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Article *in* Sri Lanka Journal of Food and Agriculture · October 2017

DOI: 10.4038/sljfa.v3i2.46

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Research Paper

Phosphate-solubilizing fungi for efficient soil phosphorus management

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Article History:
Received: 27 January 2017
Revised form received: 25 September 2017
Accepted: 30 October 2017

Abstract: Rhizosphere soil extracts obtained from eight cinnamon species and three pepper species cultivated organically were inoculated initially on Pikovskaya (PVK) medium. The twelve fungal isolates that solubilized the insoluble phosphates were further tested on the same medium added with both insoluble tricalcium phosphate and soluble dipotassium hydrogen phosphate to detect the expression of the trait in the presence of soluble phosphates in soil, for which all twelve qualified. The efficiencies of phosphate solubilization were investigated in two liquid media: PVK medium to represent soil phosphate pool and a rock phosphate (RP) medium, by determining the available phosphorus concentration in the liquid media at the end of the 24 h and 72 h incubation periods, respectively. The pH reduction in the medium was measured to ascertain the organic acid production by fungal isolates as a mechanism of solubilization. Three fungal isolates showing the highest efficiencies in phosphate solubilization were taxonomically identified based on the micromorphological characteristics and molecular techniques. Two isolates were identified as *Penicillium oxalicum* and *Trichorderma virens* and the other was belonging to the genus *Aspergillus*. A possible synergism between the three fungal species towards phosphate dissolution was detected using the broth culture procedure. *Aspergillus* sp. in combination with *P. oxalicum* dissolved both $Ca_3(PO_4)_2$ and RP recording the highest significant dissolved phosphate levels of 893.43 (±56.768) mg P/L and 309.42 (±42.52) mg P/L, respectively, within 72 h post inoculation, making them prospective candidates for increased phosphate availability of soil phosphate pool and rock phosphates.

Keywords: Phosphate-solubilizing fungi, soil phosphate pool, *in vitro*

Introduction

Absence of plant available phosphates (Pi) is a global agricultural problem, especially due to rapid fixation of applied soluble phosphates in both acidic and alkaline soils (Shen *et al*., 2011; Son *et al*., 2006). Phosphates in soil become fixed with calcium, magnesium, aluminium and ferrous ions elevating soil phosphate pool with each addition of phosphate fertilizers. Being one of the macronutrients, this imposes a significant barrier to increased agricultural production. Moreover, highly recommended phosphatic fertilizers are known to contain impurities that pose negative impacts on the environment and health (Barabasz *et al*., 2002; Son *et al*., 2006; Taylor, 2007).Value addition to rock phosphate and utilization of soil phosphate pool by using biological means were

focused in this search for an economically, agronomically and ecologically viable alternative for synthetic phosphate fertilizer (Didiek *et al*., 2001; Sahu and Jana, 2000).

Certain microorganisms, termed as phosphate solubilizing microorganisms (PSMs) play a key role in soil Pi mobilization as they possess an inherent capacity to solubilize unavailable Pi sources (Goldstein *et al*., 1999; Sahu and Jana., 2000; Vassilev *et al*., 2001). Fungi belonging to the genera *Aspergillus*, *Penicillium*, bacterial genera such as *Bacillus*, *Pseudomonas*, *Rhizobium*, *Burkholderia* and mycorrhizal fungi are known to be among the robust rock phosphate solubilizers (Iman and Azouni, 2008; Jayasinghearachchi and Senavirathne, 2006; Rodriguez and Fraga, 1999; Vassilev *et al*., 2001). The ability of Pi dissolution by microorganisms is attributed to the processes of acidification, chelation, enzymatic dissolution and ion exchange reactions (Illmer and Schinner, 1994). The use of such microorganisms as biofertilizers in agriculture is steadily on the rise with increased concern over the environmental problems associated with agrochemical usage worldwide (Chen *et al*., 2006). Therefore, search for ways of increasing plant availability of the existing fixed P pool and Eppawala Rock Phosphate (ERP), a large natural rock phosphate deposit available in Sri Lanka with little Cadmium (Cd) as a contaminant compared to triple super phosphate (Bandara *et al*., 2008; Bandara *et al*., 2011; Ranasinghe *et al*., 2013) was initiated.

Piper nigrum (pepper) and *Cinnamomum verum* (cinnamon) are two major agricultural crops contributing considerably to gross national production of South Asian countries. Being perennials, the fertilizer recommendation for these crops includes RP as the sole phosphate source (Anon, 2002). Further, there are some wild relatives that are used in breeding programmes, for which the agrochemicals are not used. Hence, it is assumed that there is a high likelihood for rhizospheres of these species to contain RP-solubilizing microorganisms. Rhizosphere competent microorganisms have a better chance in establishing in soil environment upon field introduction (Vessey, 2003). Moreover, fungi have been reported to possess a greater ability to solubilize insoluble phosphate than bacteria (Nahas, 1996). Hence, the current study was initiated with the objectives of isolating an efficient phosphate solubilizing fungus or a consortium of fungi to be used in the production of a biofertilizer to increase plant availability of both the fixed soil-P pool and rock phosphates.

Materials and Methods

Isolation of phosphate solubilizing fungi

Rhizosphere soil samples were collected from wild and cultivated species of pepper: *Piper chuvya* Miq, *P. sylvestre* Lam and *P. longum* Linn, and cinnamon: *Cinnamomum capparu-corende* Blume, *C. citriodorum* Thw, *C. dubium* Nees, *C. litseaefolium* Thw, *C. sinharajaense* Kostermans, *C. rivulorum* Kostermans, *C. verum* Presl, *C. verum* var. *srigamunu, C. verum* var. *srivijaya* and *Cinnamomum* sp. (unidentified). A dilution series of soil extracts were prepared and spread on Pikovskaya medium (PVK) containing tricalcium phosphate as the sole P source for selective screening of phosphate solubilizing microorganisms (Pikovskaya, 1948). After seven days of incubation at room temperature, phosphate solubilizing fungi (PSF) with developed clear zones around their colonies were selected and further purified by repeated sub-culturing on the same medium. As inorganic phosphate (Pi) solubilizing activity of some microorganisms is suppressed by the presence of soluble phosphates in the medium, the isolates were subjected to a secondary screening on a modified PVK medium where tricalcium phosphate was partially replaced by soluble dipotassium hydrogen phosphate (K_2HPO_4) (Mikanova and Novakova, 2002). Isolates that showed a positive response of developing clear halos around the colonies were selected and coded for convenience.

Evaluating the insoluble phosphate solubilization in broth culture

The experiment contained two approaches: investigation of efficiency of solubilizing (1) $Ca_3(PO_4)_2$, which is a close representation of the

soil P pool and (2) Eppawala Rock Phosphate (ERP), a locally available rock phosphate source. For these purposes, two testing media namely, PVK liquid medium and a modified ERP-PVK liquid medium where $Ca_3(PO_4)_2$ in PVK medium was replaced by ERP quantitatively to maintain similar P_2O_5 concentration in both media, were used. Phosphate solubilizing efficiencies of the isolates were first evaluated in PVK liquid medium where the sole P source is $Ca_3(PO_4)_2$. A volume of 100 ml PVK broth was inoculated with 1.0 ml of spore suspension containing approximately 1 x 10^7 spores from each fungus, separately. A broth inoculated with 1 ml of sterile distilled water served as the control. The triplicated treatments were incubated on a rotary shaker at 100 rpm oscillations for 24 h. As per phosphate solubilization capacity in modified ERP-PVK liquid cultures, it was noted at 24 h incubation resulted considerably low values. Hence, it was decided to increase the incubation time to 72 h. At the end of the prescribed incubation period, the cultures were filtered through Whatmann No. 1 filter papers and centrifuged at 7200 rpm for 20 min to remove any mycelial fragments. The supernatant was tested for available phosphate concentrations colorimetrically (Murphy and Riley, 1962). The pH values of the media were measured using a portable pH meter (Hach H160). To ascertain the mechanism of phosphate solubilization, change in pH (dpH) of the medium was calculated by deducting the pH after incubation from that of the initial (pH 7).

Identification of efficient phosphate solubilizing fungi

Three efficient fungal isolates that exhibited highest solubilization capacities in both PVK and ERP-PVK media were selected for further investigations. The fungi were identified up to generic level based on micromorphological and reproductive characteristics. Confirmation of the identifications were performed by extracting fungal DNA using Promega Wizard DNA extraction kit® and subjecting to PCR amplification followed by sequencing of specific region of the 18S rRNA gene. ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') served as the forward and reverse primers, respectively. The obtained sequences were blasted into Genbank accessions.

Detecting synergism among the fungal isolates

Synergistic activity towards phosphate solubilization by the three selected fungal isolates was tested by quantifying and comparing solubilized phosphate concentrations by the organisms alone and in different combinations. Three single inoculants namely, *Aspergillus* sp., *Trichorderma virens* and *Penicillium oxalicum*, and four mixed inoculants namely, *Aspergillus* sp. + *T. virens*, *T. virens* + *P. oxalicum*, *Aspergillus* sp. + *P. oxalicum*, and *Aspergillus* sp. + *T. virens* + *P. oxalicum*, were tested along with a control in PVK and ERP-PVK liquid media, separately as per the protocol mentioned earlier. Population density of the mixed inoculum was maintained at 1 x 10^7 spores per ml by taking volumes proportionately from each spore suspension. Three replicates were employed and the supernatant pH and solubilized phosphate concentrations at 72 h were quantified as mentioned earlier.

Statistical analysis

The data collected were subjected to one way ANOVA and the significant means were compared with Fisher's multiple mean comparison using one way ANOVA (P=0.05). A correlation analysis was performed to determine the relationship between pH reduction of the medium and the amount of phosphate solubilized. The statistical analysis was done by using Minitab 16.1.1 statistical package.

Results and Discussion

Isolation of phosphate solubilizing fungi

From the rhizosphere soils, 12 fungi were isolated based on the clear halo production on Pikovskaya medium as shown in Figure 1. It has been reported that phosphate solubilizing activity of microorganisms is affected by the presence of soluble phosphates in the medium (Mikanova and Novakova, 2002). Hence, testing the sensitivity to soluble phosphates is important in the selection of suitable microorganisms for practical applications (Mikanova and Novakova, 2002). Secondary screening confirmed that all twelve isolates were

capable of expressing the solubilization trait in the presence of low levels of soluble phosphates, which is an important character to be considered in a microorganism to be employed as a biofertilizer.

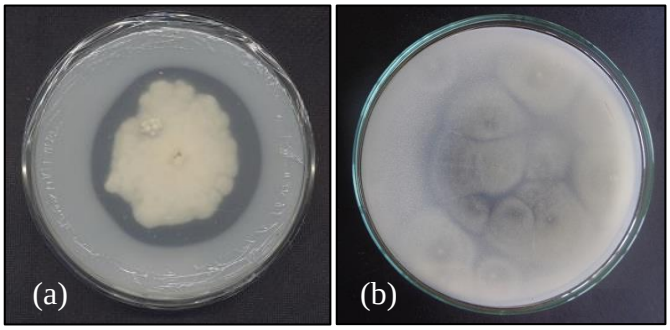

Figure 1. Clear halo produced in PVK medium by (a) *Penicillium oxalicum* (TCvgRF5) and (b) *Aspergillus* sp. (MPsRF1)

Efficiency of insoluble phosphate solubilization in broth cultures

Quantitative estimation of phosphate solubilization in PVK and modified ERP-PVK liquid media recorded a significant variability ($p<0.05$) with respect to solubilized phosphate concentrations and change in medium pH (dpH) as shown in Figures 2 and 3. Among the coded isolates, isolate MPsRF1 showed the highest phosphate solubilization values of 160.00 (±9.09) mg P/L at day 1 in PVK medium and 278.87 (±32.94) mg P/L in ERP-PVK medium at the day 3. The isolates TCvgRF5 and MCvRF1 recorded the second and third highest ERP solubilization with means of 223.86 (±13.43) and 154.89 (±39.45) mg P/L, respectively, at day 3 in ERP-PVK medium. Furthermore, the decrease of pH was evident in the tested isolates compared to the control as shown in Table 1. This supports the mechanism of medium acidulation as a means of solubilization. Organic acid production and proton extrusion have been identified as means of reducing medium pH by microorganisms (Rodriguez and Fraga, 1999). Significant pH reductions ($p<0.05$) were observed in all treatments as shown in the Figure 3. The highest pH reduction (dpH) in the PVK medium was exhibited by the fungal isolate TClRF1 with a mean value of 3.64 (±0.99), whereas in ERP-PVK medium it was the isolates MTcRF1 and MPsRF1, both recording a dpH of 4.17 (±0.06).

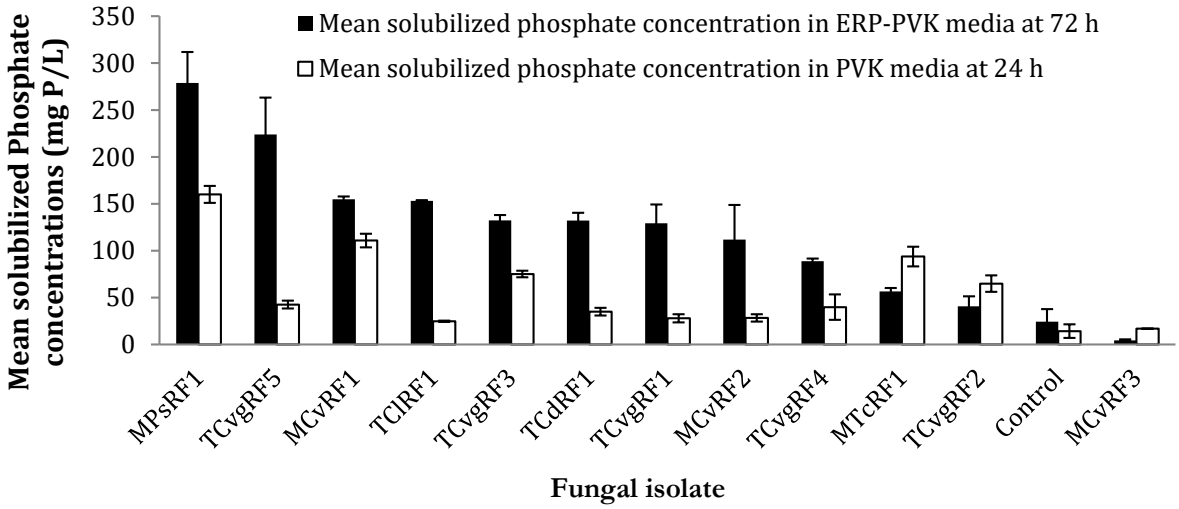

Figure 2. Mean solubilized phosphate concentration by fungal isolates in PVK medium at 24 h and in ERP-PVK medium at 72 h. Vertical lines indicate the standard deviations.

Table 1. Mean final pH in the PVK and ERP-PVK media shown by fungal isolates after 24 h and 72 h inoculation, respectively

Fungal Isolate	Final pH in PVK medium at 24 h ± SD*	Final pH in ERP-PVK medium at 72 h ± SD*
MPsRF1	4.10 ± 0.10	2.83 ± 0.06
MCvRF1	4.23 ± 0.06	3.13 ± 0.29
MTcRF1	3.73 ± 0.06	2.83 ± 0.06
MCvRF3	5.97 ± 0.06	6.33 ± 0.06
MCvRF2	4.23 ± 0.06	3.30 ± 0.00
TCdRF1	4.30 ± 0.00	3.40 ± 0.17
TClRF1	4.03 ± 0.99	3.43 ± 0.06
TCvgRF1	4.47 ± 0.06	3.50 ± 0.10
TCvgRF2	4.37 ± 0.23	3.33 ± 0.11
TCvgRF3	4.33 ± 0.06	3.37 ± 0.06
TCvgRF4	4.47 ± 0.06	3.70 ± 0.00
TCvgRF5	4.60 ± 0.10	3.80 ± 0.00
Control	5.50 ± 0.00	5.53 ± 0.15

N=3, Initial pH of the PVK and ERP-PVK media = 7.00; *SD = standard deviation

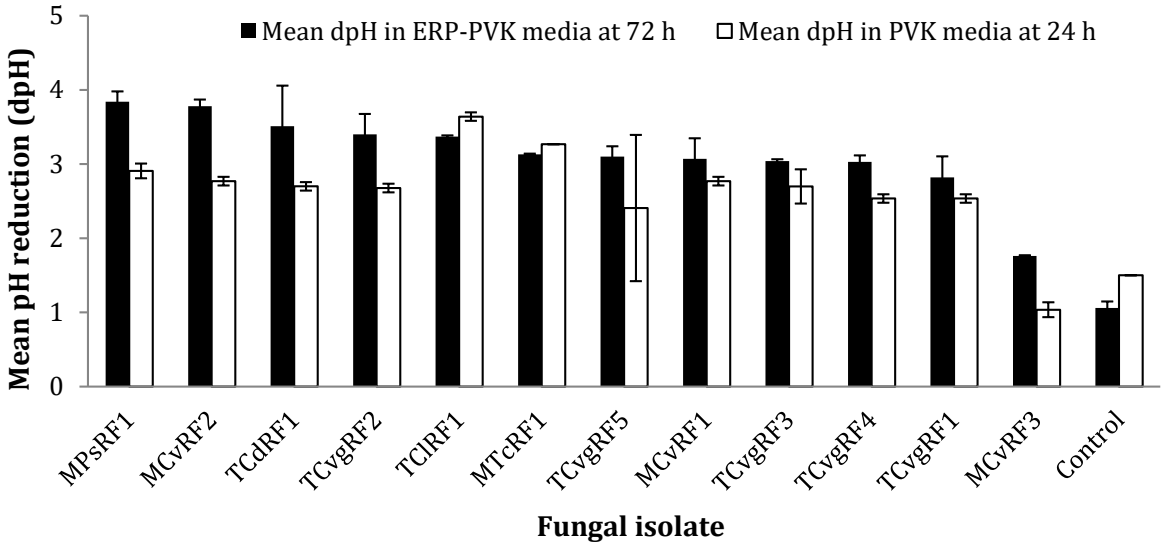

Figure 3. Mean pH reductions by fungal isolates in PVK medium at 24 h and in ERP-PVK medium at 72 h. vertical lines indicate the standard deviations

As per $Ca_3(PO_4)_2$ solubilization, a non-significant positive correlation r=0.411 (p=0.163) as illustrated in Figure 4, was observed between the amount of phosphate solubilized and pH reduction of the medium. This suggests that the medium acidification does play an important role, but it is not the only mechanism employed by the fungi under investigation for $Ca_3(PO_4)_2$ solubilization. In contrast, rock phosphate solubilization was associated with a sharp decline of pH in culture broths, which is further supported by a significant correlation as shown in Figure 5 between the two parameters with an r value of 0.614 (p=0.026). This indicates acidulation of the medium as a main mechanism of ERP solubilization, which implies that the same fungal species may adopt different strategies for Pi solubilization according to the external factors prevailing and or the source of Pi. The fact that medium acidification and solubilization of $Ca_3(PO_4)_2$ are not correlating with each other indicates that fungi may be using more than one mechanism for phosphate solubilization.

Many studies have shown that the production of soluble phosphates is not necessarily correlated with acidity suggesting that the nature of organic acids produced is more important than the total acidity (Ivanova *et al*., 2006; Sharma *et al*., 2013). In general, differences in the soluble phosphate content under different treatments could be due to quality and quantity of the acids secreted into the medium and the type of microorganism used (Mendes *et al*., 2014). In addition, fungi are known to employ proton extrusion mechanisms (Khan *et al*., 2009). Hence in this investigation, the amount of phosphates released into the culture supernatant was taken as the criterion for selecting the most efficient PSF due to the inconsistency of data obtained for the pH reduction in two media to support organic acid production as a major contributing factor for phosphate solubilization.

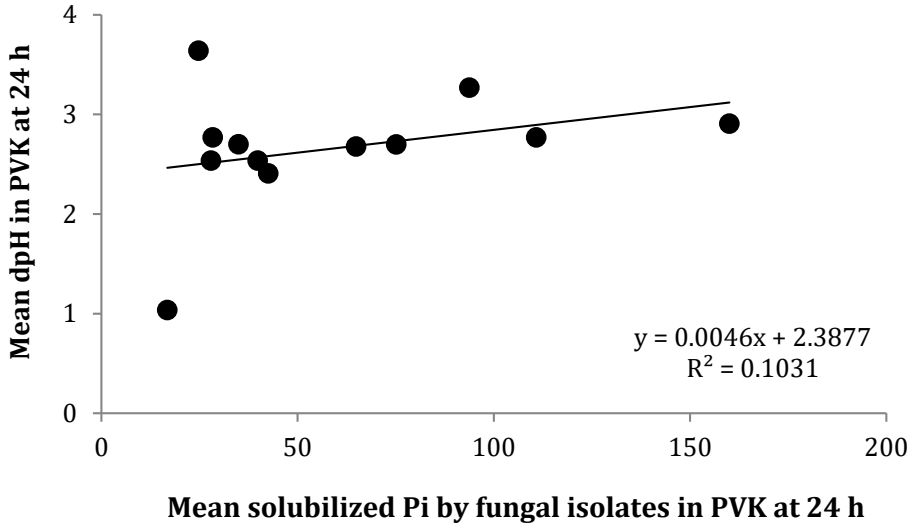

Figure 4. Scatter graph showing the relationship between mean pH reduction of culture medium and mean solubilized Pi by the fungal isolates grown in PVK medium, at 24 h

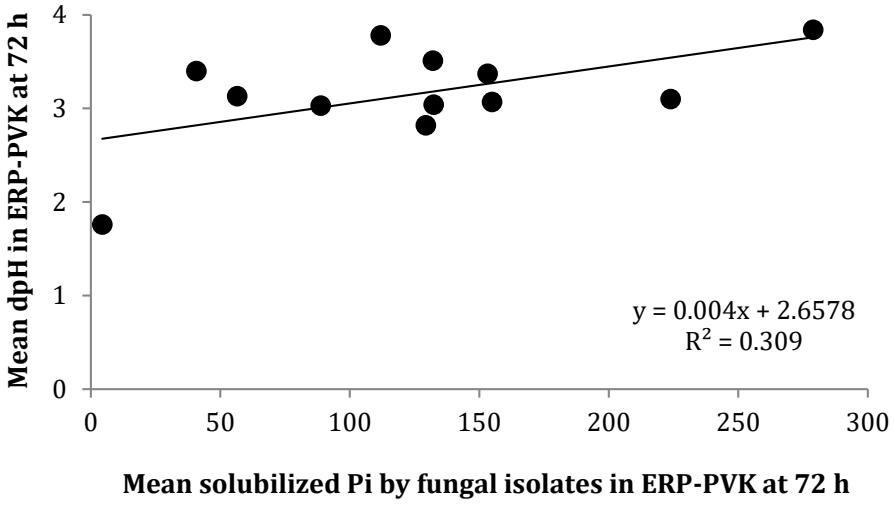

Figure 5. Scatter graph showing the relationship between mean pH reduction of culture medium and mean solubilized Pi by the fungal isolates grown in ERP-PVK medium, at 72 h

Identification of efficient isolates

Conventional identification based on micromorphological and reproductive characteristics indicated that the three efficient fungal isolates selected MPsRF1, MCvRF1 and TCvgRF5 are in the genera *Aspergillus*, *Trichoderma* and *Penicillium,* respectively. Based on the 18S rRNA sequencing data, the isolates MCvRF1 and TCvgRF5 were identified as *T. virens* and *P. oxalicum*, with 100% similarities. The sequence data are deposited in the GenBank under the accession numbers KP296798 and KP296799, respectively.

Detecting synergism among the fungal isolates

Among many research reporting the improvement of plant growth and productivity using PSF, emphasis was also placed on the use of dual application of fungal isolates (Ivanova *et al*., 2006). The test of the synergism for insoluble phosphate solubilization, the fungal combination *Aspergillus* sp. and *P. oxalicum* exhibited the highest phosphate solubilization levels of 893.43 (±56.77) mg P/L and 309.42 (±42.52) mg P/L in both PVK and PVK-ERP media, respectively, at 72 h incubation as illustrated in Figure 6. Hence, these two organisms in combination could be used in further experimentation for the development of a phosphatic biofertilizer for agricultural crops. Some *Penicillium* and *Aspergillus* species have also reported to be excellent rock phosphate solubilizers (Ivanova *et al*., 2006). Ivanova (2006) showed that pot experiments conducted using dual inoculation of *A. niger* and *P. italicum* significantly increased the dry matter and yield of soybean plants and the percentage increase in N and P contents of the plants.

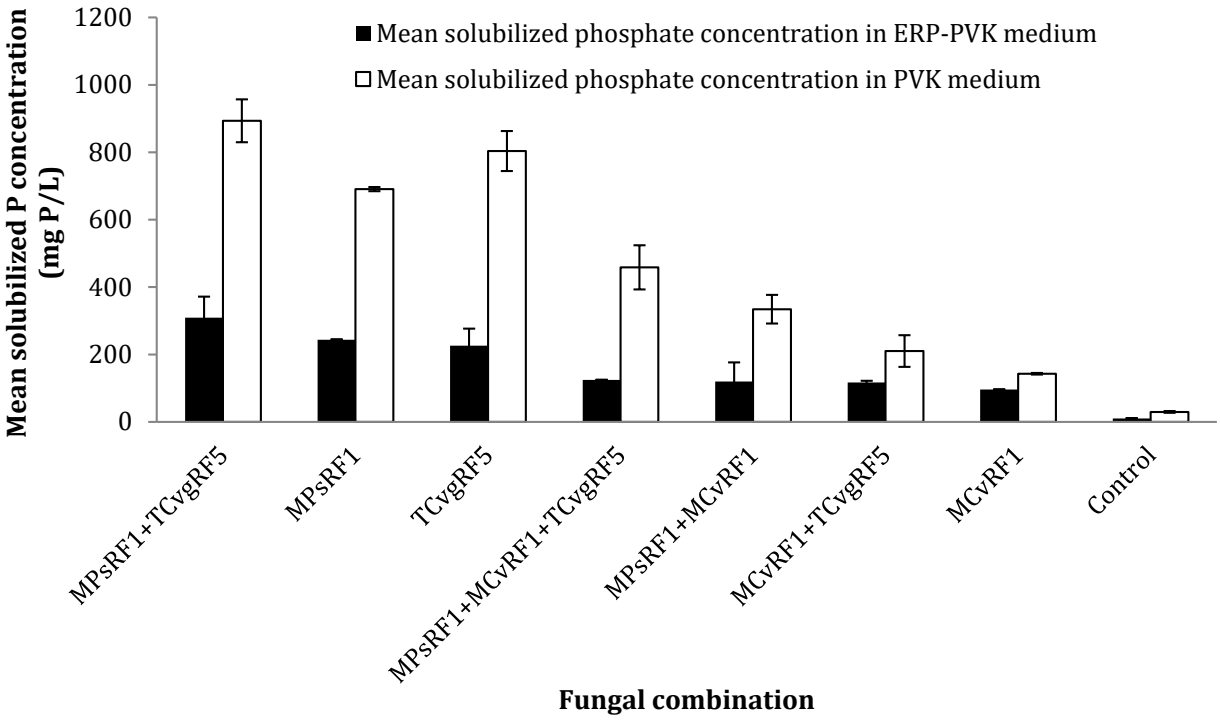

Figure 6. Mean solubilized phosphate concentrations of fungal combinations in PVK and ERP-PVK media at 72 h. Vertical lines indicate the standard deviations.

A significant positive correlation of r=0.739 (p=0.036) was observed between the solubilized phosphate concentrations and the pH reductions in ERP medium at 72 h for fungal combination as illustrated in Figure 7.

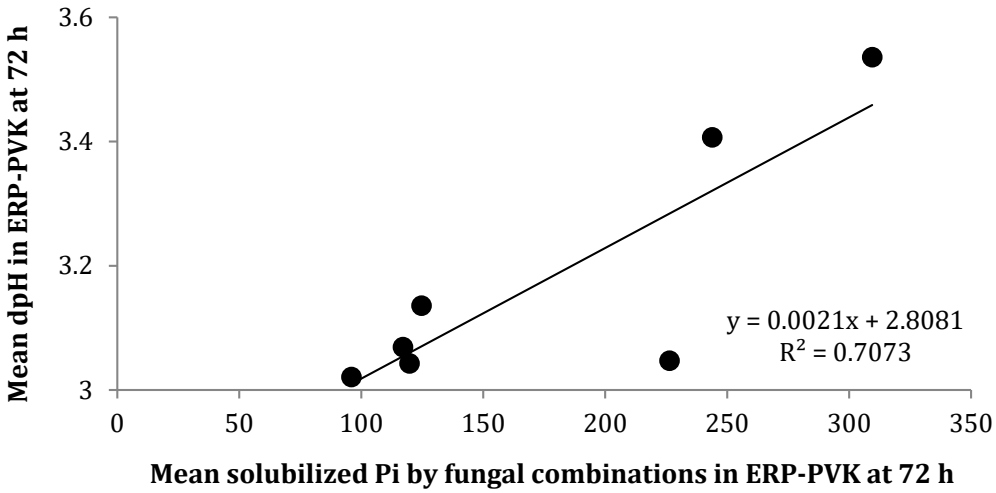

Figure 7. Scatter graph showing the relationship between mean pH reduction of culture medium and mean solubilized Pi at 72 h by fungal combinations

Conclusion

The present investigation isolated and screened *Aspergillus* sp, *Penicillium oxalicum* and *Trichoderma virens* as potential solubilizers of insoluble phosphates such as $Ca_3(PO_4)_2$ and ERP under *in vitro.* A synergism expressed towards Pi solubilization by *Aspergillus* sp. and *P. oxalicum* resulted in the highest solubilzation of tricalcium phosphate and ERP. Therefore, *Aspergillus* sp. and *P. oxalicum* are potential candidates to develop a biofertilizer to increase Pi availability in the plant rhizosphere. Furthermore, future research should concentrate on the survival, establishment and performance of introduced isolates inoculated into soil, which can be affected by the site specific environmental characters and the intense competition from native microorganisms limiting the effectiveness of the application.

Acknowledgement

The authors acknowledge the National Research Council, Sri Lanka for providing financial support (Grant No. 09-71), Drs D.N. Samaraweera and H.A. Sumanasena, Department of Export Agriculture (DEA), Sri Lanka for the cooperation extended in soil sample collection from the plantations of the DEA and for their valuable inputs, and Mrs. E.S. Karunaratne, Mrs. R.K. Maddumage and Mr. H.A.D. Indaka for the technical support.

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