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Effect of Biological and Chemical Fertilization on the Yield and Nutrients of Moldavian Balm (*Dracocephalum moldavica* L.) Seeds

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To evaluate the effect of microbial inocula application on the yield and nutrients content of Dracocephalum moldavica L. seeds, a field experiment based on randomized complete block design with four replications was conducted at the Agricultural and Natural Resources Research Center of West Azerbaijan (Urmia, Iran) in 2010. Treatments were microbial inocula [seed inoculation with nitrogen fixing bacteria (Nb), phosphate solubilizing bacteria (Pb), sulfur oxidizing bacteria (Sb), Nb+Pb, Nb+Sb, Pb+Sb, Nb+Pb+Sb], chemical fertilizer (nitrogen+phosphorus+potassium of chemical origin (NPK)) and control (no fertilizer). The highest yield of seeds (3366 kg ha^{-1}) was obtained in the NPK treatment. Maximum seed nitrogen (3.74 %) and manganese (0.0062 mg kg^{-1}) contents occurred in Nb treatment. The highest percentage of sulfur (0.56 %) and content of zinc (0.184 mg kg^{-1}) were obtained in Sb treatment, while the maximum iron content (0.62 mg kg^{-1}) was observed in the Nb+Pb+Sb. In conclusion, seed inoculation with sulfur oxidizing bacteria (Thiobacillus strains) improves nutrients availability in calcareous soils.

Keywords: Dracocephalum moldavica, inoculum, nutrients, seed yield, Thiobacillus.

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Влияние биологических препаратов и минеральных удобрений на урожайность и содержание питательных веществ в семенах змееголовника молдавского (*Dracocephalum moldavica* L.)

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Для изучения влияния предпосевной обработки инокулятом почвенных микроорганизмов на урожай и содержание питательных веществ в семенах Dracocephalum moldavica L. в 2010 году в Центре исследования сельскохозяйственных и природных ресурсов западного Азербайджана (г. Урмия, Иран) был проведен полевой эксперимент, основанный на рандомизированной блочной схеме с четырьмя повторностями. Вариантами эксперимента были: инокулят микроорганизмов [предпосевная обработка семян азотфиксирующими бактериями (N_b), фосфатсолюбилизирующими бактериями (P_b), сероокисляющими бактериями (S_b), N_b+P_b, N_b+S_b, P_b+S_b, N_b+P_b+S_b], минеральное удобрение (азот+фосфор+калий химического происхождения (NPK)) и контроль (без удобрения). Самый высокий урожай (3366 кг/га) был получен при использовании минеральных удобрений (NPK). Максимальное содержание азота (3.74 %) и марганца (0.0062 мг/кг) в семенах было обнаружено в варианте с предпосевной обработкой азотфиксирующими бактериями (N_b). Наивысшее содержание серы (0.56 %) и цинка (0.184 мг/кг) было получено после обработки сероокисляющими бактериями (S_b). В то же время максимальное содержание железа (0.62 мг/кг) наблюдалось в варианте N_b+P_b+S_b. Таким образом, при выращивании растений на известковых почвах предпосевная обработка семян сероокисляющими бактериями (штаммы Thiobacillus) повышает доступность питательных веществ.

Ключевые слова: Dracocephalum moldavica, инокулят, питательные вещества, урожай семян, Thiobacillus.

Introduction

Moldavian balm or Moldavian dragonhead (*Dracocephalum moldavica* L.) is a perennial herb belonging to the Lamiaceae (Labiatae) family, native to central Asia and naturalized in Eastern and Central Europe. In Iran it is predominantly found in the North and Northwest of the country (Dastmalchi et al., 2007).

Essential oil of dragonhead has antioxidant activity (Dastmalchi et al., 2007), antiseptic and antibacterial properties, and is used for stomach ache and bloating (Omidbaigi et al., 2010). The seeds of dragonhead are astringent, carminative, tonic, and a source of omega-3 fatty acids. It is rich in linolenic acid, one of the acids that are necessary for proper function of whole human body (Dastmalchi et al., 2007).

Using eco-friendly methods of plant nutrition, with the aim of reducing chemical inputs while sustaining yield, could allow maximizing quantitative and qualitative yield of medicinal plants (Darzi, 2012; Kapoor et al., 2004). The existence of a microorganism enhances the growth of plants by replacing soil nutrients (e.g., by biological N_2 fixation), making nutrients more available (e.g., by solubilization of phosphates), and increasing plant access to nutrients (e.g., by increasing root surface area). As long as the nutrient status of the plant is enhanced by the microorganisms, the substance that contains the microorganisms and is applied to the plant or soil is referred to as a biofertilizer (Vessey, 2003).

Biological nitrogen fixation by free-living nitrogen fixing microorganisms occurs in soils by several aerobic (*Azotobacter*, *Beijerinickia*) or micro-aerobic (*Azospirilum*) prokaryotes (Kavadia et al., 2007). These bacteria contain nitrogenase enzyme responsible for the fixation of atmospheric nitrogen (N_2), improving the soil fertility (Gothwal et al., 2008). Economic and environmental benefits of biological N_2-fixation include reducing fertilization cost, leaching of NO_3–N to ground water as well as emission of greenhouse gas (N_2O) (Mikhailouskaya and Bogdevitch, 2009).

Phosphate solubilizing microorganisms have been reported to increase the availability and uptake of soil phosphorus by converting insoluble phosphates to soluble forms through the production of various organic acids. Phosphate solubilizing microorganisms' inoculants include species of *Bacillus* and *Pseudomonas*, known to make available up to 30–35 kg ha^{-1} P_2O_5 per year (Rugheim and Abdelgani, 2012).

The biochemical oxidation of sulfur (S) produces H_2SO_2 which decreases soil pH and solubilizes $CaCO_3$ in alkaline calcareous soils. Such process makes soil conditions more favorable for plant growth, including an increased availability of soil P (Linderman et al., 1991). Also, application of S to alkaline calcareous soils could assist in correcting iron chlorosis and reclamating sodic and alkaline soils. In calcareous soils, supplying plant with sulfur would only be efficient when it is oxidized to sulphate by soil microorganisms like *Thiobacillus* species (Besharati et al., 2007).

The main objective of this research was to investigate the effect of microbial inocula (N- fixing, P- solubilizing, and S- oxidizing) application on the yield and nutrients content of Moldavian balm seeds in comparison with chemical nutrition system.

Materials and methods

Field experiment

The field experiment was conducted at the Agricultural and Natural Resources Research Center of West Azerbaijan (latitude 45º10'53'' N, 37º44'18'' E, 1338 m above sea level), Urmia, Iran in 2010. Characteristics of soil are shown in Table 1. The experiment was carried out in a randomized complete block design with four replications and 9 treatments, including: [(N_b) *Azotobacter* spp. and *Azospirillum* spp.], [(P_b) *Bacillus lentus* strain p5 and *Pseudomonas putida* strain p13], [(S_b) *Thiobacillus* spp.], (N_b+P_b), (P_b+S_b), (N_b+S_b), (N_b+P_b+S_b), [(NPK) chemical fertilizers of nitrogen+phosphorus+potassium], and untreated control. Plots were prepared in nine 3-meter long rows (40 cm inter-row spacing and 10 cm intra-row spacing), where the seeds were

Table 1. Physical and chemical properties of the experimental field soil

Mn	Zn	Cu	Fe	SO_4^{2-}	K	P	N	pH	EC (dS m^{-1})	Organic carbon (%)	Organic matter (%)	T.N.V (%)	Silt : Clay : Sand (%)	Soil texture	Soil depth (cm)
(mg kg^{-1})							(%)								
6.2	0.75	2.41	5.8	58.25	275	6	0.09	7.9	0.8	0.9	1.55	16	30:41:29	Clay-loam	0-30

sown to a depth of 2 cm. Triple superphosphate (phosphorous source, 150 kg ha^{-1}) and potassium sulfate (potassium source, 100 kg ha^{-1}) were applied before sowing, but urea (nitrogen source, 150 kg ha^{-1}) was added to soil at two stages (before sowing and at the bi-foliate stage). Seeds were inoculated with the microbial inocula before sowing. Phosphate solubilizing (*Pseudomonas putida* strain p13, *Bacillus lentus* strain p5), nitrogen fixing (*Azotobacter* spp. and *Azospirillum* spp.) and S-oxidizing (*Thiobacillus* spp.) bacteria, isolated from soils of Iran, were used for inoculation. These biological inocula were provided by Mohammad Ali Malboobi (Green Biotech Incorporation). Wet seeds were rolled into the suspension of bacteria (10^8 cfu ml^{-1}) until uniformly coated.

Seed yield

To determine the yield of seeds whole plants were harvested from 1 m^2 (seeding stage) in each plot and dried at 70 ºC until constant weight.

Measurement of macro- and micronutrients

Dried seeds were milled and homogenized before measurements. Seed phosphorus content was determined as described in Watanabe and Olsen (1965) and Onishi et al. (1975). In brief, after combustion (4 h at 500 ºC) of powdered seed sample, ashes (5 mg) were digested in concentrated hydrochloric acid (1 mL of conc. HCl). The samples were then filtered and total P was quantified as phosphate (PO_4^-) using the ascorbic acid method (Watanabe and Olsen, 1965). The amount of phosphate in solution was determined colorimetrically at 882 nm (Methods to study..., 2005).

Total nitrogen in the dry seeds was determined by the micro-Kjeldahl method (Jackson et al., 1973). About 25 mg of samples were transferred to a micro-digestion tube and digested with 1 mL of low nitrogen concentrated H_2SO_4 and a few mg of 3 : 1 $CuSO_4$-K_2S0_4 mixture. Two or three drops of 30 % hydrogen peroxide were added and the digestion was completed. The acid digest was diluted with 1 mL of ammonia-free water and washed into the distillation apparatus. After adding 7 mL of 30 % NaOH, the mixture was distilled with steam for 5 minutes. Distillates were boiled and titrated with 0.01 N NaOH to a methyl red (Stuart, 1936).

Potassium was determined by flame photometry (Chapman and Pratt, 1961). Sulfur was determined by turbidimetric method (Wall et al., 1980). Fe, Zn, and Mn were immediately analyzed following the digestion by flame atomic absorption spectrometry (FAAS) (Chapman and Pratt, 1961).

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) using the SAS 9.1 software package (SAS, 1998). Differences between the treatments were evaluated by Duncan's Multiple Range Test (DMRT) at 5 % confidence interval.

Results

The highest seed yield (3366 kg ha^{-1}) was obtained in NPK treatment, while the lowest seed yield (1485 kg ha^{-1}) occurred in the untreated control. In all inoculated treatments seed yield was higher than in untreated control, but lower than in NPK treatment (Fig. 1). There was no significant difference between NPK and N_b (*Azotobacter* and *Azospirillum*) treatments. The seed yield significantly decreased in the presence of phosphate solubilizing bacteria (Pb and Nb+Pb) and did not differ significantly from the value obtained in control (Fig. 1).

The nitrogen content varied from 3.11 % to 3.74 % in control treatment and in plants inoculated with N_b, respectively. There were no significant differences between control, P_b, S_b, P_b+S_b, and NPK treatments. Application of N_b, N_b combined with P_b and S_b, and consortium (N_b+P_b+S_b) induced the significant increases of seed nitrogen content in comparison to control. The other biological treatments caused a slight increase of seed nitrogen content in comparison to control, but it was not significantly different from those induced by the chemical treatment (NPK) (Fig. 2-I).

The highest sulfur content of seeds (0.56 %) was observed in plants inoculated with sulfur oxidizing bacteria (*Thiobacillus* sp.) and N_b+P_b+S_b (Fig. 2-II). The minimum sulfur content was observed after the chemical fertilization (0.31 %) and in untreated control (0.33 %). There were no significant differences between seed sulfur content of plants treated with N_b, P_b, N_b+P_b and control. However, all combinations of inocula containing sulphur oxidizing bacterium *Thiobacillus* sp. (S_b, N_b+P_b+S_b, N_b+S_b, P_b+S_b) induced a higher sulfur content of seeds in comparison to control.

The highest seed iron accumulation (0.62 mg kg^{-1}) occurred in plants inoculated with *Thiobacillus* sp., while the lowest amounts of seed iron (0.39 mg kg^{-1}, 0.38 mg kg^{-1}) were observed in control and NPK treatments. Seed inoculation by *Thiobacillus* sp. led to accumulation of the iron in higher concentration than in control (Fig. 3-I). Accumulation of iron in seeds of plants treated with sulfur oxidizing bacteria (S_b, N_b+S_b, P_b+S_b, and N_b+P_b+S_b) (Fig. 3-I) is similar with seed accumulation of sulfur (Fig. 2-II).

The highest Mn content in seeds (0.0069 mg kg^{-1}) resulted from the chemical fertilizer treatment and the lowest amount of seed Mn was recorded in the N_b+P_b+S_b and N_b+S_b treatments (Fig. 3-II).

Zn content in seeds of plants inoculated with *Thiobacillus* sp. (S_b, N_b+S_b, P_b+S_b, and N_b+P_b+S_b)

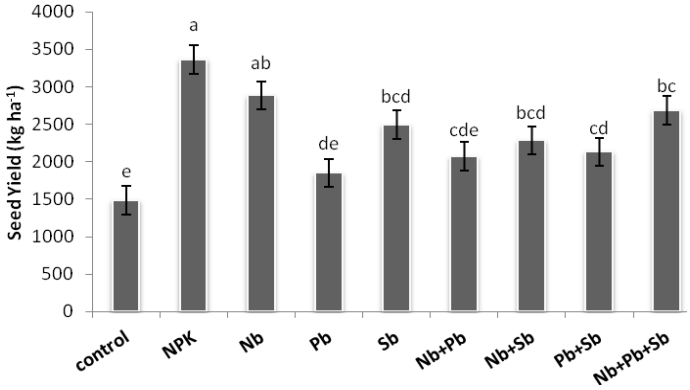

Fig. 1. Seed yield of *Dracocephalum moldavica* L. fertilized with different microbial inocula or chemical fertilizer. Means ± SD (or SEM) with the same letters are not significantly different ($P \leq 0.05$)

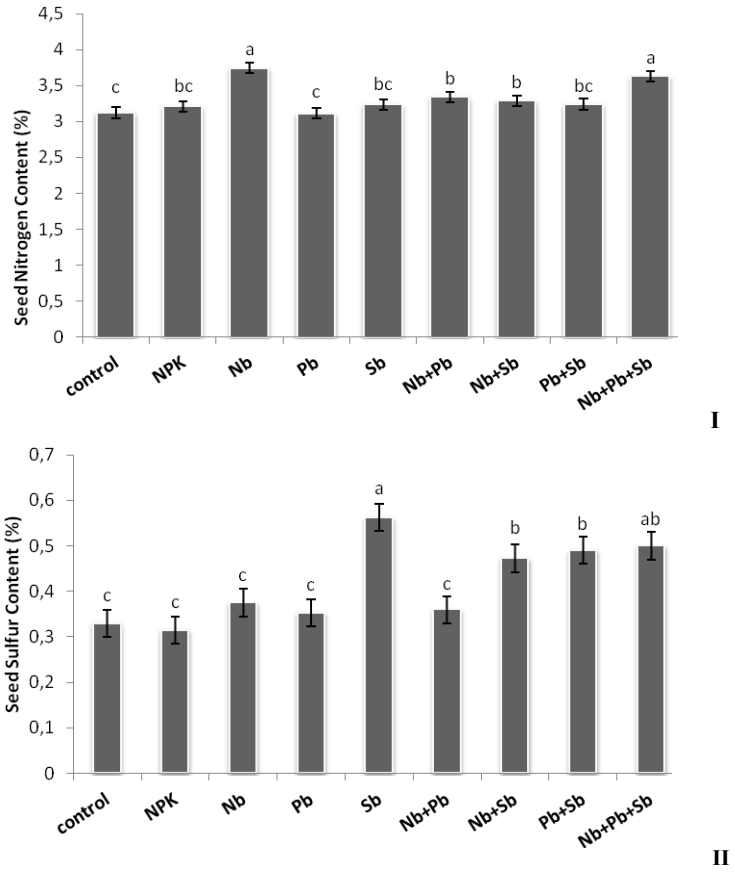

Fig. 2. Nitrogen (I) and sulfur (II) content in seeds of *Dracocephalum moldavica* L. fertilized with different microbial inocula or chemical fertilizer. Means ± SD (or SEM) with the same letters are not significantly different ($P \leq 0.05$)

was significantly higher than in untreated control. The lowest Zn concentration (0.117 mg kg^{-1} and 0.113 mg kg^{-1}) was observed in plants treated by chemical fertilizer and in untreated control, respectively. Zinc concentration in all treatments inoculated with microbial strains tended to be higher in comparison to untreated control (Fig. 3-III).

ANOVA did not show any significant effect of the treatments on the phosphorus and potassium content of seeds.

Discussion

Our results showed that all inocula noticeably increased seed yield in comparison with control (no fertilizer), but were less effective than chemical fertilization except the non- significant difference between NPK and N_b *(Azotobacter+Azospirillum*). High yield with N_b treatment indicates a beneficial effect of N fixation by non-symbiotic bacteria strains for plants nutrition. *Azotobacter* and *Azospirillum* species play important role in root development and this leads to an increase the nutrients absorption (Ardakani et al., 2011). The promotion in seed yield by microbial inocula could be attributed to enhance supply of nutrients such as N and P to the crop and production of growth promoting substances (Rokhzadi et al., 2008; Uddin et al., 2014).

Increased nitrogen content of dragonhead seeds obtained by inoculation with nitrogen fixing bacteria might be due to N-fixation, hormone

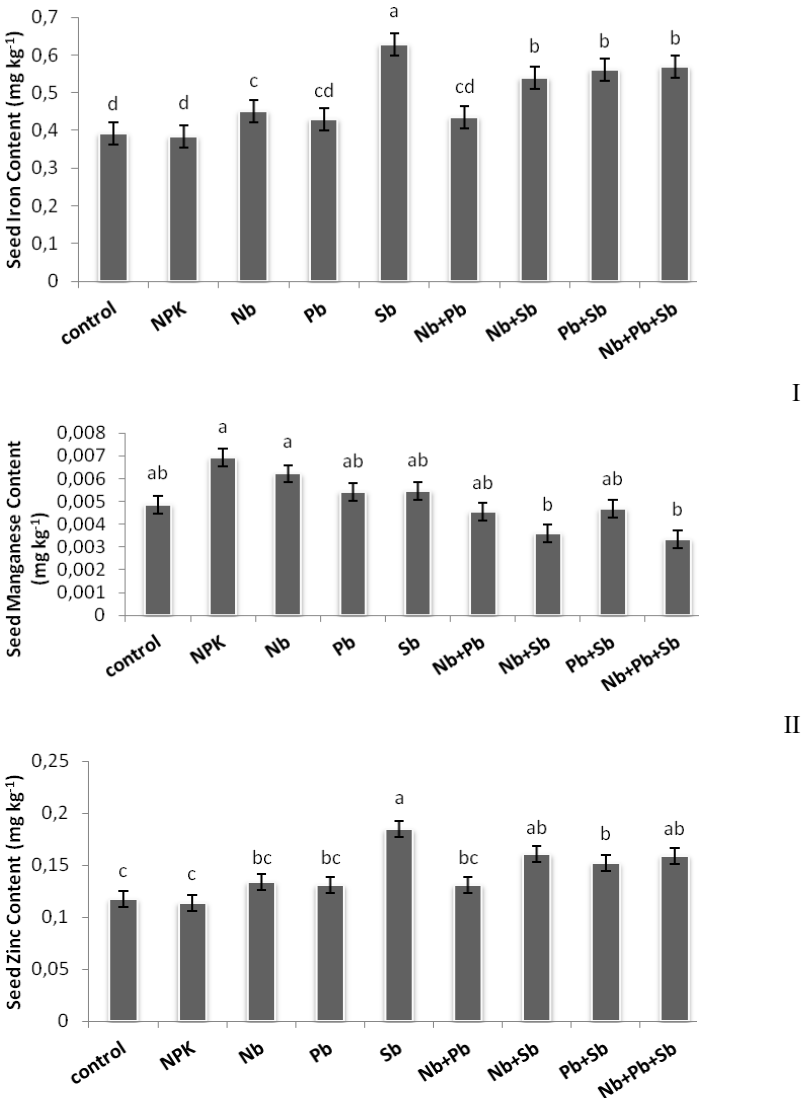

Fig. 3. Iron (I), manganese (II), and zinc (III) content in seeds of *Dracocephalum moldavica* L. fertilized with different microbial inocula or chemical fertilizer. Means ± SD (or SEM) with the same letters are not significantly different ($P \leq 0.05$)

production or more efficient N assimilation aided by the bacterial nitrate reductase (Shehata and El-khawas, 2003). In addition to direct effect of nitrogen fixing bacteria on the root growth (Ahmed et al., 2010) because of sufficient nitrogen providing, there is a synergistic effect of bacteria in N_b+P_b+S_b treatment. Some researchers noted the development of positive interactions between different microbial populations in soil (Barea et al., 2005; Das et al., 2007).

Despite high amounts of phosphorus and potassium in *Dracocephalum moldavica* L. leaves were observed after treatment with microbial inocula and chemical NPK (Rahimzadeh et al., 2013), in this work there was no significant increase of phosphorus and potassium content in seeds of dragonhead. It seems that the sink limitation of dragonhead seeds caused to same accumulation of seed phosphorus and potassium under experimental treatments.

Higher S concentration in dragonhead seeds was observed after application of *Thiobacillus* sp. (Fig. 2-II). *Thiobacillus* sp. in alkaline soils supplies SO_4^{2-} to plants and make nutrient available through oxidation (Motior et al., 2011). As a result of sulfur oxidation by *Thiobacillus* sp. and, hence, decreased soil pH (Miransari and Smith, 2007) the availability of P and micronutrients increases. Sulfur is also a necessary macronutrient for oil production in dragonhead as an essential component of fatty acids (http://canola.okstate.edu/cropproduction/fertility/sulfuroxidation.pdf).

Higher concentration of Fe and Zn in seeds produced by *Thiobacillus* sp. application (Fig. 3) might be due to soil properties. Lower soil pH and $CaCO_3$ content caused by activity of these microorganisms could have a positive influence on the concentration of Fe and Zn in dragonhead seeds. This result is in agreement with the findings of Modaihsh et al. (1989), who observed that elemental sulphur added to calcareous soil significantly decreased pH and increased Mn and Fe concentration. Mn and Fe are nutrients released by acidity promoted from the metabolic reaction carried out by *Thiobacillus* bacteria.

In conclusion, microbial inocula could be applied in production of dragonhead, and could partially substitute chemical fertilizers. Application of microbial inocula has a great potential to increase the yield and quality (nutrients) of dragonhead seeds similarly and maybe higher than chemical fertilizers. Treatments with microbial inocula produced the higher seed quality and yield than control. But they cannot modulate the chemical NPK because of direct and accessible supply of nutrients under chemical fertilizer application.

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