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Effect of Phosphate and Zinc Biofertilizers on Seed Yield and Molar Ratio of Phytic Acid to Zinc in Two Cultivars of Bean (*Phaseolus vulgaris* L.)

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***Abstract*—**In order to evaluate the effect of phosphate and Zn bio-fertilizers on the yield, phytic acid (PA), Zn concentration and PA/Zn molar ratio in bean, a field experiment was carried out for two years. The treatments included two cultivars of bean (Talash and Sadri), four levels of P (P_0, P_1: 100 kg ha^{-1} triple super phosphate (TSP), P2: 50 kg ha^{-1} TSP + phosphate bio-fertilizer, P_3: phosphate bio-fertilizer), three levels of Zn (Zn_0, Zn_1: 50 kg ha^{-1} ZnSO4, Zn_2: Zn bio-fertilizer). Phosphate bio-fertilizer consisted of inoculum of mycorrhizal fungus and Azotobacter and Zn bio-fertilizer consisted of Pseudomonas bacteria. The results revealed that there was significant difference between yield and Zn concentration between years. The effect of cultivar was significant on studied parameters. The lowest content of PA and PA/Zn were obtained from Talash. P treatment caused to significant difference on parameters in which P_2 caused to increase yield, P and Zn concentration, and decrease PA and PA/Zn by 21.8%, 38.2%, 33.4%, 17.4% and 38.6% respectively. Zn treatment caused to significant difference on studied parameters. The maximum number of parameters were obtained from Zn_1 and Zn_2. The higher Zn concentration led to lower content of PA and PA/Zn. Using of P and Zn bio–fertilizers were caused to increasing nutrient uptake, improving growth condition and reducing PA and PA/Zn molar ratio.

***Keywords*—**Mycorrhizae, phosphorus, pseudomonas, zinc.

I. Introduction

PA ($C_6P_6H_{18}O_{24}$) or myo-inositol hexakisphosphate is the main storage form of phosphorus (P) in grains of legumes and cereals [5], [11]. It includes of 50-80% of total grain P which is observed in the form of insoluble complexes with divalent cations as iron (Fe), Zn, calcium (Ca) and magnesium (Mg) in the grain growth and development process [5], [11]. Despite the high concentration of nutrient elements in bean, PA high chelating capacity strongly reduces availability of these elements and causes disturbances in the human gastrointestinal system [4]. PA is also able to form phytate-protein and phytate-mineral-protein complexes and thus impairs digestibility and bioavailability of proteins [9]. In the studies conducted on bean, PA content varied from 0.74 to 2.10% [5], [9].

World Health Organization (WHO) [19] introduced PA/Zn molar ratio as a good criterion to determine the Zn bioavailability in foods. Reference [5] showed that PA/Zn molar ratio effects on the assessment of Zn absorption, especially in cereals and legumes. WHO [19] announced that to absorb the nutrient elements in any foods, PA/Zn molar ratio must be smaller than 25. Reference [7] reported that PA/Zn molar ratio of 12 or higher caused a decrease in Zn absorption. Reference [19] reported that, if PA/Zn molar ratio in foods is less than 5, 5 - 15, and greater than 15, the food Zn content will absorb 55%, 35% and only 15% respectively.

Previous researches showed that a direct relation exists between amount of phosphorus uptake from the soil and its translocation from leaves to grains with grain PA concentration [5], [9], [14]. Also, P uptake is greatly affected by Zn deficiency [9]. Therefore, grain Zn supply can influence PA concentration. Reference [4] reported that the most abundant form of P, causing to formation of insoluble compound with other nutrients, was as a phytate or phytin. Reference [9] showed that Zn fertilizer caused to increase grain Zn concentration and decrease PA/Zn molar ratio in different chickpea genotypes. Reference [17] showed that in wheat cultivars and bread flours in Fars province of Iran, the least PA was found in Pavarus and Niknejad cultivars. The PA/Zn molar ratios were highest in Falat, Niknejad and Shiraz cultivars. The highest concentration of Zn was observed in Estar, Falat, and Niknejad, while maximum phytase activity was found in cultivars Estar, S-78-11, S-79-10, and Niknejad. The level of PA declines rapidly once the germination process starts [14]. Phytase activity in cereals and legumes increases as a result of phytase activation during germination [14].

According to restriction of product resources and reduction of soil fertility, food security is one of the major challenges facing many communities. The harmful effects of using unbalanced fertilizers especially P fertilizers has caused to produce and use of bio-fertilizers [18]. Biofertilizer is a preservative material consisting of one or several specific beneficial microorganisms or their metabolic products used to supply plant nutrients and development of root systems [18]. There are a lot of micro-organisms in soil capable to help plant nutrition and element uptake in different ways that can be mention to dual symbiotic relation between micro-organism and plant. Mycorrhizal fungus and plant growth promoting rhizobacteria (PGPR) such as *Azotobacter* and *Pseudomonas* are able to increase uptake of nutrient elements particularly when they are applied with each other [18], [16]. P bio-fertilizers contain beneficial P solubilizing fungus and bacteria causing to facilitate nutrient uptake especially elements with slow diffuse coefficient such as P, Zn and Cu. Also, they increase nutrient element availability with increasing volume

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of soil exploited by plants, spreading external mycelium, secreting organic acids, dehydrogenase and phosphates enzyme and reducing rhizosphere acidity [15], [16].

Bio-fortification is a process in which plants are allowed to take up the minerals (Fe and Zn) from the soil and immobilize them in the grains so as to produce nutritionally rich grains that support dietary requirement of humans [5]. The bio-fertilizers such as mycorrhizal symbiosis and Zn solubilizing bacteria are beneficial and economical ways to increase micronutrient in grains. The availability of micronutrients (Fe, Mn, and Zn) was positively affected by inoculation with arbuscular mycorrhizal (AM) fungi [15], [16], [18]. Reference [15] has shown that the mycorrhizal colonization facilitates acidification of rhizosphere, solubilization of tightly bound residual form Zn besides hyphal transportation of metallic micronutrients collectively contribute for the availability of theirs. Studies showed that a negative correlation exists between grain Zn and PA concentration [5], [9], [13]. *Azotobacter* bacteria that synthesis-promoting growth hormones such as indole acetic acid (IAA), gibberellin and cytokinin, causes an increase in plant growth and grain nutrient elements especially with low diffusing nutrient ions such as P, Zn and Cu [2], [12]. The main beneficial use of microorganisms is increasing of host plant growth. It can be done with increase of nutrient elements uptake. In this regard, a field experiment was conducted to evaluate the role of P and Zn bio-fertilizers on changes of some nutrient elements and decrease of PA/Zn molar ratio in the grain of two cultivars of bean.

II. Materials and Methods

A. Site Description and Weather Conditions

This study was conducted in two different sites situated in the Kiyar region of Chaharmahal va Bakhtiyari province located at 45 km southeast of Shahrekord, height 2096 m, annual precipitation 450 mm during spring and summer 2014 and 2015. The climate of this area is characterized by moderate summers and cold and humid winters. The soil famile name in two sites was fine, mixed, mesic, typic Calcixerepts. The physical and chemical characteristic of the soil experiment sites are presented in Table I.

TABLE I
Physical and Chemical Properties of Soil

Year	pH	EC	TNV	OC	N	P	K	Fe	Zn	Cu	Sand	Silt	Clay
		(dS m^{-1})		(%)				(mg Kg^{-1})				(%)	
1	7.8	0.88	24.5	0.92	0.07	6	311	4.11	0.58	0.93	20	54	26
2	8	0.50	23.4	1.57	0.1	9	362	4.02	0.55	0.89	20	45	35

B. Experimental Design and Statistical Methods

The experiment was carried out as a factorial in a randomized complete block design (RCBD) with four replications for two years. The treatments consisted of two cultivars of Pinto bean (C_1: Talash, C_2: Sadri), four levels of P (P_0: Control, P_1: Use of chemical fertilizer on the basis of soil test, P_2: 50% of P fertilizer recommendation + phosphate bio-fertilizer, and P_3: Use of phosphate bio-fertilizer), three levels of Zn (Zn_0: Control, Zn_1: 50 kg ha^{-1} $ZnSO_47H_2O$, and Zn_3: Use of Zn bio-fertilizer) (2×4×3×3 = 72 plots). The size of each plot was 3 × 4 meters. Statistical analysis was done by SAS (version 9.1.3) statistical software. The means were subjected to Duncan's multiple range test.

C. Biological Treatments and Cultural Conditions

Phosphate bio-treatment consisted of inoculums with *mycorrhizal* fungus (*Clariodeoglumus etunicatum*, *Rhizophagus intraradices and Funneliformiss mosseae* with the population of 115 viable spores g^{-1}) and *Azotobacter chroococcum* strain 5 bacteria (1.8×10^8 viable cell g^{-1} of inoculants). Zn bio-treatment consisted of inoculum with mixture of *Pseudomonas aeruginosa* strain MPFM and *Pseudomonas fluorescens* strain 187 (5×10^8 viable cell of bacteria g^{-1} of inoculant). Grain inoculation (5%) was done in shadow and after drying, inoculated grains were immediately cultivated. 2 g of mycorrhizal fungus was applied at the base of the grain hole just prior to sowing. Chemical fertilizers were applied from TSP at a rate of 100 and 50 kg ha^{-1} in P_1 and P_2 respectively, 50 kg ha^{-1} $ZnSO_4.7H_2O$ in Zn_1 and 50 kg ha^{-1} urea as a starter before planting. The bio-fertilizers are provided from soil and water research institute (SWRI), Tehran, Iran and grains from national bean research center, Khomein, Arak, Iran. During the growing season, necessary cures such as irrigation, weeds, pests and diseases control were applied identically for all treatments.

D. The Assay of PA and Nutrient Elements

Samples were powdered and sieved for determination of PA content, according to [8]. The assay was based on indirect spectrophotometric determination of phytic P in dry bean extracts. About 0.5 g ground grain sample was used for extraction of PA in 25 ml 0.2 N HCl (pH=3) for 3 hours. The extracts brought up to 50 ml with de-ionized water. 1 ml of supernatant was treated with ammonium iron(III) - sulfate solution ($NH_4Fe(SO_4)_2.12H_2O$) in a boiling water bath for 30 minutes. After cooling, samples were centrifuged and 1 ml supernatant was treated with a 1.5 ml bipyridine solution and absorbance was measured at 519 nm with spectrophotometer (Shimadzu UV 3100). The molar ratio of PA/Zn was calculated by dividing mili moles of PA to mili moles of Zn.

$$PA/Zn = PA\ (mg\ 100g^{-1})/660 / Zn\ (mg\ 100g^{-1})/65.4$$

P and Zn concentrations were measured after harvesting the grain yield. Zn was determined by an atomic absorption spectrometer and P by the spectrophotometer.

III. Results and Discussion

A. Yield

The results of analysis of variance (ANOVA) showed that the grain yield was significantly affected ($p \leq 0.01$) by the year (Table II). The maximum content of yield, 3317 kg ha^{-1}, was obtained from the second year (Table III). The best soil fertility was the reason for yield superior in the second year. Significant difference ($p \leq 0.01$) was showed between cultivars as well (Table II). As shown in Table III the grain yield in Sadri and Talash were 3188 and 3348 kg ha^{-1} respectively. It differs with using of P treatment. The highest content of yield, 3530 kg ha^{-1}, was obtained from P_2 treatment (21.9% grain yield increase). Zn treatment was significant on this parameter ($p \leq 0.01$) (Table II). The maximum content of grain yield, 3404 kg ha^{-1}, was obtained from Zn_1 treatment (Table III). Among the interaction effects, the effect of year × P ($p \leq 0.05$), C × Zn and P× Zn ($p \leq 0.01$) was significant on grain yield (Table II). The highest content of its, 3656 kg ha^{-1}, was obtained from P_2Zn_1 (42.5% grain yield increase). Our results are similar to findings of other researches [10], [13], [16]. They reported that dual inoculation increase plant productivity. In this study, Phosphate and Zn bio-fertilizers could cause to increase grain yield by increasing nutrient availability, leaf area index, chlorophyll content, photosynthesis, growth hormones and creating favourable growth conditions.

TABLE II
Analysis of Variance of Experimental Traits

Treatment	(Mean squares)					
	df	Yield	P	Zn	PA	PA/Zn
Year	1	352836**	28.4^{ns}	0.002**	1.2^{ns}	33.7^{ns}
Cultivar(C)	1	921600**	34.5**	0.003**	23**	113*
P	3	2814057**	288**	0.01**	18.4**	514**
Year×P	3	74650*	2.6^{ns}	0.0001^{ns}	1.7^{ns}	32^{ns}
C×P	3	5376^{ns}	5.7^{ns}	0.00008^{ns}	4.6*	17^{ns}
Year×C×P	3	1092^{ns}	1.8^{ns}	0.00008^{ns}	0.2^{ns}	14^{ns}
Zn	2	1377250**	5*	0.008**	5.4*	322**
Year×Zn	2	27568^{ns}	6.2^{ns}	0.00006^{ns}	4.1^{ns}	41^{ns}
C×Zn	2	124423**	10*	0.0001^{ns}	13**	140**
Year×C×Zn	2	17362^{ns}	7.6^{ns}	0.00001^{ns}	7*	66*
P×Zn	6	108648**	3^{ns}	0.0003**	3.3^{ns}	25^{ns}
Year×P×Zn	6	17362^{ns}	0.1^{ns}	0.00002^{ns}	1^{ns}	14^{ns}
C×P×Zn	6	17910^{ns}	1.1^{ns}	0.00007^{ns}	2.9^{ns}	20^{ns}
Year×C×P×Zn	6	930^{ns}	0.5^{ns}	0.000003^{ns}	0.7^{ns}	25.5^{ns}
Error	92	20480	2.2	0.00009^{ns}	14	20.3
Total	143					
CV (%)		4.3	10.4	10	12.2	13

ns, ** and * respectively: non-significant, significant in 1% and 5% area.

A. P and Zn Concentrations

According to the analysis of variance results (Table II), a significant difference was observed for P concentration between two cultivars and for Zn concentration between two years and cultivars ($p \leq 0.01$). The highest concentration of Zn, 30.3 mg kg^{-1}, was obtained from the second year (Table III). The concentration of P and Zn in Sadri was greater than Talash (Table III). It was in order to higher germination, rooting, chlorophyll content, leaf area index and better growth condition in Sadri (Unpublished data).

TABLE III
Mean Comparison of Phosphate and Zn Bio-Fertilizers Simple Effects on Studied Parameters

Treatment	Yield	P	Zn	PA	PA/Zn
	(Kg ha^{-1})	(%)	(mg kg^{-1})	(g kg^{-1})	
Year1	3218b	0.41a	28.8b	5a	17.4a
Year2	3317a	0.40a	30.3a	4.83a	16.4a
C1	3188b	0.39b	28.8b	4.52b	16.1b
C2	3348a	0.42a	30.2a	5.32a	17.8a
P0	2897d	0.34d	25.1d	5b	20.2a
P1	3217c	0.38c	29.0c	5.85a	19.8a
P2	3530a	0.47a	33.5a	4.13c	12.4c
P3	3429b	0.45b	30.6b	4.70c	15.1b
Zn0	3079b	0.43a	26.5b	5.28a	20.0a
Zn1	3404a	0.40b	31.4a	4.85ab	15.5b
Zn2	3321a	0.39b	30.7a	4.62b	15.3b

Means in each column and part by a different letter are significantly different ($P \leq 0.05$) by Duncan's Multiple range test

P treatment was caused to significant difference on these elements ($p \leq 0.01$). An increase of P (38.2%) and Zn (33.5%) was observed comparison with the control in P_2 treatment (Table III). Zn treatment was significant on grain P concentration ($p \leq 0.05$) and Zn concentration ($p \leq 0.01$). The maximum content of P (0.43 %) and Zn fortification (30.7 mg kg^{-1}) were obtained from Zn_0 and Zn_2 treatments respectively (Table III). Zn concentration was increased because of using Zn fertilizer and biological treatment. It was corresponding with the findings of [1], [5], [15]. The antagonistic effect between P and Zn was caused to decrease P concentration in Zn_1 and Zn_2 treatments [9], [12], [13], [15]. Interaction effect between P × Zn treatments wasn't significant on P concentration ($p \geq 0.05$) but significant ($p \leq 0.01$) on Zn

concentration (Table II). Nevertheless, the highest content of P (0.46%) and Zn (36.6 mg kg^{-1}) was obtained from P_2Zn_1. These changes can be related to the effect of biological treatments on increasing of nutrient element uptake [13], [16]. Studies have shown that in the most cases inoculant plants with mycorrhizal fungus and phosphate solubilizing bacteria (PSB) lead to uptake of elements with low mobility especially P and Zn on the host plant [13], [16]. P uptake was increased by mycorrhizal plants with different mechanisms such as seeking a greater volume of soil, enhancing P uptake velocity by hyphae, increasing the dissolution rate of P by secreting organic acid and phosphatase enzyme and rhizosphere acidification [10], [15]. These results consistent with findings of other researchers such as [2], [16]. They reported that the availability of nutrients was increased by dual application of *mycorrhizae* and *Azotobacte*r. Reference [12] showed that symbiosis between plants and mycorrhizal fungi can deliver up 25% of host plant Zn. Our data suggest that mycorrhizal symbiosis may be a potential factor in alleviating Zn deficiency of host plants. This process may facilitate bio-fortification of grains with micronutrients. The grain Zn concentration was increased with using of P bio-fertilizers. This increase was done due to hyphal transport, rhizosphere acidification, Fe and Zn siderophore production, solubilisation of tightly bound residual form of Zn, chelate condition improvement and increasing its availability [9], [13].

B. PA Concentration

There weren't significant differences ($p \geq 0.05$) between two years in PA concentration, but a significant difference ($p \leq 0.01$) was observed between two cultivars (Table II). Its concentration was 4.52 and 5.32 g kg^{-1} in Talash and Sadri respectively (Table III). PA concentration differs with using of P treatment. The highest content of PA (5.85g kg^{-1}) was obtained from P_1 treatment. The content of PA increased with using of P fertilizer. In this experiment, a positive relation was observed among application of phosphate chemical fertilizers, soil P, grain P and PA. This result corresponds with the findings of [5], [14]. Zn treatment was significant on this parameter ($P \leq 0.05$) (Table II). The lowest content of PA, 4.62 g kg^{-1}, was obtained from Zn_2 (Table III). As shown in Table II the interaction effect of C × P, Year × C × Zn ($p \leq 0.05$) and C × Zn ($p \leq 0.01$) was significant on this parameter. The highest and lowest of PA content, 5.92 and 4.08 g kg^{-1}, were obtained from C_2Zn_0 and C_1Zn_1, respectively (Fig. 1). Also, the highest and lowest of PA content, 6.78 and 3.80 g kg^{-1}, were obtained from C_2P_1 and C_1P_2, respectively (Fig. 2).

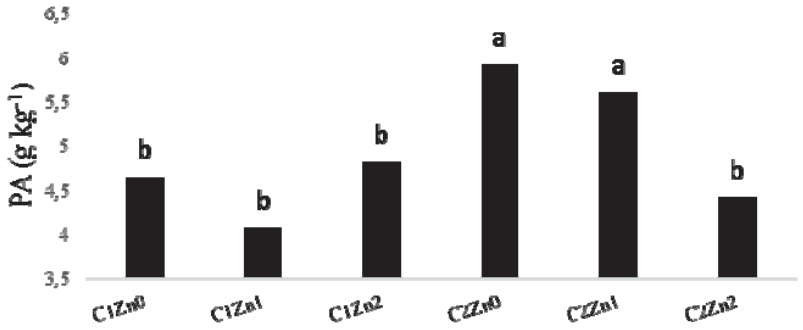

Fig. 1 Mean comparison interaction effect between cultivar and Zn on grain PA concentration ($a \leq 0.05$)

The increase of grain Zn concentration, with using chemical (Zn_1) fertilizers and bio-fertilizers (Zn_2), reduces PA concentration. Soil Zn application had a significant positive effect on grain Zn concentration and also on grain yield especially under Zn deficient conditions. Zn fertilization not only alleviates Zn deficiency but also promotes root growth.

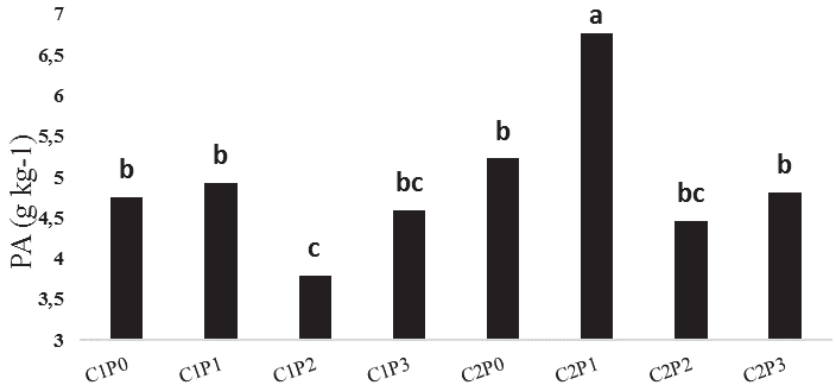

Fig. 2 Mean comparison interaction effect between cultivar and Phosphorus on grain PA concentration ($a \leq 0.05$)

This result consistent with earlier studies [5], [9], [15]. Reference [13] have reported that an indirect relation exists between the improved of Zn availability in soil and increase it concentration. Also, a negative correlation exists between wheat genotypes that rich in Zn with content of PA. Similar results have been reported in chickpea genotypes [9]. Our study has clearly shown an increase in grain Zn which may have reduced the PA concentration (Table III). The absorption, translocation and movement of P in plant were affected by the Zn and P antagonistic effect. This effect caused to reduce P absorption and consequently decrease PA content. Reference [5] reported that fertilization of twenty wheat cultivars, decrease P concentration from 0.39% to 0.35% and increase PA content from 10.7 to 10.1 mg g^{-1}. *Pseudomonas* bacteria in the Zn bio-fertilizers causes to acidification of rhizosphere and solubilization of insoluble and tightly bound residual form of Zn by increasing root colonization, secreting siderophore compounds and increasing chelating acids such as mugneic and gluconic acid [15]. Hence, the PA concentration reduces with increasing the grain Zn. One of the reasons to decrease PA with increasing grain Zn can be related to the inhibitory effect of Zn on root uptake and shoot accumulation of P [9], [12], [13]. It is reported that Zn deficient plants possess an enhanced uptake capacity for P, and supply of Zn so that reduce uptake and accumulation of P in plants [3]. Therefore, because of antagonistic effect between P and Zn, with P increase, Zn decrease which causes to PA increase (Table III). There are some different methods such as enhancing phytase activity, soaking, germination, fermentation, milling and thermal processing that decrease PA content after grain harvest [6], [11], [14], [17]. These methods aren't practical for reducing PA because reduce nutrient bioavailability especially Zn by causing chemical changes. The field enrichment of grain with using bio-fertilizers superiors to these methods because it is safer, cheaper and healthier than after harvest methods.

C. PA/Zn Molar Ratio

According to the ANOVA results (Table II) the effect of C, Year × C × Zn ($p \leq 0.05$), P, Zn and C × Zn ($p \leq 0.01$) was significant on PA/Zn molar ratio (Table II). PA/Zn molar ratio was 16.1 and 17.8 in Talash and Sadri cultivars respectively (Table III). In P treatment the lowest rate of PA/Zn, 12.4, was obtained from P_2 which was 38.6 % lower than control (Table III). The lowest rate of PA/Zn, 15.3, was obtained from Zn_2 that situated with Zn_1 in common statistical group. The highest and lowest rate of PA/Zn molar ratio was obtained from C_2Zn_0 (21.7) and C_2Zn_2 (12.7). Nevertheless, the non-significant interaction effect between P × Zn on this ratio, the lowest rate of its was obtained from P_2Zn_1 which 59.1% lower than control. The maximum of PA/Zn molar ratio, 23.6, was obtained from P_1Zn_0. The results were consistent with the findings of [9], [13]. They showed that *mycorrhizal* fungi increase the grain Zn absorption and indirectly decreases PA/Zn molar ratio. The Zn fertilizer increases Zn absorption which decreases PA/Zn molar ratio in Zn_1. Likewise, *Pseudomonas* bacteria increases Zn solubilisation in Zn_2 treatment. Zn solubilizer bacteria such as *Pseudomonas* species can solubilize Zn from low solubility and insoluble compounds with the mechanisms that explained in Section III B. Reference [1] reported that *Pseudomonas fluorescens strain* P_{19} efficiently solubilized Zn from insoluble and low soluble compounds and therefore increased its concentration in plant. Using of P and Zn bio-fertilizer in this study, with releasing Zn from unavailable Zn resources, was decreased indirectly the molar ratio of PA/Zn. The effect of Zn fertilization in deficiency soils reduces the PA/Zn molar ratio [5]. Because of soil Zn deficiency (smaller than 1 mg kg^{-1}), PA/Zn molar ratio was decreased by Zn fertilization in this study. In fact, a negative relation was observed between Zn concentration and PA content. So, PA/Zn molar ratio was reduced by using P and Zn bio-fertilizers and providing Zn from chemical fertilizer. Meanwhile these two cultivars could be introduced as suitable cultivars with low PA/Zn molar ratio.

IV. Conclusion

This study showed that the individually or combined use of P and Zn bio-fertilizers leads to increase grain yield, P and Zn concentrations in two studied bean cultivars. PA concentration showed negative and positive relation with grain Zn and P concentration, respectively. The best treatment in this study was the use of *Azotobacter* inoculants with *mycorrhizal* fungi, 50 kg ha^{-1} TSP and 50 kg ha^{-1} $ZnSO_4$ (P_2Zn_1 treatment). Therefore, with proper integration of bio-fertilizers and chemical fertilizers in addition to reduce use of chemical fertilizers caused to increase yield and bio-enrichment, improve quality and quantity of products, reduce PA and PA/Zn molar ratio and decrease environmental pollution.

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