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Isolation of phosphate solubilizing fungi from vegetable rhizosphere and their effects on growth of Tomato (*Lycopersicon* spp.)

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Key words: Phosphate solubilizing microorganisms (PSM), Phosphate solubilizing fungi (PSF) isolates, Rhizosphere, Solubilization index, Seed germination, Vigor index

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Abstract

Five (5) phosphate-solubilizing mold species isolated from the rhizosphere regimes of vegetable crops were characterized in terms of their solubilizing index (SI) and ability to grow at varying pH values. Solubilization index of isolates ranged from 1.89 (M3) to 2.56 (M6). In culture broth, PSF caused the pH dropped from 7.0 to 4.31 in 12 days with M1 producing the highest drop in pH (4.31). Two isolates with highest SI identified as *Penicillium* sp (M1) and *Rhizopus* sp. (M6) were selected to determine their effects on seed vigor and growth of tomato plants (*Lycopersicon* sp.) in a greenhouse experiment. When tested on their ability to promote seed germination, the fungal isolates increased the germination rate of seeds compared to the control. The highest germination rate was observed with Rhizopus (96%) followed by *Penicillium* (92.33%). Effect of co-inoculation did not differ significantly with single inoculation. In terms of vigor index, single and co-inoculation with fungal isolates produced the same seed vigor but improved considerably compared with the control. In pot experiments, compared with the uninoculated plants, single inoculation with fungal isolates significantly improved the height, number of leaves produced, and fresh and dry weight of tomato plant, but were comparable among the isolates. Dual or co-inoculation resulted to plant height comparable with the control. PSF a vast potential to contribute to agronomic productivity as biofertilizers for a more-environmentally friendly agricultural management.

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Introduction

Phosphorous is one of the essential mineral nutrients for plant growth and development. It is available to plant roots only in its soluble form as orthophosphates either as HPO_4^{2-} or $H_2PO_4^{1-}$. However, in most agricultural soils, the concentration of soluble orthophosphates is low, normally 1 mg kg^{-1} or lower, 10 MH_2PO_4 (Kalayu, 2019) and hence, limiting the amount of P available for plants. To meet the nutritional requirement and counterbalance P deficiency in crops, regular application of phosphate fertilizers is a conventional farming regimen in many countries. However, despite supplementation, only a low percentage of these agrochemicals can be used efficiently by plants since a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Steiner *et al.*, 2016). Therefore, the constant and continuous application of chemical fertilizers at higher rates not only is economically burdensome but also compromises soil quality.

Efforts to move towards sustainable agricultural production would investigate the role of the rhizosphere as a tool to reduce the use of chemical fertilizers and pesticides. Soil microorganisms particularly the phosphate solubilizing microorganisms (PSM) are seen as important possible alternatives for inorganic phosphate fertilizers because of their ability to release insoluble and fixed forms of phosphorus and promote subsequent uptake of P by plants to achieve maximum growth and yield (Malviya *et al.* 2011). PSMs have been shown to possess ability to bring insoluble soil phosphates into soluble forms by secreting acids such as formic, acetic, propionic, lactic, glucolic, fumaric and succinic. These acids lower the pH and bring about the dissolution of bound forms of phosphate.

There is a continuous and heightened search for efficient strains of PSM as these have prospective use as bio-inoculant (biofertilizer) components in organic agriculture, which is emerging as an alternative to chemical inputs in intensive agriculture.

A significant reduction in the use of phosphate fertilizer could be achieved if solubilization of soil-insoluble phosphorus is made available to crop plants in a more-environmentally-friendly and sustainable manner (Kalayu, 2019). Several important genera of bacteria and fungi have been reported to have the ability to solubilize phosphates, whether inorganic, organic or both.

In Cagayan Valley and most regions in the Philippines, agricultural farm management still applies the conventional regime involving the intensive use of chemical fertilizers and pesticides. However, there are efforts to shift to a more sustainable agriculture approach involving organic farming regimes. PSMs may have potential role in organic farming but there is lack of information on the use of PSM for crops. There are also very few attempts to isolate and characterize the potential PSM from the rhizosphere of crop plants in the Philippines. Hence, little information is available concerning phosphate solubilizing microorganisms and their ability to colonize locally grown crops. This study was conducted to isolate and characterize molds with phosphate-solubilizing ability (PSF) and evaluate their effects on the growth of tomato under greenhouse conditions.

Materials and methods

Isolation and Screening of Phosphate Solubilizing Fungi

Fungal strains were isolated from the soil and rhizosphere of roots of plants growing in an open field in Tuguegarao City, Cagayan, Philippines. Soil sample (10 g) was mixed with 90 mL sterile distilled water, vigorously shaken, and left to stand for 5 min. Homogeneous soil solution was serially diluted up to 10^{-6} and transferred to PDA. Fungal cultures were incubated at 28°C for 3 days. The phosphate solubilizing ability of the fungal isolates was confirmed by an agar assay on National Botanical Research Institute's phosphate growth medium (NBRIP) containing glucose 10g, $Ca_3(PO_4)_2$ 5 g, $MgCl_2 \cdot 6H_2O$ 5g, $MgSO_4 \cdot 7H_2O$ 0.25g, KCl 0.2 g, $(NH_4)_2SO_4$ 0.1g, agar 15g, and dissolved in 1000 mL of distilled water (Onyia and Anyanwu, 2013).

Colonies surrounded by discrete halo zones were assumed to be phosphate solubilizers. These were selected, purified by repeated culturing on NBRIP medium and the pure cultures were then preserved on Potato Dextrose Agar (PDA) slant at 4 °C for further investigation.

Determination of phosphate solubilization index (SI)

Phosphate solubilization index (SI) or the ability of the microorganism to solubilize insoluble phosphate was calculated using the formula of Demisie *et al.* (2013). NBRIP agar plates were point inoculated using sterile inoculating needle with 24-hr cultures of isolates and incubated at 28-30°C for 7 days. Comparative solubilization index measurement was carried out by measuring halo zone and colony diameters in centimeter. Measurements of the diameter of the colony and halo zone were done, and SI was calculated using following formula: SI = (Colony diameter + Halozone diameter)/Colony diameter. All observations were recorded in triplicate and the fungi with the highest phosphate solubilizing index were selected for evaluation.

Fungal growth and pH value of the culture medium

To determine microbial growth and change in pH value of the medium, the PSF isolates were inoculated separately in test tubes containing 10 ml of NBRIP. These were incubated for 12 days at 28-32°C. The optical density of the microorganisms and pH value of the medium were estimated at 3-day intervals using a spectrophotometer (at 600 nm) and a pH meter, respectively. Each treatment was replicated three times and data were expressed as the mean value.

Seed germination bioassay

Thirty seeds each of *Lycopersicon* tomato were dipped into 24-hr potato dextrose broth (PDB) culture of fungal isolates for 5 hr and in distilled water that served as control. Ten seeds per plate of inoculated *Lycopersicon* tomato were placed in sterile Petri plates lined with moist cotton. Plates were laid out in Complete Randomized Design (CRD) in triplicate and incubated for 3-7 days. The radicle length and plumule length of germinating seeds were measured and the germination percentages of seeds were calculated.

The seed vigor index was calculated using the formula: Vigor Index = (Mean radicle length + Mean plumule length) x Germination (%) (Baki and Anderson 1973; Kalayu, 2019).

Pot experiment

Determination of effect of fungal isolates

Inoculant preparation

Two fungal isolates (M1 and M6) were selected to evaluate their performance as individual inoculum and as co-inoculants (M1+M6) on tomato under in vitro condition. The fungal isolates were pre-inoculated in Petri dishes containing potato dextrose agar and incubated at 25°C for 7 days.

Spore suspension was prepared by scraping Petri dishes containing mycelium cultivated on potato dextrose agar for 7–10 days at 25°C. For scraping, 0.1% Tween 80 solution was used. Fungi suspensions obtained were filtered through Whatman filter paper into a sterile glass bottle to remove excess mycelium, and then washed with distilled water under aseptic condition. The pelleted cells were resuspended in distilled water. The determination of the spore concentration of each fungus was performed by using a haemocytometer. Spore suspensions were standardized to a concentration of 1 x 10^8 colony-forming units/ml (CFU mL^{-1}) (Elias *et al.*, 2016).

Seed inoculation

Tomato seeds were inoculated with the fungal isolates by soaking seeds into each suspension of potato dextrose liquid culture medium of the isolates M1, M6, and mixed suspension of M1 and M6 to deliver 1 x 10^6 CFU/mL per seed for 8 h at 25°C. For combined inoculations, the liquid cultures of each isolate were mixed in equal proportion per seed according to Elias *et al.* (2016). After the soaking period, tomato seeds were sown in pots containing previously sieved soil. The soil was obtained from a cultivated agricultural in Tuguegarao City, Cagayan, Philippines.

Treatments and experimental design

The pot experiment adopted the procedure of Elias *et al.* (2016). Black plastic bags previously sterilized with sodium hypochlorite solution were filled with 5.0 kg of non-sterile soil.

Five (5) inoculated seeds were sown per pot at 2 cm depth soil in each plastic pot. Thinning was performed after 5 days of emergence, keeping 3 plants per pot. The experiments were arranged into 5 treatments:

Group	Treatment
I	Soil only (Uninoculated)
III	Soil + M1
IV	Soil + M6
V	Soil + M1 + M6

The pots with different treatments were arranged in a complete randomized design (CRD) with three replications of each treatment. The moisture content of pots was kept around 70% of the field capacity with regular watering. The experiment was carried out until the flowering of tomato plants.

Evaluation of growth parameters and yield components

Growth parameters and yield components of tomato plant were measured following the technique of Elias *et al.* (2016). Morphological characteristics such as shoot length and number of leaves were measured from 3 plants per treatment at 16 weeks. The number of flowers produced was counted until the termination of the experiment at 12 weeks. At the end of the experiment, the whole plants were carefully uprooted from the pots, washed gently under running tap water to remove the adhering soil particles. Fresh and dry weights of shoot and roots were also recorded. For measuring the dry weight, the shoots and roots were placed in paper bags and dried in an oven at 70 °C for 48. Root and shoot dry matter weight was determined using analytical scale.

Determination of phosphorous concentration in plants

For the determination of phosphorus in plants, phosphorus concentrations in roots and shoots were determined using standard colorimetric methodology.

Statistical analysis

Mean values were Analysis of Variance (ANOVA) was done. The results were obtained for the different sets of experiments using SPSS16 at 5% probability level ($P < 0.05$).

Results and discussion

Characteristics of phosphate solubilizing fungal isolates

Five (5) phosphate solubilizing fungi (PSF) were isolated on the NBRIP containing tri-calcium phosphate. Colonies showing clear halo zones around the fungal growth were considered as phosphate solubilizers. The phosphate solubilization index (SI) of the isolates ranged from 1.89 to 2.69 (Table 1). However, results of t-test showed that isolates M1, M3, M5, and M6 have comparable solubilizing index (SI) while M4 has the lowest SI. Preliminary characterization by colony characterization and by slide culture mount revealed that the fungal isolates M1, M3, M4, and M5 were *Penicillium* species while M6 was *Rhizopus* species (Figure 1). The presence of phosphate-solubilizing microbes in the soil is related to its organic content which provides the carbon and energy source, thereby contributing to the amount of microbial activity in the soil (Elfiati *et al.*, 2021). In terms of solubility index, the higher the SI values indicate that the fungal isolate's ability to dissolve P will also be higher. In the study, the fungal isolates were able to solubilize $Ca_3(PO_4)_2$, the component of NBRIP medium. Mahamuni *et al.* (2012) obtained a solubility index range between 1.13 to 1.59 on isolated PSF from the rhizosphere of sugarcane and sugar beet. Similarly, Majumder *et al.* (2019) reported solubility index ranging from 1.42 to 2.24 of fungi isolated from Okinawa soils. Elfiati *et al.* (2021) explains that solubility index values may differ depending on the amount, type, and diffusion rate of organic acids released by the fungal isolates.

Table 1. Phosphate solubilizing index (SI) of fungal isolates on NBRIP agar

Isolates	Mean Colony Diameter (mm)	Mean Halo Diameter (mm)	Solubilizing Index
M1 (*Penicillium* sp.)	19.33	27.70	2.43[a]
M3 (*Penicillium* sp.)	4.40	6.73	2.43[a]
M4 (*Penicillium* sp.)	9.00	8.00	1.89[b]
M5 (*Penicillium* sp.)	12.30	13.83	2.12[a]
M6 (*Rhizopus* sp.)	13.50	21.1	2.56[a]

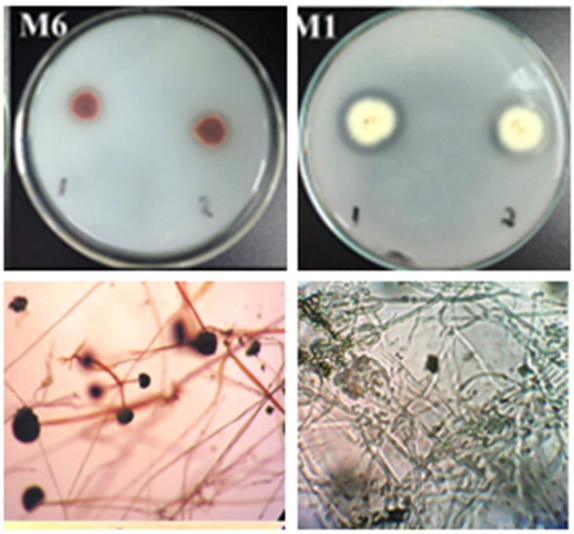

Figure 1. Growth on NBRIP agar and morphological characteristics of phosphate solubilizing fungal (PSF) isolates.

Ability of PSF to produce acids in the culture medium

To test the ability of the PSF isolates to change the pH of the culture medium and to analyze their ability to grow in a varied range of pH, the fungal isolates were inoculated separately into broth medium and incubated for 12 days. These were found to be active in lowering the pH (Table 2). Drop in pH by PSF ranged from 6.0 to 4.31 at the end of incubation period with isolate M1 resulting to the highest pH drop from 7.01 (control) to 4.31. Similar results were observed with isolates M3, M4, M5, and M6. However, M6 showed the lowest pH drop from (7.01 to 6.0). Optical density of culture medium revealed that fungal growth was either or not affected by a decrease in pH as shown by the population (cells/ml) of cells in Table 2. Growth of Isolate M1 was reduced (3 x 10^8 cells/mL) at pH 4.51 while growth of M3 and M5 were not affected (30 x 10^8 cells/mL) by decreasing pH in the medium. Li *et al.* (2016) found out that the phosphate-solubilizing ability of filamentous fungi is generally associated with the release of organic acids with the decrease in the pH values. The production of organic acids such as gluconic, 2-ketogluconic, lactic, isovaleric, isobutyric, acetic, oxalic, citric acid by phosphate solubilizing microorganisms has been well documented (Kalayu, 2019). In addition, the study of Li *et al.* (2016) also revealed that maximum fungal biomass reached also varies with the initial pH of the medium.

Table 2. Effect of pH value on growth of phosphate solubilizing microorganisms

Isolates	Cell numbers (x 10^8 cells/mL) (Day 0)	Cell numbers (x 10^8 cells/mL) (Day 12)	pH (Day 3)	pH (Day 12)
Control	0.0	0.0	7.0	7.0
M1	30	3	5.51	4.31
M3	30	30	5.70	5.49
M4	30	15	5.74	5.39
M5	30	30	5.44	6.00
M6	30	18	4.74	4.54

Although the mechanism of the bacterial phosphate solubilizing activity observed in the current study is not clear at present, most of the isolates slightly decreased the pH value of the culture medium suggesting that organic acid secretion by the fungal isolates might play a role in phosphate solubilizing activity. Several lines of evidence suggest that the principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases which play a major role in the mineralization of organic phosphorous in soil (Kalayu, 2019).

Effect of PSF inoculation on seed germination

Table 3. Effect of phosphate solubilizing fungi on germination and vigor index of *Lycopersicon* seeds

Isolates	Mean Radicle Length (cm)	Mean Plumule Length (cm)	Percentage (%) Germination	Increment Percentage (%) Germination	Mean Vigor Index	Increment of vigor index
Control	4.71	2.94	67.67^a		513.95^a	
M1	5.37	3.30	92.33^b	24.67	862.27^b	348.31
M6	4.72	2.80	96.00^c	28.33	843.39^b	329.44
M1+M6	5.28	3.27	89.33^b	21.67	784.84^b	270.89

Values with the same superscript are NOT significantly different from each other at 5% level of significance.

Fungal isolates M1 and M6 were tested for growth of tomato by moist cotton method. The effect of inoculation and co-inoculation of PSF on germination of *Lycopersicon* significantly increased percentage germination and vigor index are shown in Table 3. Percentage germination and vigor index with single inoculation of M1 (*Penicillium*) was 92.33% and 862.27, respectively, while inoculation with M6 (*Rhizopus*) yielded up to 95% and 714.40, respectively. However, co-inoculation (M1 + M6) appeared to have diminished percentage germination and vigor index to 89.33% and 784.84, respectively compared to single inoculation.

However, t-test showed that there is no significant difference between single- or co-inoculation on the germination and vigor index. Results indicate that fungal isolates were able to significantly increase the percentage of seed germination, hence, increasing seed vigor index. Increment of percentage germination occurred by 21.67% – 28.33% while tomato seeds vigor index occurred by 270.89 – 348.31 over the control, respectively. Demissie *et al.* (2013) reported the increment of bean seeds germination over control by 21.4% and 19% by growth-promoting activities of co-inoculants and single inoculants, respectively. The increment of seed germination with inoculants could be due to the isolates ability to synthesize seed germinating hormone. Gholami *et al.* (2009) demonstrated the enhancement of seed germination by inoculants due to the presence of seed germination hormone like gibberellins which triggered the activity of specific enzymes that promoted early germination, such as a-amylase that increase the availability of starch assimilation. Furthermore, co-inoculation of molds and bacteria enhanced shoot length as compared with individuals. This could be because of higher amount of growth promoting substances and biocontrol substances released by the inoculants. Birhanu Gizaw *et al.* (2018) attributed the enhancement of seed parameters to auxins (IAA) and growth promoting substances produced by the PSM inoculants. In general, the isolates have phosphate solubilizing activity as well as the ability to improve seed germination.

Effect of fungal isolates on the growth of Tomato plants

Out of the 5 phosphate solubilizing fungi (PSF) isolated from soil rhizosphere, fungal isolates M1 (*Penicillium* sp.) which showed the best phosphate solubilizing activity and M6 (*Rhizopus* sp.) were selected and evaluated for growth promoting effects in phosphate-insufficient soils. Table 4 shows that single inoculation with fungal isolates M1 and M6 the fungal strains improved the height, number of leaves produced, and fresh and dry weight of the plant. Single inoculation of the fungal isolates significantly increased the height of the plant over the uninoculated (control) at 6 weeks. Among the treatments, when inoculated singly, fungal isolate M1 promoted plant height to 20.128 cm/plant while fungal isolate M6 produced plant height of 19.111 cm/plant. However, results revealed that there is no significant difference on the effect of M1 and M6 on plant height. This implies that regardless of the microorganism, single inoculation produces the same behavior of the plant. Moreover, dual inoculation with M1 and M6 resulted to plant height comparable with the control. Hence, co-inoculation (M1M6) appeared to slow down increase in plant height. Diaz *et al.* (2021) also showed that, regardless of isolate, inoculant concentrations promoted the same fiber and seed cotton yield.

Results of the study also showed significant increase in number of leaves per plant at 6 weeks, and number of flowers produced and dry weight (g/plant) at 12 weeks with the application of single or dual inoculation of the fungal strains when compared to the control. However, there was no significant difference on the effects of M1, M6 and M1M6 with each other on the above parameters suggesting a comparable activity of the fungal isolates. Thus, the application of phosphate solubilizing fungi, particularly M1 and M6, separately from each other is a more practical way to improve growth of tomato plants under greenhouse conditions.

Table 4. Effect of PSF isolates on tomato plants grown in P-deficient soil

Treatments	Plant Height (cm)	No. of Leaves	No. of Flowers	Dry wt (g)	Fresh wt (g)	Plant Phosphorous (%)
Soil only	15.113[a]	10.333[a]	3.778[a]	27.31[a]	61.855[a]	0.468[a]
Soil + M1	20.128[b]	7.557[b]	8.889[b]	44.637[b]	84.039[b]	0.848[b]
Soil + M6	19.111[bc]	6.890[b]	12.556[b]	42.210[b]	95.158[bc]	0.645[c]
Soil + M1 + M6	16.833[a]	6.777[b]	9.000[b]	39.757[b]	89.353[bc]	0.590[d]

Values with the same superscript are NOT significantly different from each other at 5% level of significance.

Effect of PSF on phosphorous content of tomato plant

Table 4 also shows the amount of phosphorous measured from the leaves of tomato. Results revealed that single or co-inoculation with the fungal isolates produced significant differences on the phosphorous content of the plants over the control. Among the treatments, fungal isolate M1 produced the highest phosphorous content (0.848%) in tomato, followed by fungal isolate M6 (0.645%). Co-inoculation decreased the phosphorous content to 0.590%. These values are within the sufficiency range of tissue phosphorous for various crops such as corn (0.30-0.50%), soybean (0.30-0.60%) and small grains (0.2-00.50%) and canola (0.20-0.65%) (Chakraborty and Prasad, 2019). Several studies in the past showed that inoculation of PSM have resulted in significant improvement in uptake of P, K and N (Steiner, *et al.*, 2016) as well as increased grain yield (Kalayu, 2019).

Conclusion

Fungal isolates, *Penicillium* sp. and *Rhizopus* sp., have phosphate solubilizing activity. Both enhanced seed germination and seed vigor of tomato better than the uninoculated seeds. Both single and co-inoculation treatments showed better plant growth in terms of height, number of leaves, and dry weight as compared with the uninoculated control treatment. Since no significant difference exists among the inoculated treatments, single inoculation with either *Penicillium* sp. or *Rhizopus* sp. would be more practical and economical. Phosphorous uptake improved to meet a level of sufficiency in tomato plants.

From the results of the study, it can be concluded that phosphate solubilizing fungi have a vast potential to contribute to agronomic productivity as biofertilizers to alleviate soil fertility concerns as well as to limit use of chemical fertilizers for more-environmentally friendly agricultural management.

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