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Full Length Research Paper

Using sulfur oxidizing bacteria and P solubilizing for enhancing phosphorous availability to *Raphanus sativus*

Rasool Khatibi

Department of Natural Resource, University of Zabol, Iran. E-mail: r_khatibi60@yahoo.com Tel: 0098 05422232600.

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The present research was conducted in the Research Field of Agricultural Research Center of Chahnimeh, Zabol, Iran. Data related to the combined effects of rock phosphate, P solubilizing bacteria, sulfur and sulfur oxidizing bacteria on *Raphanus sativus* growth is scanty. 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.

Key words: *Raphanus sativus*, organic matter, P solubilizing bacteria, rock phosphate, sulfur oxidizing bacteria, Chahnimeh.

INTRODUCTION

The two types of phosphorous (P) in the soil are organic and inorganic. On average, proportion of P in the earth crust and agricultural soils are 0.12 and 0.06%, respectively. Different parameters such as soil pH, calcium concentration, proportion of organic matter, type and proportion 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 soil pH, high soil $CaCO_3$, low soil organic matter and drought decrease P availability to plants in the calcareous soils of Iran, with arid and semiarid 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. Under such conditions high amounts of P are turned into insoluble products and become unavailable to the plant and only about 20% of the fertilizer is soluble in the first year of use (Tisdale et al., 1993). Application of P fertilizer to enhance crop yield production has increased significantly and as a result of using organic matter improperly. Rock phosphate, the main source of P fertilizer production in Iran, is obtained from mines in Northern Africa, Iran, USA, Russia, China, and in Morocco, which produces 75% of all the world rock phosphate (van Kauwenbergh, 2001). Use of rock phosphate as a source of P fertilizer, is suitable for acidic soils, but in calcareous soils, high pH and high $CaCO_3$, decrease the fertilizer solubility (Chein et al., 1996; Abd-Elmonem and Amberger, 2000). Research has indicated that for example, acidifying rock phosphate by mixing rock phosphate with sulfur and organic matter and using rock phosphate with microorganisms including P solubilizing bacteria, sulfur oxidizing bacteria and arbuscular mycorrhiza enhances 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 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 a mixture of rock phosphate and *C. herbarum* resulted in wheat dry weights of 2.0, 2.04, 2.54, 2.8, 2.7 and 2.9 g/pot, respectively. Sundara et al. (2002) illustrated the great effects of P solubilizing bacteria on sugar beet yield when combined with rock phosphate. Schofield et al. (1981) evaluated the use of 1:5 rock phosphate and elemental sulfur with *Thiobacillus thiooxidans* 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 low to medium P levels. 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 production and 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 mm are more beneficial to plant roots (Chein 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, data related to the combined effects of rock phosphate, P solubilizing bacteria, sulfur and sulfur oxidizing bacteria on black radish growth is scanty. The hypothesis was that the biological treatment is likely to increase the efficiency of natural (rock phosphate) and synthetically (elemental sulfur) produced products. The objective was to increase black radish 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 Chahnimeh, Zabol, Iran, in 2010. The average rain per year is 150 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 to 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's 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 i) control (P fertilizer not applied), ii) 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 +

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 clay loam	7.8	0.98	48.5	0.91	49	5	104	0.58	13.2	9.4	2.6	18

Table 2. Chemical analysis of rock phosphate and tea waste.

	EC (μS 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

Thiobacillus + organic matter, viii) rock phosphate + Thiobacillus + elemental sulfur + organic matter were tested in plots measuring 3 x 7 m. Before planting, the field was cultivated, disked and levelled and a total of 24 plots were created. There was a 5 m space between the replicates, so that any likely interaction effect 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 *Raphanus sativus* PF705.91 were planted in the middle of each row, with a 20 cm interspacing. The bacterial treatments of Thiobacillus and P solubilizing bacteria were inoculated with 16 g inoculum. The Thiobacillus bacteria were neutrophobic strains with the population of 7 x 10^7 cell/g of inoculum. The P solubilizing bacteria (*B. circulans*) were isolated from Iranian soils with a population of 2.5 x 10^8 per g. All treatments were applied before planting in the furrows on both sides 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 seed and straw yield, number of plants per plot, number of pods per plot, number of seeds per pod, weight of 1000 seeds, and oil percentage were determined. Seed oil was determined using NMR. Yield relative efficiency was calculated using the following formula:

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

RESULTS

It was determined that the experimental soil was very calcareous and the levels of total P, available P, K, and Zn were too low for optimum *R. sativus* production (Table 1). The effects of experimental treatments on *R. sativus* seed and straw yield and number of pods per plant were significant at 1% level and on oil percentage at 5% level. However, the effects of different factors on the weight of 1000 seeds, and number of plants per m^2 were insignificant. The highest amounts of seed (3107 kg/ha) and straw (4178 kg/ha) yield were taken from treatment 2 (triple super phosphate), significantly higher than the control treatment with the corresponding values of 1944 and 2176 kg/ha, respectively. Treatment 8 (rock phosphate + sulfur + organic matter + Thiobacillus) producing 2677 kg/ha seed yield was the only treatment, not significantly different from the triple super phosphate treatment.

When compared to the control, treatment 8 increased seed and straw yield at 38 and 70%, respectively with the highest relative yield efficiency (Table 3). The weight of 1000 seeds ranged from 2.66 to 2.96 g, not statistically significant for different treatments. The number of seeds 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 pods per plant, respectively. Treatments 3 and 6 were not significantly different from control, while other treatments including treatment 2 (with the highest number of pods per plant) produced a significantly higher number of pods 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 seed oil at 45 and 42%, respectively.

In addition, treatment 8, including rock phosphate, elemental sulfur, organic matter and *Thiobacillus* sp. producing 4.5% 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 a higher percent of oil relative to the other treatments. There were also significant differences at P < 0.01 regarding the amount of *R. sativus* oil per ha (Table 5). Triple super phosphate with 1406, (followed by treatment 8 with 1194) and control treatment with 858

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

Treatment control (%)	Grain yield			Straw yield		
	(Kg/ha)	Increase relative to the control (0/0)	Relative yield efficiency (0/0)	Kg/ha	Increase relative to the control (0/0)	Relative yield
1	1944c	-	-	2176f	-	-
2	3107a	59.8	100	4178a	92	100
3	2227bc	14.4	24.08	2808d	29	31.47
4	2294bc	18	30.11	3663b	68.3	74.17
5	2332bc	20	33.34	3136bcd	44.1	47.85
6	2375bc	22.2	37.10	3039cd	39.6	43.01
7	2540b	30.6	51.26	3055cd	40.3	43.81
8	2677ab	37.7	63.02	3698ab	69.9	75.92

Values followed by the same letters are not statistically different at P = 0.05; using Duncan'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 numbers of plants	Numbers of pod
141.35a	165.00e
236.67ab	272.67a
336.51ab	207.00b
434.76b	232.33ab
539.84ab	227.33ab
638.02ab	204.33cd
738.49ab	244.33ab
834.21b	253.67ab

(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.

kg/ha produced the maximum and minimum amount of oil, respectively. Relative to the control, treatments 2, 7 and 8 resulted in 64, 31 and 39.0%, significantly higher oil percentage, respectively. However, regarding the other treatments, the increases were numerically but not significantly higher than the control treatment.

DISCUSSION

The optimum soil pH for P uptake is around neutral and under arid or humid conditions, its availability decreases due to the production of insoluble compounds (Rajan, 2002). 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, because of its low 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 efficient methods of using rock phosphate combined with biological methods can be of great importance. There have been many reports of the beneficial effects on plant growth of inoculation with microorganisms, including arbuscular mycorrhiza, especially when plants are under stress (Nadian et al., 1997, 1998; Miransari et al., 2007, 2008).

The experimental treatments tested in this current study significantly enhanced *R. sativus* 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 *R. sativus* 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 (Chein et al., 1996).

Hence in the current study, we proposed and tested a collection of different methods (chemical and biological), which enhance P availability to *R. sativus* 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

Table 5. Effects of different treatments on *Raphanus sativus* 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 Duncan'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.

organic matter) higher yield increase (20%) resulted when *Raphanus sativus* 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 effective on the intensity of sulfur oxidation (Tisdale et al., 1993). As a result of sulfur oxidation by *Thiobacillus* sp., and hence decreased soil pH (Miransari and Smith, 2007) the availability of P in rock phosphate increases. Sulfur is also a very necessary macronutrient for oil production in *R. sativus,* as it is a necessary component of fatty acids. Low soil nutrient levels and low microbial populations can decrease the effects of treatments. There must be enough time for sulfur oxidizing bacteria to oxidize sulfur and enhance plant growth (Tabatabai, 1986; Agrifacts, 2003).

We examined the effects of different combinations of chemical and biological methods (Wu et al., 2005) on *R. sativus* 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. 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. The use of 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.

Our results show that the interactions between soil, fertilizer and microbial populations can very much determine the optimal situations for *R. sativus* yield and oil production. Hence, the right combinations of chemical and biological resources can greatly contribute to the enhanced black radish yield and oil production, while being agriculturally sustainable. There are very little data regarding the effects of such resources on *R. sativus* yield and oil production and this is the contribution of this study, to our knowledge in this field. It can also be interesting to evaluate how such interactions can affect *R. sativus* performance under different conditions. The other interesting aspect related to this research work is that using such treatments in the field is economically very recommendable, as recycling such organic wastes can also be very favorable to the environment.

Conclusions

Mixing rock phosphate with sulfur and organic matter significantly increased *R. sativus* growth and oil production relative to the control treatment. Meanwhile, inoculation of the aforementioned treatments with P solubilizing bacteria and *Thiobacillus* sp. resulted in higher plant growth and oil production. 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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