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ORIGINAL ARTICLE

Ability of *Emericella rugulosa* to mobilize unavailable P compounds during Pearl millet [*Pennisetum glaucum* (L.) R. Br.] crop under arid condition

B. K. Yadav · J. C. Tarafdar

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Abstract Phosphate solubilizing microorganisms are ubiquitous in soils and could play an important role in supplying P to plants where plant unavailable P content in soil was more. A phosphatase and phytase producing fungus *Emericella rugulosa* was isolated and tested under field condition (Pearl millet as a test crop) in a loamy sand soil. In the experimental soil 68% organic phosphorous was present as phytin; less than 1% of phosphorous was present in a plant available form. The maximum effect of inoculation on different enzyme activities (acid phosphatase, alkaline phosphatase, phytase, and dehydrogenase) was observed between 5 and 8 weeks of plant age. The depletion of organic P was much higher than mineral and phytin P. The microbial contribution was significantly higher than the plant contribution to the hydrolysis of the different P fractions. A significant improvement in plant biomass, root length, seed and straw yield and P concentration of root and shoot resulted from inoculation. The results suggest that *Emericella rugulosa* produces phosphatases and phytase, which mobilize P and enhance the production of pearl millet.

Key words · *Emericella rugulosa* · pearl millet · acid and alkaline phosphatase · phytase · P mobilization.

B. K. Yadav (✉) · J. C. Tarafdar
Central Arid Zone Research Institute,
Jodhpur - 342 003,
India.
e-mail: bkyadav74@yahoo.co.in
Tel.: +91 / 291 / 2740666

Introduction

Phosphorus is a major plant nutrient. Soil phosphorus exists in bound or dissolved inorganic or organic forms. The concentration of soluble P in soil is usually 1 ppm or less.[1] A large proportion of P that is applied to soil as fertilizer rapidly becomes unavailable to plants, accumulating in inorganic P fractions that are fixed by chemical adsorption and precipitation, and organic P fractions that are immobilized in soil organic matter[2]. Since crops require 10 to 100 Kg P ha^{-1}, the ability of microorganisms to solubilize and mineralize P in soil is vital. Phosphate availability in soil is greatly enhanced through microbial production of metabolites leading to lowering of pH and release of phosphate from organic and inorganic complexes. The rate of phosphorus (P) mineralization depends on microbial activity[3] and on the activity of free phosphatase and phytase enzymes[4], which is controlled by the soluble P concentration[5].

The ability of soil microorganisms to solubilize various forms of insoluble P fractions is well-documented[6, 7]. However, the potential of soil fungi to mediate P availability to plants from otherwise poorly available sources under field condition is less clear. The importance of soil microorganisms in increasing the available P of phytate and glycerophosphate to plant roots has been suggested by Tarafdar and Marschner[8]. They showed that the P nutrition of wheat grown in soil supplied with phytate was increased when the plant was co- inoculated with the mycorrhizal fungus *Glomus mosseae* and *Aspergillus fumigatus*, a non-mycorrhizal fungus with known phytase activity[9]. Yadav and Tarafdar[10] reported that fungal isolates differed in their abilities to hydrolyze different organic P compounds.

An examination of the breakdown of unavailable P compounds suggested the importance of identifying potential phosphatase and phytase releasing organisms, which can

exploit the substantial amounts of less available soil P. In addition, little information is available on the partitioning of plant and microbial contributions to P hydrolysis. We therefore isolated a phosphatase and phytase releasing fungus, *Emericella regulosa*, and examined the efficiency of its enzymes in releasing available phosphorus from unavailable P sources for plant nutrition under field condition.

Materials and Methods

Isolation and identification of fungi: Fungi were isolated from twenty-seven varied soils, using a dilution plate technique[11] on Martin's Rose Bengal agar containing streptomycin sulphate[12]. Twenty phosphatase and phytase-producing fungi were isolated, purified from the single spore in slants, identified by Agharkar Research Institute, Pune, India. The pure cultures were maintained on potato dextrose agar (PDA) medium. Based on their intra and extracellular acid phosphatase, alkaline phosphatase and phytase activity (data not shown) the best fungus *Emericella rugulosa* was selected.

Seed inoculation: The pearl millet seeds were surface sterilized with acidified 0.05% $HgCl_2$ for 2 minutes thereafter washed with sterilized deionized water for 5 to 6 times. The inoculation with *Emericella rugulosa* was carried out in the slurry of carrier-based culture in sterilized (20%) jaggery (gur) solution and seeds were treated with *Emericella rugulosa* (100g kg^{-1} seed), dried under shed (to avoid direct sun rays) and shown immediately. For uninoculated treatment seed was inoculated with sterilized culture of the same amount (100g kg^{-1} seed).

Field experiment: Pearl millet (*Pennisetum glaucum* (L.) R. Br.; cv, HHB 67) was cultivated under rainfed condition during *Kharif* 2004 at Central Research Farm, CAZRI, Jodhpur, to evaluate the performance of fungi under field condition. The area is located at latitude of 26°18 N and longitude of 73°01 E. A randomized block design with three replications was used. There were two treatments; with inoculation and without inoculation. The plot size of each replicate was 5×4 m. The pearl millet seeds were inoculated with *Emericella rugulosa* (2×10^6) before sowing in inoculated treatment. Crops were grown under rainfed condition. No fertilizer and irrigation was applied during the growth period; only 273 mm rainfall was received during the growth period. The experimental soil was a typial Caborthid (Table 1). Four plants of each replicate with intact roots were carefully freed from soil at 28, 35, 42, 49 and 56 days after planting. We harvested the crops after maturity (63 days).

Processing and biochemical analysis: After each harvest, roots were thoroughly washed free of soil in tap water followed by deionized water. The roots were separated from the soil and the root lengths were measured using a modified line-intersect method of Tennant[13]. The P-content of the plant was determined using the vanadomolybdophosphoric acid yellow colour method[14]. The fungal population was counted in Martin's Rose Bengal agar medium[12]. Acid and alkaline phosphatases were assayed by adopting the standard procedure of Tabatabai and Bremner[15] using acetate buffer (pH 5.4) and sodium tetraborte- NaOH buffer (pH 9.4), respectively. The enzyme substrate (4- nitrophenyl phosphate) mixture was incubated at 25°C for 1 h and the enzyme assay was expressed as enzyme units (EU). One unit is the amount of enzyme which hydrolyses 1.0 µM of p-nitrophenyl phosphate per sec at pH 5.4 (acid phosphatase) or 9.4 (alkaline phosphatase) at 35°C. Phytase activity was assayed by measuring inorganic phosphate (Pi) hydrolyzed from sodium phytate in acetate buffer (pH 4.5) incubating at 37°C for 1h[16]. The activity was expressed in terms of enzyme unit (EU). One unit of phytase activity was defined as the amount of enzyme, which liberated 1µM Pi per second. Dehydrogenase activity, a measure of total microbial activity, was assed by the method of Tabatabai[17].

The microbial contribution to P hydrolysis was defined as the mineral, organic and phytin P depletion from the plots due to inoculation of *Emericella rugulosa*, in plots without plants. The plant contribution was defined as the additional

Table 1 Characteristics of the soil used in the study.

Parameter	Characteristics*
pH (soil: water)	7.9±0.05
EC (dSm^{-1})	0.2±0.01
Organic matter (%)	0.2±0.02
Sand (%)	85.1±0.1
Silt (%)	5.5±0.1
Clay (%)	7.9±0.05
Total P (mg kg^{-1})	1260.9±13.4
Mineral P (mg kg^{-1})	884.3±8.5
Organic P (mg kg^{-1})	366.6±5.4
Olsen's P (mg kg^{-1})	10.5±1.1
Water soluble Pi (mg kg^{-1})	1.6±0.01
Phytin P (mg kg^{-1})	252.2±7.6
Acid phosphatase activity (EU × 10^{-4})	0.05±0.01
Alkaline phosphatase activity (EU × 10^{-4})	0.08±0.01
Phytase activity (EU × 10^{-4})	1.52±0.18
Dehydrogenase activity (p kat g^{-1})	1.11±0.20
Fungi (×10^{-4})	15±1.2

*Mean value; ± indicate the standard errors of mean.

depletion of different forms of unavailable P after introduction of plants to inoculated plots. We estimated pH (1:2), EC (1:2), partical size distribution and available P (Olsen's) using standard methods[18]. The data were subjected to analysis of variance and vertical bars are standard errors of the differences between means[19].

Results

Characteristics of the soil under field, before start of the experiment, were presented in Table 1. In general 70% of the total P was present in organic form, 29% was present in unavailable inorganic form and approximately 1% of the total P was present in available form. Organic P in the soil mainly present as phytin form, which was measured 68% of total organic phosphorus. The best organism to exploit the unavailable P was identified as *Emericella rugulosa* (Fig. 1). This was based on its phosphatase and phytase activity and its ability to hydrolyze unavailable P, as compared to other isolated fungi (results not shown).

The highest acid phosphatase activity was observed after 7 weeks of pearl millet growth (Fig. 2). Inoculation with *Emericella rugulosa* significantly enhances the acid phosphatase secretion from 4 to 8 weeks. The inoculation effect was more between 4 to 6 weeks and 20% improvement in acid phosphatase secretion due to inoculation *Emericella rugulosa* was observed.

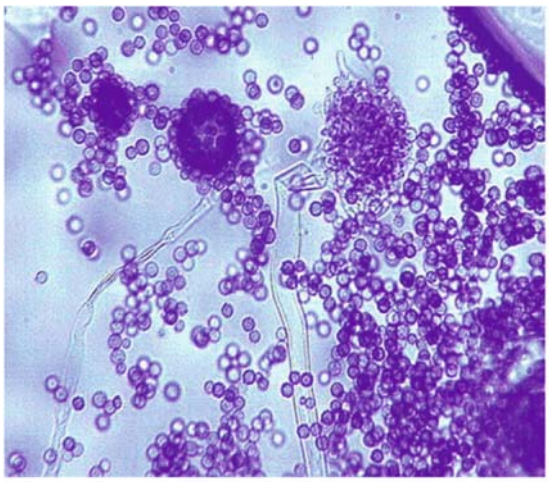
Alkaline phosphatase activity between 4–8 weeks period in the rhizosphere of pearl millet (Fig. 2) resulted a significantly higher activity throughout the period, which was maximum after 7 weeks. Alkaline phosphatase activity increased in efficiency (45%) under the *Emericella rugulosa* treatments.

Fig. 2 shows the increase in phytase activity with crop age in pearl millet. A significantly higher phytase activity was observed after 4 weeks onward due to inoculation of *Emericella rugulosa*. The phytase activity was increased by 46% after inoculation of *Emericella rugulosa*.

Significant increase in dehydrogenase activity in pearl millet rhizosphere was observed, which was progressively increased with crop age up to 8 weeks, after inoculation of *Emericella rugulosa* (Fig. 2). The increase in activity was observed (98%) due fungal inoculation.

The depletion of unavailable P was partitioned into the plant and microbial contribution. A gradual increase in depletion of different forms of unavailable P with the inoculation of *Emericella rugulosa* in pearl millet with plant age was observed (Table 2). With increase in crop age the plant contribution was more, whereas the microbial contribution was declining with plant age in hydrolysis of all the three unavailable P fractions (mineral P, organic P, phytin P). In general, plant contribution varies between 18–49% to hydrolyze mineral P, 35–62% to hydrolyze organic P and 18–56% to hydrolyze phytin P. The microbial contribution varies between 82–51% for mineral P, 38–65% for organic P and 44–82% for phytin P.

(A)



(B)

Fig. 1 *Emericella rugulosa* and its spores (A) growth in Petriplate (B) spore (× 312.5).

A significant increase in dry matter ($p < 0.01$, $n = 6$) was observed in pearl millet (Table 3) due to inoculation of *Emericella rugulosa* as compared to control (without inoculation). Increase in dry matter varies between 21–52 % after inoculation of fungi.

Root length of pearl millet at different crop growth period after inoculation with fungi was presented in Table 3. In general, root length was increased significantly ($p < 0.01$, $n = 6$) with inoculation, which varies between 19–26 %.

At crop harvest, the seed and straw yield of pearl millet was increased significantly ($p < 0.01$, $n = 6$) by 23% and

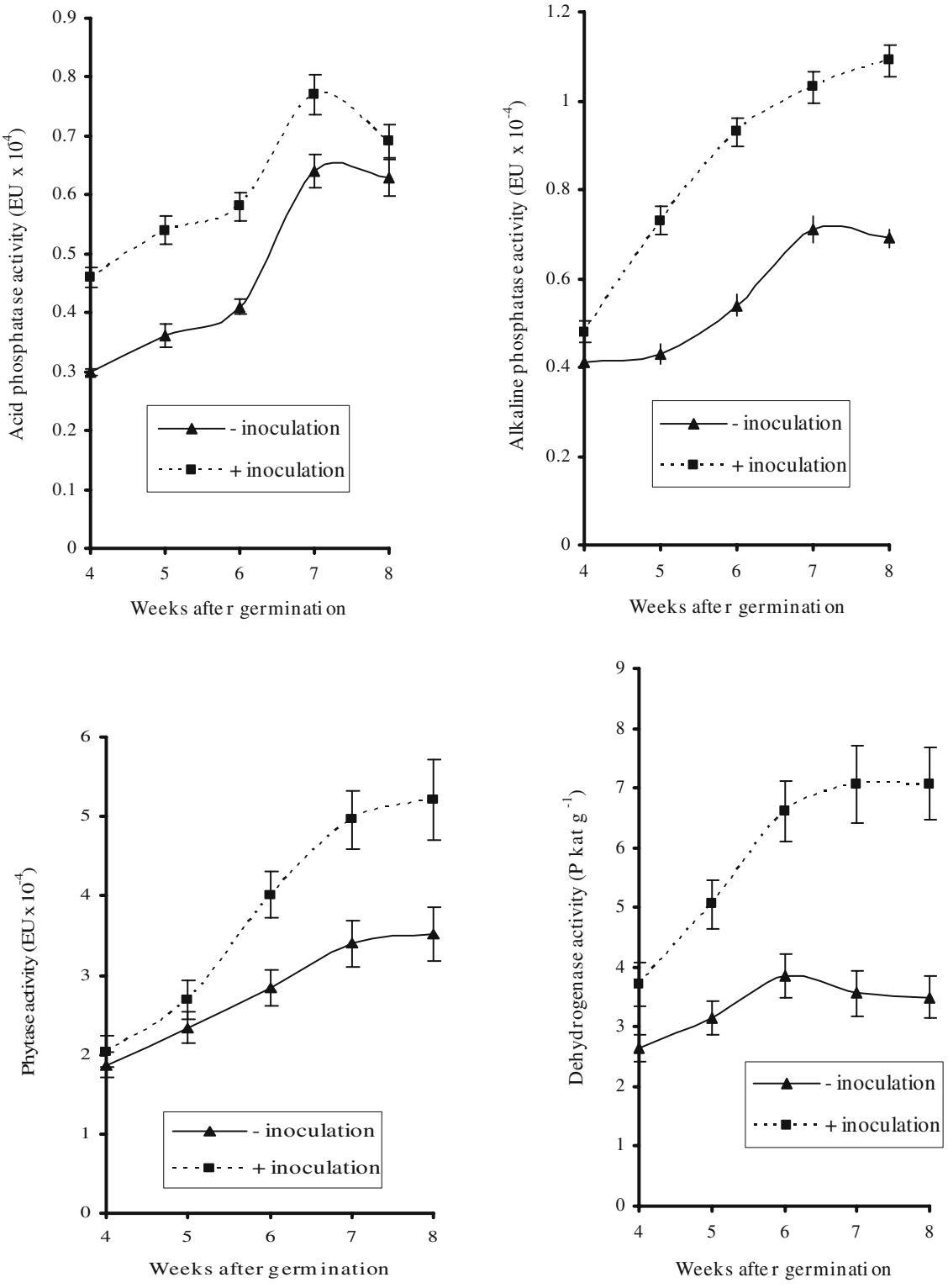


Fig. 2 Soil enzyme activities at different growth periods in pearl millet under field condition.

39% respectively due to inoculation of *Emericella rugulosa* (table 4). A significant improvement ($p < 0.01$, $n = 6$) in shoot P concentration (20%) and marginal improvement in root P content (5%) was also observed.

Discussion

Plants acquire phosphorus as phosphate anions from the soil solution. It is probably one of the least available plant

nutrients found in the rhizosphere. In particular, plant growth promoting fungi have been reported to be key elements for plant establishment under nutrient-imbalance conditions. Use of those fungi use in agriculture can favor a reduction in agro-chemical use and support more crop production[20,21,22].

The availability of phosphorus (P) to plants depends on the activity of microorganisms present in the rhizosphere[23] and the strategies of plant for taking up P[24]. A major process is the decrease in the concentration of phosphate ion (PO_4) in the soil solution, which occurs within the rhizosphere as a direct consequence of the removal of P by the root uptake. Such a depletion of rhizosphere P has been reported for various soils and plants[25,26]. This depletion results in a replenishment of P from the solid phase[27], with growing period and P concentration and is influenced by the physicochemical conditions of the soil.

In the experimental soil, 68% of the total organic P was present as phytin P (Table 1). The largest fraction of organic P in the soil being in the form of phytin and its derivatives is consistent with the literature[28].

In this study, maximum enzyme activity was observed in the 5–8 week period after germination (Fig. 2), this coincided with the maximum rate of root exudates secretion[29]. Plants and microorganisms[30] can release enzymes, therefore under field microbial build up due to root exudates resulted in higher enzyme activity (Fig. 2). The introduction of plant does not automatically increase alkaline phosphatase activity, as microorganisms may be the only contributor of alkaline phosphatase in soil[30]. Both plant and microorganisms, however, influence phytase activity in pearl millet (Fig. 2). Enhanced secretion of phytase[31] by plant roots and rhizosphere microorganisms[32] may contribute to inorganic P acquisition through the hydrolysis of organic esters in the rhizosphere. Dehydrogenase activity is an indicator of total microbial activity[33]. The increase in dehydrogenase activity reflected a considerable increase in microbial activity in the rhizosphere. Specific stimulation of phosphatase producing microorganisms by root exudates has been previously reported[34].

The microbial contribution was much higher than the plant contribution to the hydrolysis of different unavailable P fractions (Table 2). In addition to the cleavage of the C-O-P bond by microbial phosphatases and phytases, the microorganisms may also produce organic acids such as malate, citrate, oxalate, which may also help in the release of Pi[35]. Enhanced secretion of phosphatases and phytase[31,36] by plant roots and rhizosphere microorganisms[32] may contribute to Pi acquisition through the hydrolysis of organic P esters in the rhizosphere. Richardson[37] reported the potential of soil microorganisms to increase the availability of P from phytate both through phytase activity and perhaps by affecting the availability of phytate itself. However, they identified that the extent to which microorganism's activity release P from phytase in soils for its subsequent uptake by plant roots, remained to be determined. In this experiment, our results clearly demonstrated the efficiency of *Emericella rugulosa* in releasing p from phytate (phytin-P) and other unavailable P sources.

A positive influence of inoculation on plant biomass, root length, (Table 3), straw yield, seed yield and P concentration of shoot and root was observed (Table 4). Microbial activity results in quantitative and qualitative alterations of root exudates composition due to the degradation of exudates compounds and the release of microbial metabolites[38]. Microbial activity is a central factor in the soil organic P cycle and affects the transformations of inorganic P[39]. Higher enzyme activity is soils indicated the potential

Table 2 Contribution by pearl millet and *Emericella rugulosa* to hydrolyze different unavailable P fractions.

Plant age (days)	Depletion of total unavailable P (mg kg^{-1})					
	Mineral P		Organic P		Phytin P	
	PC*	MC**	PC	MC	PC	MC
28	2.9±0.17 (17.9)	13.3±0.8 (82.1)	11.5±0.97 (34.6)	21.7±1.2 (65.4)	3.8±0.27 (17.8)	17.5±1.2 (82.2)
35	11.9±0.75 (21.9)	42.4±2.5 (78.1)	17.8±1.12 (38.0)	29.0±1.4 (62.0)	10.5±0.79 (36.8)	18.0±2.1 (63.2)
42	23.2±1.27 (37.3)	39.0±1.7 (62.7)	27.1±1.35 (51.0)	26.0±1.6 (49.0)	17.0±1.20 (41.2)	24.3±2.2 (58.8)
49	31.1±1.56 (43.5)	40.4±2.2 (56.6)	33.5±1.75 (55.2)	27.2±1.3 (44.8)	23.5±1.82 (47.7)	25.8±2.4 (52.3)
56	38.5±2.15 (48.5)	40.8±2.3 (51.5)	44.6±2.54 (62.4)	26.9±1.4 (37.6)	32.3±2.54 (56.2)	25.2±2.2 (43.8)

* Plant contribution ** Microbial contribution; Figure in parenthesis denotes the per cent of total mineral/organic/phytin-P depleted.

Table 3 Effect of *Emericella rugulosa* on plant biomass and root length of Pearl millet under field condition.

Days after germination	Treatments	Plant biomass (g plant^{-1})	Root length (cm)
	- Inoculation	5.4	2017
28	+ Inoculation	8.2	2427
	LSD(p=0.05)	0.63	191.4
	- Inoculation	7.6	3245
35	+ Inoculation	10.6	3868
	LSD(p=0.05)	0.86	283.6
	- Inoculation	10.2	3736
42	+ Inoculation	14.0	4487
	LSD(p=0.05)	1.21	339.08
	- Inoculation	13.4	4072
49	+ Inoculation	16.6	5098
	LSD(p=0.05)	1.41	413.20
	- Inoculation	15.9	4468
56	+ Inoculation	19.2	5630
	LSD(p=0.05)	1.58	428.66

Table 4 Straw yield, Grain yield and P concentration of Pearl millet after harvesting of crop.

Treatment	Yield (q ha^{-1})		P concentration(mg g^{-1})	
	Straw	Grain	Shoot	Root
-Inoculation	19.4	8.8	4.82	3.44
+Inoculation	27.0	10.8	5.76	3.61
L.S.D.(p=0.05)	3.87	1.42	0.31	0.21

of soil to affect the biochemical transformations necessary for the maintenance of soil fertility[40]. The results presented clearly demonstrate the positive influence of *Emericella rugulosa* on pearl millet production apparently as a result of the increased release of phosphatases and phytase.

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