Results were calculated by using a standard protein solution (5 g/dL) and expressed as milligrams of total protein per gram of dry weight (mg TP/g dw).

Determination of hexose (glucose) concentration: 25 mg O-dianisidine completely dissolved in 1 mL of methanol. Then 49 mL of 0.1 M phosphate buffer (pH 6.5), 5 mg of peroxidase, and 5 mg of glucose oxidase added to the above prepared O-dianisidine solution. The mixture was incubated at 37^{0}C for 40 min. The reaction was terminated by the addition of 2 mL of 6 N-HCl and, resultant color was measured at 540 nm (Raabo and Terkildsen, 1960). Glucose (Glc) concentrations were calculated by using a standard glucose solution (100 mg/dL), and expressed as milligrams of Glc per gram of dry weight (mg Glc/g dw).

Determination of total phenolic concentration: 500 mg of grounded vetch samples were mixed with 10 ml of 80% ice-cold acetone, and vortexed. Subsequent, samples were incubated for 1 hr in the dark and centrifuged at 5.000 x g for 10 min, and supernatants were collected. To 0.1 ml of supernatant, 2.5 ml Folin-Ciocalteau reagent (1:10 v/v diluted with dH_2O) was added, and incubated at 25^{0}C for 5 min. Later, 2.0 ml of 7.5% of sodium carbonate was added, and mixtures were incubated at 25^{0}C for 45 min. After incubation, absorbances of the samples were measured at 765 nm using spectrophotometer. The total phenolic content was calculated using a standard tannic acid solution (100 mg/dL), and results were expressed as milligrams of tannic acid equivalents per gram of dry weight (mg TAE/g dw) (Javanmardi et al., 2003).

Statistical analyses: Analysis of variance (ANOVA) was performed to evaluate the effects of different type of P applications on the carbohydrate, protein, and lipid parameters with SPSS 20.0 software (IBM SPSS 2012). Levels of significance were determined at

p<0.05 according to Duncan, and data shown as mean values ± SD.

RESULTS and DISCUSSION

P concentration: The maximum P concentration was observed in BP_1M (662.00±15.19 µg P/g DW) (p<0.05) (Table 2). Whereas, the minimum P concentrations were observed at high P levels combine with or without bacterial inoculation; BP_2M, P_2M, BP_2, and P_2 (558.00±8.01, 573.90±7.04, 562.93±13.29, and 584.10±8.90 µg P/g DW, respectively) (p<0.05) (Table 2).

Plant absorbs phosphorus through root hairs, root tips, and root cells. Mycorrhizal fungi that grow in association with the roots also facilitate uptake. Plants release organic acids such as citric, and malic acids, from roots (Armstrong, 1999), and by this way, accelerate the mobilization of P - increase the organic P level (Hayes et al., 2000). Eventually, as a combination of these effects, plants with higher P supply through either chemical fertilization or chemical fertilization with PSB inoculation, will not cause an increase in P concentration in their tissues, and cause a reduction in P concentration in plant. The changes on P concentrations may result from high P concentrations that cause reductions in excretion of organic acids; root hair density, and vesicular-arbuscular mycorrhizae (Bar-Yosef, 1996; Bates and Lynch, 1996). Therefore, BP_1M (~80 kg ha^{-1} P with 10^{-8} CFU ml^{-1} PSB) was considered optimum dose and/or combination in terms of P uptake for Hungarian vetch.

Table 2. Metabolic profiles of Hungarian vetch at different P levels with or without *B. megaterium* inoculation

	TP	P	Glc	TG	TPhe
	mg TP/g dw	µg P/g dw	mg Glc/g dw	mg TG/g dw	mg TAE/g dw
Ctrl	8.90 ± 0.12^{b}	620.83 ± 15.57^{c}	2.24 ± 0.06^{bc}	0.98 ± 0.09^{bcd}	2.94 ± 0.07^{b}
B	8.91 ± 0.33^{b}	643.93 ± 8.66^{cde}	2.23 ± 0.04^{bc}	1.07 ± 0.06^{cde}	2.85 ± 0.10^{b}
M	8.88 ± 0.26^{b}	632.73 ± 7.00^{cd}	2.20 ± 0.10^{bc}	1.05 ± 0.06^{cde}	2.95 ± 0.11^{b}
BM	8.97 ± 0.26^{b}	650.97 ± 15.22^{de}	2.16 ± 0.11^{b}	1.10 ± 0.11^{de}	2.85 ± 0.06^{b}
P_1	8.96 ± 0.24^{b}	634.77 ± 9.67^{cd}	2.17 ± 0.08^{b}	1.09 ± 0.09^{cde}	2.65 ± 0.07^{a}
BP_1	9.82 ± 0.12^{c}	653.57 ± 30.25^{de}	1.93 ± 0.07^{a}	1.15 ± 0.06^{e}	2.55 ± 0.11^{a}
P_1M	9.70 ± 0.35^{c}	645.00 ± 10.01^{cde}	1.90 ± 0.07^{a}	1.12 ± 0.04^{e}	2.64 ± 0.08^{a}
BP_1M	9.86 ± 0.20^{c}	662.00 ± 15.19^{f}	1.80 ± 0.07^{a}	1.16 ± 0.07^{e}	2.48 ± 0.10^{a}
P_2	8.46 ± 0.17^{a}	584.10 ± 8.90^{b}	2.26 ± 0.11^{bcd}	0.97 ± 0.08^{bc}	2.98 ± 0.15^{b}
BP_2	8.48 ± 0.15^{a}	562.93 ± 13.29^{ab}	2.40 ± 0.07^{d}	0.87 ± 0.07^{ab}	3.16 ± 0.06^{c}
P_2M	8.38 ± 0.25^{a}	573.90 ± 7.04^{ab}	2.34 ± 0.08^{cd}	0.90 ± 0.03^{ab}	3.21 ± 0.12^{c}
BP_2M	8.25 ± 0.11^{a}	558.00 ± 8.01^{a}	2.41 ± 0.10^{d}	0.80 ± 0.05^{a}	3.21 ± 0.11^{c}

[1]Values were presented as mean±SD.
[2]Different superscripts within the same columns differ p<0.05 for each parameter.
[3]TP: Total protein, P: Phosphorus, Glc: Glucose, TG: Triglyceride, TPhe: Total phenolics
Ctrl: Control, B: *Bacillus megaterium*, M: Poultry manure, P; Phosphorus fertilizer

TG concentration, and lipid profile: The highest TG concentrations were observed in BP_1M, BP_1, and P_1M (1.16±0.07, 1.15±0.06, and 1.12±0.04 mg TG/g dw, respectively) (p<0.05). On the other hand the lowest TG concentration was observed at the highest P concentrations combine with bacterial inoculation (BP_2M, 0.80±0.05 mg TG/g dw) (p<0.05) (Table 2). Influence of different P fertilization on the percentages of DAG, FFA, PL, and STR in total lipids of Hungarian vetch was shown in Table 3. The highest DAG, and PL, and the lowest FFA, and STR percentages in total lipids were determined in BP_1M (p<0.05) (Table 3). Instead, the highest FFA percentage in total lipids was determined in BP_2M (p<0.05) (Table 3). Moreover, the lowest DAG percentages in total lipids were determined in BP_2M, BP_2, and the lowest PL percentages in total lipids were determined in BP_2M, P_2M, BP_2, and P_2 (p<0.05) (Table 3).

De novo fatty acid synthesis starts with activation of cytosolic carbon sources to acetyl-CoA by activity of acetyl - CoA synthetase in an ATP-dependent reaction (Kuhn et al., 1981). Then malonyl-CoA required for acyl-chain elongation is synthesed from acetyl-CoA by acetyl-CoA carboxylase - another ATP-dependent reaction (Murray et al., 2009). The final reaction includes combination of the fatty acid with glycerol backbone which generating is also required ATP (Murray et al., 2009). The synthesis of fatty acids involves well balanced amounts of ATP and acetyl - CoA, and NADPH (Murray et al., 2009). In plants, NADPH and ATP are resulting from photosynthesis

(Armstrong, 1999). ATP also generated by substrate level phosphorylation in the reactions of glycolysis (Murray et al., 2009). On the other hand, cytosolic carbon sources for fatty acid synthesis such as glucose 6-phosphate (Glc6P), glucose, dihydroxyacetone phosphate, malate and pyruvate are created during glycolysis (Murray et al., 2009). A decrease in phosphate concentration due to limited Pi supply inhibits both glycolysis, and carbon flux that required for key anabolic pathways such as fatty acid synthesis, through glycolysis (Murray et al., 2009). In addition, PLDz1 and PLDz2 are PSI phospholipases that catabolize phospholipids during Pi deprivation (Li et al., 2006). In contrast to PLDz function in roots, a nonspecific phospholipase C5 is responsible for phospholipid degradation in leaves during Pi starvation (Gaude et al., 2008).

The changes in TG concentrations and lipid profiles were indicated that proper P supplementation increase lipid biosynthesis, and fatty acid incorporation with DAG to form TG, and decrease PL degradation. Acetyl CoA is the main source of sterol biosynthesis, and derived from both lipids, and carbohydrates (Murray et al., 2009). Relative changes in sterol percentage in total lipid without a reduction in TG, and Glc concentration may be a result of increase in the other lipid constituent in total lipids.

Table 3. Lipid profiles of Hungarian vetch at different P levels with or without *B. megaterium* inoculation

	FFA %	DAG %	STR %	PL %
Ctrl	23.20 ± 1.81^{d}	20.21 ± 1.09^{c}	29.66 ± 1.65^{cd}	26.92 ± 0.99^{b}
B	19.80 ± 1.37^{c}	19.60 ± 1.45^{bc}	31.95 ± 0.56^{de}	28.65 ± 1.69^{b}
M	21.26 ± 0.87^{cd}	18.60 ± 0.97^{bc}	31.10 ± 1.63^{cde}	29.05 ± 1.78^{b}
BM	15.86 ± 1.28^{ab}	23.87 ± 0.98^{d}	25.59 ± 1.05^{b}	34.68 ± 1.89^{cd}
P_1	17.68 ± 1.47^{b}	19.84 ± 1.13^{bc}	32.75 ± 1.21^{e}	29.73 ± 0.90^{b}
BP_1	14.73 ± 1.19^{a}	23.71 ± 0.76^{d}	25.13 ± 1.43^{b}	36.43 ± 2.00^{d}
P_1M	16.58 ± 0.74^{ab}	23.41 ± 0.81^{d}	26.52 ± 2.08^{b}	33.49 ± 1.57^{c}
BP_1M	14.61 ± 0.64^{a}	25.78 ± 1.35^{e}	22.54 ± 0.84^{a}	37.08 ± 1.31^{d}
P_2	28.07 ± 1.00^{e}	17.85 ± 0.38^{b}	30.70 ± 1.23^{cde}	23.37 ± 0.94^{a}
BP_2	30.36 ± 1.20^{f}	15.31 ± 1.22^{a}	31.18 ± 1.17^{cde}	23.14 ± 2.59^{a}
P_2M	28.22 ± 1.58^{e}	18.59 ± 1.02^{bc}	31.46 ± 0.86^{cde}	21.73 ± 1.27^{a}
BP_2M	34.38 ± 1.21^{g}	15.61 ± 1.35^{a}	29.11 ± 0.87^{c}	20.90 ± 0.82^{a}

[1]Values were presented as mean±SD.
[2]Different superscripts within the same columns differ $p<0.05$ for each parameter.
[3]FFA: Free fatty acids, DAG: Diacylglycerols, STR: Sterols, PL: Phospholipids.
Ctrl: Control, B: *Bacillus megaterium*, M: Poultry manure, P; Phosphorus fertilizer

Protein concentration: The maximum protein concentrations were observed in BP_1M, P_1M, and BP_1 (9.86±0.20, 9.70±0.35, and 9.70±0.35 mg TP/g dw, respectively) ($p<0.05$) (Table 2). Oppositely, the minimum protein concentrations were observed at high P levels combine with or without bacterial inoculation; BP_2M, P_2M, P_2, and BP_2 (8.25±0.11, 8.38±0.25, 8.46±0.17 and 8.48±0.15 mg TP/g dw, respectively) ($p<0.05$) (Table 2).

Phosphorus plays a significant role in N metabolism. Commonly, inadequate P causes decrease in NO_3-absorption by roots, and NO_3-translocation from roots to shoots (Pilbeam et al., 1993). The changes on total protein concentrations can be explained by higher P levels than optimum levels in soil cause a reduction in NO_3 - assimilation, and finally decrease the concentrations of proteins (Sánchez et al., 2004). Moreover, the unfavorable effects of high P concentrations on N metabolism may due to the antagonism between PO_4 and NO_3^- at absorption level (Marschner, 1995), and/or P toxicity on mycorrhizal fungal growth (Schwab et al., 1983). Additionally, PSB inoculation with P fertilizers at critical or high concentrations can render P toxic and/or increase the toxic effects on plant by increasing cumulative P concentration in soil (Silber et al., 2002).

Hexose (Glucose) concentration: The minimum Glc concentrations were observed in BP_1M, P_1M, and BP1 (1.80±0.07, 1.90±0.07, and 1.93±0.07 mg Glc/g dw, respectively) ($p<0.05$) (Table 2). Whereas, the maximum Glc concentrations were observed at higher

P levels combine with or without bacterial inoculation; BP_2M, P_2M, BP_2, and P_2 (2.41±0.10, 2.40±0.07, 2.34±0.08, and 2.26±0.11 mg Glc/g dw, respectively) ($p<0.05$) (Table 2).

The most important chemical reaction in nature is photosynthesis (Armstrong, 1999). Optimal photosynthesis involves an exceptionally leveled Pi concentration in the cytosol of plant cell (Usuda and Edwards, 1982). Hexokinase (HXK) catalyzes the first enzymatic step of sugar metabolism, the formation of hexose phosphates - glucose 6 - phosphate (Glc6P). HXK activity and hexose concentrations are affected by P level in the plant, and P - deficient plant has depressed HXK activity (Silber et al., 2002). Consequently, inadequate P reduces carbohydrate utilization, while carbohydrate production continues through photosynthesis (Armstrong, 1999), which is defined by low concentrations of hexose phosphate - high concentrations of hexose in plant cell (Silber et al., 2002).

Total phenolics concentration: The minimum total phenolics concentrations were observed in BP_1M, BP_1, P_1M, and P_1 (2.48±0.10, 2.55±0.35, 2.64±0.08, and 2.65±0.07 mg TAE/g dw, respectively) ($p<0.05$) (Table 2). In contrast, the maximum concentrations were observed at high P levels combine with or without bacterial inoculation; BP_2M, P_2M, and BP_2 (3.21±0.11, 3.21±0.12, and 3.16±0.06 mg TAE/g DW, respectively) ($p<0.05$) (Table 2).

In plants, reactive oxygen species are formed even during regular metabolism in plants, and electron transport is the major source of active oxygen production (Asada and Nakano, 1978). In similar, abiotic stress factors including UV radiation, low temperatures, wounding, low nutrients, and exposure to metal ions can induce the production of harmful reactive oxygen species within the cells (Alscher et al., 1997; Sakihama et al., 2000). Superoxide, the primary produced reactive oxygen species. It is detoxified to hydrogen peroxide (H_2O_2) by superoxide dismutase (Asada, 1994). H_2O_2 is not very harmful when compared with other reactive oxygen species, but in the presence of transition metals, H_2O_2 produces the most reactive oxygen species - the hydroxyl radical. Therefore, scavenging of H_2O_2 is essential to avoid oxidative damage of plant cells. Normally, the H_2O_2 is detoxified to water by the ascorbate-glutathione system (Foyer, 1993). In this system ascorbate acts as the electron donor for ascorbate peroxidase to remove H_2O_2 (Asada, 1997). However, under severe stress conditions including abiotic stress, plastid ascorbate pools are become oxidized and levels of the reactive oxygen species are exceed the scavenging capacity of the chloroplast. This phenomenon can induce accumulation of phenolics in plants (Yamasaki et al., 1995). The flavonoids are capable of scavenge H_2O_2 by serve as electron donors for guaiacol peroxidases (GuPXs) (Sakihama et al., 2000), and function as a backup defense mechanism to support the principal ascorbate-dependent detoxification system for vascular plants (Yamasaki et al., 1995). The changes on total phenolic concentrations were parallel with total P concentration, and may be a result of excess and/or inadequate P doses in soil cause a stress in Hungarian vetch - increase the accumulation of phenolic compounds (Alscher et al., 1997).

CONCLUSION

This study was aimed to determine the effects of different P fertilizers at different P concentrations on biochemical parameters of Hungarian vetch where PSB was used as phosphorus solubilizer. Due to significant improvement in the metabolic parameters, BP_1M (~80 kg ha^{-1} P with 10^{-8} CFU ml^{-1} PSB) was considered optimum dose or combination without negative effect for Hungarian vetch. On the other hand, PSB inoculation without chemical fertilizer was not more effective on biochemical parameters. However, PSB increased the positive effects of P on metabolism when used with P fertilizers (e.g. M vs BM or P_1 vs BP_1). As a conclusion, prior to inoculation of PSBs with P fertilizers, essential P and PSB concentrations for each crop should be determined precisely by using biochemical and crop yield parameters.

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