

Phosphate Solubilization and Growth Promotion of Rubber Tree (*Hevea brasiliensis* Muell. Arg.) by *Trichoderma* Strains

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Abstract

The role of *Trichoderma* species as phosphate solubilizing microorganism was studied *in vitro*, by means of the Modified Pikovskaya's Broth medium (MPB) and *in vivo*, under greenhouse conditions using Rock Phosphate (RP) as a source of phosphorus (P). In broth medium, *Trichoderma* strain FR-NST-009 gave the highest P-solubilization (80.25%), followed by FR-NST-353 (77.51%), CB-Pin-01 (66.91%), RB-NST-028 (46.05%), and RB-NST-003 (21.20%) as compared with a control (broth medium non-inoculated with *Trichoderma* strain) after incubation at room temperature ($27 \pm 2^\circ\text{C}$) for 7 days. In addition, the treatments with *Trichoderma* species provided the final pH of broth culture lower than the control. Three kinds of organic acids including citric acid, lactic acid and succinic acid were detected by High Performance Liquid Chromatography (HPLC) in all treatments inoculated with *Trichoderma* strain. *Trichoderma* species produced citric acid in higher than lactic acid and succinic acid. Production of organic acids by *Trichoderma* species is one of the mechanisms for phosphate solubilization. In greenhouse conditions, at 60 days after planting, the treatments with *Trichoderma* strain FR-NST-009+RP provided the percentage of available phosphorus increasing more than the control 1 (with only RP) to 14.91%. However, after 60 days, the available phosphorus in the planting medium continually decreased in all treatments. At 180 days after planting, the treatments with FR-NST-009+RP increased plant height (22.19%), stem circumference (13.81%), leaf number (71.43%), total phosphorus in the rubber tree leaves (18.90%), shoot fresh weight (43.95%), root fresh weight (19.36%), shoot dry weight (39.96%), and root dry weight (21.13%), as compared with the control 1 (with only RP). Furthermore, the treatments with FR-NST-009+RP provided the population of *Trichoderma* species in the planting medium with 1.78×10^5 Colony-Forming Units (CFU) per gram planting medium and gave the root colonization percentages with 100.00%. The selected indigenous strain, FR-NST-009 was *Trichoderma harzianum* identified by using morphological characteristics.

Keywords: *Trichoderma* spp., phosphate solubilization, growth promotion, rubber tree

1. Introduction

Phosphorus (P) is one of macronutrients required for the growth and development of the plant. Generally, plants need phosphorus in 2,000 μg per 1 g dry weight or 0.2%. Phosphorus is the component of nucleic acids, phospholipids and adenosine triphosphate, ATP (Schachtman et al., 1998). Plants absorb phosphorus in inorganic form, dihydrogen phosphate ion (H_2PO_4^-) (and hydrogen phosphate ion (HPO_4^{2-})). The concentration of both H_2PO_4^- and HPO_4^{2-} depends on the pH of soil. However, phosphate ion was also absorbed by soil particle or fixed by the other element such as calcium (Ca), magnesium (Mg), aluminum (Al), and iron (Fe). Thus, insoluble phosphate forms, $\text{Ca}_3(\text{PO}_4)_2$, AlPO_4 , and FePO_4 , are not available to the plant (Altomare et al., 1999).

Trichoderma species are antagonistic fungi that have high efficacy to biological control of numerous plant diseases both in Thailand and in other countries, such as stem rot of tomato (Inwang & Chamswarn, 1986), damping-off of cotton, Chinese kale, cucumber, yard long bean, okra, soybean and tomato (Chamswarn &

Inwang, 1987; Chamswarnng & Intanoo, 2002; Intana, 2003; Sangsridum, 2003; Promwee, 2008), *Sclerotium* blight of barley (Chamswarnng et al., 1990), root rot of tangerine (Kitjaideaw et al., 2000), root rot of durian (Chamswarnng et al., 1997), anthracnose of mango (Yenjit et al., 2004), root rot of lettuce (Lamool, 2006), flower rusty spot of *Dendrobium* orchid (Thienkhao, 2007), peanut brown root rot (Rojo et al., 2007), chickpea wilt (Dubey et al., 2007), sheath rot of rice (Sattasakulchai, 2009), avocado white root rot (Rosa & Herrera, 2009), and ber fruit rot (Nallathambi et al., 2009).

Trichoderma species are not only having the ability in biological control of plant diseases, but also have high efficiency to promote plant growth and increase yield in several plants such as cucumber (Intana, 2003), chili (Inbar et al., 1994), marigold (Ousley et al., 1994), cabbage (Rabeendran et al., 2000), papaya (Morales-Payan & Stall, 2004), vegetable amaranth (Chakhatrakan et al., 2006), and Chinese kale (Promwee, 2008). Phosphate solubilization of *Trichoderma* species is one of the mechanisms of these fungi as the plant growth promoting fungi. Moreover, *Trichoderma* species can produce phosphatases and several organic acids; both phosphatases and organic acids were found to solubilize insoluble phosphate. However, the ability of *Trichoderma* species depends on the kind and strain of *Trichoderma* and source of phosphate (Akintokun et al., 2007; Kapri & Tewari, 2010; Promwee, 2011). In this sense, Thailand imported 5,583,276 tons of chemical fertilizer as valued 83,947 million Baht in 2012 (Office of Agricultural Economics, 2014). Consequently, application of high phosphate solubilizing fungi, *Trichoderma* species, in an organic farming system is an alternative method to reduce using chemical fertilizer for agricultural development in a sustainable agriculture system of Thailand.

Nevertheless, there are only a few reports about using *Trichoderma* species to solubilize insoluble phosphate both in Thailand and in other countries. Furthermore, the environments of southern part of Thailand are different from other parts; for instance, there are the high humidity and high level of rain, as a result, *Trichoderma* species habiting in soil of southern part may be different in the biodiversity. Hence, centring on southern Thailand, the objectives of this study were 1) to study the efficiency of indigenous *Trichoderma* species to solubilize insoluble phosphate both *in vitro* and under greenhouse conditions 2) to determine the organic acids produced by *Trichoderma* species and 3) to evaluate the efficacy of *Trichoderma* species for growth promotion of rubber tree under greenhouse conditions.

2. Materials and Methods

2.1 Strains of *Trichoderma* Species

Five strains of *Trichoderma* species used in this study include two indigenous strains (RB-NST-003, RB-NST-028) isolated from rubber planting soil samples in Nakhon Si Thammarat (Thailand), two indigenous strains (FR-NST-009, FR-NST-353) isolated from forest soil samples in the same study (Sattasakulchai, 2009), and *Trichoderma harzianum* strain CB-Pin-01, the commercial strain of Thailand.

The *Trichoderma* species were isolated from collecting rubber tree soil samples by soil dilution spread plate technique using Martin's medium (Martin, 1950), *i.e.*, KH_2PO_4 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, peptone 5.0 g, dextrose 10.0 g, rose bengal 0.033 g, and agar 15.0 g, dissolved in 1,000 ml distilled water and supplemented with 100 mg/L streptomycin. Ten grams of each soil sample were added into a 250 ml flask containing 90 ml of sterile water and mixed by a shaker (GFL 3020, Germany) at 120 RPM for 30 min. Then, the soil suspension was diluted at 10^{-2} and 10^{-3} fold before 0.1 ml of dilution was dropped on the surface of Martin's medium in a Petri dish. Soil suspension was spread on the surface of the medium with sterile glass rod before the dishes were sealed with paraffin film and incubated at room temperature ($27 \pm 2^\circ\text{C}$) for 3-7 days.

The *Trichoderma* species growing on agar was selected and sub-cultured on Potato Dextrose Agar (PDA), *i.e.*, potato 200.0 g, dextrose 20.0 g, agar 15.0 g, and distilled water 1,000 ml (Intana, 2003; Promwee, 2008). A pure culture of *Trichoderma* species was identified based on its morphological and reproductive characteristics (Samuels et al., 2014).

2.2 Testing of Phosphate Solubilization *in vitro*

2.2.1 Qualitative Estimation

All strains of *Trichoderma* species were tested for their ability to solubilize inorganic phosphate on Modified Pikovskaya's Agar (MPA), *i.e.*, rock phosphate 2.5 g, glucose 13 g, $(\text{NH}_4)\text{SO}_4$ 0.5 g, NaCl 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, KCl 0.2 g, yeast extract 0.5 g, MnSO_4 0.0002 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0002 g, agar 15 g, pH adjusted to 7.2, and dissolved in 1,000 ml distilled water (Pikovskaya, 1948).

One mycelial disc (0.7 cm in diameter) of *Trichoderma* strain was put on the center of agar plate and incubated at room temperature for 3 days. Phosphate Solubilization Index (PSI) was measured and calculated by a formula as follows (Alam et al., 2002; Afzal & Bano, 2008):

$PSI = [\text{colony diameter} + \text{halozone diameter}] / \text{colony diameter}$

2.2.2 Quantitative Estimation

All strains of *Trichoderma* species were tested for their ability to solubilize inorganic phosphate in Modified Pikovskaya's Broth (MPB), i.e., rock phosphate 2.5 g, glucose 13 g, $(\text{NH}_4)_2\text{SO}_4$ 0.5 g, NaCl 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, KCl 0.2 g, yeast extract 0.5 g, MnSO_4 0.0002 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0002 g, pH adjusted to 7.2, and dissolved in 1,000 ml distilled water] (Pikovskaya, 1948).

Five mycelial discs (0.7 cm in diameter) of *Trichoderma* strain were inoculated into 250 ml Erlenmeyer flask containing 100 ml of broth medium and incubated at room temperature in a shaker (GFL 3020) at 120 RPM for 7 days. Then, spores and mycelia of *Trichoderma* strains were removed from broth culture by filtration through 0.45 μm Whatman No.1 and centrifuged by centrifuge (Z 200 A, HERMLE Labortechnik GmbH) at 5,000 RPM for 10 min.

The supernatant of each culture was analyzed for pH by using a pH meter (CyberScan pH 510, Singapore). Phosphate concentration in the supernatant was estimated by spectrophotometric method (Fiske & Subbarow, 1925; Saravanakumar et al., 2013). An aliquot of 750 μl culture supernatant was mixed with 750 μl of color reagent containing ammonium molybdate $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ 1.5% (w/v), sulfuric acid (H_2SO_4) solution 5.5% (v/v) and ferrous sulfate (FeSO_4) solution 2.7% (w/v) and then measured by a spectrophotometer (U-1800, Hitachi, Japan) at 600 nm. The level of phosphate concentration was determined by using a standard graph of potassium dihydrogen phosphate (KH_2PO_4) and expressed as equivalent phosphate in mg-P/L.

2.3 Studying of Organic Acids Produced by *Trichoderma* Species

2.3.1 Qualitative Assessment

All strains of *Trichoderma* species were tested for acid production on modified Pikovskaya's agar supplemented with bromocresol purple (100.0 mg/L). One mycelial disc (0.7 cm in diameter) of *Trichoderma* strain was put on the center of agar plate and incubated at room temperature for 7 days. The phosphate solubilization activity of acid production was observed that *Trichoderma* strain turned the agar plate from purple to yellow in zones of acidification (Vazquez et al., 2000; Hoyos-Carvajal et al., 2009).

2.3.2 Quantitative Assessment

Eleven of organic acids such as acetic acid, citric acid, fumaric acid, gluconic acid, glutaric acid, lactic acid, maleic acid, malic acid, oxalic acid, succinic acid and tartaric acid were analyzed by high performance liquid chromatography (HPLC) (Waters alliance 2690, Waters) and compared with the standard (AOAC, 2000). Supernatant of samples in broth medium was passed through 0.45 μm syringe filters and injected with 20 μl injection loop into the column. These were determined by Metacarb H plus column (Varian), mobile phase 0.001 N sulfuric acid (H_2SO_4) at the flow rate of 0.4 ml/min. The column was set at 50 °C temperature. Each of organic acids produced by *Trichoderma* species was expressed in mg/L.

2.4 Testing of Phosphate Solubilization in vivo (Under Greenhouse Conditions)

Two strains of *Trichoderma* species (FR-NST-009 and CB-Pin-01) were tested for phosphate solubilization under greenhouse condition. Fresh culture of *Trichoderma* strain was prepared according to the method of Chamswarnng and Intanoo (2002) and applied at 100 g/pot into the planting medium, i.e., 10 kg of soil (pH = 5.05, Total N = 0.17%, Available P = 10.72 mg/kg, and Exchangeable K = 62.88 mg/kg) collected from rubber planted area in Nakhon Si Thammarat (Thailand), mixed with 2 kg of cow manure (Total N = 1.10%, Total P = 0.48%, Total K = 1.68%), and supplemented with 200 g/pot of Rock Phosphate (RP). The 6-month-old rubber tree clone RRIM 600 was then planted in 15 inch diameter plastic pot.

The treatments were FR-NST-009+RP, CB-Pin-01+RP, control 1 (with only RP) and control 2 (without RP). Completely Randomized Design (CRD) with 4 replications per treatment and 3 plants per replication was used in this study. The parameters were estimated on this item as follows.

2.4.1 Growth and Development Parameters

At 180 days after planting, plant height of rubber tree was measured from the planting medium surface of the uppermost leaf sheath with a meter stick and expressed in cm, stem circumference of rubber tree was estimated with tape measure and shown in cm, and leaf number of rubber tree was counted and shown in the leaves per plant. For shoot and root fresh weights, these were harvested, weighed with an electronic analytical balance (Sartorius CP32032S, Germany), and shown in gram per plant. For shoot and root dry weights, shoot and root of rubber tree were air dried in hot air oven (WTE binder, Germany) at 60 °C for 7 days. Then, dried shoot and root of rubber tree were weighed with an electronic analytical balance and shown in gram per plant.

2.4.2 Population and Root Colonization of *Trichoderma* Species

The population of *Trichoderma* species in the planting medium was studied by a dilution plate technique using Martin's medium at 0, 6, 120 and 180 days after planting according to the method of Intana (2003) and Promwee (2008). For root colonization of *Trichoderma* species, at 180 days after planting, root colonization of *Trichoderma* species was studied using Martin's medium according to the method of Intana (2003); Promwee (2008).

2.4.3 Chemical Analysis

Available phosphorus in the planting medium was extracted using 0.03 M NH_4F in 0.10 M HCl (Bray II solution), and its concentration was analyzed using the molybdenum blue method (AOAC, 2000), expressed in mg/kg. The pH of the planting medium was determined using soil: H_2O at 1:2.5 w/v by pH meter (CyberScan pH 510, Singapore) at 0, 6, 120 and 180 days after planting. Finally, total phosphorus in rubber tree leaves was determined by the vanadomolybdate method (AOAC, 2000) and expressed in mg/kg at 180 days after planting.

2.5 Identification of *Trichoderma* Strain to Species Levels

The high efficacy strain of *Trichoderma* obtained from the *in vitro* and *in vivo* tests was identified to species level by using morphological characteristics under compound microscope (Nikon YS100, Japan) and scanning electron microscope, SEM (JEOL, JSM5600LV, England) according to interactive key to species of Samuels et al. (2014) and compared with previous reports (Intana, 2003; Promwee, 2008; Sattasakulchai, 2009).

2.6 Statistical Analysis

All data were subjected to analysis of variance (ANOVA) followed by a comparison using Duncan's Multiple Range Test at $P < 0.05$.

3. Results and Discussion

3.1 Testing of Phosphate Solubilization *in vitro*

For the qualitative estimation of phosphate solubilization, all strains of *Trichoderma* species did not show any clear zone on Modified Pikovskaya's Agar (MPA) after incubation at room temperature for 0-7 days (Figure 1-a). Similarly, the study of Rawat and Tewari (2011) reported that *Trichoderma* species revealed good mycelial growth, but no halo-zone formation on the solid medium containing insoluble inorganic phosphorus source. In addition, Nautiyal (1999) reported that the criterion for isolation of phosphate solubilizers based on the formation of a visible halo-zone on Pikovskaya's agar is not a reliable technique because many isolates of Phosphate Solubilizing Microorganisms (PSM), which did not show any clear zone on agar plates, could be able to solubilize insoluble inorganic phosphates in liquid medium.

For the quantitative estimation of phosphate solubilization, all strains of *Trichoderma* species significantly provided P-solubilization in Modified Pikovskaya's Broth medium (MPB) as compared with a control (broth medium non-inoculated with *Trichoderma* strain) after incubation at room temperature for 7 days. Especially, *Trichoderma* strain FR-NST-009 gave the highest P-solubilization (80.25%), followed by FR-NST-353 (77.51%), CB-Pin-01 (66.91%), RB-NST-028 (46.05%), and RB-NST-003 (21.20%), as compared with a control (Table 1). This study indicated that *Trichoderma* species have the ability to solubilize insoluble phosphate into soluble phosphate. Phosphate solubilization by *Trichoderma* species have been reported in a few researches (Akintokun et al., 2007; Kapri & Tewari, 2010; Saravanakumar et al., 2013).

Table 1. Final pH, phosphorus concentration and P-solubilization percentage of *Trichoderma* species in the Modified Pikovskaya's Broth medium (MPB) supplemented with Rock Phosphate (RP) after incubation at room temperature for 7 days

Treatments	Final pH	Phosphorus (mg-P/L)	P-solubilization (%)
RB-NST-003	5.98 c ^{1/}	6.25 c ^{1/}	21.20 d ^{1/}
RB-NST-028	6.42 b	7.54 b	46.05 c
FR-NST-009	4.68 f	9.31 a	80.25 a
FR-NST-353	5.12 d	9.17 a	77.51 a
CB-Pin-01	5.00 e	8.62 a	66.91 b
Control	7.26 a	5.15 d	0.00 e

^{1/}Values in the same column followed by the same alphabet are not significantly different from each other as analyzed by Duncan's Multiple Range Test at $P < 0.05$.

In addition, the treatments with *Trichoderma* species provided the final pH of broth culture lower than the control. The treatment with *Trichoderma* strain FR-NST-009 gave the lowest final pH (4.68), followed by CB-Pin-01 (5.00), FR-NST-353 (5.12), RB-NST-003 (5.98), and RB-NST-028 (6.42), while the final pH in control treatment was 7.26 (Table 1). The pH drop in PSM broth cultures has been reported in several researches which supports the pH change in the present study (Vazquez et al., 2000; Alam et al., 2002; Rashid et al., 2004; Pradham & Sukla, 2005; Akintokun et al., 2007; Kapri & Tewari, 2010; Yadav et al., 2011; Saravanakumar et al., 2013).

3.2 Studying of Organic Acids Produced by *Trichoderma* Species

For the qualitative estimation of organic acids produced by *Trichoderma* species, all strains of *Trichoderma* species could turn the Modified Pikovskaya's Agar (MPA) supplemented with bromocresol purple from purple to yellow in zones of acidification after incubation at room temperature for 7 days. Especially, *Trichoderma* species strains FR-NST-009, FR-NST-353, and CB-Pin-01 provided the yellow zones more than *Trichoderma* species strains RB-NST-003 and RB-NST-028 (Figure 1-b). This study supported by the experiment of Hoyos-Carvajal et al. (2009), *Trichoderma* species including *T. harzianum*, *T. asperellum*, *T. virens*, *T. viridescens*, *T. longibrachiatum* showed phosphate solubilization activity by acid production on agar media used tri-calcium phosphate as a phosphate source, with bromocresol purple (100.0 mg/L).

For the quantitative estimation of organic acids produced by *Trichoderma* species in broth medium, 3 kinds of organic acids including citric acid, lactic acid, and succinic acid were detected in all treatments inoculated with *Trichoderma* strain. However, the quantity of organic acids was significantly different when separated by the kind of organic acid and the strain of *Trichoderma*. Thus, *Trichoderma* species produced citric acid in higher than lactic acid and succinic acid. For citric acid, *Trichoderma* strain FR-NST-009 gave the highest value (612.23 mg/L), followed by CB-Pin-01 (514.01 mg/L), FR-NST-353 (264.24 mg/L), RB-NST-003 (216.61 mg/L), and RB-NST-028 (191.53 mg/L). *Trichoderma* strain FR-NST-009 also gave the highest lactic acid (73.47 mg/L), followed by RB-NST-028 (52.69 mg/L), RB-NST-003 (51.46 mg/L), FR-NST-353 (49.80 mg/L), and CB-Pin-01 (42.20 mg/L). Finally, *Trichoderma* strain RB-NST-003 gave the highest succinic acid (16.37 mg/L), followed by CB-Pin-01 (16.31 mg/L), FR-NST-009 (14.54 mg/L), RB-NST-028 (13.67 mg/L), and FR-NST-353 (13.58 mg/L) (Table 2, Figure 2).

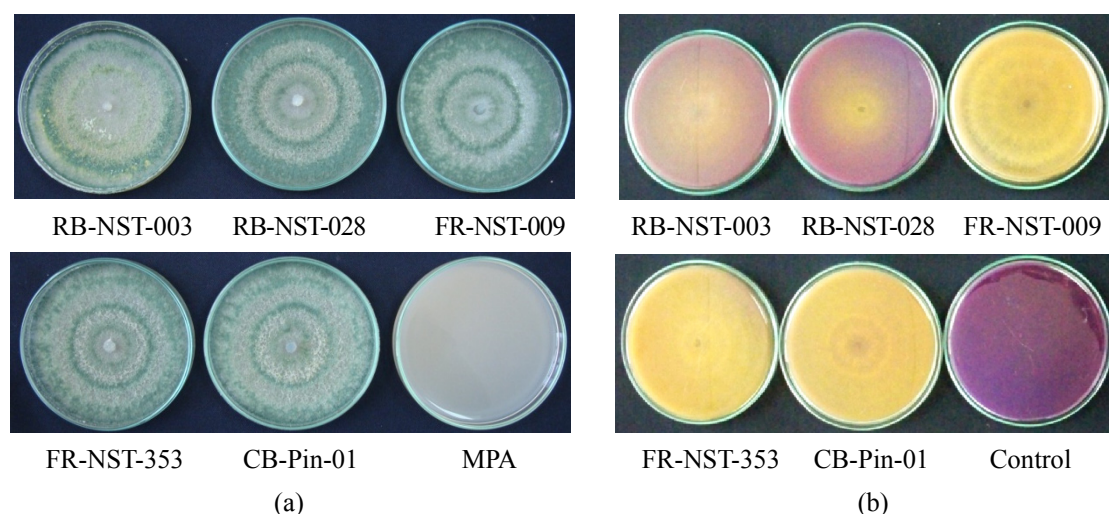


Figure 1. Growth of *Trichoderma* species on Modified Pikovskaya's Agar (MPA) (a), phosphate solubilization activity by acid production of *Trichoderma* species on Modified Pikovskaya's Agar supplemented with bromocresol purple (b) after incubation at room temperature for 7 days

The previous results were supported with many reports that phosphate solubilizing microorganisms could produce several of organic acid for phosphate solubilization. For example, *Aspergillus flavus*, *A. niger*, and *Penicillium canescens* produced oxalic, citric, gluconic, and succinic acid (Rashid et al., 2004); *P. rugulosum*, *P. radicum*, and *P. variable* produced gluconic acid (Reyes et al., 1999; Whitelaw et al., 1999; Vassilev et al., 1996); *A. awamori*, *A. foetidus*, *A. terricola*, *A. amstelodemi*, *A. tamari*, *Bacillus polymyxa*, and *B. licheniformis* produced oxalic and citric acid (Gupta et al., 1994). For organic acids produced by *Trichoderma* species, only the report of Akintokun et al. (2007) showed that *T. isridae* can produce acetic acid, citric acid, fumaric acid, gluconic acid, glutaric acid, lactic acid, maleic acid, malic acid, succinic acid, and tartaric acid. However, Alam et al. (2002) and Rashid et al. (2004) reported that oxalic and citric acids were two major acids produced by PSM.

In addition, the treatments with *Trichoderma* species significantly provided the final glucose concentration in broth culture lower than the control. The treatment with *Trichoderma* strain FR-NST-009 gave the lowest final glucose concentration (3,722.30 mg/L), followed by FR-NST-353 (3,894.43 mg/L), CB-Pin-01 (4,050.36 mg/L), RB-NST-003 (4,204.15 mg/L), and RB-NST-028 (5,529.28 mg/L); the glucose concentration in control treatment was 12,131.59 mg/L (Table 2, Figure 2). This result indicated that *Trichoderma* species used glucose as a carbon source for organic acid production; the glucose concentration decreasing correlated with organic acids increasing produced by *Trichoderma* strain. In this sense, Rashid et al. (2004) showed that PSM used glucose as a carbon source to solubilize insoluble phosphate by secreting organic acid. Furthermore, Nautiyal (1999) and Pradham and Sukla (2005) reported that glucose was the best carbon source of PSM for phosphate solubilization as compared with arabinose, fructose, galactose, sorbitol, mannitol, xylose, sucrose, maltose, lactose, and raffinose.

Table 2. Organic acids produced by *Trichoderma* species and glucose concentration in modified Pikovskaya's Broth (MPB) supplemented with Rock Phosphate (RP) after incubation at room temperature for 7 days

Treatments	Citric acid (mg/L)	Lactic acid (mg/L)	Succinic acid (mg/L)	Glucose (mg/L)
RB-NST-003	216.61 d ^{1/}	51.46 bc ^{1/}	16.37 a ^{1/}	4,204.15 c ^{1/}
RB-NST-028	191.53 e	52.69 b	13.67 b	5,529.28 b
FR-NST-009	612.23 a	73.47 a	14.54 b	3,722.30 f
FR-NST-353	264.24 c	49.80 c	13.58 b	3,894.43 e
CB-Pin-01	514.01 b	42.20 d	16.31 a	4,050.36 d
Control	0.00 f	0.00 e	0.00 c	12,131.59 a

^{1/}Values in the same column followed by the same alphabet are not significantly different from each other as analyzed by Duncan's Multiple Range Test at P < 0.05.

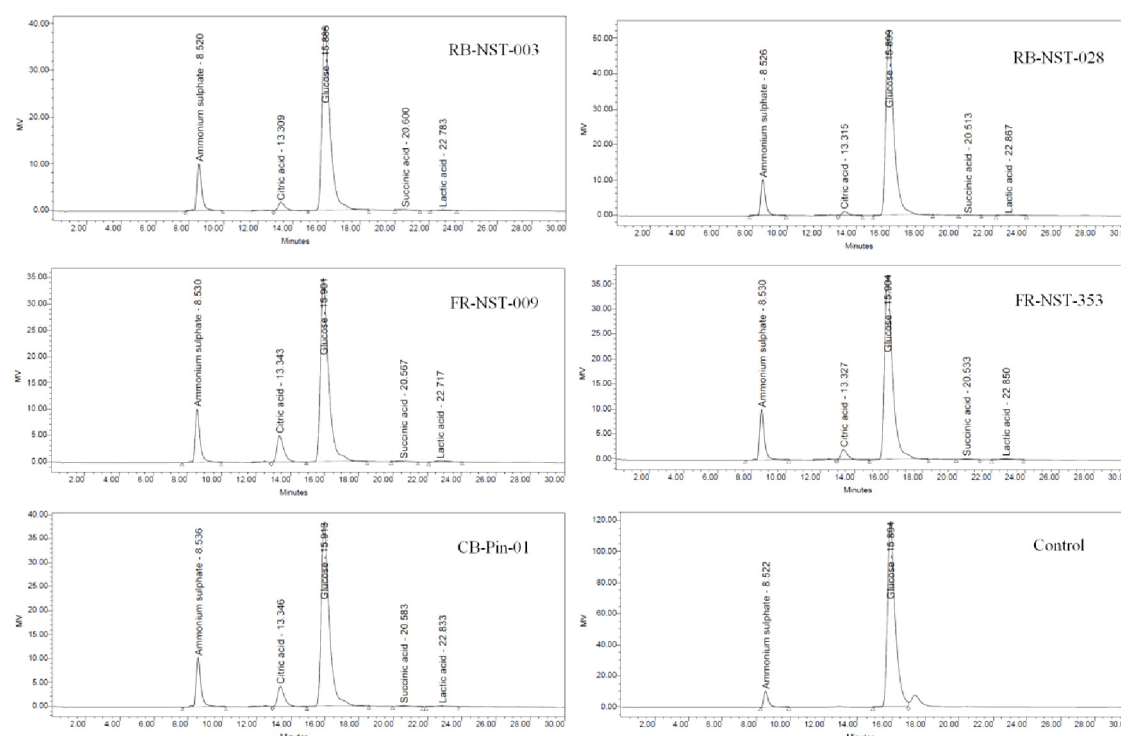


Figure 2. Chromatogram of organic acids produced by *Trichoderma* species and glucose utilization in the Modified Pikovskaya's Broth (MPB) supplemented with Rock Phosphate (RP) after incubation at room temperature for 7 days as compared with a control

Although the mechanisms of phosphate solubilization by microorganisms are still not fully understood, several mechanisms have been implicated in the operation. The organic acid production is an important mechanism in phosphate solubilization, but not the sole mechanism (Nautiyal et al., 1999; Alam et al., 2002). Production of organic acids results in acidification of microbial cell and its surroundings, and consequently, inorganic phosphate (PI) may be released from the insoluble phosphate forms, $\text{Ca}_3(\text{PO}_4)_2$ by the proton substitution for Ca^{2+} (Rodríguez & Fraga, 1999). Other mechanisms include lowering of pH as a result of acid production, and ion chelation and exchange reaction (Akintokun et al., 2007). Additionally, phosphate solubilization of *Trichoderma* species may be occurred from phosphatase and phytase enzyme activity (Kapri & Tewari, 2010; Saravanakumar et al., 2013).

3.3 Testing of Phosphate Solubilization in vivo (Under Greenhouse Conditions)

The efficacy of *Trichoderma* species and Rock Phosphate (RP) on the growth of rubber tree was conducted under greenhouse conditions between July, 2013-December, 2013. The plant height, stem circumference, and leaf number of 6-month-old rubber tree clone RRIM 600 used in the experiment in all treatments were not significantly different on the initial day of testing. The means of plant height, stem circumference, and leaf number were 60.56 cm, 3.84 cm, and 32 leaves per plant (data not shown), respectively.

At 180 days after planting, the treatments with *Trichoderma* species and Rock Phosphate (RP) showed significant differences in growth and development parameter, population and root colonization of *Trichoderma* species, and chemical analysis as compared with control 1 (with only RP) and control 2 (without RP). The treatments with FR-NST-009+RP increased plant height (22.19%), stem circumference (13.81%), leaf number (71.43%), as compared with the control 1. Moreover, the treatments with FR-NST-009+RP provided the total phosphorus in the rubber tree leaves more than the control 1, with 18.90%, while the treatments with CB-Pin-01+RP provided the total phosphorus in the rubber tree leaves more than the control 1, with 2.68% (Table 3). In addition, the treatments with FR-NST-009+RP increased shoot fresh weight (43.95%), root fresh weight (19.36%), shoot dry weight (39.96%), and root dry weight (21.13%), as compared with the control 1 (Table 4).

Table 3. Plant height, stem circumference, leaf number and total phosphorus in the leaves of the rubber tree after planting for 180 days in the testing of phosphate solubilization under greenhouse conditions

Treatments	Plant height		Stem circumference		Leaf number		Total P in Leaves	
	(cm)	(%)	(cm)	(%)	leaf/plant	(%)	mg/kg()(%)
FR-NST-009+RP	113.50 a ^{1/}	24.38	6.58 a ^{1/}	21.18	129 a ^{1/}	84.29	2,886.25 a ^{1/}	30.47
CB-Pin-01+RP	102.50 b	12.33	6.40 a	17.86	103 ab	47.14	2,527.62 b	14.25
Control (1+RP(93.25 c	2.19	5.83 b	7.37	79 bc	12.86	2,468.26 bc	11.57
Control 2-RP(91.25 c	0.00	5.43 b	0.00	70 c	0.00	2,212.27 c	0.00

^{1/} Values in the same column followed by the same alphabet are not significantly different from each other as analyzed by Duncan's Multiple Range Test at $P < 0.05$.

Table 4. Shoot and root fresh weights, shoot and root dry weights of rubber tree after planting for 180 days in the testing of phosphate solubilization under greenhouse conditions

Treatments	Shoot fresh weight		Shoot dry weight		Root fresh weight		Root dry weight	
	(g/plant)	(%)	(g/plant)	(%)	(g/plant)	(%)	(g/plant)	(%)
FR-NST-009+RP	208.40 a ^{1/}	54.52	59.54 a ^{1/}	54.29	140.52 a ^{1/}	22.61	45.99 a ^{1/}	23.36
CB-Pin-01+RP	175.15 ab	29.87	53.48 ab	38.59	135.16 ab	17.93	44.05 a	18.16
Control (1+RP(149.13 b	10.57	44.12 bc	14.33	118.34 b	3.25	38.11 b	2.23
Control 2-RP(134.87 b	0.00	38.59 c	0.00	114.61 b	0.00	37.28 b	0.00

^{1/} Values in the same column followed by the same alphabet are not significantly different from each other as analyzed by Duncan's Multiple Range Test at $P < 0.05$.

On the one hand, the treatments with *Trichoderma* strain FR-NST-009 provided the population of *Trichoderma* species in the planting medium with 2.73×10^7 , 2.15×10^6 , 5.00×10^5 and 1.78×10^5 Colony-Forming Units (CFU) per gram planting medium at 0, 60, 120 and 180 days after planting, respectively. For the other hand, the treatments with *T. harzianum* strain CB-Pin-01 provided the population of *Trichoderma* species in the planting medium with 2.55×10^7 , 2.13×10^6 , 4.75×10^5 and 1.63×10^5 CFU/g planting medium at 0, 60, 120 and 180 days after planting, respectively. However, the both treatments with *Trichoderma* strain FR-NST-009 and *T. harzianum* CB-Pin-01 provided the root colonization percentages with 100.00% (Table 5).

Table 5. Root colonization percentages at 180 days after planting, the population of *Trichoderma* species in the planting medium at 0-180 days after planting in the testing of phosphate solubilization under greenhouse conditions

Treatments	Root colonization (%)	Population of <i>Trichoderma</i> spp. (CFU/g planting medium)			
		0 day	60 days	120 days	180 days
FR-NST-009+RP	100.00 a ^{1/}	2.73×10^7 a ^{1/}	2.15×10^6 a	5.00×10^5 a	1.78×10^5 a
CB-Pin-01+RP	100.00 a	2.55×10^7 a	2.13×10^6 a	4.75×10^5 a	1.63×10^5 a
Control (1+RP(0.00 b	0.0 b	0.0 b	0.0 b	0.0 b
Control 2-RP(0.00 b	0.0 b	0.0 b	0.0 b	0.0 b

^{1/} Values in the same column followed by the same alphabet are not significantly different from each other as analyzed by Duncan's Multiple Range Test at $P < 0.05$.

At 60 days after planting, the treatments with *Trichoderma* strain FR-NST-009 provided the percentage of available phosphorus increasing more than the control 1 at 14.91%, while the treatments with *T. harzianum* strain CB-Pin-01 provided the percentage of available phosphorus increasing more than the control 1 at 11.08%. However, after 60 days, the available phosphorus in the planting medium continually decreased in all treatments

(Figure 3-a). The pH of the planting medium in all treatments continually decreased from the initial day of testing to 180 d after planting. The treatments with *Trichoderma* species and Rock Phosphate (RP) and control 1 gave the pH in planting medium higher than control 2 (Figure 3b).

These results indicated that *Trichoderma* species had ability to promote growth of rubber tree through phosphate solubilization. Growth promotion of *Trichoderma* species has been reported by many researchers. Thus, Yedidia et al. (2001) reported that *T. harzianum* strain T-203 increased root area (95.00%), cumulative root length (75.00%), dry weight (80.00%), shoot length (45.00%), and leaf area (80.00%) of cucumber after sowing for 28 days in soil amended with *T. harzianum*. Additionally, Yadav et al. (2011) showed that *T. harzianum* significantly enhanced the shoot length (61.56%), root length (69.95%), shoot dry weight (38.71%), and root dry weight (57.58%) of chickpea as compared with the control. Moreover, Saravanakumar et al. (2013) reported that *Trichoderma* strain TSK8 enhanced total mangrove seedling biomass significantly by 48.00%, 19.00%, and 11.00% when supplemented with soluble super phosphate (KH_2PO_4), without any phosphate and insoluble tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$), respectively.

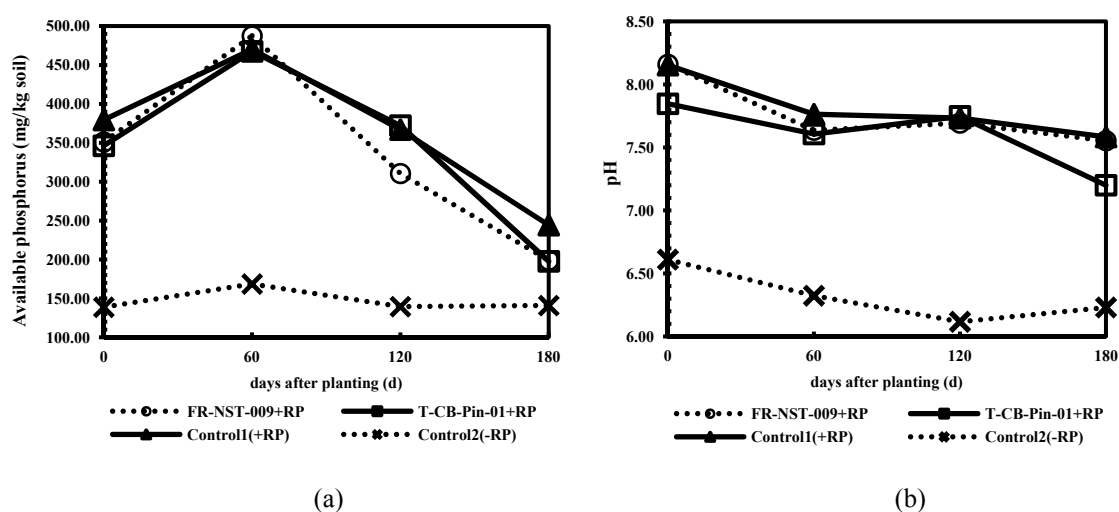


Figure 3. Available phosphorus (a), the pH of the planting medium (b) in the testing of phosphate solubilization under greenhouse conditions

Phosphate solubilization is one of the mechanisms of *Trichoderma* species for plant growth promotion, although other mechanisms of *Trichoderma* species to increase growth and yield of plants have been reported. For example, Windham et al. (1986) reported that *Trichoderma* species produced growth regulating factors, which enhanced seed germination and plant growth. Baker (1988) reported that such increases in plant growth and development may result from control of minor pathogens or increased nutrient uptake through enhanced root growth and promoted the availability of necessary nutrients. Also, Harman (2000) reported that *T. harzianum* strain T-22 was as effective as a commercial rooting hormone in inducing roots of plant, such as tomato and potato. Intana (2003) reported that inoculation of cucumber seedling with certain mutant strains derived through UV irradiation and wild type isolates of *T. harzianum* or treatment of cucumber seeds only with their purified metabolites (pentyl pyrone, harzianic acid or harzianic acid isomer) resulted in significant increases in the shoot and root fresh weights of cucumber seedlings. Yadav et al. (2011) reported that *T. harzianum* could produce Indole Acetic Acid (IAA) which effected on the growth of chickpea.

Furthermore, *Trichoderma* species had an ability to uptake phosphorus in plant. Yedidia et al. (2001) noted that *T. harzianum* strain T-203 increased the concentration of phosphorus in the shoots of cucumber with 90.00% after sowing for 28 days in soil amended with *T. harzianum*. Rudresh et al. (2005a, 2005b) reported that *Trichoderma* spp. (*T. harzianum*, *T. viride*, and *T. virens*) could increase the quantity of phosphorus in chickpea when compared with the control both in greenhouse and field conditions.

Finally, *Trichoderma* strain FR-NST-009 was identified by morphological characteristics according to interactive key to species of Samuels et al. (2014). The results showed that *Trichoderma* strain FR-NST-009 was *Trichoderma harzianum*, which grew rapidly on PDA (0.90 mm/h), showing white mycelia and green conidia.

Conidia were sub-globose to ovoid, 2.7-3.5 μm in length and 2.5-3.0 μm in width, dry and smooth. Conidiophores were long central axis and lateral branches typically paired. Phialides were flask shape, 5.1-6.8 μm in length, enlarged in the middle, sharply constricted below the tip to form a narrow neck and slightly constricted at the base, and held in cruciate whorls of 2-4 (Figure 4). This study indicated that *T. harzianum* was the plant growth promoting and phosphate solubilizing fungi. Accordingly, *T. harzianum* has been used in bio-products in many countries such as USA, Japan, Canada, Germany, England, Denmark, Israel, and Thailand (Harman, 2000; Intana, 2003; Chamswarng, 2006).



Figure 4. Characteristics of *Trichoderma harzianum* strain FR-NST-009; colony grew on potato dextrose agar after incubation at room temperature for 5 days (a), conidiophores, phialides and conidia observed under Scanning Electron Microscope-SEM (b)

4. Conclusions

Trichoderma species could solubilize insoluble phosphate into available phosphate through organic acid production and they could promote growth of rubber tree under greenhouse conditions, especially indigenous *T. harzianum* strain FR-NST-009.

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