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Fertilization of *Phaseolus vulgaris* with the Tunisian rock phosphate affects richness and structure of rhizosphere bacterial communities

Darine Trabelsi[a,*], Alaeddine Cherni[a], Ameni Ben Zineb[a], Sana Fitouri Dhane[b], Ridha Mhamdi[a]

[a] Centre of Biotechnology of Borj-Cedria, Laboratory of Legumes, BP 901, Hammam-Lif 2050, Tunisia
[b] National Institute of Agronomy of Tunisia, Laboratory of Forage Production, 43 Charles Nicolle Avenue, Tunis-Mahrajène 1082, Tunisia

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ABSTRACT

Tunisian rock phosphate (TRP) extracted from the region of Gafsa is one of the most promising rock phosphates for soil fertilization. Its appropriate use as a source of phosphate nutrition can substitute chemical fertilizers for sustainable agriculture. The main objective of this study is to investigate the impact of P fertilizers on soil bacterial communities in the rhizosphere of bean (*Phaseolus vulgaris*). Application of chemical triple superphosphate (TSP) or TRP at similar P rates induced a significant increase in alkaline phosphatase and fluorescein diacetate hydrolysis activities. T-RFLP analysis of 16S rDNA revealed that P fertilization affected soil bacterial richness. Application of TRP at the same P rate as TSP was characterized by the stimulation of Actinobacteria both in the rhizosphere and in the uncultivated soil. Some of these Actinobacteria are known by their ability to produce organic acids thus promoting the dissolution of calcium and phosphore. Another important feature was the stimulation of 'mycorrhiza helper bacteria' like Comamonadaceae, Bradyrhizobacteriaceae and Oxalobacteraceae and other plant growth promoting bacteria such as Enterobacteriaceae and Pseudomonodaceae. These bacteria may contribute to the solubilization of phosphate through the production of organic acids (i.e., citric acid) and phytohormones (i.e., indol acetic acid) that stimulate plant root development and help P uptake by increasing the absorptive surface area. However, many putative plant growth promoting bacteria (mainly Sphingomonadaceae and Rhizobiaceae) were found to be inhibited by the chemical TSP fertilizer.

1. Introduction

Agricultural practices over-exploiting the natural resources have decreased soil fertility particularly nitrogen and phosphorus (Duponnois et al., 2005). Phosphorus is, after nitrogen, a major essential macronutrient for plant growth and development and a most important limiting factor for crop production in the majority of soils (Richardson et al., 2009). However, in order to reach optimum crop yields, phosphate fertilizers are applied at high rates, causing unexpected environmental impacts on soil structure, composition, microflora and other soil properties (Akande et al., 2005). Because of this, extension services should search for an alternative, naturally-occurring phosphate source, stimulating the reconstruction of soil texture and productivity. Using rock phosphate, a cheaper source of P, to surmount P deficiency was frequently reported (Kolawole and Tian, 2007). It is supposed to be agronomically more useful and environmentally more feasible than soluble P (Nelson and Janke, 2007). However, not all of the rock phosphate resources are readily plant-available and agronomically effective when applied directly to soils (Verma et al., 2012). The agronomic effectiveness of phosphate rocks is related to their reactivity (rate of P release under favorable soil conditions). Differences in agronomic effectiveness observed among phosphate rocks are largely due to chemical, crystallographic and mineralogic composition of their apatites. Moreover, the availability of P from the rock is influenced by fertilizer management, soil properties and crop type (Hammond et al., 1986). Basically, the availability of P from the rock depends on soil pH, amount of soluble Fe, Al, Mn and Ca, amount of organic matter and microbial activity. A large number of organic acids including aliphatic and aromatic acids have been tested for their ability to dissolve dicalcium and tricalcium phosphates and it was confirmed that action of these acids on their structural characteristics is not only pH dependent. In the rhizosphere, the solubilization of rock phosphate is

* Corresponding author.
E-mail address: trabelsi_darine2@yahoo.fr (D. Trabelsi).

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accelerated in the presence of large microbial communities (acidogenic autotrophs and heterotrophs) through the production of organic acids, phenolic compounds, protons and siderophores (Bojinova et al., 2008; Delvasto et al., 2008) which could improve plant P nutrition (Khan et al., 2014).

Tunisia is the second largest phosphate producer in Africa, turning out about 8 Mt of rock on average in recent years. More than 90% of the phosphate rocks are currently mined from the region of Gafsa (Southwest of Tunisia). The rocks consist mostly of insoluble calcium phosphate, known as apatite. The general formula is $Ca_5(PO4)_3(OH, F, Cl)$ and depending on the last functional group, apatite is referred to as hydroxy, fluoro or chloro apatite (Pattanayak et al., 2007). Tunisian rock phosphate belongs to the francolite (carbonate-fluorapatite) group. This structure tends to make rock phosphates more reactive when directly applied to soil. Consequently, the apatite of reactive rocks has a greater ability to dissolve in the soil and to release available P to the plants than unreactive rocks. According to standard solubility tests for reactivity assessment and various agronomic trials, Tunisian rock phosphate (TRP) is classified as one of the most reactive phosphates and most efficient natural P fertilizer in the world with 30% P. TRP appears to be an efficient source of calcium with regard to plant nutrition because of its high CaO (45–50%) and its high reactivity. Its advantages have been reported agronomically, environmentally and economically (Yingben et al., 2012). Numerous studies were aimed on rock phosphate application and often focused on its impact on shoot biomass and mineral (N, P and K) content (Hosseini et al., 2010; Ndiayei et al., 2009). Other studies explored the effect of different organic and mineral fertilizers containing equal P amounts on bacterial and fungal populations (Beauregard et al., 2010). DGGE analysis demonstrated that these communities varied according to the fertilizer type applied. However, the impact of TRP on microbial communities in contemporary agricultural sites compared with the chemical triple superphosphate (TSP) fertilizer is still poorly investigated. The aim of this study is to investigate the short live effects of TRP fertilizer on (i) grain yield of *P. vulgaris*, (ii) key enzyme activities (FDA, urease and phosphatase), (iii) to detect bacterial T-RFLP profile changes, and (iv) to compare the effect of TRP fertilization to the conventional use of TSP in cultivated and non-cultivated soils.

2. Material and methods

2.1. Experimental design

The experiment was carried out with soil collected from the top layer (0–30 cm) from an agricultural field in the region of Cap Bon in Northeast Tunisia (10° 51′ 19” E, 36° 43′ 32” N). Immediately after collection, the soil sample was air-dried at room temperature and sieved through a 2-mm mesh to remove root residues. The soil was silty clay loam with 3.17% organic matter, 1.41 g/kg total nitrogen, 34.3 mg/kg available P, 91.5 mg/kg available K and pH 7.5. The experimental design consisted of polystyrene trays (40/30/10 cm) containing 8 kg dry soil. Each tray received 9 seeds of *Phaseolus vulgaris* cv. coco (common bean). Non-planted trays were also used as control. The commercial triple superphosphate (TSP) and Tunisian rock phosphate (TRP) were separately used as P fertilizers. The commercial TSP contained 45% P_2O_5 and 15% Ca. TRP contained 30% P_2O_5 and 35% Ca. The detailed chemical composition of TRP had been reported in other studies (Galai and Sliman, 2014; Raja et al., 2014). The contamination level with heavy metals was reported as being below the European Commission limit. TSP was used at the rate of 50 kg P ha^{-1}. TRP was used at two different rates, 50 kg P ha^{-1} (TRP 1X) and 250 kg P ha^{-1} (TRP 5X). Control treatments with no P fertilization were also included. All treatments were conducted in triplicate in a glasshouse (16/8 h photoperiod, 25 °C). Plants were naturally nodulated. After a growing period of 10 weeks, rhizosphere soil and also bulk soil from uncultivated trays were sampled. The rhizosphere soil was collected by gently shaking off the soil closely adhering to the roots (Barillot et al., 2012). Soil samples were passed through a 2-mm sieve and stored at −80 °C after shock-freezing in liquid nitrogen for later DNA extraction and enzyme activity analysis.

2.2. Grain yield determination and enzyme activity assays

At final harvest (75 days), plants were collected and used for grain yield determination. The relative agronomic effectiveness (RAE) of TRP compared to TSP was calculated

as: $RAE = (\gamma_{TRP} - \gamma_{Control})/(\gamma_{TSP} - \gamma_{Control}) \times 100$

where γ_{TRP} is bean grain yield from TRP, γ_{TSP} is bean grain yield from TSP and $\gamma_{Control}$ is bean grain yield from non-fertilized control (0 P).

At this stage, rhizosphere soil samples were collected and used for estimation of some enzyme activities commonly used as indicators of soil quality. The alkaline phosphatase activity (PHOSH) was assayed according to the method of Nannipieri et al. (2011). The released p-nitrophenol (*p*-NP) was measured at 400 nm and reported as μg PNP $h^{-1}g^{-1}$ dried soil. The urease activity was assayed as previously reported (Kizilkaya et al., 2004). The released ammonium was determined at 578 nm and expressed as μg NH3-N g^{-1} dried soil. The determination of Fluorescein diacetate hydrolysis activity (FDA) was carried out as previously reported by Adam and Duncan (Adam and Duncan, 2001). Fluorescein concentration was measured at 490 nm. Results were reported as mg fluorescein g^{-1} soil.

Statistical analysis was performed using the STATISTICA software by the ANOVA/MANOVA module of the STATISTICA program. The HSD Tukey test ($p < 0.05$) was used for mean comparison.

2.3. T-RFLP profiling and data processing

Total community DNA was extracted directly from 0.5 g soil samples using the Power Soil DNA extraction kit (Mobio Laboratories). The DNAs extracted were checked on 1% agarose gel and quantified using the standard quantification marker 1 kb of Qiagen. DNA extracts were then stored at −20 °C until use. Soil bacterial 16S rRNAs were amplified using 27 F (5′ end labeled with 6-carboxyfluorescin (FAM)) and 1492R as previously described (Mengoni et al., 2004). PCR amplification products were digested with *Alu*I (Fermants) and *Hae*III (Fermants) and resolved by capillary electrophoresis on ABI 3130 DNA Genetic Analyzer using GS500 (Applied Biosystems) as size standard (Trabelsi et al., 2011). Chromatogram files from automated sequencer sizing were imported into GeneMapper v4.0 software (Applied Biosystems) by filtering with the default options of the AFLP module. Peaks in the size range 50–500 nt with a peak height above 20 fluorescence units were considered for profile analysis. The non-reproducible TRFs (peaks) that appeared once over the three replications were ignored. Statistical analyses were performed on a binary matrix obtained by linearly combining data from both restriction enzymes. Past 2.02 software package (Hammer et al., 2001) was used to compute Non-Metric Multidimensional Scaling (N-MDS) based on Jaccard distance. Taxonomic interpretation of TRFs was done by the MiCA3 web tool (http://mica.ibest.uidaho.edu/pat.php) by confronting the results from both enzymes. The taxonomic classification was retrieved from NCBI using the taxonomy status