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Bacillus thuringiensis Strains Isolated from Agricultural Soils in Mali Tested for Their Potentiality on Plant Growth Promoting Traits

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To screen of the multiple plant growth promoting activities of some *Bacillus thuringiensis* strains isolated from Malian Agricultural soils and evaluate their ability to improve maize seed germination and seedling vigor *in vitro*.
Study Design: Strains of *Bacillus thuringiensis* (*Bt*) used in this study belong to collection of the Laboratory of Research in Microbiology and Microbial Biotechnology (LaboREM-Biotech) isolated from different agricultural soils of Mali.
Methodology: Different tests, namely: Phosphate solubilization, Siderophore production, Indol acetic acid cellulase and chitinase production tests were performed to confirm the PGP characteristic of the insecticidal *B. thuringiensis* strains screened. *In vitro* test was performed in the laboratory to confirm the capacity of these bacteria to enhance maize germination and seedling vigor.
Results: All tested *Bacillus* strains solubilize efficiently insoluble phosphate, but *Bt*I4″ showed the

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highest clearance zone around its colony. In this study, except in *Bt*I4', the siderophore production was significantly elevated in the other Bt strains tested. Only *Bt*D5 was able to produce Indol acetic acid. Contrary, except *Bt*D5, all the isolates produce chitinase and cellulase. Exept IAA, the isolate *Bt*I4″ produce all the tested compounds and showed the highest % seed germination and seedling vigor.

Conclusion: In the current study, plant growth promotion analysis of three *B. thuringiensis* strains from Malian agricultural soils were assessed in relation with maize seed germination and seedling vigor. These tested *Bt* strains showed several plant growth-promoting characteristics. These activities may allow the use of these isolates for plant growth promotion. Future work will address the applications of the selected bacteria in biocontrol and plant growth promotion. Insect pest biocontrol, enhancement of plant nutrition and production of phytohormon are the mechanisms involved.

Keywords: Bacillus thuringiensis; PGPR; maize; biocontrol; Mali.

1. INTRODUCTION

Plant beneficial microorganisms are known to antagonize phytopathogens through competition for niches (e.g. iron through siderophores synthesis); parasitism, that may involve production of hydrolytic enzymes such as chitinase, β-1,3glucanase, protease and cellulose, that lyse pathogen cell walls, inhibit the pathogens by secreting anti-microbial compounds and induce systemic resistance in host plants [1]. There are several species of *Bacillus* known as plant growth and health supporting in nature because of beneficial characteristic features which act directly and indirectly [2,3]. The principal mechanisms of plant growth promotion include: production of phytohormones such as indole acetic acid (IAA), solubilization of phosphate, siderophore production, antibiosis, inhibition of plant ethylene synthesis, production of volatile compounds such as HCN and induction of plant systemic resistance to pathogens [3-5]. One or more of these mechanisms may contribute to the increases obtained in plant growth and development that are higher than normal for plants grown under standard cultivation conditions.

Bacillus thuringiensis strains can enhance biomass production and protect plants against insect pests [6]. In recent years, studies about *Bacillus thuringiensis* (*B. thuringiensis* or Bt) and their insecticidal activity coupled with plant growth promotion of cultivated crops have attracted the attention of several investigators [7]. These bacteria can promote plant growth under stress conditions through siderophore production, indole acetic acid production, phosphate solubilisation and antimicrobial enzyme production [8]. Recent studies have revealed that these bacteria could promote plant growth and protect plants against important crop microbial pathogens [9]. The present study was designed to screen of the multiple plant growth promoting activities of some *Bacillus thuringiensis* strains isolated from Malian Agricultural soils and selected for their high insecticidal activities against maize and rice insect pests [2,10] and evaluate their ability to improve maize seed germination and seedling vigor *in vitro*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

*Bacillus thuringiensis Bt*D5*, Bacillus thuringiensis Bt*I4' *and Bacillus thuringiensis Bt*I4″ in this study was isolated from Malian agricultural soils [2], and maintained in the LaboREM-Biotech, Faculty of Sciences and Techniques, bacterial culture collection in Mali. These bacteria, selected for their high insect pest biocontrol capacity, was assessed for their Plant Growth Promotion (PGP) activities.

2.2 Plant Growth Promotion Activities

2.2.1 Phosphate solubilization

The ability of tested *B. thuringiensis* strains to solubilize phosphate was evaluated qualitatively using NBRIP solid medium with tricalcium phosphate or rock phosphate as sole source of phosphorus. Each bacterial culture was spot inoculated in the center of the plate and incubated at 28±2℃ for 10 days. Phosphate solubilization was assessed by measuring the clear/halo zone around each colony. The halo zone was calculated by subtracting bacterial colony diameter from the total halo zone diameter [3].

2.2.2 Celulase test

Isolated bacteria were tested for the ability to degrade Carboxymethyl cellulose (CMC) according to the method described by [11]. Plates with single colonies were tested with the CMC assay on solid media by covering the petri dishes with Congo red dye. After an appropriate incubation period at 37℃, the agar medium was flooded with an aqueous solution of Congo red (1 mg/ml) for 15 min. The Congo red solution was then poured off, and plates were further treated by flooding with 1 M NaCl for 15 min. The visualized zones of hydrolysis were stabilized by flooding the agar with 1 M HCl, which changes the dye color to blue and inhibits further enzyme activity. CMC degradation was indicated by a clear zone around the colonies. Enzyme activity was indexed as the diameter of the colony plus the clear zone around it divided by the diameter of the colony. Two measurements were taken from each colony, with at least two colonies from the same bacterial isolate.

2.2.3 Indole acetic acid production

Production of Siderophore production as determined as described by [11]. The bacterial test cultures were grown separately on nutrient broth enriched with 50 mg/ml of L-tryptophan at 30℃ for 48 h. Fully grown cultures were centrifuged at 8000 rpm for 10 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid and 1 ml 0.5 M $FeCl_3$ solution). The tubes were kept at room temperature for 20 minutes. Development of pink color indicates IAA production.

2.2.4 Siderophore production

The universal CAS assay [12] was used to test the siderophore production ability of *Bacillus thuringiensis* isolates. Plates containing the universal CAS solid medium were inoculated with the *Bt* isolates and were incubated in the dark at 28℃ for 6 days. Bacterial growth was monitored daily to observe change in color from blue to orange color in the CAS medium. The colonies with orange zones were considered as siderophore producing isolates.

2.2.5 Determination of seed germination and seedlings vigor activities

To evaluate the role of the studied *Bacillus thuringiensis* (*Bt*) strains in the growth of maize seedlings, an experiment was conducted with the strains *Bt*D5, *Bt*I4' and *Bt*I4″ [9]. Treatments included an un-inoculated maize control, maize + *Bt*D5, maize + *Bt*I4' and maize + *Bt*I4″. Bacterial strains were used as maize seed treatments. Seeds of maize (cv. Dembanyuman) were surface-sterilized with 0.02% sodium hypochlorite for 2 min, and rinsed thoroughly in sterile distilled water. For inoculation seeds were coated with 20% cassava starch as an adhesive and rolled into the suspension of bacteria (10^8cfu/ml) with powder of charcoal uniformly coated. Germination tests were carried out by the paper towel method. 25 seeds for each treatment with three replications in completely randomized design and incubated at 28℃. After 7 days the number of germinated seeds was counted. Root and shoot length of individual seedling was measured to determine the vigor index with following formula:

> Vigor index= (mean root length +mean shoot length) × % germination [13].

A two-factor analysis of variance (treatment and repetitions) for each parameter was performed using the general linear models procedure of SAS [14]. The Least Significant Difference (LSD) test at probability level 0.05 was used to separate the means when the ANOVA F-test indicated a significant effect of the treatments.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Plant growth promoting parameters

Plant growth parameters namely siderophoric activity, indole acetic acid production, phosphate solubilization, cellulase and chitinase production were observed qualitatively. Results from these observations are presented in Table 1.

Analyses of data in the Table 1 showed an ability to solubilize insoluble phosphate source by all the tested *B. thuringiensis* strains. But, the strains *Bt*I4″ showed the highest phosphate solubilizing activity (Table 1, Fig. 1A).

Except in BtI4', the siderophore production was significantly elevated in BtI4" and BtD5. But BtI4" produce more siderophore than the strain BtD5 (Table 1).

In this study the BtD5 was the only strain who produce significant quantity of Indol Acetic Acid

(Table 1). IAA production by microorganisms enhances plant growth by promoting cell elongation, cell division, root initiation, and/or altering the expression of specific genes under stress conditions [8]. Elevated levels of heavy metals in soils and variation in soil pH also interfere with P uptake by maize plant, but PGP bacteria can compensate for this deficiency by solubilizing phosphorus [15]. Cellulase and chitinase production are important traits in pathogen biocontrol by PGP bacteria. Our result showed that except the BtD5 who didn't produce cellulase, all the tested bacterial strains produce these to enzymes. The highest cellulase production activity was recorded for the strain *Bt*I4″ (Fig. 1B), while the strain BtD5 produce the highest quantity of chitinase (Table 1).

3.1.2 Maize seed germination and seedlings vigor

Inoculation with *Bacillus thuringiensis* strains significantly affected seed germination, root and shoot growth, and seedling growth and vigor of maize cv. Dembagnuma (Table 2). No significant effect was observed between the different repetitions. This indicates, for example, that the % germination of seed treated or not by *Bacillus thuringiensis* will not be affected differently by the repetitions.

The germination percentage, growth and vigor of maize seedlings as influenced by the application of Plant growth promoting *Bacillus thuringiensis*; such as control, *Bt*I4', *Bt*I4'' et *Bt*D5 from Malian agricultural soils are presented in Table 3.

Seed Inoculation significantly enhanced seed germination and seedling vigor of maize. However, the rate of enhancement varied with bacterial strains. All bacteria, significantly increased seed germination over non treated control. The highest percentage germination (94%), seedling growth (36.08 cm/seedling) and vigor index (3391.52) were recorded in maize seedlings grown with BtI4'' treatment. The lowest germination percentage (72%) seedling growth (20.67 cm/seedling), vigor index (1488.24) were recorded in maize seedlings grown without Plant Growth Promoting *B. thuringiensis* treatment (control). But, all the PGPRs *B. thuringiensis* showed higher positive effect, on all measured parameters, compared to control (Table 3).

Table 1. Phosphate solubilization, siderophore and enzymes (cellulase and chitinase) production by the *B. thuringiensis* (*Bt*) strains studied

B. thuringiensnsis strains	Phosphate solubilization	Cellulase	Chitinase	Siderophore	Indole acetic acid
BtD5	+	-	++	+++	++
BtI4'	+	+	+	-	±
BtI4″	++++	++++	+	++++	-

- No halo, ± Halo not very clear, + Halo < 1 mm, Halo 1 < 2 mm, +++ Halo =2 mm, ++++ Halo > 2 mm.

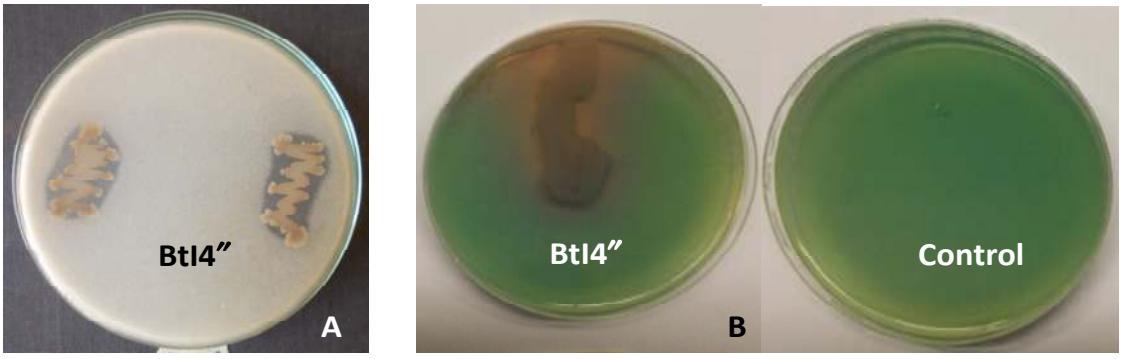

Fig. 1. Phosphate solubilization (A) and siderophore production (B) by the *B. thuringiensis* strains isolated from agricultural soils of Mali

Table 2. Summary from the analyses of variance for germination (%), root length (cm), shoot length (cm), seedling length (cm) and vigor index of maize cv. Dembagnuma inoculated with PGPRs *B. thuringiensis*

Source of variation	Degree of freedom	Mean squares				
		% germination	Root length (cm)	Shoot length (cm)	Seedlings length (cm)	Vigor index
Treatments	3	2466.67**	75.07**	262.93**	1326.86**	18842803,70**
Repetitions	24	0.83NS	3.77NS	0.10NS	0.67NS	8439,19NS
Error	72	1,72	3.82	0.105	0.739	7224,731

**, **, Significant at P<0.05 and P<0.01, respectively.*
NS: Statistically not significant.

Table 3. Maize cv. Dembagnuma germination, root and shoot length, and seedling length and vigor index as influenced by single inoculation with PGPR *B. thuringiensis* strains

Treatments	% germination	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Vigor index
Control	72d	13.26c	7.42d	20.67c	1488.24d
BtI4’	92b	20.69b	14.22a	34.91b	3211.72b
BtI4’’	94a	22.54a	13.54c	36.08a	3391.52a
BtD5	86c	20.70b	13.85b	34.55b	2971.30c

For each PGPR Bt treatment (uninoculated or Bt) within each column means followed by the same letter are not statistically different according to the Fisher protected Lsd test (P<0.05).

3.2 Discussion

Qualitative test for P solubilization on NBRIP medium containing tricalcium phosphate as sole source of P [16] indicated that all the *Bt* isolates, were positive for phosphate solubilisation and isolate *Bt*I4*″* formed zone of clearance on NBRIP medium (Fig. 1A). This result confirm the findings of several researchers who showed high P solubilization by soil and rhizosphere bacteria [17,18,5]. The tested Bt isolates produce chitinase and cellulase which antifungal activity activity coupled with auxin production and phosphate solubilisation are desirable for a potential plant growth promoting agent. [19] reported enhancement of biomass production and heavy metal tolerance of plants under the stress environment after inoculation of *Bacillus thuringiensis*. The genus Bacillus has been reported to produce siderophores [20]. Our results are in accord with this, as except the isolate BtI4’, all the insecticidal *Bacillus thuringiensis* isolates screened, produced significant quantity of siderophore on CAS medium. Our results showed that inoculation of maize seeds with PGP *B. thuringiensis* strains significantly affect seedlings root and shoot lengths compared to the non-inoculated maize seedlings (Table 3). The highest root length (22.54 cm) was observed with *Bt*I4*″*, the highest shoot length (14.22 cm) was observed with *Bt*I4’ and the lowest root (13.24 cm) and shoo (7.42) lengths were observed with non-inoculated seedlings. Plant growth promoting effects of PGPR strains in different crops were clearly demonstrated [5]. Bacterial inoculants are able to increase plant growth and germination rate, improve seedling emergence, responses to external stress factors and protect plants from disease [21]. This present investigation confirms the earlier works. It revealed that under *in vitro* conditions, seed treatment with PGPR *B. thuringiensis* strains improved seed germination and seedling vigor over the control. Similar improvement of seed germination parameters by plant growth promoting rhizobacteria has been reported [22,23], sorghum [24] and pearl millet [25,26]. The improvement in seed germination by PGPR was also found in work with wheat [3] and rice [27,28], where it was found that some PGPR induced increases in seed emergence, in some cases achieving increases up to 100% greater than controls. These findings may be due to the increased synthesis of hormones like gibberellins, which would have triggered the activity of specific enzymes that promoted early germination, such as amylase, which have brought an increase in availability of starch assimilation. Beside, significant increase in seedling vigor would have occurred by better synthesis of auxins [22].

4. CONCLUSION

The results obtained in laboratory tests showed that the *Bacillus thuringiensis* strains (*Bt*I4', *Bt*I4'' and *Bt*D5) from Agricultural soils of Mali possess important plant growth promoting traits. Seed Inoculation significantly enhanced seed germination and seedling vigor of maize, likely due to production of IAA, production of siderophore, and by P-solubilization. These results may allow the use of these *B. thuringiensis* strains for plant growth promotion. Future works will address the application of these bacteria in biofertilization and bio-control.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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