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Citric acid production and rock phosphate solubilization by Co-inoculation with cellulolytic and phosphate solubilizing fungi

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Abstract

In this study water hyacinth and pineapple fermentation wastes were treated with 0%, 5% and 10% rock phosphate and inoculated by three different P-solubilizing fungi individually and in dual culture with cellulolytic fungus *Tricoderma ressi*. Results indicated that the dual inoculation of phosphorus solubilizing fungus and cellulolytic fungus and carbon substrates (water hyacinth and pineapple) can increase water soluble and citrate soluble P and provide the best alternative method of using poor graded rock phosphate in the soil.

Keyword: P-solubilizing fungi, tricoderma reesi, water hyacinth

Phosphorus (P) is the second most important nutrient required for growth and development of plants and is applied to soil in the form of phosphatic fertilizers (Chen *et al*. 2006). Phosphorus is essential for plant biomass, the metabolic process of energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis, and respiration chain reactions (Shenoy and Kalagudi, 2005). However, P is one of the least available and the least mobile mineral nutrients in the soil (Takahashi and Anwar, 2007) since more than 70-90% of the applied phosphatic fertilizers get fixed in soil with cations (Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}) or get adsorbed to Fe-oxides and Al-oxides, Al-silicates, and Ca-carbonates depending on the soil properties. Thus, several studies involving P- solubilizing soil and rhizosphere microorganisms have been reported so far for their relative abilities to dissolve calcium phosphate and apatites in pure culture and in association with plant roots.

Rohr *et al.* (1983) reported that *Aspergillus niger* can increase P mobilization via production of citric acid through fermentation. Subsequently, nutritional composition of media, environmental condition, deficiency of Mn and other metals, P^H and dissolve oxygen tension were reported as crucial parameters for the production of citric acid. El- Holi and Al- Delaimy (2003) observed 92.86 g

l^{-1}citric acid production in whey media containing 15% sucrose. However, when the media was amended with 1-5% tricalcium phosphate, citric acid production was reduced to 45.6%. The purpose of the present investigation is to investigate whether a cellulolytic fungi *Tricoderma reesi* in combination with P-solubilizing fungi (PSF) *Aspergillus* sp or *Penicillium* sp. is able to increase solubilization of rock phosphate than individual PSF. This study will provide the basis for future studies on the effect of microbial interaction on P-solubilization and how they affect both microbial and plant growth.

Materials and Methods

Isolation of P-solubilizing Fungi (PSF): Strains of *Tricoderma reesi, Aspergillus* sp1(T2), *Aspergillus* sp2(F8) *and Penicillium* sp.(F2) were isolated from the soil samples collected from Jalpaiguri, Rashik bill and Tufanganj of Coochbehar and Kharibari of Darjeeling district in West Bengal. All PSF strains were maintained in PDA medium. The plates ware incubated up to 2 weeks at 28 ± 2^{O}C and preserved at 4 ^{o}C.

Solid state fermentation (SSF) and determination of pH: One green biomass (i.e. water hyacinth) and one agro-industrial waste (i.e. pineapple waste) were taken as substrates for solid state fermentation. Fresh water hyacinth and pineapple waste were collected and macerated (size range from 0.1-0.5 cm) in mixer grinder. Subsequently, on dry weight basis 8 g of each substrate was added to 250 ml conical flask and amended with 0, 5 and 10% P_2O_5 as Udaipur rock phosphate (RP). All the flasks were sterilized and subsequently spore suspensions (~10^7 ml^{-1}) of various fungal cultures were added to each flask as per the treatments of SSF. The inoculated flasks were then incubated at 30 ^{o}C for specified time. In this study, two sets of treatments were considered with triplicates as follows: i) both substrates were amended with three levels of RP and subsequently inoculated with T2, F2, and F8 followed by 28 days incubation and ii) all the amended substrates were first inoculated with *Trichoderma reesi* for 12 days, sterilized at 121 ^{o}C for 30 minutes and subsequently inoculated with T2, F2, and F8 followed by 16 days incubation**.** The details of different treatments were as follows:T1-Substrate with 0, 5 and 10% P_2O_5 as RP, inoculated with T2 (*Aspergillus* sp.1); T2-Substrate with 0, 5 and 10% P_2O_5 as RP, inoculated with F2 (Penicillium sp.); T3- Substrate with 0, 5 and 10% P_2O_5 as RP, inoculated with F8 (*Aspergillus* sp.2); T4-Substrate with 0, 5 and 10% P_2O_5 as RP, inoculated with *Trichoderma reesi* and T2; T5- Substrate with 0, 5 and 10% P_2O_5 as RP, inoculated with *Trichoderma reesi* and F2; T6- Substrate with 0, 5 and 10% P_2O_5 as RP, inoculated with *Trichoderma reesi* and F8; and T7-Substrate with 0, 5 and 10% P_2O_5 as RP, without inoculation (Control).

Estimation of water soluble and citrate soluble phosphate: Briefly, 2.5 g of fermented material was transferred to 250 ml conical flask followed by addition of 25 ml distilled water and one spoonful of charcoal. The filtrate was tested for water soluble–P per Jackson (1967). Following the extraction of water soluble phosphorus, the filter paper and the residues on filter paper were transferred to a 250 ml conical flask. After adding 50 ml of previously heated (65 ^{o}C) ammonium citrate solution, mouth of the conical flask was closed with rubber cork and was shaken vigorously till the filter paper was reduced to pulp. The flask was then heated in a water bath at 65 ^{o}C for 1 h. The material was filtered very quickly using a suction pump. The filtrate was considered as citrate soluble phosphorus which was estimated per Jackson (1967).

Citric acid analysis: Citric acid extraction was carried out by mixing 5g fermented sample with 50 ml of distilled water using magnetic stirrer for 10 min and filtering through Whatman 42 (Prado *et al*. 2005). Citric acid was determined titrimetrically (AOAC, 1995) by using 0.1N NaOH and phenolphthalein as indicator. An equal volume of extracted sample was passed through cation-exchange chromatography and eluted sample was titrated with 0.1 N NaOH to assay for total citric acid and the citrate content (Tan *et al*. 1981). Citric acid content was calculated as % according to Eq. (1):

$$\% CA = \frac{\text{Normality} \times \text{Volume of NaOH} \times \text{Equiv. wt. of CA}}{\text{Weight of sample (g)} \times 10} \quad (1)$$

Results and Discussion

This study investigates the mobilization of P along with production of organic acids via fungal isolates T2, F2 and F8 individually and dual inoculation of T2, F2, F8 along with cellulolytic *Tricoderma reesi*. It was apparent that F8 and F2 isolates produced highest citric acid (18.83g kg^{-1}) and citric acid + citrate (51.10 g kg^{-1}) respectively from water hyacinth in absence of rock phosphate (Table 1). Moreover, highest water soluble –P (12.32 g kg^{-1}) and citrate soluble-P (25.62 g kg^{-1}) were obtained from *Tricoderma* + F8 with 5% of rock phosphate and Tricoderma + F2 with 10% rock phosphate, respectively ; clearly indicating the synergistic effect of dual inoculation.

Table 1: Citric acid (g kg^{-1}), Citric acid + Citrate (g kg^{-1}), water soluble-P (g kg^{-1}) and citrate soluble-P (g kg^{-1}) produced from water hyacinth treated with fungi and rock phosphate

Treatments	Citric acids (g kg-1)				Citric acid +Citrate (g kg-1)				Water soluble-P (g kg-1)				Citrate soluble-P (g kg-1)			
	RP0	RP5	RP10	Mean	RP0	RP5	RP10	Mean	RP0	RP5	RP10	Mean	RP0	RP5	RP10	Mean
T2	12.81	11.36	10.76	11.64	51.10	38.13	33.26	40.83	10.40	10.50	5.55	8.82	12.50	15.44	19.61	15.85
F2	11.95	11.66	9.22	10.94	29.89	22.48	19.98	24.11	5.23	7.03	5.75	6.01	13.97	16.49	21.17	17.21
F8	18.83	15.37	10.89	15.03	45.72	35.86	28.31	36.63	9.19	8.95	5.75	7.96	8.23	18.63	23.33	16.73
Tr+T2	16.14	14.79	12.30	14.41	32.28	34.07	21.86	29.40	8.06	10.31	10.40	9.59	17.76	24.96	12.74	18.49
Tr+F2	14.60	10.25	5.73	10.19	28.39	25.62	20.32	24.78	9.18	9.23	7.25	8.56	17.23	21.08	25.62	21.31
Tr+F8	11.53	10.76	6.92	9.73	23.05	30.48	20.75	24.76	12.16	12.32	6.59	10.36	13.12	23.04	24.21	20.12
Control	4.16	3.84	3.07	3.69	1.67	1.84	2.31	1.94	2.32	2.64	2.02	2.33	0.48	0.88	1.76	1.04
Mean	12.86	11.15	8.41		30.30	26.93	20.97		8.08	8.71	6.19		11.90	17.22	18.35	

Table 2: Citric acid (g kg^{-1}), Citric acid + Citrate (g kg^{-1}), water soluble-P (g kg^{-1}) and citrate soluble-P (g kg^{-1}) produced from pineapple waste treated with fungi and rock phosphate

Treatments	Citric acids (g kg-1)				Citric acid +Citrate (g kg-1)				Water soluble-P (g kg-1)				Citrate soluble-P (g kg-1)			
	RP0	RP5	RP10	Mean	RP0	RP5	RP10	Mean	RP0	RP5	RP10	Mean	RP0	RP5	RP10	Mean
T2	7.68	24.21	10.76	14.22	35.22	35.86	36.12	35.73	5.12	12.66	11.62	9.80	6.98	8.06	9.40	8.15
F2	7.49	5.16	4.62	5.76	12.81	14.73	18.47	15.34	4.10	3.39	3.43	3.64	2.71	4.44	5.64	4.26
F8	6.66	19.21	14.99	13.62	24.14	27.15	29.42	26.90	4.72	11.08	9.88	8.56	9.24	14.56	17.52	13.77
Tr+T2	7.49	9.22	10.33	9.02	20.81	22.29	36.63	26.58	5.42	6.87	10.08	7.46	3.98	4.56	9.84	6.13
Tr+F2	12.49	6.92	5.76	8.39	19.15	19.21	19.85	19.40	3.84	5.50	3.95	4.43	6.47	7.28	8.46	7.40
Tr+F8	11.24	19.15	15.21	15.20	21.61	31.64	27.38	26.88	5.91	9.62	7.87	7.80	9.26	12.90	13.23	11.80
Control	5.44	6.76	9.10	7.10	10.52	11.27	11.45	11.08	2.09	2.34	2.17	2.20	0.31	0.65	1.29	0.75
Mean	8.36	12.95	10.11		20.61	23.16	25.62		4.46	7.35	7.00		5.57	7.49	9.34	

Conversely, in case of pineapple SSF the results indicated that T2 with 5% rock phosphate and *Tricoderma* + T2 with 10% rock phosphate produced highest citric acid (24.21 g kg^{-1}) and citric acid + citrate (36.63 g kg^{-1}) (Table 2). The production of citric acid was higher with 5% rock phosphate (12.95 g kg^{-1})

and acid + citrate with 10% rock phosphate (25.52 g kg^{-1}) whereas, mean water soluble-P (7.35 g kg^{-1}) and citrate soluble –P (9.34 g kg^{-1}) were higher with 5% and 10% rock phosphate.

While comparing water hyacinth and pine apple SSF for their suitability for production of citric acid, citric acid + citrate, water soluble P and citrate soluble- P, it was found that water hyacinth SSF produced higher mean citric acid + citrate and citrate soluble P and lower citric acid and water soluble p as compared to pine apple SSF (Table 3). While the increasing the concentration of rock phosphate (0%, 5% and 10%) decrease the citric acid production with water hyacinth, in case of pine apple SSF increase all fractions exhibited increase concentration in 5% rock phosphate as compared to the absence of rock phosphate.

Table 3: Comparison of mean Citric acid (g/kg, Citric acid + Citrate (g/kg), water soluble-P (g P_2O_5/l) and citrate soluble-P(g P_2O_5/l) produced from water hyacinth and pineapple waste treated with fungi and rock phosphate.

Fungal treatments	Water hyacinth				Pine apple			
	Citric acids (g/kg)	Citric acid +Citrate (g/kg)	Water soluble P2O5 (g/kg)	Citrate soluble-P2O5 (g/kg)	Citric acids (g/kg)	Citric acid +Citrate (g/kg)	Water soluble P2O5 (g/kg)	Citrate soluble-P2O5 (g/kg)
T2	11.64	40.83	8.82	15.85	21.83	15.50	17.73	18.35
F2	10.94	24.11	6.01	17.21	15.78	13.00	15.33	14.70
F8	15.03	36.63	7.96	16.73	20.44	15.05	17.41	17.63
Tr+T2	14.41	29.40	9.59	18.49	19.16	15.75	17.80	17.57
Tr+F2	10.19	24.78	8.56	21.31	18.21	16.03	18.52	17.59
Tr+F8	9.73	24.76	10.36	20.12	18.42	16.30	18.28	17.66
Control	3.69	1.94	2.33	1.04	1.77	1.71	1.51	1.66
Rock phosphate treatments								
RP0	12.86	30.30	8.08	11.90	8.35	20.61	4.45	5.56
RP5	11.15	26.93	8.71	17.22	12.94	23.16	7.35	7.49
RP10	8.41	20.97	6.19	18.35	10.11	25.61	6.99	9.34

Conclusion

In the present study it was observed that increase in the level of rock phosphate decreased production of citric acid with water hyacinth, while in case of pineapple it showed some high trends of P release with 5% rock phosphate. It was also found that increasing the level of rock phosphate increase production of citric acid + citrate with pineapple whereas a reverse trend was observed in water hyacinth. The maximum water soluble P was produced from both the substrate with 5% rock phosphate and citrate soluble P. It can be concluded from the results that the isolated organisms can effectively be used for the production of citric acid and subsequent rock phosphate solubilization using biomass waste. Both water hyacinth and pineapple act as a good substrate for the phosphorus solubilizing fungus. The combined use of phosphorus solubilizing fungus and cellulolytic fungus and carbon substrates (Water hyacinth and pineapple) can increase water soluble and citrate soluble P and provide the best alternative method of using poor graded rock phosphate in the soil.

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