Australian Journal of Crop Science AJCS
AJCS 4(5):330-334 (2010) ISSN:1835-2707

Enhancing phosphorous availability to canola (*Brassica napus* L.) using P solubilizing and sulfur oxidizing bacteria

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Abstract

Enhancing the availability of soil nutrients to crop plants such as canola (*Brassica napus* L.) for oil and grain production is of great significance. Data related to the combined effects of rock phosphate, P solubilizing bacteria, sulfur and sulfur oxidizing bacteria on canola growth is scanty. The present research was conducted in the Research Field of Agricultural Research Center of Safi-Abad, Dezful, Iran. The experimental design was a completely randomized design with eight treatments in three replicates. Treatments including: (1) control, (2) triple super phosphate (80 kg/ha), (3) rock phosphate (160 kg/ha), (4) rock phosphate + organic matter (tea waste, 1000 kg/ha), (5) rock phosphate + organic matter + P solubilizing bacteria, (6) rock phosphate + elemental sulfur (1000 kg/ha) + *Thiobacillus* sp., (7) rock phosphate + *Thiobacillus* sp. + organic matter, (8) rock phosphate + elemental sulfur + *Thiobacillus* sp.+ organic matter were tested in plots measuring 3 x 7 m. At harvest crop yield and the related components were determined. Treatment 2 resulted in the highest amount of yield and stover with a 60 and 92% increase, relative to the control treatment, respectively. The next highest corresponding values were related to treatment 8 at 38 and 70%, respectively, not significantly different from treatment 2. Treatment 8 produced the highest oil percentage, followed by treatment 2 (39% increase) relative to the control. Sulfur treatments resulted in the highest oil percentage. The combination of chemical and biological methods (biofertilizers) can be a favorable method to increase the efficiency of naturally (rock phosphate) and synthetically (elemental sulfur) produced resources and hence optimization of chemical fertilization for crop production.

Keywords: canola (*Brassica napus* L.), organic matter, P solubilizing bacteria, rock phosphate, sulfur oxidizing bacteria, triple super phosphate

Introduction

The two types of phosphorous (P) in the soil are organic and inorganic. On average, the amounts of P in the earth crust and agricultural soils are 0.12 and 0.06%, respectively. Different parameters such as soil pH, calcium concentration, amount of organic matter, type and amount of clay, soil moisture, soil texture, root density and exudates can affect the availability of soil P to the plant (Tisdale et al., 1993; Barber, 1995). Parameters including high pH, high amount of $CaCO_3$, little amount of organic matter and drought decrease P availability to plants in the calcareous soils of Iran, with arid and semi-arid climates. Using P fertilizers, especially superphosphate, as a very common method of providing plant P requirement, is not very efficient in calcareous and alkaline soils. Because under such conditions high amounts of P are turned into insoluble products and become unavailable to the plant, as only 20% of the fertilizer is soluble in the first year of use (Tisdale et al., 1993). Application of P fertilizer has increased significantly to enhance crop yield production and as a result of using organic matter improperly. Rock phosphate, which is the main source of P fertilizers production, is not recyclable and its mines are found in North of Africa, Iran, USA, Russia, China, and Morocco, which produce 75% of the all world rock phosphate (van Kauwenbergh, 2001). Use of rock phosphate as a source of P fertilizer, which is a simple and in the meanwhile not expensive method, is recommendable for acidic soils, because in calcareous soils, high pH and high amount of $CaCO_3$, decreases the fertilizer solubility (Chein et al., 1996; Abd-Elmonem and Amberger, 2000). Different researchers have indicated that it is likely to increase P availability in soil. For example, acidizing rock phosphate, mixing rock phosphate with sulfur and organic matter and using rock phosphate with microorganisms including P solubilizing bacteria, sulfur oxidizing bacteria and arbuscular mycorrhiza are among the methods, used for enhancing P availability (Chien, 1996; Vessey, 2003). Plant residues can be used as a source of C for soil fungi and heterotrophic bacteria, which produce organic acids enhancing P availability in the rock phosphate through protonization and chelating. Acid strength, the amount of soluble calcium and type and properties of chelating ligands are among the parameters affecting P availability (Chein et al., 1996). Phosphate solubilizing microorganisms (10% of total soil microorganisms), which include a large number of soil micro-flora (Whitelaw, 1997; Sundara, 2001), can solubilize inorganic phosphate (including soil phosphate) with the production of inorganic (carbonic and sulfuric) and organic (citric, butyric, oxalic, malonic, lactic and etc.) acids and phosphatase enzyme (Whitelaw, 1997; Sundara et al., 2001). The activities of such microorganisms are affected by different soil parameters including soil fertility, temperature, moisture, organic matter, and soil physical properties (Kim et al., 1998). Sing and Kapoor (1992) indicated that mixing rock

Table 1. Soil physical and chemical properties

Texture	pH	EC (dS/m)	T.N.V.	O.C.	SP	P	K	Zn	Fe	Mn	Cu	CEC cmolc/kg
				%				Mg/kg				
Silty caly loam	7.8	0.98	48.5	0.91	49	5	104	0.58	13.2	9.4	2.6	18

phosphate with P solubilizing bacteria, increased wheat yield between 32 to 42%, relative to the control treatment. In the same experiment, treatments of control, rock phosphate, *Bacillus circulans* and *Cladosporium herbarum* inoculants, mixture of rock phosphate and *B. circulans*, and mixture of rock phosphate and *C. herbarum* resulted in the wheat dry weights of 2.017, 2.04, 2.54, 2.76, 2.7 and 2.89 g/pot, respectively. Sundara et al. (2002) illustrated the great effects of P solubilizing bacteria on sugar beet yield when combined with rock phosphate. Scofield et al. (1981) evaluated the use of 1:5 rock phosphate and elemental sulfur with *Thiobacillus tiooxidance* as a source of P fertilizer (biosuper) in three calcareous soils in the greenhouse. They found that similar to superphosphate the biosuper fertilizer also increased trifolium yield and P uptake. They accordingly indicated that the biosuper fertilizer can be used as a useful source of P for crop production in soils with little to medium amount of P. The enhanced P availability in rock phosphate combined with elemental sulfur has also been indicated by other researchers, in which *Thiobacillus* sp. with elemental sulfur (biosuper) has been used (Stamford, 2002). A large part of sulfur is biologically oxidized in the soil (Tabatabai, 1986). Parameters affecting the P availability of rock phosphate when used in combination with elemental sulfur include the type of rock phosphate, the ratio of rock phosphate to elemental sulfur and crop and soil conditions (Rajan, 2002). Elemental sulfur must be inoculated with *Thiobacillus* to enhance the P solubility of apatite; and hence plant biomass (Stamford et al., 2003). Soil P availability is determined by the following factors: (1) the reaction time between apatite and organic acid, (2) the rate of organic acid dissociation, (3) the type and place of the functional (chemical) group, (4) the affinity of the chelating compound for cations, (5) time and method of using rock phosphate, (6) soil chemical and physical properties especially the ability for P fixation, (7) crop plant species and their nutritional requirements, (8) soil particles size and their surface area, (9) mineralogy and chemical properties of rock phosphate, (10) the activity and solubility of rock phosphate, and 11) soil organic matter (Grover, 2003). Apatite particles with size less than 0.15 are more beneficial to plant roots (Chien et al., 2003). In India microorganisms are used for the enhanced availability of rock phosphate and experiments with P solubilizing bacteria and rock phosphate have indicted their significant effects on crop yields such as wheat, rice and potato (Rajan, 2002). The combination of the affordable rock phosphate, elemental sulfur, and recyclable organic matter with P solubilizing and sulfur oxidizing bacteria indicate the applicability and significance of the present research work. Although there are data related to the effects of rock phosphate and P solubilizing bacteria on the growth of different crop plants, however data related to the combined effects of rock phosphate, P solubilizing bacteria, sulfur and sulfur oxidizing bacteria on canola growth is scanty. The hypothesis was that it is likely to increase the efficiency of naturally (rock phosphate) and synthetically (elemental sulfur) produced products, through biologically treating them. The objective was to increase canola oil and yield production through enhancing P availability in a calcareous soil using rock phosphate and elemental sulfur inoculated with P solubilizing and sulfur oxidizing bacteria, respectively.

Materials and methods

The present research was conducted in the Research Field of the Agricultural Center of Safi Abad, Dezful, Iran, in 2002. The average rain per year is 250 mm and the temperature and moisture patterns are hyperthermic and ustic, respectively. Before conducting the experiment, composite soil samples were collected from the field (0-30 cm depth) and after air drying and sieving with a 2 mm sieve, the samples were analysed for soil physical and chemical properties (Table 1). Soil texture was determined using the hydrometric method, pH (pH meter, Metrom model) and electrical conductivity of the saturated paste, soil organic carbon (Walkley and Black, 1934), total and available P using the wet oxidation and Olsen method (Olsen, 1982), respectively, available K using ammonium acetate method, neutralizing material and cation exchange capacity using the titration method (Rhoades, 1982), soil saturation percentage and the amount of available iron, zinc, copper and manganese were also determined using DTPA. The experimental design was a completely randomized block with eight treatments in three replicates. Treatments including control (P fertilizer not applied), triple super phosphate (80 kg/ha), iii) rock phosphate (160 kg/ha), iv) rock phosphate + organic matter (1000 kg/ha), v) rock phosphate + organic matter + P solubilizing bacteria, vi) rock phosphate + elemental sulfur (1000 kg/ha) + *Thiobacillus*, vii) rock phosphate + *Thiobacillus* + organic matter, viii) rock phosphate + *Thiobacillus* + elemental sulfur + organic matter were tested in plots measuring 3 x 7 m. Before planting, cultivation, disking and leveling the field was conducted and total of 24 plots were created. There was a 5m-sapce between the replicates so that any likely interaction effects would be inhibited. Water streams and drainage were created for each plot. Each plot was irrigated, separately. Plots were fertilized, according to soil testing, with urea, potassium and zinc sulfate at 30, 180 and 40 kg/ha (mixed with the soil using disk), respectively to provide N, K and Zn, necessary for plant growth. Using a furrower, rows, which were 60 cm wide, were created in each plot and seeds of canola PF705.91 were planted in the middle of each row, with a 20 cm interspacing. The bacterial treatments of *Thiobacillus* and P solubilizing were inoculated at 16 g. The *Thiobacillus* bacteria were neutrophobic strains with the population of 7×10^7 cell/g of inoculum. The P solubilizing bacteria (*Bacillus circulans*) were isolated from the Iranian soils (unpublished data) with the population of 2.5×10^8 per gram. All treatments were used before planting in the furrows on the two side of each row. During the different stages of plant growth, practices such as weeding, irrigation and pest control were performed for all plots. The sulfur treatment (elemental sulfur with the purity of 98%), was used with the waste of tea factories and rock phosphate. Some of the chemical properties of these two products including electrical conductivity, pH, total P, available P, total N, organic C and soluble P were determined (Page, 1982) (Table 2). After harvesting the plants, the amounts of grain and straw yield, number of plant per plot, number of pod per plot, number of grain per pod, weight of 1000 grain, and oil percentage were determined. Grain oil was determined using NMR. Yield relative efficiency was calculated using the following formula.

Table 2. Chemical analysis of rock phosphate and tea waste

	EC dS m^{-1}	pH	OC (%)	TN (%)	Total P_2O_5 (%)	Available P
Rock phosphate	5.1	7.6	0	0	38	2
Tea waste	6.6	4.6	32.4	3.34	0	0

$$\text{Yield relative efficiency} = \frac{\text{Plant dry mater in the treated plots – plant dry matter in the control}}{\text{Plant dry matter (treated with super phosphate triple) - plant dry matter in the control}}$$

Data were analysed using MSTATC. Significant differences were determined by Duncan's Multiple Comparison Test.

Results

It was determined that the experimental soil was very calcareous and the amounts of P, K, and Zn were less than necessary for canola production (Table 1). As seen from Table 1, it could be said that there is very little amount of available P for plant uptake. According to the analysis of variance the effects of experimental treatments on canola grain and straw yield and number of pod per plant were significant at 1% level (data not shown) and on oil percentage at 5 % level. However, the effects of different factors on the weight of 1000 grains, and number of plants per m^2 were insignificant. The highest amounts of grain (3107 kg/ha) and straw (4178 kg/ha) yield were taken from treatment 2 (triple super phosphate), significantly different from the control treatment with the corresponding values of 1944 and 2176 kg/ha, respectively. Treatment 8 (rock phosphate + sulfur + organic matter + *Thiobacillus*) producing 3677 kg/ha grain yield was the only treatment, not significantly different from the triple super phosphate treatment. When compared to the control, treatment 8 increased grains and straw yield at 37.70 and 70%, respectively with the highest relative yield efficiency (Table 3). The weight of 1000 grains ranged from 2.66 to 2.96 g, not statistically significant for different treatments. The number of grains per pod in different treatments was between 19 and 21, and was not significantly affected by different treatments (Table 4). Treatments 2 and control were significantly different and produced the maximum and minimum of 272 and 165 pod per plant, respectively. Treatments 3 and 6 were not significantly different from control, while other treatments including treatment 2 (with the highest number of pod per plant) produced significantly higher number of pod per plant relative to the control treatment. The number of plants/m^2 for different treatments ranged not significantly from 34 to 41 (Table 4). Treatments 2 (super phosphate triple) and 5 (rock phosphate + organic matter + P solubilizing bacteria) were significantly different and produced the maximum and minimum percent of grain oil at 45.27 and 42.82%, respectively. In addition, treatment 8, including rock phosphate, elemental sulfur, organic matter and *Thiobacillus* sp. producing 44.57% oil was the second highest oil producing treatment. The oil percent was not significantly different between the control and other treatments. It is also interesting to mention that treatments with sulfur produced higher percent of oil relative to the other treatments, significantly different for some treatments. There were also significant differences at $P < 0.01$ regarding the amount of canola oil per hectare (Table 5). Triple super phosphate with 1406, followed by treatment 8 with 1194 and control treatment with 858 kg/ha produced the maximum and minimum amount of oil, respectively. Relative to the control, treatments 2, 7 and 8 resulted in 63.86, 31.35 and 39.04% significantly higher oil percentage, respectively. However, regarding the other treatments the increases were numerically and not significantly higher than the control treatment.

Discussion

The optimum pH for P uptake is around the neutral and under arid or humid conditions its availability decreases due to the production of insoluble compounds. Accordingly it is pertinent to look for methods, which may result in higher P availability under such conditions. For example, although rock phosphate is the main source for P fertilizer production, however because of its little P solubility, especially in calcareous soils (the conditions in the present experiment), its direct application in the field is not economically recommendable. Hence, testing and suggesting some efficient methods of using rock phosphate especially when combined with biological methods can be of great importance. The great effects of using microorganisms, as biological methods, including arbuscular mycorrhiza on plant growth, especially under stress was reported by some authors (Nadian et al., 1997; 1998; Miransari et al., 2007; 2008). The experimental treatments tested in the current study significantly enhanced canola yield and oil, indicating that the right combinations of treatments have been suggested. The great advantages of plant growth promoting rhizobacteria (PGPR) including P solubilizing bacteria on enhanced plant growth have been previously observed by different scientists (Kim et al., 1998; Fernandez et al., 2007). However, there is very little data related to the effects of P solubilizing bacteria on canola growth, fertilized with rock phosphate, particularly when combined with sulfur oxidizing bacteria. According to the results of this experiment, treatment 2 (triple super phosphate) resulted in the highest amount of plant yield and oil. Although relative to the other sources of P, triple super phosphate is of higher solubility, as a chemical source it is more expensive and not very favorable to the soil environment. Hence in the current study we proposed and tested a collection of different ways (chemical and biological), which to our opinion and with regard to the literature, can enhance P availability to canola and hence its growth and yield. Crop residues such as tea waste are a favorable source of organic carbon (Table 2) to soil microorganisms and can also significantly improve soil properties including soil structure, and hence increase plant growth and yield (Tisdale et al., 1993). Thus, when combined with microbial treatments, organic matter can enhance their performance (treatment 4), relative to the control and treatment 3 (rock phosphate) (Table 3). Compared with treatment 4 (18%) (rock phosphate and organic matter) higher yield increase (20%) was resulted when canola plants were also inoculated with P solubilizing bacteria (treatment 5). In addition to enhanced P solubility through producing organic acids, and phosphatase enzymes, P solubilizing bacteria are also able to produce other plant growth promoting metabolites such as siderophores, plant hormones and lytic enzymes inhibiting pathogen activities (Rodríguez and Fraga, 1999; Vassilev et al., 2006; Fernández et al., 2007). According to the results treatment 2 produced the highest amounts of yield and oil, followed by treatments with sulfur and *Thiobacillus* sp. Soil conditions such as soil fertility and the population of oxidizing microorganisms are very much affective on the intensity of sulfur oxidation.

Table 3. Effects of different treatments on grain yield and straw

	Grain yield			Straw yield		
Treatment	Kg/ha	Increase relative to the control (%)	Relative Yield Efficiency (%)	Kg/ha	Increase relative to the control (%)	Relative Yield Efficiency (%)
1	1944 c	-	-	2176 f	-	-
2	3107 a	59.8	100	4178 a	92	100
3	2227 bc	14.4	24.08	2808 d	29	31.47
4	2294 bc	18	30.11	3663 b	68.3	74.17
5	2332 bc	20	33.34	3136 bcd	44.1	47.85
6	2375 bc	22.2	37.10	3039 cd	39.6	43.01
7	2540 b	30.6	51.26	3055 cd	40.3	43.81
8	2677 ab	37.7	63.02	3698 ab	69.9	75.92

Values followed by the same letters are not statistically different at P= 0.05 using Dunkan's Multivariate test. (1) control, (2) triple super phosphate (80 kg/ha), (3) rock phosphate (160 kg/ha), (4) rock phosphate + organic matter (tea waste, 1000 kg/ha), (5) rock phosphate + organic matter + P solubilizing bacteria, (6) rock phosphate + elemental sulfur (1000 kg/ha) + *Thiobacillus* sp., (7) rock phosphate + *Thiobacillus* sp.+ organic matter, (8) rock phosphate + elemental sulfur + *Thiobacillus* sp.+ organic matter

Table 4. Effects of different treatments on the number of pod per plant and number of plants/ m^2

Treatment	Number of pod	Number of plants
1	165.00 e	41.35 a
2	272.67a	36.67 ab
3	207.00 b	36.51 ab
4	232.33 ab	34.76 b
5	227.33 ab	39.84 ab
6	204.33 cd	38.02 ab
7	244.33 ab	38.49 ab
8	253.67 ab	34.21 b

(1) control, (2) triple super phosphate (80 kg/ha), (3) rock phosphate (160 kg/ha), (4) rock phosphate + organic matter (tea waste, 1000 kg/ha), (5) rock phosphate + organic matter + P solubilizing bacteria, (6) rock phosphate + elemental sulfur (1000 kg/ha) + *Thiobacillus* sp., (7) rock phosphate+ *Thiobacillus* sp.+ organic matter, (8) rock phosphate+ elemental sulfur + *Thiobacillus* sp.+ organic matter

As a result of sulfur oxidation by *Thiobacillus* sp., and hence decreased soil pH (Miransari and Smith, 2007) the availability of P in the rock phosphate increases. Sulfur is also a very necessary macronutrient for oil production in canola as it is a necessary component of fatty acids. The little amounts of soil nutrients and also the little microbial population can be the reasons for the situations, where plant parameters were not significantly affected by different treatments. There must be enough time for sulfur oxidizing bacteria to oxidize sulfur and enhance plant growth (Tabatabai, 1986 ; Agrifacts, 2003). As previously mentioned, we examined the effects of different combinations of chemical and biological methods (biofertilizers, Wu et al., 2005) on canola growth in the presence of rock phosphate. It was accordingly indicated that soil microorganisms including P solubilizing bacteria and sulfur oxidizing bacteria are very important components (biofertilizers) of the treatments tested in the experiment. Because, P solubilizing bacteria are able to enhance P solubility of rock phosphate and hence its availability to the plant. Sulfur oxidizing bacteria can also act similarly through oxidizing sulfur and hence decreasing soil pH. Using very affordable sources of P (rock phosphate) and elemental sulfur combined with the related microorganisms tested in this experiment can have very favorable economical and environmental advantages, resulting in the optimal application of chemical fertilizers. According to the results the interactions between soil, fertilizer and microbial populations can very much determine the optimal situations for canola yield and oil production. Hence, the right combinations of chemical and biological resources can greatly contribute to the enhanced canola yield and oil production, while agriculturally sustainable. There are very little data regarding the effects of such resources on canola yield and oil production and this is the part that can contribute to our knowledge regarding the field. It can also be interesting to evaluate how such interactions can affect canola performance under different conditions. The other interesting aspect related to this research work is that using such treatments in the field are economically very recommendable as recycling such treatments can also be very favorable to the environment.

Table 5. Effects of different treatments on canola oil concentration and extractable oil

Treatment	Amount of grain oil (%)	Amount of grain oil (kg/ha)
1	44.14 ab	858 c
2	45.27 a	1406 a
3	43.13 bc	960 bc
4	43.07 bc	988 bc
5	42.82 c	998 bc
6	44.05 bc	1046 bc
7	44.38 ab	1127 bc
8	44.57 b	1193 b

Values followed by the same letters are not statistically different at P= 0.05 using Dunkan's Multivariate test. (1) control, (2) triple super phosphate (80 kg/ha), (3) rock phosphate (160 kg/ha), (4) rock phosphate + organic matter (tea waste, 1000 kg/ha), (5) rock phosphate + organic matter + P solubilizing bacteria, (6) rock phosphate + elemental sulfur (1000 kg/ha) + *Thiobacillus* sp., (7) rock phosphate + *Thiobacillus* sp.+ organic matter, (8) rock phosphate + elemental sulfur + *Thiobacillus* sp.+ organic matter

Conclusions

According to the present research, mixing rock phosphate with sulfur and organic matter significantly increased canola growth and oil production relative to the control treatment. In the meanwhile, inoculation of the above mentioned treatments with P solubilizing bacteria and *Thiobacillus* sp. resulted in higher plant growth. According to the results, and with respect to the presence of high amount of sulfur and rock phosphate resources in the country and other parts of the world, it is suggested that in soils with different buffering capacities, and for different crop plants different combinations of sulfur and rock phosphate with P solubilizing and sulfur oxidizing bacteria be used. It is also very important to compare their efficiencies, when combined with chemical P fertilizers so that the use of P fertilizer can be optimized.

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