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THE EFFECTIVENESS OF PHOSPHATE SOLUBALIZING BACTERIA AS BIOCONTROL AGENTS

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ABSTRACT

Phosphorus is considered among most vital macro-elements, which is mandatory for successful plant growth. Although it is plentiful in soil both in its organic and inorganic form but its larger fraction is unavailable to plants. Different phosphate solubilizing micro-organisms (PSMs) residing in soils can change the chemical nature of these organic and inorganic phosphate compounds and make them available to plants. Only bacteria are focus of this study which investigates the antifungal potential of these phosphate solubilizing bacteria (PSB). These These PSB can perform important role in defending plant roots from pathogenic fungi in an environment friendly mode. Hence, help in minimizing the dependence on environmentally harmful and expensive phosphate fertilizers and fungicides. From a plenty of purified PSB isolates only elite strains with higher phosphate solubilization index have been selected for identification and for bio control studies. *Alternaria alternate (A. alternate), Macrophomina phasiolina (M. phasiolina), Fusarium oxysporum (F. oxysporum) and Sclerotium rolfsii (S. rolfsii)* had been grown simultaneously along with six selected PSB strains including *Burkholederia cepacia (B. cepacia), Citrobacter freundii (C. freundii), Enterobacter aerogenes (E. aerogenes), Klebsiella pneumonia (K. pneumonia), Proteous vulgaris* (*P. vulgaris)* and *Acinetobacter lwoffi (A. lwoffi)*. The completely restricted growth of mycelia of these plant pathogenic fungi was observed in the presence of selected bacterial strains which have been found as effective bio control agents. The results of the presented work strongly encourage the need to explore more PSB strains with higher phosphate solubilizing potential and deadly effective against broader range of plant pathogenic fungi.

Key words: Bio control, Phosphate solubilizing bacteria, rhizosphere soil, plant pathogens.

INTRODUCTION

Phosphorus (P) is an important plant nutrient required in minor amount but is limiting factor for the growth of plants as it is a part of many biomolecules (Saber *et al.,* 2005). Naturally it can be categorized as soluble inorganic phosphorus and insoluble organic phosphorus. However, due to low solubility and fixation in soils, only a small fraction of phosphorus exists in soil solution (1 ppm or 0.1%), which is readily available to plants (Mahidi *et al.,* 2011). The content of phosphorus in soil varies from 200 to 2000 kg phosphorus/ha of the upper 15 cm of soil, with an average of about 1000 kg (Ahmed *et al.,* 2009., Hinsinger, 2001). The quantity accessible to plants is generally a lesser proportion of this over-all Phosphorous. Less availability of this element to plant roots is due to low solubility and rapid fixation in soils particles. The reduced availability of phosphorous pushes towards extensive use of expensive inorganic phosphatic fertilizers. Higher prices of phosphatic fertilizers in combination with other agricultural inputs reduce the agricultural profitability. It also aids to higher salt accumulation in agricultural soils, resultantly, precious arable lands are gradually getting salt stressed.

The reported study has been carried to find out the possibility of releasing locked phosphorus with the help of phosphate solubilizing microorganism (bacteria). Phosphate solubilizing microorganism can work efficiently to enhance the fraction of available phosphate to plants by solubilizing inorganic as well as organic phosphates (Anamika *et al*., 2007). This process of phosphates solubilization is associated to the production and release of organic acids of low molecular weight. It is accepted fact that their carboxyl and hydroxyl groups inter-chelate in the phosphate bound cations, resulting in their conversion to soluble forms (Alam *et al.,* 2002). Phosphate solubilizing bacteria are also produce plant growth hormones, participate in bio control activities and effect the process of nitrogen fixation (Neelam ana Meenu., 2003).

Bacillus, *Achromobacter*, *Agrobacterium Micrococcus*, *Burkholderia*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, and *Rhizobium*, are some well-known phosphate-solubilizing bacterial strains which are used as biofertilizers and bio-control agents for agriculture improvement (Tamilarasi *et al.,* 2008; Srivastava and Shalini 2009). These PSB genera form an important group of microorganisms that augment the nutrient excellence of soil which finally assist plant growth and

enlargement. Microbial activity of phosphate solubilizing bacteria produce organic acid by lowering pH, hence take part in biological control against soil borne phytopathogens (Botelho *et al*., 2006; Compant *et al*., 2005). These bacteria can be grown in laboratory on calcium phosphate containing growth medium. They solubilize insoluble precipitates of calcium phosphate and this activity can be observed as a halo or clear zone on the plate. The diameter of these halo zones are used to estimate the phosphate solubilizing efficiency of these bacteria (Sadiq *et al*., 2013; Pikovskaya, 1948).

Pathogens are potential threat to plant health. Thus, they decrease the crop yield as well as affect food quality. A number of chemicals have been introduced to control the plant disease; however, they pose many side effects. Alternatively, bio-control has emerged as an effective technique to overcome the negative impact of pathogens on plant growth. To this end, phosphate solubilizing bacterial (PSB) are being considered as bio-control agent (Mamaghani *et al*., 2009; Yaqub and Shahzad, 2011). Thus, this study aims to investigate the potential to control pathogenic attack on the plant, and thus, to enhance growth. The conditions to isolate and to identify PSBs from rizosphere of different plants are optimized. The efficiency of PSBs is tested against soil borne phytopathogens which potentially inhibit the plant growth.

MATERIALS AND METHODS

Sampling: A lot of soils were sampled from rhizospheric zones of pea, sugarcane and mustard plants of CAMB trial fields and agricultural fields in Okara and Lahore districts. The soils samples were collected in disposable autoclave-able bags from the depth of 5 inches in the month of March 2012 and stored at 4 ± 1°C temperature until used. The method of Walkely and Black (1934) was opted for the analyses of organic carbon and pH of collected soil samples. Olsen's method (1954) was employed for estimation of available and total 'P' content of soil samples.

Solubilization index (SI) of purified PSB strains: Bacterial strains were isolated using 10-fold serial dilutions (Johnson and Curl; 1972). Serially diluted soil samples were spread on Pikovaskya's agar plates for growth and incubated at 28°C for 48 hours (Pickovaskaya1948). Pikovskaya solid medium contained glucose (ICN Biomedicals,Inc, CAT 152527)10g, Ca_3 $(PO_4)_2$(LAB CHEM,CAT A2140-M) 5g, $(NH_4)_2SO_4$(Biobascic, CAT PCRB55) 0.5g, NaCl (Merck, CAT 7782-63-0)0.2 g, $MgSO_4 \cdot 7H_2O$(SIGMA-ALDRICH,CAS 7487-88-9)0.1g, KCl (Fisher Scientific, CAT P 330-500) 0.2g, yeast extract (Biochemika, CAT 70161-500G) 0.5g, $MnSO_4 \cdot H_2O$ (SAI CHEMICALS,CAT 7786-87-7) 0.002g, $FeSO_4 \cdot 7H_2O$ (Fisher Scientific, CAT 7782-63-0) 0.002g and Agar (Biotechnology Grade, CAT 40100044-1) 18g per liter of the medium. Pikovskaya broth medium contained all above mentioned ingredients except Agar. All culture media (Pikovskaya, Plate count agar, Potato Dextrose Agar, and MacConkey agar) were sterilized at 121^0C and 15 pounds per square inch pressure for 15 minutes. For bacterial strain purification a single colony of PSB strains was streaked repeatedly on fresh plate of Pikovaskya medium on same incubation conditions until pure culture was raised (Sadiq *et al*; 2013). For estimation of P solubilizing efficiency, 10 µl of freshly grown purified broth culture of 1 OD concentration was inoculated in the center of PVK plate (pin point inoculation), one strain per plate for zone formation and inoculated for 7 days at 28°C. Solubilization index was measured using following formula (Edi Premono *et al*; 1996 and Sadiq *et al*; 2013):

$$SI = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

Morphological studies of PSB isolates: The phosphate solubilizing bacterial colony was streaked on plate count agar (Sigma, CAS 9002-18-0) and incubated at 28°C for 48 hours to study the morphological aspects of colony (Johnson and Curl, 1972 and Sadiq *et al*; 2013). Gram staining of purified PSB strains was performed according to Vincent method (Vincent J.M., 1970) and was observed under Microscope (Van Guard 1400 series).

Catalase and oxidase test: For the biochemical identification of phosphate solubilizing bacteria catalase test was performed and a drop of 30% hydrogen peroxide was dropped on a glass slide. 24 hour's old pure bacterial colony was taken from PVK plate and gently mixed with drop of hydrogen peroxide. The breakdown of hydrogen peroxide into water and oxygen indicates the presence of catalase in the bacteria. Oxidase test was performed to determine the presence of oxidase enzyme in all bacterial isolates (MacFaddin,1980 and Sadiq *et al*; 2013).

Biochemical tests for PSB Identification: PSB isolates were undergone for Biochemical identification. The method of bacterial identification described by Yasmin and Bano (2011), who had used 25 biochemical tests as indicators to identify different gram negative bacteria, has been followed in this study. For this purpose selected bacterial strains with higher SI were streaked on MacConkey agar (Fluka, P1400) and incubated overnight at 30°C. Single colonies from this 24 hours old culture were used in a stripped test kit of DESTO Laboratories Karachi, Pakistan according to directions given in the kit named as QTS-24 miniaturized identification system. The results of reactions were observed frequently but were recorded and considered after 18-24 hours of commencement of experiment which was provided 30°C atmospheric temperature.

Antagonistic Activities: The rhizobacterial isolates were screened qualitative for their ability to suppress growth of the common fungal pathogen, *Fusarium oxysporum, Sclerotium rolfsii, Alterneria alternatae* and *Macrophomina phasiolina.* Potato Dextrose Agar (PDA) was used to study the antagonistic activity of all 6 rhizobacterial isolates. A mycelial plug of fungus was taken and each placed at the centre of the PDA plates. The rhizobacterial isolates were then individually pin point inoculated at 2.5cm away from the mycelial plug at opposite locations around the periphery of the plate and provided 28 ± 2°C incubation temperature. After 3 days of incubation, the distance in-between fungal mycelial growth and the edges of the bacterial colony was measured in cm and recorded as zone of inhibition of fungal growth (Panhwar *et al*; 2012).

Phytopathogenic fungal culture was collected from the culture bank of Plant Pathology department of Punjab University, Lahore. The fugal cultures viz *Macrophomina phasiolina, Sclerotium rolfsii, Alterneria alternatae* and *Fusarium oxysporum* which were coded as MP (1076), SR (737), AA (1129), and FR (975) respectively; and were refreshed by transferring them to potato dextrose agar slants.

RESULTS AND DISCUSSION

Solubilization index (SI): Isolated bacterial strains have been designated as phosphate solubilizing bacteria on the basis of possessing capability to solubilize the insoluble precipitates of tri-calcium phosphate Ca_3 $(PO4)_2$ present in Pikovaskya's agar medium. This is observed by appearing of visible halos which are formed around bacterial colony on PVK agar plates (Figure-1 A to D). These results are in close accordance of results reported by El-Komy and Sadiq *et al* ., (2013) have also found similar behavior of PSB (El-Komy, 2005 and Sadiq *et al*; 2013). All PSB were compared for their potential of P solubilization and six excellent isolates were selected for further studies. The superiority was designated on the basis of higher SI value. These strains included PR1, PR2, PR4, BR1, BR2 and SR1 and the value of their SI was calculated as 2.0, 2.18, 2.16, 2.11, 2.23, and 2.08 cm respectively. The recorded observations depicted that among 6 studied isolates, the BR2 showed maximum solubilization index of 2.23cm. Similar results were also reported by Alam *et al* (2002), Yasmin *et al* (2012), and Sadia *et al* (2002). According to De Freitas (1997) good phosphate-solubilizes produce halos around their colonies with diameters higher than 1.5cm.

Description of PSB colonies: Morphological studies revealed that all six PSB strains, included PR1, PR2, PR4, BR1, BR2 and SR1, formed round colonies which had shiny, smooth colony surfaces with raised elevation and were odorless. PR2, BR1 and BR2 were observed to have wavy margins, whereas, PR1, PR4, and SR1 had entire margins. Colonies of PR4, BR1, BR2 and SR1 had off-white shade and PR1and PR2 were skin colored. Staining experiments revealed that all six selected strains belonged to gram negative groups of bacteria and cellular shape was rod like (Figure. 2. (A to B).

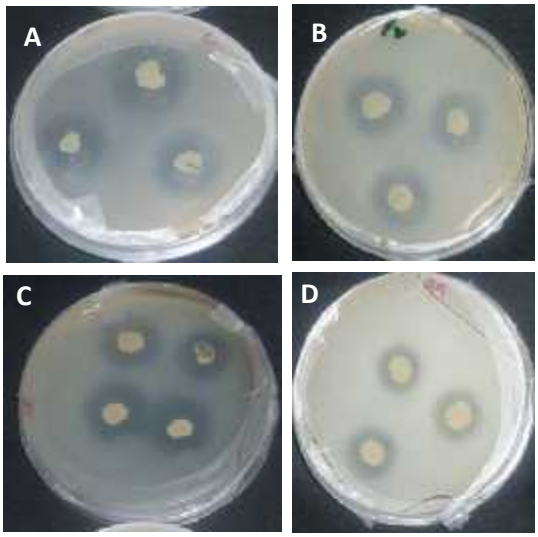

Fig.1 Halo formation around the colony due to solubiliziation of phosphorous on PVK agar plate is measured as 2.5cm, 2.3cm, 2.6 cm and 1.9 cm in **(A)** *Burkholederia cepacia* or BR1, **(B)** *Citrobacter freundii* or BR2, **(C)** *Enterobacter aerogenes* or PR4, and **(D)** *Acinetobacter lwoffi* or SR1 respectively.

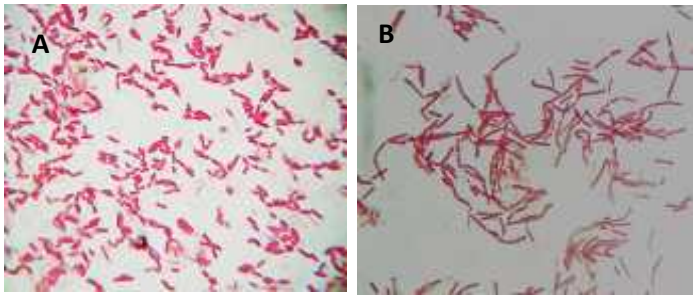

Fig. 2 (A) Rod shape BR1 (100X under Olympus CX31) **(B)** Long chain SR1 (100X under Olympus CX31)

Catalase and Oxidase test: BR1, BR2, PR2, and PR4 showed catalase activity while PR1 and SR1 were negative for catalase test. BR1 and BR2 depicted positive results for oxidase test but PR4, PR2, PR1, and SR1 were negative for oxidase.

Identified PSB isolates: QTS- 24 test results were analyzed using key provided in the kit (Yasmin and Bano, 2011). The purified strains were identified as *Proteous vulgaris* (PR1), *Klebsiella pneumonia* (PR2),

Acinetobacter lwoffi (SR1), *Enterobacter aerogenes* (PR4), *Burkholederia cepacia* (BR1) and *Citrobacter freundii* (BR2) (Table 1). Many other scientists have also reported similar results relating to purification and identification of PSB isolates (Narsian and Patel, 2006., Sadiq *et al*; 2013 and Chung, 2005). Similarly Haque and Dave (2005) isolated the *Acinetobacter lwoffi* and reported the rhizosphere isolates of different regions and their effect on enhanced plant growth.

No.	Biochemical tests	PR1	PR2	PR4	BRI	BR2	SRI
Gram test		-	-	-	-	-	-
Catalase Test		-	+	+	+	+	-
1	ONPG	-	+	+	-	+	-
2	CIT	-	+	-	+	+	-
3	MALO	-	+	+	-	+	-
4	LDC	-	+	+	+	+	-
5	ADH	-	-	-	-	-	-
6	ODC	-	-	+	-	+	-
7	H_2S	-	-	-	+	-	-
8	UREA	+	+	-	-	-	+
9	TDA	-	+	-	+	+	+
10	IND	+	-	-	-	-	+
11	VP	-	-	-	-	-	-
12	GEL	-	-	+	+	+	+
13	GLU	+	+	+	-	+	+
	NO_3/N_2	+	-	+	+	+	-
14	MALT	+	+	+	+	+	+
15	SUC	+	+	+	+	+	+
16	MANN	+	+	+	-	+	+
17	ARAB	+	+	+	+	+	+
18	RHAM	+	+	+	+	+	+
19	SORB	+	+	+	-	-	-
20	INOS	+	+	+	-	-	-
21	ADO	+	-	+	-	-	+
22	MEL	+	+	+	-	-	-
23	RAF	+	-	+	-	-	-
24	MOT	-	-	-	-	-	-
25	CO	-	-	-	+	+	-

TABLE 1

Biochemical test for the identification of PSB isolates by the QTS-24 Miniaturized Quantification System

(ONPG: Ortho nitrophenyl β-D-galactopyranoside; CIT: Sodium citratrate; MALO: Sodium malonate; LDC: Lysine decarboxylase; ADH: Arginine dihydrolase; ODC: Ornithine decarboxylase; H2S: H_2S production; URE: Urea hydrolysis; TDA: Tryptophan deaminase; IND: Indole; VP: (Vogesproskauer): Acetion; GEL: Gelatin hydrolysis; GLU: Acid from glucose; MAL: Acid from maltose; SuC: Acid from sucrose; MAN: Acid from mannitol; ARA: acid from arabinose; RHA: Acid from rhamnose; SOR: Acid from sorbitol; INO: Acid from inositol; ADO: Acid from adonitol; MEL: Acid from melibiose; RAE: Acid from raffinose. Where, (-) stands for negative in the test and (+) stands for positive in the test.)

Bio control activity against fungi: *Proteous vulgaris* (PR1), *Klebsiella pneumonia* (PR2), *Acinetobacter lwoffi* (SR1), *Enterobacter aerogenes* (PR4), *Burkholederia cepacia* (BR1) and *Citrobacter freundii* (BR2) was treated with four different fungal species (*Macrophomina phasiolina, Alterneria alternatae, Fusarium oxysporum and Sclerotium rolfsii*) to check and evaluate the bio control activity (Figure. 3. (A to D).

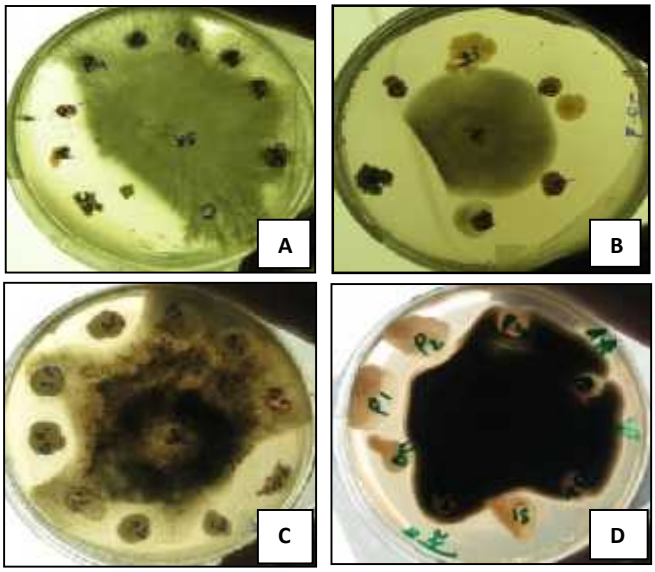

Fig.3 (A) *C. freundii, K. pneumonia, E. aerogenes* inhibit growth of *S. rolfsii* on PDA **(B)** *C. freundii, K. pneumonia, E. aerogenes, B. cepacia* inhibits growth of *F. oxysporum* on PDA **(C) C**. *freundii, K. pneumonia***,** *E. aerogenes B. cepacia, A. lwoffi* inhibit growth of *M. phasiolina* on PDA **(D)** *C. freundii, K. pneumonia, B. cepacia, P. vulgaris* inhibit growth of *A. alternate* on PDA

Phosphate solubilizing rhizobacterial isolates (Khan *et al*; 2007, Leon *et al*; 2009 and Nur Azura, 2008) were tested qualitative for their ability to inhibit the growth of Fungi on PDA plates. All six phosphate solubilizing rhizobacterial isolates, *Proteous vulgaris* (PR1), *Klebsiella pneumonia* (PR2), *Acinetobacter lwoffi* (SR1), *Enterobacter aerogenes* (PR4), *Burkholederia cepacia* (BR1), and *Citrobacter freundii* (BR2) showed antifungal activity towards *Fusarium oxysporum, Sclerotium rolfsii*, *Alterneria alternatae* and *Macrophomina phasiolina* fungi by induced growth free inhibition zones. After three days of incubation, fungal hyphae were unable to reach the bacterial culture and inhibition zone was established with the dimension of inhibition circle (Figure. 3. (A to D). While control plates not treated with the PSB isolates were completely covered by the pathogen having no antagonistic effect (Fig. 4). Nur Azura *et al.*, (2008) have previously reported similar results that PSB strains

Sr No	Bacterial Isolates	*M. phasiolina (cm)*	*A. alternatae (cm)*	*F. oxysporum*(cm)	*S. rolfsii*(cm)
1	*Citrobacterfreundii*(BR2)	1.9±0.033961c	1.8±0.058823c	2.1±0.033961d	1.7±0.058823[d]
2	*Klebsiella pneumonia* (PR2)	1.3± 0.033961b,c,d	1.5±0.033961a,c,d	1.6±0.058823a,b	1.6±0.058823a,b,c
3	*Enterobacteraerogenes*(PR4)	1.3±0.033961b	_a,c,d	1.2±0.089547b	1.3±0.033961
4	*Burkholederiacepacia*(BR1)	1.5±0.033961b.c,d	1.2±0.089547a,d	1.2±0.089547a,d	_a,b,c
5	*Acinetobacterlwoffi*(SR1)	1.2±0.089547b,c,d	_a	_a	_a
6	*Proteous vulgaris*(PR1)	_b	0.9±0.058823a,c,d	_b	_b

Table-2 Zone of inhibition by phosphate solubilizing bacteria (Each value is an average of three replicates; ± denotes standard error means. The means followed by different letters within each column are significantly different at $P< 0.005$)

exhibited the best antagonistic properties against the pathogenic fungus.

Zone of inhibition was measured for all fungus and *Citrobacter freundii* (BR2) showed best result against all four funguses (*Macrophomina phasiolina, Alterneria alternatae, Fusarium oxysporum and Sclerotium rolfsii*). Similar results were observed by (Ahlem *et al.,* 2011) that PSB isolates revealed the best antagonistic activity against the pathogenic fungi.

Citrobacter freundii (BR2) made highest zone of Inhibition of approximately 2.1cm against *Fusarium oxysporum. Citrobacter freundii* (BR2) and *Klebsiella pneumonia* (PR2) showed bio control activity against all four funguses and measured zone of inhibition is given in (Table 2 Fig. 4). Similar antifungal activity of bacterial isolates was reported by Salem *et al.* (2012).

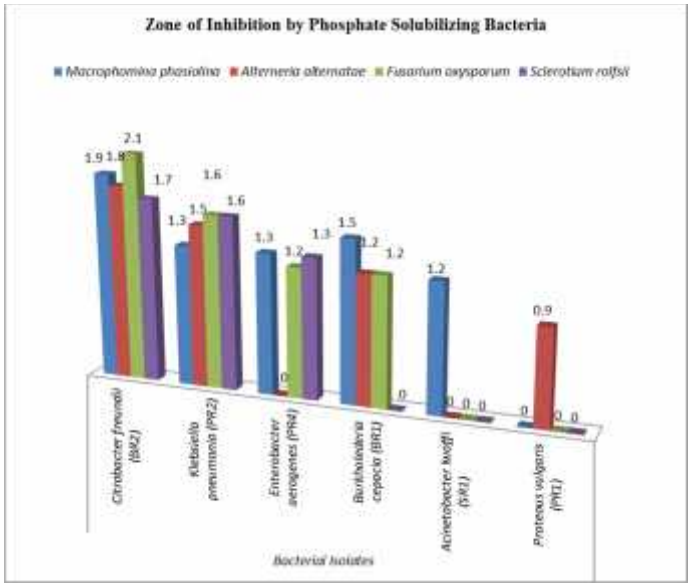

Fig. 4: Fungal growth inhibition zone by the bio-control activity of Phosphate solubilizing bacteria

Acinetobacter lwoffi also showed bio control activity against *Macrophomina phasiolina* and zone of inhibition was measured 1.2cm while *Acinetobacter lwoffi* did not show biocontrol activity against *Fusarium oxysporum, Alterneria alternatae and Sclerotium rolfsii.* While *Proteous vulgaris* (PR1) did not show bio control activity against *Macrophomina phasiolina, Fusarium oxysporum and Sclerotium rolfsii* and made zone of inhibition of 0.9cm against *Alterneria alternatae* (Figure. 3. A to D, Table 2). Leon *et al*., 2009 reported that the antagonistic activity of Phosphate solubilizing bacteria from rhizosphere soil may be due to secretion of antifungal compound.

Statistical Analysis: The data were evaluated statistically by analysis of variance (one way ANOVA) and its POST HOC test using SPSS software version 20. The means followed by different letters within each column are significantly different at P< 0.005.

Significant difference P<0.005 was observed between the values obtained for Bio control as compared to other bacterial isolates (Table 2).

Conclusion: The present investigation has revealed that the phosphate solubilizing bacterial strain has the potential to be applied in fields as a bio control agent against fungal pathogens like *F. oxysporum, S. rolfsii, A. alternatae and M. phasiolina*. The finding of present investigation highlighted that PSB from local soil could be easily isolated and may be exploited for local use**.** Phosphate solubilizing bacteria are environment friendly and bear superior agronomic utility to compensate the expensive inorganic sources of P fertilizers so these isolates may promote plant growth directly, indirectly or synergistically in the soil environment by using Phosphate Solubilizing bacteria as Bio control. Furtehermore, it can be demonstrated that the natural *P. sativum, B. campestris* and *S. officinarum* rhizosphere soils can be the rich source for isolation of phosphate

solubilizing microorganism. Large scale investigation is required to isolate and identify more efficient PSB for commercial production of inoculum.

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