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Bacillus subtilis Strains Isolated from Cocoa Trees (*Theobroma cacao L.*) Rhizosphere for their use as Potential Plant Growth Promoting Rhizobacteria in Côte d'Ivoire

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Abstract

Plant growth promoting rhizobacteria (PGPR) are important for agriculture through their activity in stimulating and facilitating plant growth. The rhizobacteria were screened for molecular characterization and followed by their indole acetic acid (IAA) production, phosphate solubility, antibiosis activity. In this study, 162 soil samples were collected from the cocoa rhizosphere to isolate *Bacillus subtilis* strains using Mössel agar medium with an additional egg yolk and identified by sequencing the *ytcP* gene. The ability of each strain to form biofilms was obtained in a tube. Indole-3-acetic acid (IAA) production was estimated in Yeast Peptone Dextrose (YPB) broth. Phosphates were solubilized by each strain on Pikovskaya agar medium. The detection of lipopeptide genes using the molecular method has established the possession of isolates by antimicrobial genes. Fifty (50) *B. subtilis* strains were isolated and identified using the *ytcP* gene. Ninety percent (90%) of the strains were able to form a biofilm. All isolates produced an IAA. Forty (40 (80%)) of *B. subtilis* were solubilized phosphate with phosphate solubilizing index (PSI) of 0 to 97.33 ± 0.70%. Of all *B. subtilis* strains, 45 (90%) have the *srfAA* gene, 19 (38%) have the *fenD* gene and 12 (24%) have the *ituC* gene. *B. subtilis* strains from cocoa rhizosphere would be beneficial for agricultural production by their PGPR activities.

Introduction

Plants rhizosphere has an abundant microbial population. Many microbial communities in the rhizosphere are capable to exert beneficial, neutral, or harmful effects on plant growth [1]. Plant growth influenced by PGPR can have a direct or indirect effect on plant growth. They can directly stimulate plant growth by increasing nutrient absorption, inducing, and producing plant growth regulators. They can activate induced resistance mechanisms in plants. Indirect effects are related to the production of antibiotics such as lipopeptides (fengycin, iturin, surfactin) by *B. subtilis* [2]. In addition, biofilm formation is important for rhizobacteria to adhere to plant roots. Phosphorus is the second most important nutrient limiting plant growth after nitrogen. It is widely available in soil in both organic and inorganic forms [3]. It has an important role in all major plant metabolic processes, including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration [4]. Plants are not able to use phosphate because 95% of the phosphate is insoluble, immobilized, and precipitated form. Plants can absorb phosphate in the monobasic (H_2PO_4) and basic ($HPO_4{}^2$) ion forms [5]. Microbial solubilization of phosphate is a significant factor in the conversion of insoluble phosphate to soluble phosphate. Physiologically active auxins such as indole-3-acetic acid (IAA) are the most important plant hormones. They improve many aspects of plant growth and development throughout the plant's cell cycle, cell division, cell elongation, and differentiation. IAA is a common product of the metabolism of L-tryptophan produced by several microorganisms including PGPR [6]. IAA increases the surface area and length of the root and thus provides the plant with better access to soil nutrients.

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In addition, the production of IAA by rhizobacteria releases the plant's cell walls and facilitates an increase in root exudation [7]. Some rhizobacteria such as *Bacillus subtilis* are involved in the solubilization of insoluble phosphates, the formation of biofilm and the production of IAA [2, 8]. *B. subtilis* strains are associated with plant protects against infections [8]. This study was carried out to detect *B. subtilis* strains from the cocoa rhizosphere that could produce IAA, form a biofilm, solubilize phosphate, and possess antimicrobial genes.

Results

Isolation and Identification of *B. subitilis* from Cocoa Rhizospheres

The exploration of biodiversity within microorganisms derived from cocoa soils has revealed different population of *B. subitilis* strains. There was more population of *B. subtilis* ($2.50 \pm 1.71 \times 10^{8}$ cfu/g) from cocoa fields without cocoa diseases than from cocoa fields infested by fungal or viral diseases. There is no significant difference at 5% of the *B. subtilis* population between soil samples from cocoa trees with fungal and viral diseases (Table 1). In this study, 50 strains of *B. subitilis* were isolated and identified and characterized by amplification of *ytcP* genes specific to *B. subitilis* strains (Fig. 1). Twenty-nine (29) out of fifty strains were isolated from cocoa soil samples without cocoa disease. However, 10 strains come from soil samples from cocoa fields with fungal diseases and 11 strains from soil samples with viral diseases (Fig. 2). Phylogenetic tree of some *B. subitilis* strains are summarized in Fig. 3.

PGPR Activities

Lipopeptide Detection

In this study, the srfAA gene was the most dominant in B. subtilis isolates. The results showed that 45 (90%) out of 50 strains of *B. subtilis* isolates had the *srfAA* gene. Antifungal genes were also detected in 19 (38%) and 12 (24%) *fenD* and *ituC* genes, respectively. The results also showed that some isolates had both sfrAA and fenD genes (36%). Few strains had both *srfAA* and *ituC* gene (16%), *fenD* and *ituC* (14%). 14% of *B. subtilis* strains had all three lipopeptide genes (Fig. 4).

Table 1 Population level of *B. subtilis* from cocoa trees rhizospheres

Type of soil	Rhizobacteria density (cfu.g^{-1})
Soil from cocoa trees with virus diseases	$2.14 \pm 1.55 \times 10^{4a}$
Soil from cocoa trees with fungy diseases	$1.24 \pm 0.43 \times 10^{4a}$
Soil from cocoa trees without disease	$2.50 \pm 1.71 \times 10^{8b}$

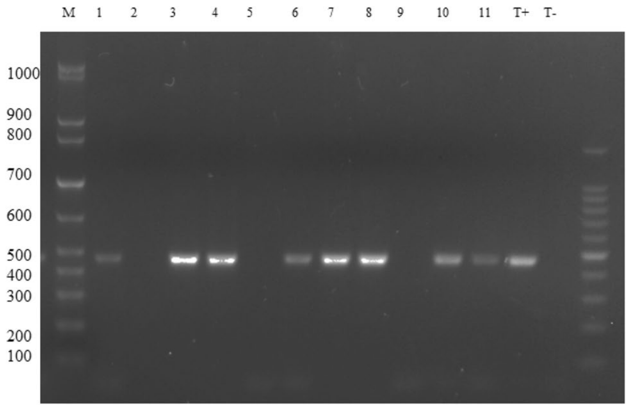

Fig. 1 Electrophoresis profile of amplification for *ytcP* gene. M marker (100 bp); 1 to 11: *B. subtilis* strains; T+positive control (*B. subtilis* GA1) and T-: negative control

Biofilm Production

The ability of bacterial isolates to form biofilms is very crucial for their adhesion to plant roots for better management in biological control of plant pathogens. The biofilms formed in tubes by the adhesion of bacteria were revealed by the appearance of colored rings (Fig. 5).

The thickness of the rings was proportional to the biofilm formed on the tube walls. These thicknesses varied from 0 to 70 mm. Among the isolates tested, 5 (10%) were unable to form biofilms compared to 45 (90%) with the ability to form biofilms. Of these strains, 28 (56%) had a biofilm thickness of between 10 and 30 mm. 13 (26%) of the strains had a biofilm thickness that varied from 31 to 50 mm and 4 (8%) from 51 to 70 mm. Strains of *B. subtilis* BT 1, BK 8, BT 8, BL 11, and BL 19 could not formed biofilms.

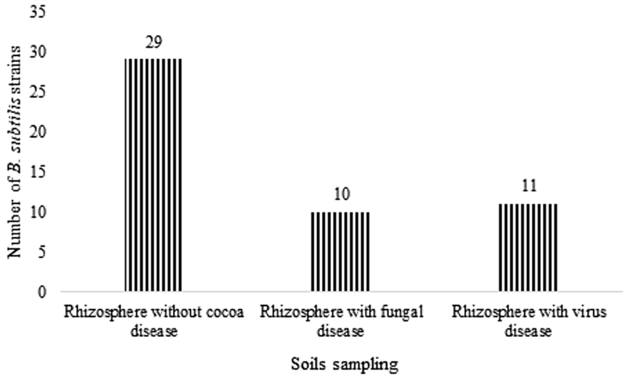

Fig. 2 Proportion of *B. subtilis* isolates from cocoa trees rhizospheres

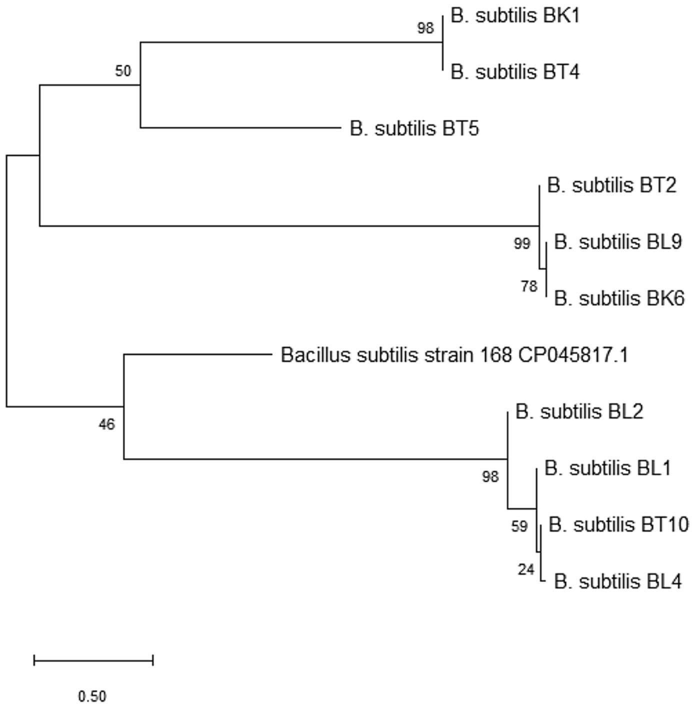

Fig. 3 Phylogenic tree of *B. subtilis* isolates

Position of the primer used within the ytcP-gene (**seq_ref_CP045817.1**)

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ctgaaaaaccgtctgtttgatatgttgatttatggtttcttgctgatgttcgctttaatatg
cgtacttccgttcattcatgttatcgcagcatcctttgccacagtagaagaagtcgtgtcga
aaaaatttattttaataccgaccacttttcgctagatgcttatcgctacatttttcaaca
gatattatttataagagtttgcttgtttctgtgtttgtgacagtgataggcactgcggtcag
catgtttctttcgtcactgatggcttacgggttatcccgccgtgatttaatcggccggcagc
cgctcatgtttctcgtcgtatttacgatgctgtttagcggcggcatgattccgactttcctt
gtggtcaaatcgcttggattgctcgattcttactgggcgcttattttgccgacagccattaa
tgcctttaacctgatcattctgaaaaacttctttcaaaatatcccgtcaagcctggaagagt
ccgcgaaaattgacgggtgcaatgatctgggcatattctttaaaattgtgctgccgctgtct
cttcctgcgatcgcaacgatttcactattttatgcggtcacgtattggaacacgtatatgac
agcgatcttgtacttaaatgattcagcaaaatggccaattcaggtgcttctgcgccaaatcg
tcattgtatcaagcggtatgcaggggatatgtctgaaatggggtcgggcagcccgccgcct
gagcaaaccattaaaatggcggtcatagtggtggcgaccatccctgttctgcttgtctatcc
gtttatacaaaagcattttgcaaaaggagctttgctgggatctgtcaaaggataa

Features : ytcP-F    : [271 : 288] ; ytcP-R    : [709 : 730]
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IAA Production

IAA production by *B. subtilis* isolates was qualitatively observed by changes in red color when the isolate filtrate was mixed with the Salkowski's reagent (Fig. 6). Out of the 50 isolates, 39 had produced IAA without the addition of L-trp in the YPD broth. 11 isolates were not able to produce IAA without the addition of L-Trp. All isolates were able to produce IAA by addition of L-trp to the YPD broth. The addition of L-Trp increases the number of isolates capable of producing the IAA (Fig. 7). The best production was obtained by the strains BK 3, BT 5, BK 15, and BT 10.

Phosphates Solubilization

The ability of *B. subtilis* isolates to solubilize tricalcium phosphate (Ca_3PO_4) on Pikovskayas agar was demonstrated by the production of a clear zone around the colony after 4 days of incubation at 30 °C (Fig. 8). The Phosphate solubilization index (PSI) of *B. subtilis* strains was between 0 and 97.33%. 10 (20%) of the strains were unable to solubilize phosphorus. Of these, 80% come from cocoa-farmed soils showing symptoms of cocoa diseases. From among the 50 isolates, 40 isolates (80%) formed a clear area on Pikovskayas agar. The best phosphorus solubilization rates were observed with strains come from cocoa-farmed soils

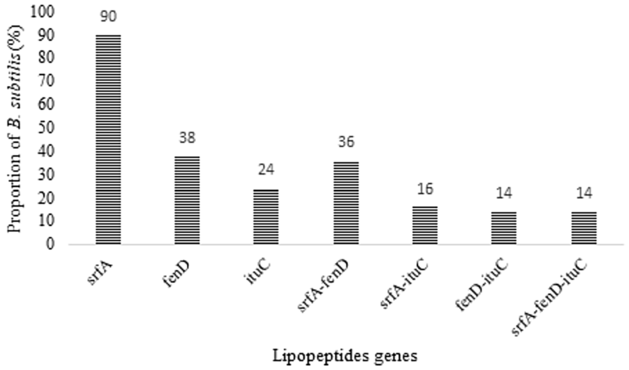

Fig. 4 Proportion (%) of lipopeptide genes biosynthesis of *B. subtilis* isolates

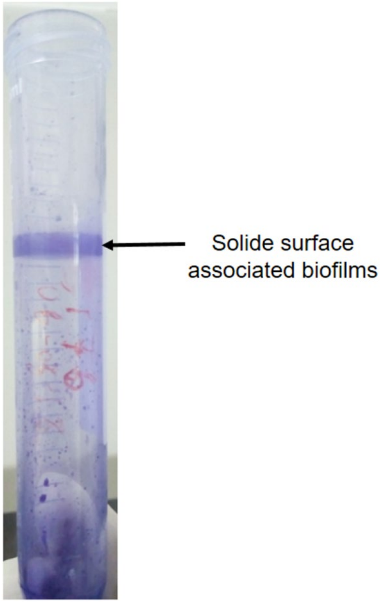

Fig. 5 Biofilm formation by *B. subtilis*

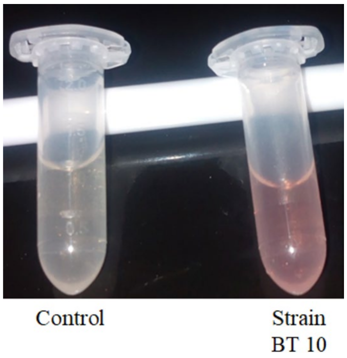

Fig. 6 IAA production by *B. subtilis* BT 10

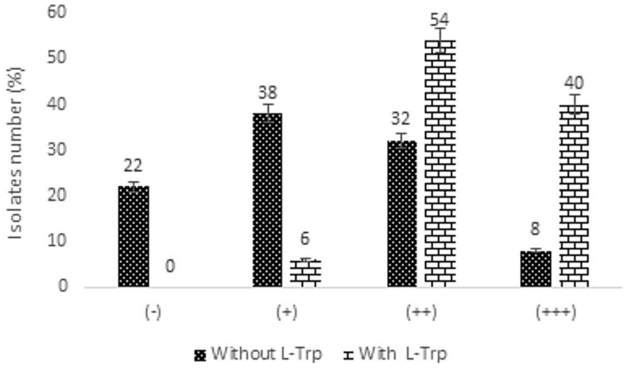

Fig. 7 Proportion (%) of *B. subtilis* produce IAA with and without L-Trp. (-): Absence production of IAA, (+): low production of IAA, (++): mean production of IAA, (+++): Excellent production of IAA

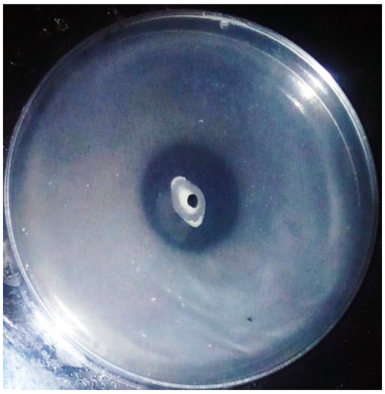

Fig. 8 Phosphate solubilization by *B. subtilis* isolates from cocoa trees rhizosphere on Pikovskayas Agar

without cocoa diseases with PSI that ranged between 42.91 and 97.33%.

Discussion

Species isolated from the rhizosphere area was widely studied previously. In this study, it was to have many informations including *B. subtilis* species and plant growth promoting activity of these isolates indigenously isolated from cocoa rhizosphere in Cote d'Ivoire. This study showed that cocoa fields without cocoa diseases have a higher density of *B. subtilis* ($2.50 \pm 1.71 \times 10^8$ cfu/g). Molecular identification by *ytcP* gene was used to identify fifty strains of *B. subtilis*. The *B. subtilis* isolates from the cocoa trees rhizosphere without cocoa disease were the most abundant (29 isolates). Experimental results suggest that plant protection could be observed when the density of rhizobacteria is higher. According to Ongena [2], plants are better protected when the density of rhizobacteria is about 10^6 cfu/g. In our study, 45 out of 50 isolates possess the *srfAA* gene for surfactant biosynthesis. These strains are excellents for bacterial and virus plant diseases control by their antibacterial and antiviral activities [9, 10]. 38% of the isolates had the *fenD* gene, responsible for the biosynthesis of fengycin and 24% had *ituC* gene for iturin biosynthesis. Experimental results suggest that these isolates would be the best candidates for biological control of moulds using their antifungal genes [2]. Isolates identified with the *srfAA* gene and the *fenD* gene could be used to induce systemic resistance in plants [11]. In this study, the formation of biofilms was estimated for 45 isolates. In India, studies conducted by Saha et al. [12] showed the ability of *B. subtilis* strains AI01 and AI03 to form biofilms. The formation of biofilm is important for rhizobacteria to adhere to plant roots. Their ability to form a biofilm could come from their ability to produce surfactin [2]. 39 *B. subtilis* isolates that have been able to produce IAA in the presence of L-trp could be independent of L-trp, but the other 11 isolates would be dependent on L-trp. All isolates of *B. subtilis* were able to produce IAA in the presence of L-trp. A corresponding result was recorded by Wahyudi et al. [13]. According to Patten and Glick [14], tryptophan is the main precursor to IAA biosynthesis. The production of IAA by isolates could be used to boost seed germination, growth and plant production. The ability of 40 isolates to solubilize phosphate in vitro reveals the possible application of strains of *B. subtilis* in the field as a biofertilizer.

Bacillus sp. isolated from rhizosphere soils of groundnut have shown their ability to solubilize phosphate [15].

Material and Methods

Sampling and Collection

Soils samples were obtained from the rhizosphere of cocoa fields in the Soubre area, Côte d'Ivoire (5°43′10,31″ N et 6°48′9,74 W). Three types of soil samples were collected from the cocoa tree rhizosphere. These soil samples were from cocoa fields that were healthy or infested with fungal or viral diseases (Cocoa Swollen Shoot Virus). Soil samples are collected from three villages of Soubre. In each village, three cocoa fields were selected. In each selected field, 3 cocoa trees by disease with a distance of 10 cocoa trees were selected. Samples were collected by inserting a sterile spatula into the roots of the cocoa trees [8]. These samples were taken from soils that adhere strongly to the roots of cocoa trees 5 cm above the ground. Two soil samples were collected by cocoa tree. The soil samples collected were placed in aseptic tubes. 162 soil samples were collected and stored in aseptically polyethylene bags and maintained in the laboratory at + 4 °C for microbiological analysis.

Isolation of *B. subtilis* Strains

Soils samples (1 g) were transferred to 9 ml of sterile distilled water [16]. The samples were serially diluted from 10^{-1} to 10^{-6}. Serial dilutions were heat-treated at 80 °C for 12 min to kill residual vegetative cells. 100 µl of each dilution was inoculated three times on Mossel agar medium with addition of egg yolk. After incubation for 24–48 h at 30 ± 1 °C, yellow colonies without halos characteristic of *B. subtilis* were counted and isolated.

Molecular Identification of *B. subtilis* Strains

Bacterial DNA extraction was performed by the phenol–chloroform method. The *ytcP* (460 bp) gene specific for *B. subtilis* strains has been amplified with ytcP-F primers (5′-GCTTACGGGGGGTTTTATCCCGCGC-3′) and ytcP-R 5′-CCGACCCCATTTCAGACATCAGACATC-3′) [17]. PCR amplification were carried out in a 50 µl reaction mixture containing 28.8 µl of ultrapure sterile water, 5 × reaction buffer, 25 mM of MgCl2, 10 µM of dNTPs, 10 pmol of each primer, 1 U Go Taq DNA polymerase (Promega, USA) and 5 µl of DNA. PCR amplification was done in a thermocycler GENEAMP7900 (Applied Biosystem, USA) as following: an initial denaturation at 94 °C for 5 min followed by 40 cycles of Denaturation: 94 °C for 30 s, Annealing: 55 °C for 1 min, Elongation: 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR amplicons were revealed with a Gel Doc Ez system (Biorad, USA) after electrophoresis on 2% agarose containing SYBER Safe DNA stain Gel (Thermofisher). The PCR products were purified with QIAquick Gel purification kit (QIAGEN, Germany), according to the manufacturer's instructions and then sequenced with an ABI 3500XL (Applied Biosystem, Japan) DNA analyzer. The sequences obtained were cleaned and aligned with software MEGA 7 in the Genbank database by the NCBI BlastN search.

The evolutionary history was inferred using the Neighbor-Joining method [18]. The optimal tree with the sum of branch length = 7.70760101 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [19] and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 861 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [20].

Table 2 Oligonucleotide primers used to detect lipopeptides genes in *B. subtilis* strains

Gene	primer	Sequence: 5′-3′	Product size	References
Iturin	ITUC-F	GGCTGCTGCAGATGCTTTAT	423 bp	Mora et *al.* (2011)
	ITUC-R	T CGCAGATAATCGCAGTGAG		
fengycin	FEND-F	GGCCCGTTCTCTAAATCCAT	269 bp	
	FEND-R	GTCATGCTGACGAGAGCAAA		
surfactin	SRFA-F	TCGGGACAGGAAGACATCAT	201 bp	
	SRFA-R	CCACTCAAACGGATAATCCTGA		

Lipopeptide Detection

Bacterial DNA was amplified by molecular characterization of the *ituC, fenD* and *srfAA* genes described by Mora et al. [21] using the primer described in Table 2. These genes are respectively responsible for biosynthesis of iturin, fengycin and surfactin. Multiplex PCR were carried out according to Kakar et al. [22]. PCR amplification were carried out in a 25 µl reaction mixture containing 9.3 µl ultrapure sterile water, 5X reaction buffer, 25 mM MgCl2, 10 µM dNTPs, 10 pmol of each primer, 1 U Go Taq DNA polymerase and 5 µl DNA. PCR amplification was performed in a thermocycler GENEAMP7900 (Applied Biosystem, USA) as following: an initial denaturation at 94 °C for 5 min followed by 40 cycles of Denaturation: 94 °C for 45 s, Annealing: 60 °C for 1 min, Elongation: 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR amplicons were revealed with a Gel Doc Ez (Biorad, USA) system after 2% agarose electrophoresis containing SYBER Safe DNA.

Biofilm Formation

Biofilm formation was estimated by the crystal violet (CV) staining tube method [23]. The isolates were grown in tube (15 ml) containing 10 ml of yeast peptone dextrose broth (Glucose 20 g l^{-1}, yeast extract 10 g l^{-1}, Casein peptone 10 g l^{-1}) and incubated for 24 h at 30 ± 1 °C. Each tube was rinsed with phosphate buffer saline (PBS) and the remaining cells and matrices were stained with 1% CV solution for 20 min at room temperature. The tube was washed twice with distilled water. Biofilm formation is positive when a doubles ring is observed in the tube.

Detection of Phytohormone Indole-3-Acetic Acid (IAA)

IAA production by isolated strains was determined using Ehmann's [24] methods. The isolates were grown in a YPB, enriched with or without 1 g l^{-1} L-tryptophan (L-trp). They were incubated at 30 ± 1 °C for 96 h at 120 rpm. The broth was centrifuged at 10,000×*g* for 15 min at 4 °C to collect the supernatant. 800 µl of Salkowski reagent (60 ml sulfuric acid in 100 of sterile distilled water and 3 ml of $FeCl_3$) was added to 300 µl of supernatant. The production of IAA was positive by appearance of red color [25].

Phosphate Solubilization

Phosphate solubilization ability by isolates strains was evaluated on Pikovskaya agar medium [26]. A culture bacterial suspension of 24 h was placed on Pikovskaya agar. The Petri dishes in triplicate were incubated at 30 ± 1 °C for 4 days. The clear area around the bacterial colony indicates the isolate's ability to solubilize phosphorus. The Colony diameter and halozone were measured to estimate phosphate solubilization index (PSI). PSI was calculated as PSI $(\%) = Z - C/C \times 100$ where $Z =$ halozone diameter, $C =$ colony diameter.

Statistical Analysis

Analysis of variance (ANOVA) was used to analyze the data. The difference between the treatments was considered significant at the 5% level ($p = 0.05$). Turkey test was carried out to determine the various classes of homogeneity through software R × 64 3.5.2 for Windows.

Conclusion

Fifty (50) strains of *B. subtilis* were isolated on soil samples from cocoa rhizosphere in Côte d'Ivoire. These isolates have been confirmed for their PGPR characteristics. The PGPR activities of each *B. subtilis* were evaluated by in vitro tests. These isolates would have a role as a plant growth promoter. Some isolates have antibacterial, antiviral and/or antifungal genes. They showed potential activity in the production of IAA, phosphate solubilization, and biofilm formation. These strains have some properties that would be beneficial for agriculture.

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