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Influence of acidulation and bioactivation of rock phosphate along with AM fungus on growth of maize and pigeon pea in an Alfisol

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Abstract
A greenhouse investigation was conducted to determine the effect of acidulation and bio-activation of North Carolina rock phosphate (NCRP) and inoculation of soil with arbuscular mycorrhizal (AM) fungus on growth of maize and pigeon pea in an Alfisol. This investigation consisted of 24 treatments resulting from factorial combination of two plant species, two levels of AM fungus (*Glomus aggregatum*) inoculation and six levels of phosphorus amendment. Based on titrable acidity data the acidification potential of *Penicillium* sp. was found to be higher than that of *Bacillus* sp. The extent of mycorrhizal fungal colonization in roots of pigeon pea was higher than that maize. Inoculation of soil with AM fungus caused significant increase in total shoot and dry weight of pigeon pea as well as maize. The results of this study suggest that application NCRP bio-acidulated with *Bacillus* sp. and *Penicillium* sp. increases growth of maize and pigeon pea and inoculation of soil with AM fungus augments that effect.

Keywords: North Carolina rock phosphate, Phosphate solubilizing microorganisms, Acidulation, Bioactivation, *Glomus aggregatum*

Introduction
Sequioxide - rich alfisols are one of the major group of soils of South India. The available P status of these soils is low which often limits the productivity of plant species. Further, soluble phosphate added to these soils get converted into insoluble forms by reacting with iron and aluminum hydroxides (Sanchez and Uehara, 1980; Holford, 1997) [15, 7] It is suggested that these soils need a different kind of management, particularly with reference to maintaining their available P status, in order to make crop production profitable. Goenadi *et al*. (2000) [5], Geel *et al*. (2016) [3] and Zwetsloot *et al*. (2016) [17] explored the possibility of using slow-release phosphorus fertilizers, bone-char and rock phosphate along with either bio-char or organic matter to increase the available phosphorus status of P-fixing soils. In such situations and wherever phosphate rock is available it may be economical to apply phosphate rock as source of P. Although, total P content of phosphate rock is high (> 13% P) It's water soluble P content is low. Phosphate solubilizing microorganisms (PSMs) influence plant growth and development by enabling plants to grow in soils amended with insoluble sources of P.
Goenadi *et al.* (2000) [5] used a culture of phosphate solubilizing fungus (PSF), *Aspergillus niger* to bio-activate Morrocan rock phosphate (MRP) and observed significant increase in its citrate soluble phosphorus content. In this context, the bio-activation of rock phosphate with culture of PSMs before application, will not only increase phosphorus availability soon after application and may also sustain it, since these organisms have assured supply of energy substrates. It is possible that P availability from bio-activated rock phosphate may be enhanced due to inoculation of soil with arbuscular mycorrhizal (AM) fungi. AM fungi are a group of soil fungi which are known to have symbiotic association with plants species and the colonization of roots with these fungi is known to enhance the uptake of diffusion-limited nutrients of which phosphorus is most important (Habte and Osorio, 2001) [6]. Therefore, Therefore, this investigation was conducted to determine the effect of application of bio-activated North Carolina rock phosphate (NCRP) on phosphorus nutrition and growth of two indicator plant species, with distinct root morphological traits, maize and pigeon pea in the presence and absence of AM fungus, *Glomus aggregatum* in an Alfisol.

Materials and Methods
Phosphate solubilizing bacterium, *Bacillus sp.* and phosphate solubilizing fungus, *Penicillium*

sp. were obtained from the culture collection of Biofertilizer production laboratory of the Department of Agricultural Microbiology, GKVK, Campus. They were tested for phosphate solubilization efficiency using Sperber's medium. The zone of phosphorus solubilization on Sperber's medium was noticed with both the organisms, hence they were used to bio-activate rock phosphate in this study (Fig 1).

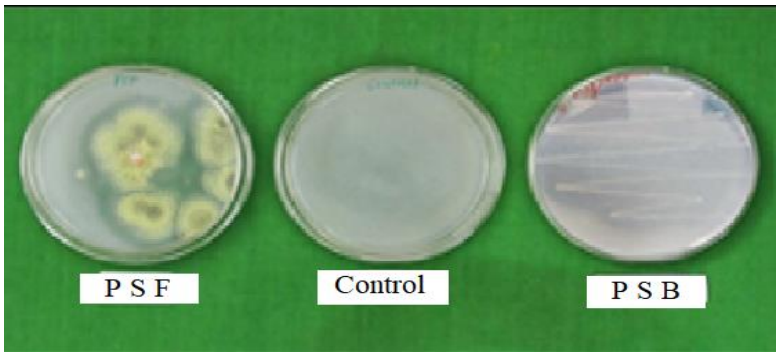

Fig 1: Phosphate solubilizing microorganisms grown on Sperber's medium. PSF= Phosphate Solubilizing Fungus (*Penicillium* sp.), PSB= Phosphate Solubilizing Bacteria (*Bacillus* sp.)

Determination of phosphorus solubilizing efficiency

Phosphate solubilizing microorganisms (PSMs) were grown in 100 ml of modified Sperber's broth. In this culture medium, North Carolina Rock Phosphate (NCRP) was used as sole source of phosphorus which was added at the rate of 0.36 per cent P. One ml of 24 hours old culture of PSMs were inoculated to culture medium and grown for 15 days. After inoculation, flasks were incubated on a temperature controlled shaker at 30°C. There were three replicates for each PSM. The uninoculated broth served as control. After the incubation period, the culture filtrate was obtained by filtering broth using Whatsman 1- grade filter paper. The filtrate of medium in which different PSMs were grown was titrated against 0.05N of NaOH using phenolphthalein indicator. At the end, the volume of NaOH needed to completely neutralize the acidity of culture filtrates was recorded after confirming the stability of pink colour.

Table 1: Acid produced by PSMs used in this study

PSMs	Titrable acidity
Bacillus sp.	0.005 N
Penicillium sp.	0.012 N

PSMS- Phosphate solubilizing microorganisms

Preparation of acidulated and bio-activated NCRP

Three hundred grams of NCRP was placed in four 500 ml glass beakers. Fifty three ml distilled water was added to one of these NCRP samples. The remaining 3 samples of NCRP received 53 ml of HCl solution, homogenized culture of *Bacillus* and or homogenized culture *Penicillium* respectively. The acidity level of these three solutions was similar. The volume of liquid added brought the moisture content of NCRP to 50% of its maximum water holding capacity. NCRP samples were incubated at 30°C for 15 days in an incubator and during incubation period, 30 ml of distilled water was added to all samples to compensate evaporation loss during incubation. These NCRP samples were mixed uniformly at the rate of 200 mg of phosphorus per kg of soil a day before planting.

Mycorrhizal inoculum production

The culture of AM fungus was obtained from Soil Microbiology Laboratory of the Department of Agronomy and Soil Science, University of Hawaii, Honolulu, USA. The crude inoculum of AM fungus *Glomus aggregatum*, was prepared by following the procedure proposed by Habte and Osorio (2001) [6]. This fungus is an obligate symbiont and was mass multiplied by using maize (*Zea mays* L.) as host. After 75 days of plant growth, plants were allowed to wilt. Shoot portions were discarded and sand containing dried root pieces of maize colonized with *Glomus aggregatum*, extrametrical chlamydospores and mycorrhizal hyphal bits served as crude inoculum. Soil samples in pots were mixed with this crude inoculum at the rate of 50 g per kg soil.

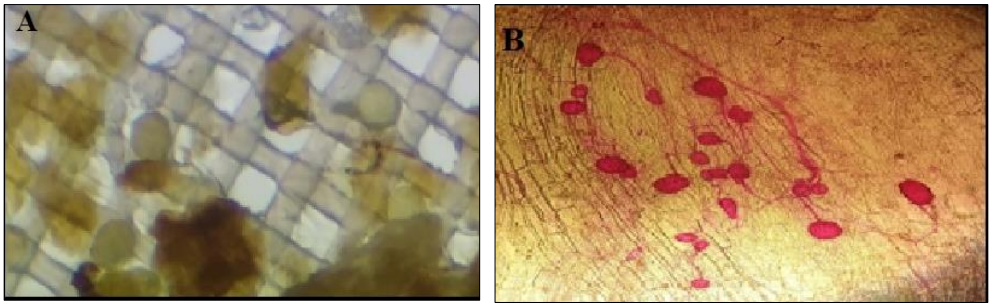

Fig 2: Extramatrical spores of *Glomus aggregatum* (a) and vesicles formed in roots of maize colonized with *G. aggregatum* (b)

This experiment consisted of 24 treatments resulting from factorial combinations of 2 plant species (maize and pigeon pea), 2 levels of GA (*Glomus aggregatum*) inoculation (GA- and GA+) and 6 types of phosphorus amendment [Unamended Control, Untreated NCRP, Acidulated NCRP, NCRP bio-activated with PSB, NCRP bio-activated with PSF and KH_2PO_4]. Each treatment was replicated thrice, hence there were 72 experimental units in this factorial randomized complete block design experiment. Plants were grown under natural light conditions in a greenhouse. Moisture content of soil in pots was maintained near field capacity by adding water at regular intervals. Plants were harvested after 60 days of growth under greenhouse conditions. Shoots were separated by cutting at collar region and roots samples were thoroughly washed under gentle stream of water.

Determination of per cent AM fungal colonization in roots

Fresh root samples (200 mg) collected after harvesting, washed roots were used to determine the extent of mycorrhizal colonization. Gridline intersect method outlined by Giovannetti and Mosse (1980) [4] was followed to determine extent of mycorrhizal colonization in roots of maize and pigeon pea after staining with acid fuchsin. Fresh root samples were immersed in 10% KOH solution placed in test tubes. These test tubes were placed in simmering water bath for 45 minutes to cause partial hydrolysis of root tissue. Alkali solution was decanted and 10% HCl solution was added to neutralize alkali and to cause acidification of roots. After decanting acid solution, acidified roots samples were stained with 0.2% acid fuchsin dissolved in lacto-glycerol. Excess staining solution was decanted and roots were de-stained with lacto-glycerol solution. The stained roots were placed on grid-line plates and observed using stereomicroscope at 40X magnification. Number of root intersections with AM fungal colonization were recorded and the per cent of mycorrhizal colonization was calculated by using formula.

$$\text{Mycorrhizal Colonization (\%)} = \frac{\text{Number of root intersections +ve for AM fungal colonization}}{\text{Total number of root intersections observed}} \times 100$$

Determination of dry weight and Total biomass

Shoots and roots samples were placed in an oven at 70°C and their dry weights were recorded after drying them to constant weight. Total biomass was computed as the summation of dry weight of shoot and root.

Statistical analysis

The data were subjected to two way Analysis of Variance by factorial randomized complete block design and means were separated by the Duncan's multiple range test (DMRT) (Little and Hills, 1978) [10].

Results

Laboratory investigations were conducted to determine acidification potential of two phosphorus solubilizing microorganisms (PSMs), *Bacillus* sp and *Penicillium* sp. Based on titrable acidity data presented in Table 1, it can be concluded that the acidification potential of *Penicillium* sp is higher than that of *Bacillus* sp.

A greenhouse investigation was conducted to determine the effect of application of NCRP bio-activated with these PSMs and acidulated NCRP on growth of maize and pigeon pea in the presence and absence of AM fungus, *Glomus aggregatum*. Data on shoot dry-weight, root dry-weight, total biomass and the extent of AM fungal colonization in roots were determined. due to acidification as well as bio-activation were calculated. Data were analyzed using factorial randomized complete block design and means were separated by the DMRT.

Mycorrhizal colonization in roots

The extent of AM fungal colonization in roots of pigeon pea was higher than that of maize (Table 2). The extent of mycorrhizal colonization in roots of pigeon pea and maize grown in soil amended with NCRP formulations were significantly higher than those with KH_2PO_4 amendment. Further, the per cent mycorrhizal colonization in roots of plants grown in soil without NCRP amendment was significantly lower than those grown in soil with NCRP application.

Table 2: Mycorrhizal colonization in roots of maize and pigeon pea as influenced by inoculation of soil with *Glomus aggregatum* and application of different sources of phosphorus

Treatments	Mycorrhizal colonization (%)*	
	Maize	Pigeon pea
Unamended control	82.28b	89.23c
NCRP- Untreated	91.34a	95.05a
NCRP- Acidulated	90.80a	95.90a
NCRP-PSB (Bioactivated)	91.63a	92.64b
NCRP-PSF (Bioactivated)	91.75a	93.34b
KH_2PO_4	79.11c	84.07d

*Means followed by the same letter within a plant species do not differ significantly at the 5% level of significance.

Means for main effect

Main effect	Mycorrhizal colonization (%)**
Plant species	
Maize	87.82b
Pigeon pea	91.70a
Treatment	
Unamended control	85.76b
NCRP- Untreated	93.19a
NCRP- Acidulated	93.35a
NCRP-PSB (Bioactivated)	92.13a

NCRP-PSF (Bioactivated)	92.55a
KH_2PO_4	81.58c

**Means followed by the same letter within a main effect do not differ significantly at the 5% level of significance. -GA = Un-inoculated; +GA = Inoculated with *Glomus aggregatum*; NCRP = North Carolina rock phosphate; PSB = Phosphate solubilizing bacterium; PSF = Phosphate solubilizing fungus.

Table 3: Shoot dry weight of maize and pigeon pea as influenced by inoculation of soil with *Glomus aggregatum* and application of different sources of phosphorus

Treatments	Shoot dry weight (g/plant)*			
	Maize		Pigeon pea	
	- GA	+ GA	- GA	+ GA
Unamended control	0.74e	1.35de	0.28c	0.46c
NCRP- Untreated	2.16cd	4.10b	0.31c	0.90b
NCRP- Acidulated	2.86c	4.01b	0.41c	1.21b
NCRP-PSB (Bioactivated)	2.64c	3.87b	0.37c	0.98b
NCRP-PSF (Bioactivated)	2.86c	4.32b	0.35c	1.14b
KH_2PO_4	11.15a	11.64a	0.89b	3.59a

*Means followed by the same letter within a plant species do not differ significantly at the 5% level of significance.

Means for main effects

Main effect	Shoot dry weight (g/plant)**	
	Maize	Pigeon pea
VAM inoculation		
- GA	3.74b	0.43b
+ GA	4.88a	1.38a
Treatments		
Unamended control	1.04c	0.37c
NCRP- Untreated	3.13b	0.60bc
NCRP- Acidulated	3.44b	0.81b
NCRP-PSB (Bioactivated)	3.25b	0.68b
NCRP-PSF (Bioactivated)	3.59b	0.75b
KH_2PO_4	11.39a	2.24a

** Means followed by the same letter within a main effect do not differ significantly at the 5% level of significance. -GA = Un-inoculated; +GA = Inoculated with *Glomus aggregatum*; NCRP = North Carolina rock phosphate; PSB = Phosphate solubilizing bacterium; PSF = Phosphate solubilizing fungus.

Table 4: Root dry weight of maize and pigeon pea as influenced by inoculation of soil with *Glomus aggregatum* and application of different sources of phosphorus

Treatments	Root dry weight (g/plant)*			
	Maize		Pigeon pea	
	- GA	+ GA	- GA	+ GA
Unamended control	0.48g	0.48g	0.09e	0.10e
NCRP- Untreated	0.82fg	2.04c	0.10e	0.18cde
NCRP- Acidulated	1.05ef	1.79cd	0.11e	0.25bc
NCRP-PSB (Bioactivated)	1.01ef	1.43de	0.10e	0.21bcde
NCRP-PSF (Bioactivated)	1.08ef	1.62cd	0.12de	0.33b
KH_2PO_4	4.12a	3.35b	0.25bc	0.75a

*Means followed by the same letter within a plant species do not differ significantly at the 5% level of significance.

Means for main effects

Main effect	Root dry weight (g/plant)**	
	Maize	Pigeon pea
VAM inoculation		
- GA	1.43b	0.13b
+ GA	1.78a	0.30a
Treatments		
Unamended control	0.48c	0.10c
NCRP- Untreated	1.43b	0.14bc
NCRP- Acidulated	1.42b	0.18bc
NCRP-PSB (Bioactivated)	1.22b	0.16bc
NCRP-PSF (Bioactivated)	1.35b	0.23b
KH_2PO_4	3.73a	0.50a

** Means followed by the same letter within a main effect do not differ significantly at the 5% level of significance. -GA = Un-inoculated; +GA = Inoculated with *Glomus aggregatum*; NCRP = North Carolina rock phosphate; PSB = Phosphate solubilizing bacterium; PSF = Phosphate solubilizing fungus.

Dry weight of shoot and root

Inoculation of soil with AM fungus significantly increased shoot as well as root dry weight of maize and pigeon pea. The magnitude of increase with pigeon pea was higher compared to maize (Tables 3 and 4). Application of NCRP formulations did not significantly influence dry weight of shoot and root. The highest dry weight of shoot and root was noticed with maize and pigeon pea grown in soil amended with KH_2PO_4 (Fig 4 and 5).

Total biomass

Trends noticed with total biomass were similar to dry weight of shoot and root (Fig 3). Inoculation of soil with *Glomus aggregatum* increased biomass of pigeon pea (3 times) more than that of maize (1.3 times). Application of NCRP formulations did not significantly total biomass of both plant species either under mycorrhizal or under non mycorrhizal condition. The highest total biomass was noticed with plants grown in soil amended with KH_2PO_4.

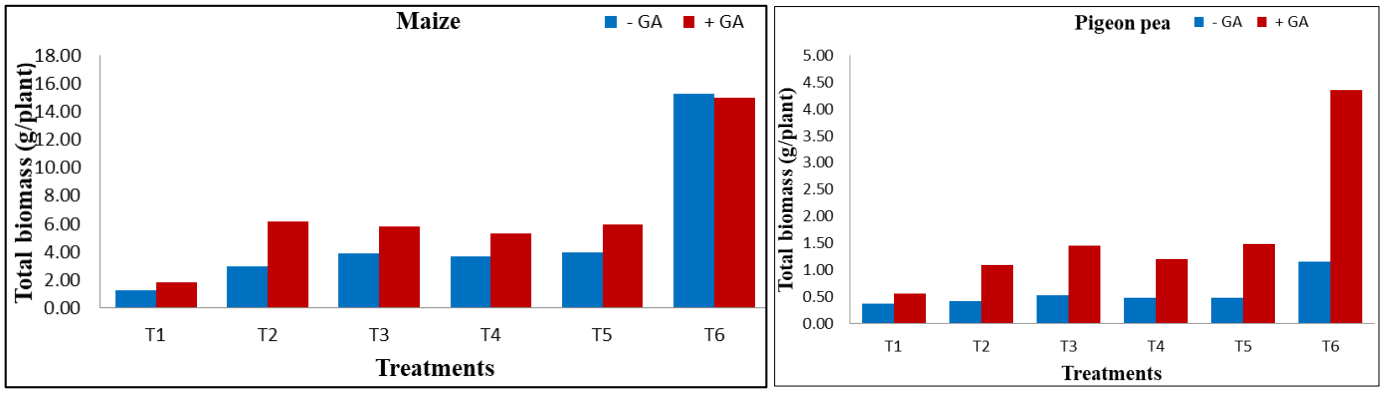

Fig 3: Total biomass of maize and pigeon pea as influenced by inoculation of soil with *Glomus aggregatum* and application of different sources of phosphorus

Discussion

Phosphate solubilizing efficiency of PSMs

Titrable acidity data presented in Table 1, clear halo zone indicating the solubilization of tricalcium precipitate (Fig 1). PSMs were grown in modified Sperber's medium which contains NCRP as the sole source of phosphorus. NCRP was treated with homogenized culture media of both these PSMs (Plate 3) clearly suggest that isolates of PSMs used in this study solubilize phosphorus from insoluble sources and also differ in their ability to solubilize phosphorus. This supports the findings of numerous investigators that phosphorus solubilization is due to decrease in pH of the medium which is mainly due to the production of organic acids. The presence of organic acids such as, citric acid, oxalic acid, butyric acid, malonic acid, lactic acid, succinic acid, tartaric acid and gluconic acid have been determined in the culture filtrates of PSMs (Bhattacharyya and Jain, 2000 [2]; Illmer and Schinner, 1992) [2, 8]. The presence of water soluble phosphorus in acidulated NCRP (treated with mineral acid) conclusively suggests that increase in available phosphorus status of NCRP is due to acidification of medium. In this context, it may be more appropriate to designate bio-activated rock phosphate as bio-acidulated because PSMs were responsible for acidification, irrespective of the mechanism by which acidification was caused.

Effect of application of bio-acidulated NCRP and inoculation of soil with *Glomus aggregatum* on growth of maize and pigeon pea

Data on the extent of mycorrhizal colonization in roots of maize and pigeon pea observed in this study is in good agreement with earlier studies. Mycorrhizal colonization in root of pigeon pea was significantly higher than that of maize. Plant species have been observed to differ in their susceptibility to colonization by AM fungi (Rengel, 2002; Janos, 1987) [14, 9].

In a greenhouse study, involving four species of *Leucaena* and four species of *Sesbania*, Manjunath and Habte (1991) [12] observed reduction in mycorrhizal colonization in roots of *Sesbania* only at soil solution phosphorus levels higher than 0.08 mg/l. This clearly indicates that application of bio-acidulated NCRP at the rate of 200 mg of phosphorus per kg soil will not increase soil solution phosphorus concentration to a level that would interfere with mycorrhizal colonization of root.

In the present study, significant increase was noticed in shoot dry weight, root dry weight and total biomass of maize in response to application of NCRP formulations, in the presence as well as in the absence of mycorrhizal fungus. Such a response trend was noticed with pigeon pea under nonmycorrhizal condition. This is possibly related to their internal phosphorus (tissue phosphorus concentration) as well as external phosphorus (soil solution phosphorus concentration) requirement and to their root morphological traits. Plant species are known to differ not only in their phosphorus requirement but also in their phosphorus utilization efficiency (Manjunath and Habte, 1992; Tisdale *et al*., 1985) [11, 16]. However, inoculation of soil with AM fungus caused significant increase in biomass of both plant species. The effect of AM fungal colonization in increasing the plant growth has been well documented by many workers in many plant species (Habte and Osario, 2001) [6]. The beneficial effect of AM fungal colonization on plant growth has been attributed to an increase in uptake of diffusion-limited nutrients, particularly that of phosphorus and also that of zinc and copper (Mosse *et al*., 1973; Bethlenfalvay, 1992) [3, 1].

Conclusion

Based on titrable acidity data, the acidification potential of *Penicillium* sp. is higher than that of *Bacillus* sp. The extent of AM fungal colonization in roots of pigeon pea was higher than that of maize. The extent of mycorrhizal colonization in roots of pigeon pea and maize grown in soil amended with NCRP formulations were significantly higher than those with

K_2HPO_4 amendment.

Inoculation of soil with AM fungus significantly increased shoot as well as root dry weight of maize and pigeon pea. The magnitude of increase with pigeon pea was higher compared to maize. Application of NCRP formulations did not significantly influence dry weight of shoot and root. Trends noticed with total biomass were similar to dry weight of shoot and root. The highest total biomass was noticed with plants grown in soil amended with KH_2PO_4. The results of this study suggest that applicaiton NCRP bio-acidulated with culture media of *Bacillus* sp. and *Penicillium* sp. increases growth of maize and pigeon pea campare to NCRP without bioactivation and inoculation of soil with AM fungi augments that effect.

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