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Isolation of Six Phosphate Dissolving Rhizosphere Bacteria (*Bacillus subtilis*) and Their Effects on the Growth, Phosphorus Nutrition and Yield of Maize (*Zea mays* L.) in Mali

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Abstract: The disadvantages of the long term application of Tilemsi natural phosphate (TNP) on maize (*Zea mays* L.) production has come out because of its low P availability. Some functional soil microbes, such as phosphate dissolving bacteria, have great potential in improvement of P solubility from TNP and P uptake by plants. The present study aimed to isolate and characterize typical phosphate dissolving bacterial strains (*Bacillus subtilis*) from Malian soils, and investigate their role in P uptake by maize grown in soils amended with TNP. The experimental design was a split plot with three main plots of fertilizers sources, i.e., natural phosphate, commercial fertilizer and without fertilizer, and with seven sub-plots of six microorganisms plus the control. The field experiment results have shown that the maize inoculated with the phosphate dissolving bacteria was improved in seed germination, plant growth, plant production (increase yield by 42%), grain and aerial dry biomass (P) content of 34% and 64%, respectively. They have also shown that the locally available TNP can be used by the Malians farmers in maize culture and have comparable production to the one obtained with the costly imported commercial phosphate fertilizer, like the complex cereal. The project has provided information for the combined use of the Mali TNP and phosphate dissolving bacteria *Bacillus subtilis* subsp. *subtilis* (T): DSM10 in improvement of maize production in the country.

Key words: Maize, phosphate, microorganisms, characterization, growth, production, bacteria.

1. Introduction

In Mali, as in almost all arid and semi-arid areas of West Africa, one of the constraints to food security lies in part in the poverty of phosphorus (P) element in soils, which determines the agricultural and forest production [1]. Despite the efforts of the Malian government to ensure sustainable agriculture, the productions are insufficient and limited by external factors, such as the inaccessibility of farmers to agricultural inputs, particularly fertilizers because of their prohibitive cost. Maize production faces declining soil fertility due to land overuse and the high input cost that limit the sustainability. The depletion of soil nutrients is huge, i.e., 25 kg N, 3 kg P and 20 kg K per ha per year and 2%-4% for organic matter [2]. It has been reported that Malian farmers annually invest 40% of their income in soil fertilization [3]. To solve this problem, different approaches have been

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used, such as, the use of fallow, long term crop rotation and contribution of organic manure (garbage, manure, compost, etc.), but these measures have proved either inadequate or impractical because of multiple socio-economic and technical constraints related to the production systems. In addition, mineral fertilization was often used inappropriately. Another alternative was the use of the natural phosphate rock from Tilemsi Valley (NTP) in Northern Mali. This NTP, which is a local resource, could be a solution to soil fertility problem. It is one of the best P fertilizers in West Africa that has good chemical and mineralogical characteristics suitable for direct use. Since 1977, the Institute of Rural Economy (IER) of Mali and its partners have conducted a lot of research work to improve NTP physica-chemical characteristics and P solubility in soil [4-9]. Despite the positive results of research and extension efforts, the NTP is still poorly adopted by the farmers [8]. However, some research results have indicated that a group of microorganisms have shown themselves capable of dissolving rock phosphate from organic acid secretions to put P at the disposal of plant [10-13]. These microorganisms responsible for this dissolution include some bacteria, such as *Bacillus megaterium*, *B. subtilis*, *B. polymyxa*, *Pseudomonas striata*, and some fungi including *Aspergillus awamori*, *Penicillium bilaii*, *P. digitatum* and *Trichoderma* sp.. Due to scientific progress, new perspectives have emerged. The green revolution has enabled the production of a lot of grain to feed millions of humans, the progress of medicine has improved the life span, and other movements of science have made notable advanced in genetics and cell biology. The success of biosciences in the field of sustainable agriculture should be considered in the short and long term. In addition to C and N cycles, P adds an additional interest in improving the biological soil fertility. Indeed, many researchers have shown the solubilizing effect of natural P by soil microorganisms. The higher is the P content of a soil, the higher is the P dose required to maintain fertility [14]. In addition, crops are not taking all the same amounts of P. The changes in its content in soils will also depend on cropping systems. Apart from fertilization and enzymatic decomposition of organic compounds, microbial mobilization of available P would be the only possible way to increase the amount of P available to plants [15]. Thus, inoculation with soil microorganisms capable of solubilizing the phosphates could avoid fixing P [10, 16]. There are some rhizosphere bacteria which are considered as plant growth stimulator, called plant growth-promoting rhizobacteria (PGPR) or plant growth regulators, which are capable of dissolving rock phosphate and insoluble forms of inorganic phosphate by the production of organic acids and proton H^+ ions (acidotic). It is the same for the enzymes (phosphatase) produced by these bacteria. [17]. Among the microorganisms in soil, mycorrhizal fungi are an essential component. It has been shown that their role is particularly important in the uptake of P by plants. If the phosphate can serve as a source of P and optimize plant growth, it is necessary that the conditions of the phosphate use stimulate microbial activity [18]. The soil texture and structure act directly or indirectly on microbial activity. Thus, in a sufficiently moist sandy soil, there is a rapid spread of microbial activity, while in clay soil, clay form with the organic substances of the organo-mineral complexes, in which these substances become less available to the microorganisms causing slowing microbial activity [19]. The TNP dissolving microorganisms naturally present in soil will make the P available to the growing crops. The objective of this study was to investigate the six isolated high phosphate dissolving bacterial strains (*Bacillus subtilis* subsp. *subtilis* (T), DSM10) as bio-fertilizers using DNA sequencing methods on the growth, P nutrition and yield of maize in Mali. It was assumed that some microorganisms isolated from the rhizosphere of maize in Mali can effectively dissolve TNP, and stimulate the growth and improve P nutrition of maize.

2. Materials and Methods

2.1 Materials

2.1.1 Study Site

The research work was done in the experiment station of Samanko, located at 15 km from Bamako district in Mali. It has a latitude of 12°31′552″ N, a longitude de 8°04′906″ W and an altitude of 316.8 m. The soil is of tropical ferruginous type slightly leached of texture sandy loam with 76% sand, 15% silt and 9% clay [20].

2.1.2 Crops

Sotubaka variety of maize from IER was used with an intermediate cycle (110-120 d), average yield of 5-6 ton/ha and is permitted by the producers.

2.1.3 Fertilizers

NTP (30% P_2O_5) was natural phosphate rock from Tilemsi Valley in Northern Mali. It is soluble in citric acid (38.46%), formic acid (61.21%) and water (3.87%) [21]. And the commercial fertilizer, complex cereal (NPK 15:15:15) was used.

2.1.4 Soil Samples

Soil samples were collected from farmers' fields of Kangaba, Bancoumana and Gouani in the Koulikoro region located in the main maize growing zones of the country.

2.1.5 Microorganisms

They were six isolated and selected bacterial strains I_1, I_2, I_3, I_4, I_5 and I_6 capable of dissolving the TNP.

2.2 Experiment Design

The effects of the isolated bacterial strains on the growth, P nutrition and yield of maize were investigated through using a split-plot experimental design on the field with three main plots: fertilization sources from TNP, commercial fertilizers and the control without P. The sub-plots were composed of a non-inoculated control and inoculated with the six TNP dissolving bacterial strains (I_1, I_2, I_3, I_4, I_5 and I_6). The experiment was conducted in three replicates. The isolation and characterization of the microorganisms were carried out as the methods described in Refs. [22, 23], and their ability to dissolve P was tested according to Refs. [24, 25]. The phosphate dissolution mechanism by the production of organic acids and enzymes was described in Ref. [26]; and then the producted substances, i.e., siderophores and indole acetic acid (IAA), can promote the protection and growth of the plant [27, 28].

Identification of bacterial strains was done using the DNA sequencing methods, which is an identification technique of the microorganisms through a genbank. It is composed of the below following steps:

(1) The preparation of bacteria growth culture medium;

(2) Extraction of total DNA using the Fast Biogene soil DNA extraction kit;

(3) Analyze the DNA extract on agarose gel to determine the quality and quantity;

(4) DNA concentration measurement;

(5) Bacteria genotype checking using the Box-PCR (pre-primer and post-primer; 5'-CAA CGG CTA GGC GAC GAC GCT G-3');

(6) DNA preparation for sequencing;

(7) Identification of bacterial strains from the chromosomal gene bank.

Plant growth was measured through leaf number and plant height at 30 d and 60 d after seed germination. Plant and grain (P) content were done through chemical analysis in IER laboratory of Mali and Horticulture Research Center of Laval University, Québec, Canada.

2.3 Statistical Analysis

SAS software (Statistical Analysis Institute System, inc., 1990) with the general linear model (GLM) program was used to increase the accuracy of procedures of variance analysis software. The homogeneity of variance was tested for the variables using Barrtlett tests [29]. Treatments with inhomogeneous medium have been transformed prior to analysis. The number of microorganisms (CFU) of

soil and rhizosphere of maize was a logarithmic transformation in log(CFU), because it has been noticed that the standard deviation increases proportionally with the average. Whenever the calculated *F* is significant, the test of significant difference or protected Fisher's least significant difference (LSD) was used to compare the means of this experiment.

3. Results

3.1 Isolation of Phosphate Dissolving Bacteria

After 7 d of growth of bacterial colonies on the phosphate growth culture medium of National Botanical Research Institute (NBRI), those bacteria surrounded by clear or halo areas, indicating the P dissolution, were selected (Fig. 1).

3.2 Morphological Characterization of Bacterial Strains Isolates

Six pure colonies of bacterial strains (I_1, I_2, I_3, I_4, I_5 and I_6) with gram (-) and (+) were all *Bacillus* and selected according to their TNP dissolving capacity. These colonies were observed under the electronic microscope, and the contained bacteria were photographed with a numeric camera as illustrated in Fig. 2.

3.3 Organic Phosphate (Phytate) Dissolution by the Bacteria

All the six pure colonies of bacterial strains (I_1, I_2, I_3, I_4, I_5 and I_6) have high phytate dissolution potential according to the clear halo diameter surrounding the colonies, but only I_5 and I_6 were selected (Fig. 3).

3.4 Biological Characterization of the Bacteria Strains

3.4.1 Organic Acids Produced by the Bacteria

The presence of the organic acids produced by the bacteria in a culture medium is illustrated by the orange coloration in the Pétri dish, when these acids are in contact with the bromothymol blue.

The orange colored substance chemical analysis has shown the presence of organic acids, like lactic acid, gluconic acid, oxalic acid, succinic acid, and enzymes such as acid phosphatase and pyrophosphatase, which were produced by the selected microorganisms (Fig. 4).

3.4.2 Produced Siderophores and IAA for the Protection and Production of Plant

The pathogenic fungi use soil Fe to cause the damage to crop. The lowest concentrations of Fe for the optimal growth of many microorganisms are approximately from 10^{-5} to 10^{-7} μmol [30]. To maintain the level of Fe, most of the soil microorganisms secrete siderophores to the iron complex, which can facilitate the Fe uptake by bacteria [31]. The orange blue area surrounding the colonies in the Petri dish is an indication of the presence of siderophores (Fig. 5).

The reddish points surrounding the bacterium colony is an indication of the presence of the IAA in the Petri dish (Fig. 6).

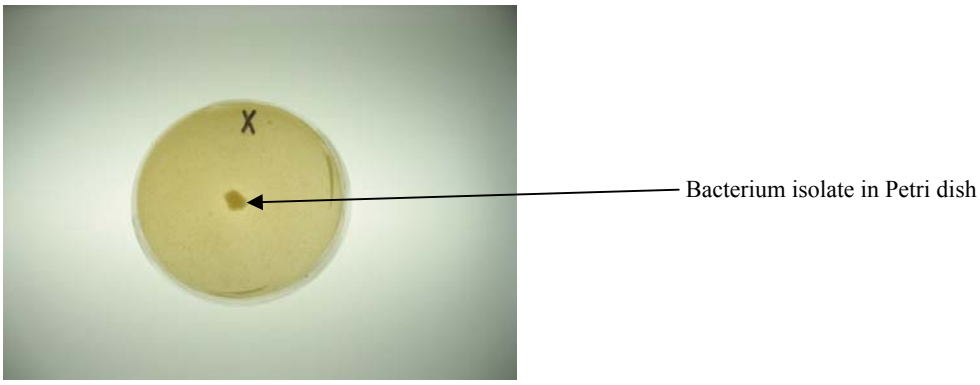

Fig. 1 The bacterium colony surrounded by clear or halo zone, indicating P dissolution.

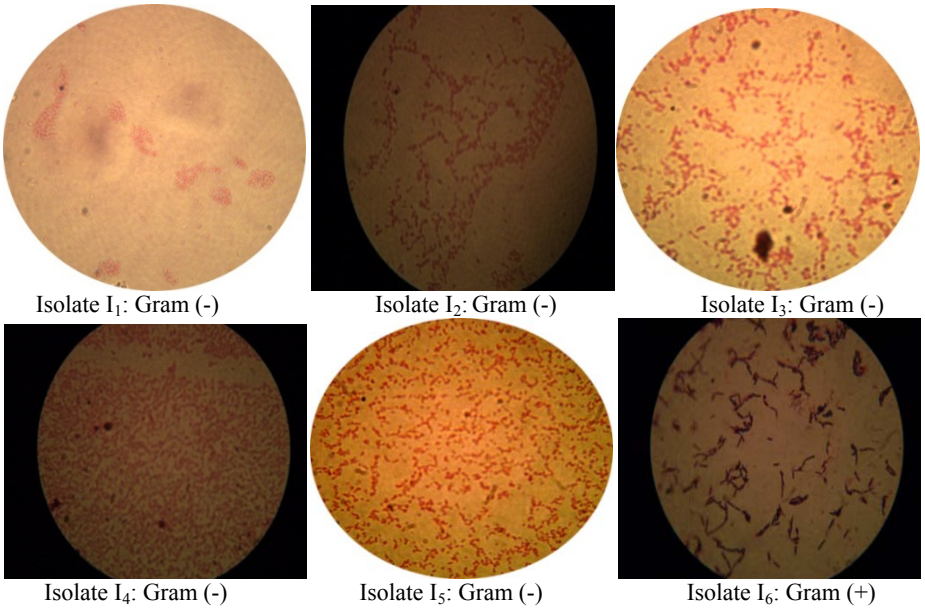

Fig. 2 Six bacteria isolates observed under the microscope.

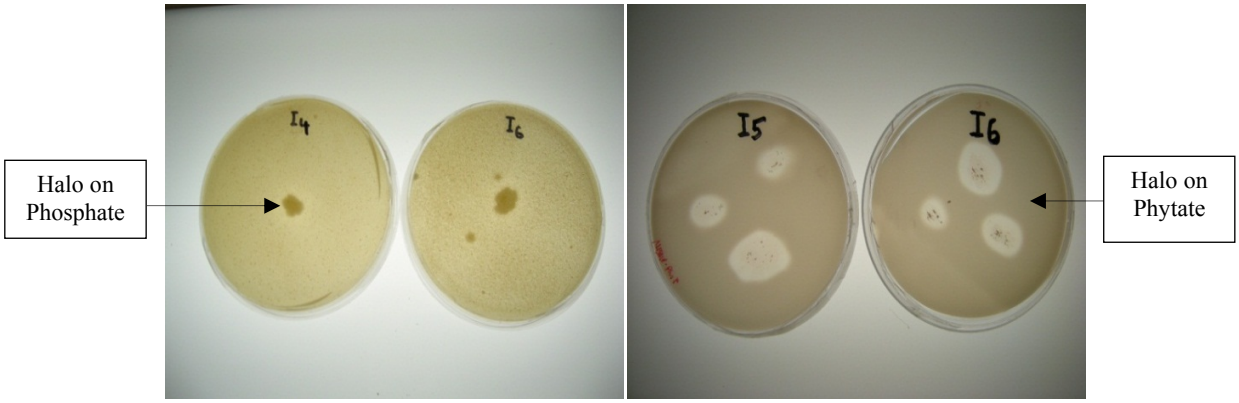

Fig. 3 Phosphate and phytate dissolution by the bacteria strains in the Petri dishes.

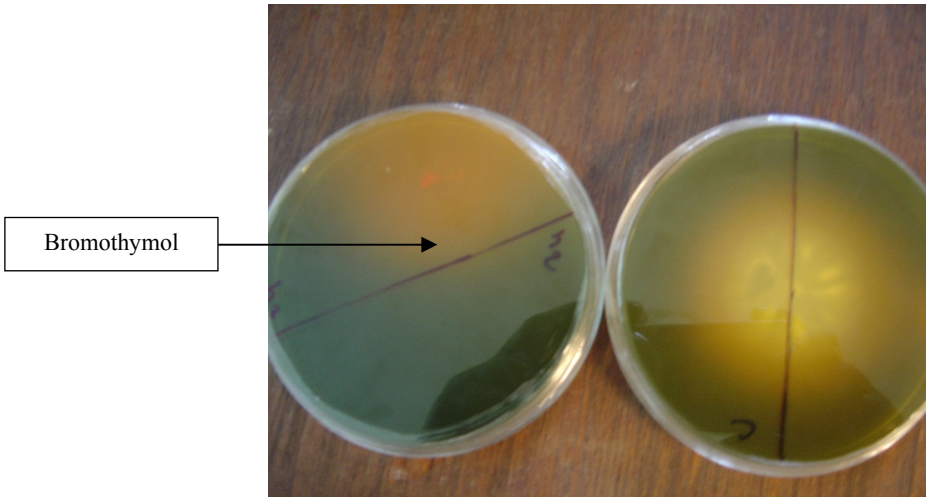

Fig. 4 Organic acids produced by the bacteria isolates.

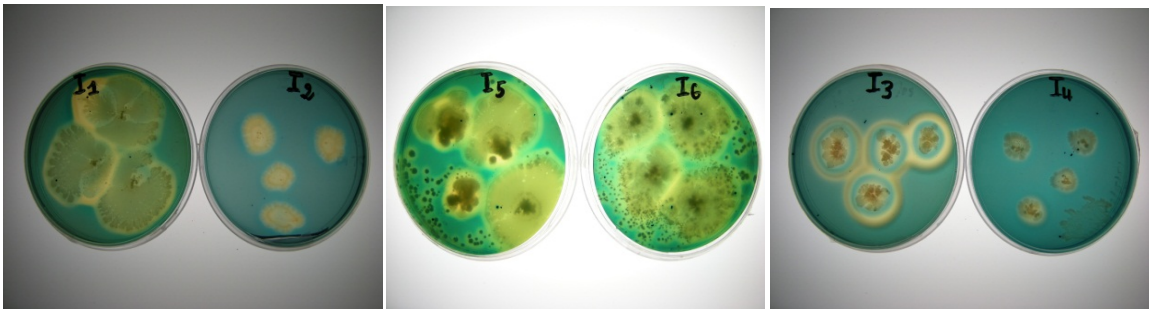

Fig. 5 Siderophores produced by the bacteria isolates.

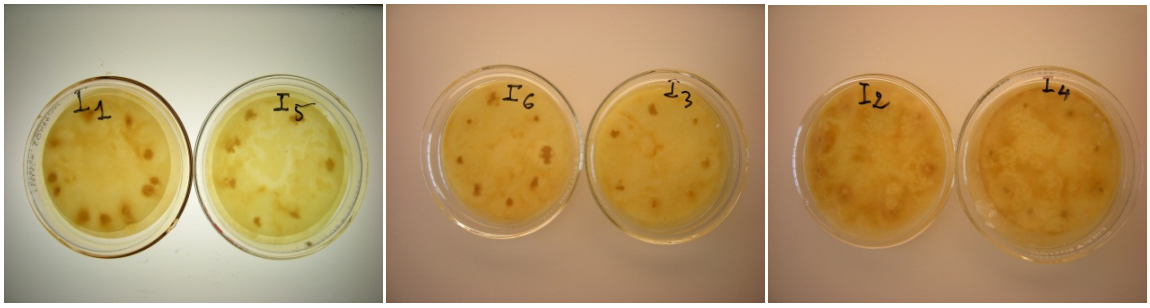

Fig. 6 IAA produced by the bacteria isolates, represented by the reddish points in the Petri dishes.

Table 1 The properties of selected bacteria.

Bacteria strains isolates	Produced organic acids	Produced enzymes	Produced growth substances	TNP solubilization rates (%)
I_1	Lactic, gluconic, oxalic acids	Acid phosphatase, pyrophosphatase	Siderophore, IAA	31.09
I_2	Lactic, gluconic, oxalic acids	Acid phosphatase, pyrophosphatase	Siderophore, IAA	21.74
I_3	Lactic, gluconic, oxalic acids	Acid phosphatase, pyrophosphatase	Siderophore, IAA	17.37
I_4	Succinic acid	Acid phosphatase, pyrophosphatase	Siderophore, IAA	14.67
I_5	Lactic, gluconic, oxalic acids	Acid phosphatase, pyrophosphatase	Siderophore, IAA	12.01
I_6	Lactic, gluconic, oxalic acids	Acid phosphatase, pyrophosphatase	Siderophore, IAA	14.59
Dissolution average				18.58

The selected bacteria are capable of producing organic acids, enzymes, plant growth substances and phosphate solubilization as indicated in the Table 1.

3.5 Effects of Bacterial Inoculation on Growth, P Nutrition and Maize Yield in the Field

Maize seeds were inoculated with strains of rhizospheric phosphate dissolving bacteria. The inoculation volume was determined by the formula of the cell growth curve for each bacterium, as Eq. (1):

$$Y = ax + b \quad (1)$$

where, x is equal to the optical density of the bacterial suspension. Thus, the formula obtained for each bacterial strain is:

I_1: $Y = 2.442x + 4.415$;

I_2: $Y = 1.300x + 6.391$;

I_3: $Y = 1.864x + 3.862$;

I_4: $Y = 0.748x + 6.152$;

I_5: $Y = 1.090x + 6.216$;

I_6: $Y = 1.331x + 6.619$.

After the seeds germination, the bacterial strains colonize the roots in contact with the applied phosphate, and they dissolve the phosphate by the production of organic acids.

3.5.1 Effects of Bacterial Inoculation on Growth

The interaction effect of P source and bacterial strains inoculation was more effective with the TNP than with the commercial fertilizer (complex cereal) and no P treatment at $P < 0.05$ (Table 2).

3.5.2 Effect on P Nutrition of Maize

During the two phases of plant growth (30 d and 60 d), the P uptake by maize was higher in soils treated with TNP compared to those of treated with complex and control treatments (Figs. 7 and 8).

3.5.3 Effect of the Bacteria on Grain Yield Production and Stover Yield of Maize

The isolates I_5, I_2 and I_6 showed the highest ability to improve plant growth with grain yield of 3,911, 3,793 and 3,466 kg/ha, respectively. I_2 and I_6 had an increasing effect of 545 kg/ha and 218 kg/ha, respectively, in TNP conditions compared to the control, while I_5 has increased up to 604 kg/ha in complex conditions (Fig. 9).

The isolates I_5, I_1 and I_2 have the best effects with the TNP, while I_5, I_6 and I_3 have the same effects with the complex cereal (Fig. 10).

The remarque was that maize inoculated with selected phosphate dissolving bacteria in the presence of TNP can produce grain yields (kg/ha) and dry stover (kg/ha) comparable to those produced by the imported chemical P fertilizers.

Table 3 showed the consistent and remarkable effect of NTP in the presence of inoculation on grain yield, 1,000 kernel weight, dry stover, P content and root volume. The NTP in the presence of inoculation made the difference compared to other factors for the measured parameters.

Table 2 Interaction of P sources and bacterial strains inoculation on maize growth parameters.

P sources	Height at 30 d of growth (cm)	Height at 60 d of growth (cm)	No. of leaf at 30 d of growth	Root at 30 d of growth (kg/ha)	Root at 60 d of growth (kg/ha)	Stalk at 30 d of growth (kg/ha)	Stalk at 60 d of growth (kg/ha)
TNP + B	61.11^{a}	74.68^{a}	6.37^{a}	32.25	130.00	215.00^{a}	850.70
CC + B	51.00^{b}	69.87^{a}	6.32^{ab}	45.00	147.50	245.00^{a}	793.27
WP + B	55.32^{b}	70.81^{b}	6.00^{ab}	17.50	147.50	115.00^{b}	800.80
LSD (0.05)	4.93	ns	0.33	10.00	ns	42.25	ns

TNP = Tilemsi natural phosphate; CC = complex cereal; WP = without P; B = bacterium.

LSD = least significant difference; ns = not significant. The numbers followed by the same letter are not statistically different ($P < 0.05$).

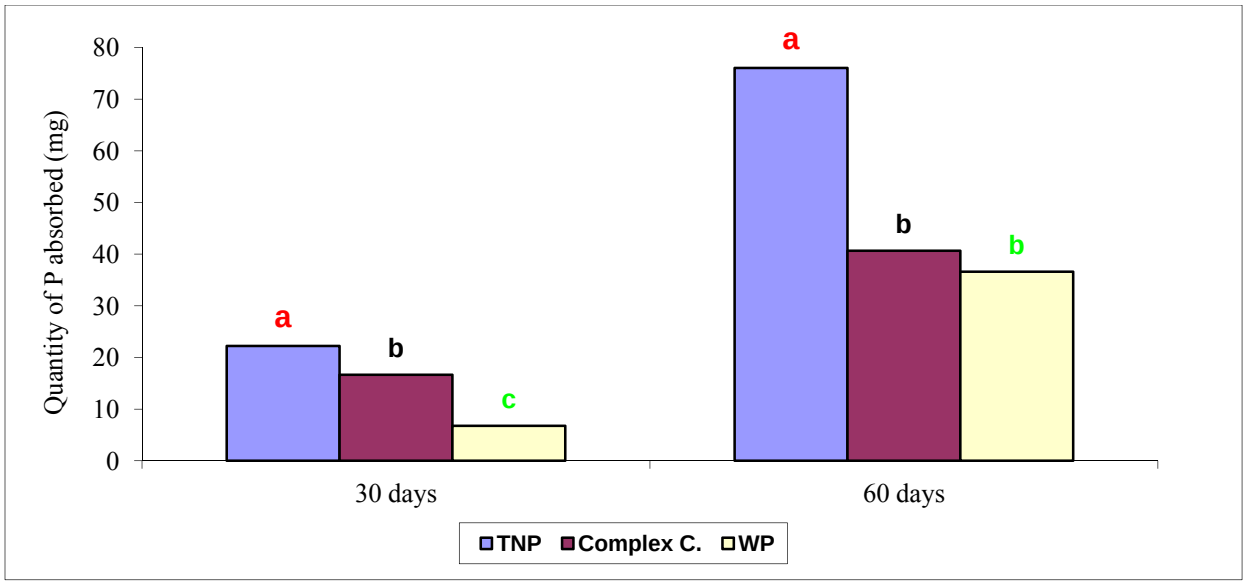

Fig. 7 P source and inoculation with bacteria effect on P absorption (mg) by maize plant after 30 d and 60 d of growth.

Means followed by the same letters are not statistically different at $P < 0.05$.

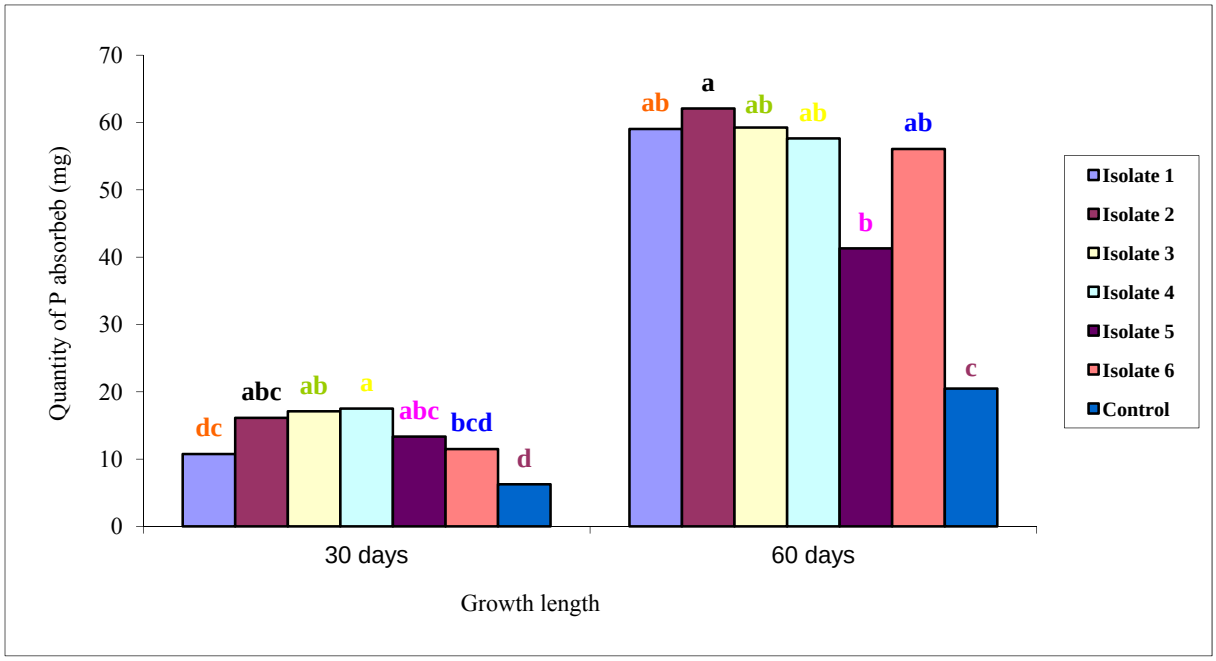

Fig. 8 Inoculation effect on P absorption (mg) by maize plant after 30 d and 60 d of growth.
Means followed by the same letters are not statistically different at $P < 0.05$.

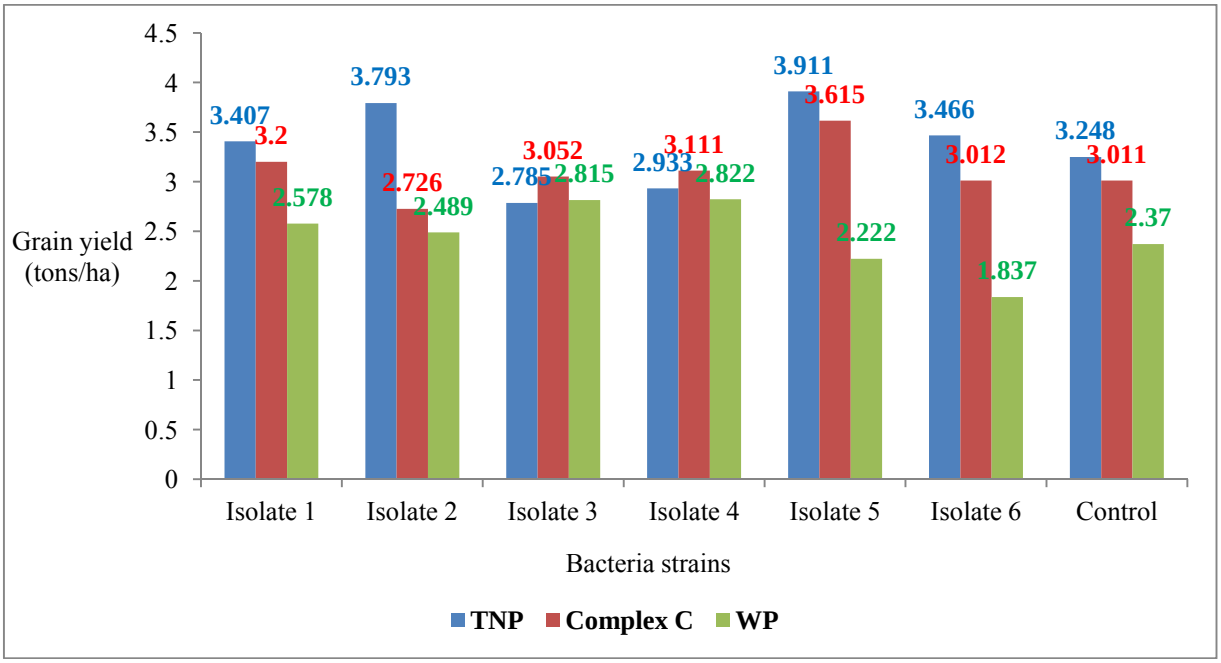

Fig. 9 Inoculation effect on the maize Sotubakagra in yield (kg/ha).

Table 3 P sources effect on stover, P content, grain yield, 1,000 grains weight, dry stover and root volume.

Factors	P content (kg/ha)	Grains yield (kg/ha)	1000 grains weight (g)	Dry Stover (kg/ha)	Roots volume (cm^3)
TNP	3.992[a]	3,377.70[a]	229.13[a]	3,606.00[a]	145.55[a]
CC	3.859[a]	3,119.60[b]	219.17[b]	3,552.80[a]	117.56[b]
WP	3.172[b]	2,447.60[c]	209.50[b]	2,662.60[b]	110.35[b]
LSD	3.52	253.56	9.27	425.55	26.36

[a-c] Number followed by the same letter is not significantly different ($P < 0.05$).

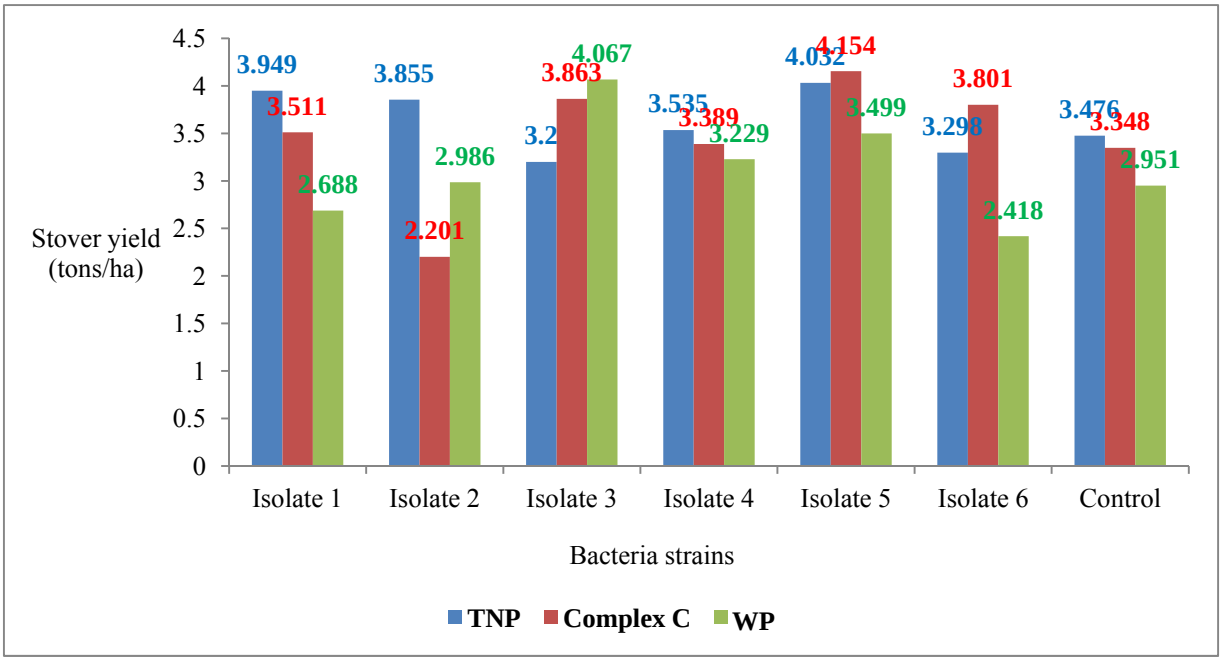

Fig. 10 Inoculation effect on the maize Sotubaka dry stover (kg/ha).
TNP = Tilemsi natural Phosphate; C = cereal; WP = without phosphorus.

3.5.4 Identification of Bacteria Lines by Molecular Biology Methods

Based on the partial sequence of the 16S ribosomal DNA bands, all the bacteria isolates were shown much closer to *Bacillus subtilis* subsp. *subtilis* DSM10 (Ehrenberg 1835) Cohn 1872 with 99% similarity.

3.5.4.1 PCR Products Run on Agarose Gel to Compare Fingerprints

The PCR product showed that all the bacteria had similar genotype, except for the isolate I_6 strain, which appeared to have a different genotype (Fig. 11).

3.5.4.2 Extracted Plasmid DNA

After confirming the presence of the proper sized cloned insert using PCR (3 kb for vector and 1.5 kb for template), one clone from each isolated was selected and plasmid DNA was extracted to be used

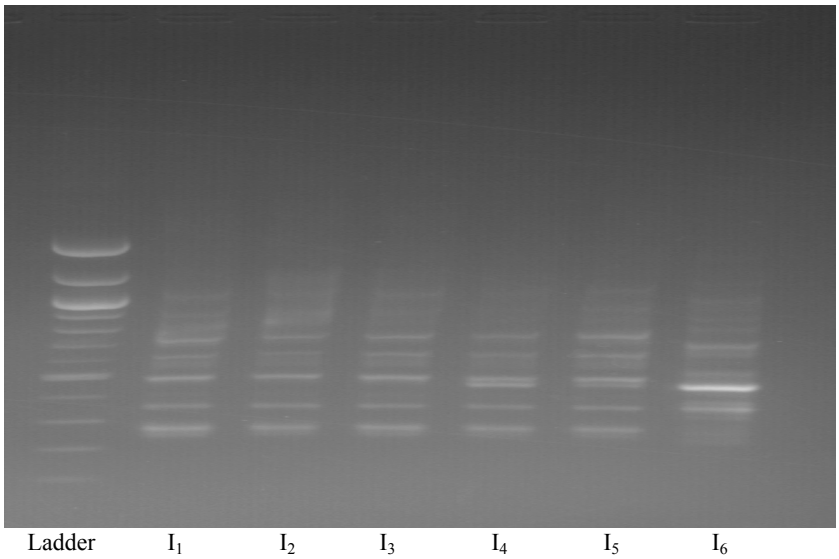

Fig. 11 PCR product on agarose gel.

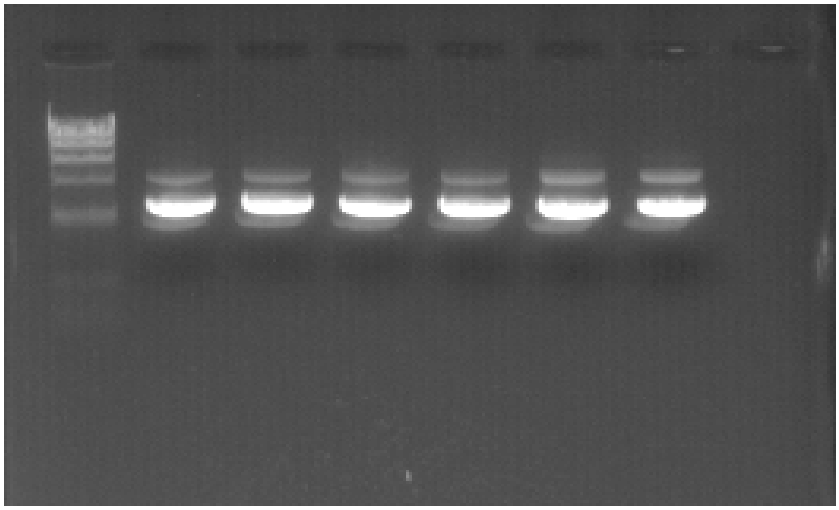

Fig. 12 Electrophoresis of plasmid DNA.

Table 4 Best matching scores to sequences deposited in the GenBank database.

Isolate	BLAST match	GenBank ID	Score bits	Identity (base matches)	% identity
I_1	*Bacillus subtilis* subsp. *subtilis* strain SB 3130	GU191916.1	2837	1540/1542	99
	Bacillus subtilis BSn5	CP002468.1	2832	1539/1542	99
	Uncultured *Bacillus* sp. clone TCCC 11231	EU567055	2839	1539/1540	99
I_2	*Bacillus subtilis* subsp. *subtilis* strain SB 3130	GU191916.1	2833	1538/1540	99
	Bacillus subtilis BSn5	CP002468.1	2828	1537/1540	99
I_3	*Bacillus subtilis* subsp. *subtilis* strain SB 3130	GU191916.1	2826	1538/1542	99
	Bacillus subtilis BSn5	CP002468.1	2820	1537/1542	99
I_4	*Bacillus subtilis* BSn5	CP002468.1	2832	1539/1542	99
	Bacillus subtilis subsp. *subtilis* strain SB 3130	GU191916.1	2826	1538/1542	99
I_5	*Bacillus subtilis* BSn5	CP002468.1	2820	1537/1542	99
	Bacillus subtilis subsp. *subtilis* strain SB 3130	GU191916.1	2815	1536/1542	99
I_6	*Bacillus subtilis* BSn5	CP002468.1	2832	1539/1542	99
	Bacillus subtilis subsp. *subtilis* strain SB 3130	GU191916.1	2826	1538/1542	99

Table 5 Best matching scores to sequences from bacterial type strains deposited in the RDP database.

Isolate	Type strain match (RDP)	GenBank ID	Similarity score	Identity (base matches)	% identity
I_1	*Bacillus subtilis* subsp. *subtilis* (T); DSM10	AJ276351	0.990	1515/1517	99
I_2	*Bacillus subtilis* subsp. *subtilis* (T); DSM10	AJ276351	0.996	1516/1517	99
I_3	*Bacillus subtilis* subsp. *subtilis* (T); DSM10	AJ276351	0.983	1513/1517	99
I_4	*Bacillus subtilis* subsp. *subtilis* (T); DSM10	AJ276351	0.985	1513/1517	99
I_5	*Bacillus subtilis* subsp. *subtilis* (T); DSM10	AJ276351	0.978	1511/1517	99
I_6	*Bacillus subtili* ssubsp. *subtilis* (T); DSM10	AJ276351	0.978	1514/1517	99

for sequencing as represented in Fig. 12.

3.5.4.3 Sequenced Isolates

Sequence analyses of the isolates most closely related sequences from the databanks are listed in Table 4 (using GenBank) and the best matching type strains are in Table 5 (using RDP database).

Table 4 shows that all isolated strains of phosphate dissolving bacteria from Mali closely matched the same sequences that have been deposited into the GenBank database. Sequence identity is 99% to

Bacillus subtilis subsp. *subtilis* strain SB 3130 and *Bacillus subtilis* BSn5. Neither of these are type strains, but the whole genome sequence has been determined for *Bacillus subtilis* BSn5. If this strain also has the ability to dissolve phosphate, it may be possible to identify potential genes involved in this process. The closest match to a type strain is also to *Bacillus subtilis* subsp. *subtilis* DSM10 (Ehrenberg 1835) Cohn 1872. The match to this sequence is also at 99%. This did not score higher when searched in GenBank, because the deposited sequence length for this strain is shorter. The scores using the BLAST algorithm is based both on number of sequence matches and length of the sequence. However, since *Bacillus subtilis* subsp. *subtilis* DSM10 is the type strain, this provides further strength to the argument that these phosphate solubilizing bacteria are also strains of *Bacillus subtilis*.

There were three genotypes found by Box-PCR. Two of the genotypes are highly similar, suggesting that the genomes are very similar. Only isolate I_6 strain appears to have a different genotype. All isolates match one species, *Bacillus subtilis*, at 99% identity based on almost the whole gene sequence.

4. Discussion

Six isolates are allowed be to retained by the selection of microorganisms based on their ability to dissolve the NTP and the amount of soluble P product, and they were classified according to their TNP dissolution efficiency in solid medium varying from 114.29% to 300% P dissolved. By studying *Pseudomonas fluorescencelines*, *Bacillus megaterium* and *Azospirillum* spp., El-Komy [32] obtained values of 128% to 150% on the *in vitro* dissolution of the calcium phosphate. Mallaiah and Sridevi [33] has demonstrated the effectiveness of solubilizing tricalcium phosphate on solid agar, and found that five isolates from 46 rhizobia was of the order of 33% to 150%. According to Kuccey et al. [34], the solubilization of insoluble phosphates depends on a multitude of factors, such as the decrease of pH, the type of microorganisms, the nature of organic acids produced by microorganisms and the nature of insoluble phosphate. In general, the isolates stimulated germination at plot level, positively because it has changed from 60% to 90%, suggesting that the isolates stimulated germination and seedling growth. Antoun and Kloepper [17] bind this action to the production of activation of the germination and growth hormone substances. In addition, in this work it was observed that all the isolates produced siderophores and indole acetic acid. Dommergues et al. [35] reported that certain microorganisms of the rhizosphere produce vitamins, such as thiamine, nicotinic acid, pantothenic acid and others that stimulate the germination and plant growth. It is therefore possible that the stimulation of the germination observed with these bacteria was due to the production of these substances or to the increase of P concentration of the soil by these microorganisms. Rodriguez and Fraga [36] also showed that soil P deficiency increases not only the germination time but also the time between the emergence of the first leaf to tillering. This may explain the latency observed with the bacterial isolates. It has been established for some time that plant growth can be enhanced by certain plant growth promoting rhizobacteria or regulatory bacterial plant growth that colonize the roots of crops [37, 38]. However, the mechanism for regulating the growth of plants by plant growth promoting bacteria has not yet been sufficiently elucidated. From the analysis of the field trials results, it was observed that phosphate fertilization and inoculation with these bacteria significantly influenced the growth parameters, such as plant height after 30 d of growth, dry biomass and the root volume after 60 d of growth. The isolate effect in the presence of NTP resulted in an average increase in the maize Sotubaka height of 18.88 cm, and 24.16 cm in the presence of complex cereal in the first 30 d of growth. This indicated that the phosphate fertilization and inoculation have a

positive effect on plant height during that period. These results are consistent with those of Rock et al. [39], who reported that certain microorganisms, such as *Enterobacter* sp., *Pseudomonas* sp., *Serratia* sp., are effective in the solubilization of inorganic phosphates used for the cultivation of maize. Babana and Antoun [40] have shown that inoculation with microorganisms in the presence of P fertilizer significantly influenced the height of wheat after eight weeks of growth. Glick [41] also showed that rhizosphere bacteria may accelerate plant growth by different mechanisms, including the dissolution of insoluble phosphates in the soil. The plant species as well as cultivars of the same species can have different behavior vis-à-vis soil microorganisms. Thus it was noted that the phosphate fertilization in the presence of inoculation increased root volume of 23 cm^3 and 7 cm^3 for complex cereal conditions compared to the non-inoculated control. Hinsinger and Gilkes [42] indicated that the maximum agronomic effectiveness of phosphate on crops is partially translated by acidifying the soil type and the high root density. A high root density facilitates the use of a larger volume of soil for P due to the presence of a high number of fine roots by unit volume of soil. As for the production of dry matter, inoculation in the presence of NTP caused a grain yield increase of 12.33% compared to the complex cereal and 42.72% compared to treatment without P. Similarly, an increase of 8.87% and 23.51% were recorded for dry biomass after 60 d of growth, 12.28 g and 31.55 g for the 1,000 grains weight. So, it was observed that inoculation in the presence of NTP improved grain yield and dry biomass per hectare compared to the complex cereal and the control without inoculation. These results agree with those obtained by Hameeda et al. [43], who reported that an experiment on five bacteria lines with the power to dissolve the phosphate caused an increase in the maize dry biomass production of 20% to 40%. Peix et al. [44] found a similar result on barley inoculated with the rhizobia strain (*Mesorhizobium mediterraneum* PECA21) in the presence of insoluble phosphate. Chung et al. [45] found that with the addition of insoluble phosphate in soil inoculated with the microorganisms dissolvent, the phosphate significantly increased the dry matter production of crops from 4% to 18%. Khan, M. R., and Khan, M. S. [46] noted that the yield of tomato increased significantly by 23%, when it was inoculated with *Aspergillus awamori*, a solubilizing fungus phosphates. After 30 d and 60 d of growth, maize plant inoculated with NTP dissolving bacteria significantly increased P absorption by plants. By the inoculation in the presence of NTP, the maize Sotubaka showed an increase in the grains P content of 25.11% compared to non-inoculated control and 13.28% in the presence of the complex cereal, and 54.57% and 10.92% for the dry biomass. These results are consistent with those obtained by Gaur [11] and Chung et al. [45] in barley. This has allowed to say that maize inoculated in the presence of NTP can produce grain yields (kg/ha), dry biomass (kg/ha) and P content (%) comparable to those produced by the complex cereal.

By the method of molecular biology, six identified bacteria were *Bacillus* spp., including three producing the spores (I_3, I_5 and I_6). This production of spores can be advantageous in the context of an industrial formulation of the inoculum on one hand and on the other hand by their resistance to drought. The other three (I_1, I_2 and I_4) which do not produce spores are more adapted to the formulation of the liquid inoculum. These results are consistent with that in Refs. [10-13], which showed the *Bacillus megaterium*, *B. subtilis*, *B. polymyxa* and *Pseudomonas straita* among phosphate solubilizing microorganisms.

5. Conclusions and Recommendation

The present field experiments have shown that the TNP can be used by Malian farmers for growing maize, which has even better function compared with that of high-cost phosphate fertilizer. Inoculation with

the selected *Bacillus subtilis* in the presence of TNP improves the growth and the production of maize of 12.33% compared to the commercial chemical fertilizer. These *Bacillus subtilis* strains can also produce growth promoting substances (siderophores and IAA) for plant protection and production. Thus, they can be used in agriculture as bio-inoculants. Finally, by developing microbial inoculum products, cereals producers can inoculate theirs crops with functional rhizosphere bacteria to improve soil fertility and production.

However, the on-station trials and on-farm tests should be implementd in different agricultural regions of Mali on the combination of TNP and selected microorganisms including mycorrhizae, as well as in West Africa sub-region countries having natural phosphate deposits as Mali, Burkina Faso, Niger, Nigeria, Togo and Senegal. The efficiency of inoculation in the presence of TNP in crops rotation systems also need to be studied. Besides, develop locally an inexpensive and simple method for the production of inoculum containing microorganisms and TNP or cereal seeds inoculation techniques for farmers. Proponents results of this sub-regional study can be a solution to the problem of the non-use of phosphate rocks in the sub-region in agriculture. An extensive sub-regional research program will benefit not only farmers but also economic operators in the West African sub-region.

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