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Primed Expression of Defense-Related Genes by *Streptomyces cameroonensis*-Based Bioformulation (SCaB) on Cocoa Seedlings in a Nursery Challenged with *Phytophthora megakarya*

Dzelamonyuy Aristide[1,2], Téné Tayo Paul Martial[1*], Alain Bopda Waffo[2], Effa Onomo Pierre[1,3,4], Ewané Cécile Annie[1,3,4*], Boudjeko Thaddée[1,3+]

[1]Laboratory of Phytoprotection and Valorization of Genetic Resources, Biotechnology Center, University of Yaounde I, Yaounde, Cameroon
[2]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, USA
[3]Department of Biochemistry, Faculty of Science, University of Yaounde 1, Yaounde, Cameroon
[4]Laboratory of Plant Physiology, Department of Biological Sciences, Higher Teacher's Training College, Yaounde, Cameroon
Email: *martialtene@gmail.com, *ccilany.ewane@gmail.com

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Abstract

A *Streptomyces cameroonensis* based bioformulation (SCaB) has been developed and shown to be stable and effective in controlling the early proliferation of *P. megakarya* and promoting the growth of cocoa seedlings in nursery. This study was carried out to explore the molecular mechanisms associated with the interaction of SCaB, cocoa seedlings, and the pathogen during the early stages of seedling growth in the nursery. For this purpose, seedling treatment with 10% W/W SCaB under greenhouse conditions evaluated SCaB's capacity to stimulate the defense mechanisms in cocoa. Agronomic growth parameters and the level of induction of defense-associated compounds were analyzed. Real-time (rt) PCR was used to assess the level of expression of defense genes. Here, we showed that the application of SCaB as a seedling treatment enhanced the growth of cocoa seedlings in the nursery by an average of 15.6% after 30 days of growth and led to an average reduction in disease severity of 64% when challenged with *P. megakarya*. The latter led to an increased synthesis of total phenolic compounds, flavonoids, chitinases, peroxidases, and β-1,3-glucanases and an induced up-regulation of TcChiB, TcGlu-1, TcPer-1, and TcMYBPA genes. This research provides a basis for the optimization of beneficial microorganisms as a viable alternative

*Corresponding author.
#The supervisor and original initiator of this project.

to chemical fungicides used in disease suppression.

Keywords

Cocoa Seedlings, *Phytophthora megakarya*, *Streptomyces cameroonensis*, Bioformulation, Priming

1. Introduction

Cocoa (*Theobroma cacao*), cultivated for its beans, is the source of the raw material for the global chocolate industry, valued at over US $103 billion [1]. In Cameroon, cocoa culture is an important economic activity that generates over 1.2% of the national gross domestic product (GDP) and provides employment to hundreds of thousands of Cameroonians [2]. Parasitic attacks are a major threat to cocoa production in Cameroon and worldwide [3] [4]. Black pod disease, caused by the soil-borne fungal pathogen *Phytophthora megakarya* (Brasier and Griffin), is the main disease of cocoa in Cameroon as well as in Central African countries and can cause losses of over 80% in farms and nurseries if unchecked [3] [5]. Healthy and available seedlings constitute the basis for plants to grow and flourish well in the future, hence the necessity to deter the effects of these parasitic attacks at an early stage. Chemical control is often used, but the latter causes a wide range of challenges, including the proliferation of diseases with heightened resistance, the degradation of soil, and concerns about human health and the environment. This therefore highlights the necessity for more environmentally friendly methods of disease control. Prospects for biological control in Phytoprotection have been regularly explored in the eradication of plant diseases in recent decades, and it is frequently employed in contemporary agriculture. Biological control involves the use of microbial inoculants or naturally fermented products to suppress the activities and populations of one or more plant pathogens. Worldwide, intensive research on plant growth-promoting rhizobacteria (PGPR) is being conducted to develop biofertilizers and biocontrol agents [2] [6]. These beneficial microbes provide cross-protection in plants against multiple stress factors in a plethora of ways. These include the production of antibiotics like geldanamycin, cell wall-degrading enzymes like chitinases and β-1,3-glucanases, stress-alleviating enzymes such as the 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile compounds, the synthesis of siderophores and phytohormones, the solubilization of inorganic phosphorus (P), the formation of biofilm, and induced mechanisms of host resistance, among others [7].

The molecular mechanisms by which these biocontrol agents and biofertilizers initiate resistance in plants associated with pathogen interaction have been linked to priming. Priming is the phenomenon whereby plant tissues are preconditioned to respond in a more rapid and robust manner to very low levels of stimulus [8]. Priming confers important fitness benefits to the plant by setting it in a state of readiness to respond faster and stronger to attacks compared to

plants not exposed to priming agents. Studies on the genetic control of cocoa resistance to *Phytophthora* species have shown quantitative trait loci (QTLs) located in many genomic regions associated with *Phytophthora* species, thus offering the possibility of improving resistance in cocoa by a possible accumulation of many different resistance genes, such as those coding for pathogenesis-related proteins (PRs) [9]. Several studies have reported the systemic induction of these defense gene transcripts, including those that code for chitinases (PR-3, 8, and 11), glucanases (PR-2), peroxidases (PR-9), or transcription factors during *Phytophthora* species interaction with plants [10] [11] [12]. Induced systemic resistance leads to the rapid production of these PR proteins and other defense-related biomolecules when plants are attacked [13] [14]. The mechanism of priming is based on induced systemic resistance, often observed in the interaction between plants and beneficial microorganisms [15]. Several beneficial microorganisms and their products have been identified as priming agents in plants. Seedlings of *Arabidopsis* and rice exposed to bacterial cultures of *Streptomyces* species have been shown to significantly reduce disease severity [5] [7] [16]. Our previous work focused on developing and testing a bioformulation based on the biocontrol agent *Streptomyces cameroonensis* (labeled as ScaB) against *Phytophthora megakarya* and its effects on the growth and resistance of selected varieties of cocoa seedlings in a nursery [2]. *Streptomyces cameroonensis,* like other actinomycetes and other classes of plant growth-promoting rhizobacteria, has been shown to ameliorate the growth and resistance of plants using various similar mechanisms [7]. Recent studies have shown formulations of these biocontrol agents to be better suited for agricultural systems and effective in field applications in improving the growth and yield of seedlings [2] [6] [17] [18]. In this study, we investigate the ability of our SCaB treatment to induce growth and resistance in cocoa seedlings in the nursery against *P. megakarya* by quantifying the primed induction of defense metabolites and expression of stress response genes in cocoa leaves using rt-PCR.

2. Material and Methods

2.1. Material

The soil used to grow the cocoa seedlings was black humus soil, often used by cocoa seedling producers in the environs of Yaoundé, Centre Region, Cameroon. The soil collected was air dried, sieved through a 10 mm sieve to get rid of hard material and debris, and then mixed with river sand in a ratio 3:1 w/w. The *Streptomyces cameroonensis* and *Phytophthora megakarya* PM5 strains were obtained from the microorganism bank of the Laboratory of Phytoprotection and Valorization of Genetic Resources (LPVGR) of the Biotechnology Centre (BTC) of the University of Yaoundé 1, Cameroon. Hybrid pods of *Theobroma cacao* (♀) SNK413 × (♂) T79/467) produced by hand pollination were obtained from the SODECAO ("Société de Développement du Cacao") experimental biclonal farm in Mengang, Center Region, Cameroon.

2.2. Culture of *S. cameroonensis* Spores and Storage Conditions

This strain was cultivated on the International *Streptomyces* Project-2 medium (ISP-2 medium). This medium was prepared and autoclaved at 121˚C for 15 minutes, then poured onto sterile Petri dishes. Spores from the stock were streaked on freshly prepared ISP-2 media on Petri dishes and incubated at 30˚C for 7 days for full sporulation. The spores were later collected using sterile glass beads over sporulating colonies. The beads were washed in glycerol (20% v/v) and stored at −20˚C. After freezing for 24 hours at −20˚C, the purity of the inventory was checked for possible contamination. The sporal solution was then adjusted to 10^9 CFU/ml and stored in a 20% glycerol solution at −20˚C for further use.

2.3. Culture of *S. cameroonensis* Spores and Storage Conditions

The *P. megakarya* PM5 strain was cultured on PDA medium (Potato Dextrose Agar). The medium was prepared, autoclaved at 121˚C for 15 minutes, and then poured into sterile Petri dishes. The 5 mm-diameter mycelial discs taken from the surface of the stock culture of *P. megakarya* were transferred to the Petri dishes containing PDA medium and incubated at 26˚C ± 1˚C in the dark. A sporal solution of *P. megakarya* was obtained according to the protocol described by [19]. The number of zoospores was calibrated at 10^6 zoospores/ml and stored at −20˚C for further use.

2.4. Powder-Based Bioformulation of *S. cameroonensis* (SCaB)

Streptomyces cameroonensis based bioformulation (SCaB) was prepared as described [2]. The viability of the spores in the powder bioformulation was determined using the spread plate technique, and the number of CFU/g of powder was calculated.

2.5. Evaluation of the Effect of SCaB on Growth of Cocoa Seedlings

Freshly harvested cocoa pods were dehusked, the seeds were washed with sand and distilled water to remove the mucilage. The washed seeds were surface sterilized with 70% ethanol for 5 minutes, then later with 0.2% sodium hypochlorite solution for another 5 minutes. The sterilized seeds were then rinsed with sterilized distilled water. A water suspension of SCaB (0.1 mg/mL) was prepared, while sterilized cocoa seeds were divided into three equal groups. The first group was treated with 0.1 mg/ml of the bioformulation suspension as described by [2]. The second and third groups served as positive and negative controls respectively. The positive control consisted of seedlings treated with the chemical fungicide CALLOMIL SUPER 66 WP following the manufacturer's protocol, while the negative control was made up of untreated seedlings. Each treatment consisted of 60 seedlings. The different groups of seedlings were planted in polythene bags each containing a mixture of 1 kg of soil and river sand mixed in the ratio of 3:1, kept in the greenhouse for a period of 30 days and watered consis-

tently with sterilized distilled water. At the end of this period, stem length, leaf surface area, number of leaves, root and shoot fresh weights were sampled and measured.

2.6. Evaluation of the Effect of SCaB on Disease Severity

After 30 days of growth, leaves were harvested from each treatment, conditioned in plastic sachets, and transported to the lab. The leaves were washed, then surface sterilized with 70% v/v ethanol and rinsed with sterilized distilled water. Leaf discs were cut out with a 15-mm-diameter cock borer and placed on their abaxial surface in randomized Petri dishes lined with tissue paper soaked in sterilized distilled water (8 discs per dish and two dishes per treatment). One group of leaf discs was inoculated on their abaxial surface with 10 µL of 10^6 zoospore/ml suspension of *P. megakarya,* while the other group, which serves as the control, was inoculated with an equivalent amount of sterilized distilled water. The Petri dishes were covered and incubated under darkness at 25˚C ± 2˚C. Disease expression was rated six days later using a rating scale described by [20]. This experiment was repeated twice, and the severity of the disease was determined for each treatment by calculating the ratio of the sum of individual scores to the total number of discs used. The disease severity index used to express the resistance level was done following the scale described by [21] as follows: $0 < \text{Index} \leq 1$: very resistant; $1 < \text{Index} \leq 2$: resistant; $1 < \text{Index} \leq 2.5$: moderately resistant; $2.5 < \text{Index} \leq 3$: susceptible; $\text{Index} \leq 3.5$: very susceptible.

2.7. Evaluation of the Effect of SCaB on Induction of Biochemical Markers

For each treatment, two groups of 10 leaves per treatment were selected, sterilized, and arranged separately in lightproof trays as described by [2]. One group of leaves was inoculated on their abaxial surface with 10 µL of 10^6 zoospore/ml suspension of *P. megakarya,* while the other group, which serves as the control, was inoculated with an equivalent amount of sterilized distilled water. The trays were hermetically sealed and incubated under darkness at 25˚C ± 2˚C. Six days after inoculation, samples for biochemical and molecular analysis were collected about 1 cm away from the infected area on the leaves.

2.7.1. Extraction and Quantification of Total Polyphenols and Flavonoids

The extraction and quantification of phenolic compounds were done following the modified protocol described by [23]. One g of tissue extract (leaf) was ground in 5 mL of 80% methanol (V/V). The sample was incubated at 4˚C and centrifuged at 10,000 g for 5 min at room temperature using the Beckmann-Coulter microfuge 20 R centrifuge. The supernatant was collected, and the precipitate was re-suspended in 3 mL of methanol and incubated at room temperature for 15 min, followed by another centrifugation. The second supernatant was collected and mixed with the first to form the extract.

The quantification of phenolic compounds was determined spectrophotomet-

rically at 725 nm according to the protocol set by [24] using the Folin-Ciocalteu reagent. In a test tube, 10 µL of phenolic extract was added to 500 µL of distilled water, followed by 75 µL of Folin-Ciocalteu and 500 µL of Na_2CO_3. The mixture obtained was incubated for 30 minutes at 25˚C. In the presence of phenolic compounds, the Folin-Ciocalteu reagent appears blue. The color intensity is proportional to the quantity of phenolic compounds present in solution. Absorbance was measured using the Shimadzu UV-1605 Spectrophotometer at 725 nm against a standard where the extract was replaced by distilled water. Three repetitions were carried out. The quantity of phenolic compounds is expressed in mg/g of fresh matter equivalent to a standard curve of gallic acid (0.1 g/mg).

Flavonoid content was determined in phenolic extract according to the method described by [25] with some modifications. Briefly, 400 µL of phenolic extract, 200 µL of HCl (50%) and 200 µL of formaldehyde (8 mg/L) were incubated for 15 min at 4˚C and centrifuged at 3000 ×*g* for 5 min at 4˚C. The supernatant was collected and used for non-flavonoid quantification spectrophotometrically at 725 nm [24].

2.7.2. Extraction of Total Proteins and Estimation of the Enzymatic Activities of Peroxidases, *β*-1,3-Glucanasea, and Chitinases

The extraction and quantification of proteins were carried out following the modified protocol of [22]. Initially, 1 g of the fresh plant leaves were ground in 5 ml of the Tris-Maleate buffer (Tris-HCl 10 mM, Triton X-100 1%, pH 7.5) using a pestle and mortar on ice. After grinding, the mixture was vortexed for 10 minutes and kept on ice. It was then centrifuged at 10,000 g for 25 min under cold conditions using the Beckmann-Coulter microfuge 20 R centrifuge. The supernatant was collected and conserved, while the remaining residue was centrifuged again at 20,000 g for 20 min. The two supernatants obtained were added and constituted our extract, which was conserved at −20˚C pending quantification of protein. The protein content was determined according to the standard Bradford assay. The absorbance was read at 595 nm using the Shimadzu spectrophotometer UV-1605 against control in which the extract was replaced by distilled water. For each extract, three repetitions were carried out. The concentration of the total protein present was expressed in mg/g of fresh matter, reference to an extrapolation on a standard curve realized under the same conditions as those with the samples using BSA (Bovine Serum Albumin) at 0.1 mg/mL.

The activity of peroxidase (POD) was measured following the method described by [23] with some modifications. 925 µL of Tris-Maleate buffer (0.1 M, pH 7.2, 1 g Guaiacol), was added to 25 µL protein extract, followed by 50 µL H_2O_2 (10%). After homogenization, the reaction mixture was incubated at ambient temperature for 3 minutes. The activity of peroxidase was determined following the formation of tetraguaiacol with an absorbance of 470 nm using the Shimadzu UV-1605 spectrophotometer. The enzymatic activity was expressed in enzymatic units per gram of fresh weight (Δ_{470}/min (EU)/g FW). The control was done using the same procedure as the samples, except that the plant extract

was replaced with water. The enzymatic unit corresponds to the quantity of enzyme, which leads to a 0.1 variation in optical density.

The activity of β-1,3-glucanases was determined according to [26] using laminarine as a substrate. 500 µL of phosphate buffer (pH 7, 0.66 M), was added to 150 µL of catechin (10 mM), followed by 35 µL of protein extract. The mixture was incubated at 25˚C for 30 s. The quantity of glucose released was determined spectrophotometrically, as changes in absorbance were measured at 540 nm using the Shimadzu UV-1605 spectrophotometer. The amount of reducing sugars released was calculated from a standard curve prepared with glucose, and the glucanase activity was expressed in units (g of glucose equivalent per g of fresh weight). The enzymatic activity was calculated from the standard graph of glucose.

Chitinase activity was determined by colorimetric assay using a biopolymeric substrate, colloidal chitin-RBV, following the modified protocol of [27]. 200 µL of colloidal chitin was added to 300 µL protein extract, followed by 300 µL Tris HCl (10 mM, Triton 1%, pH 7.5). This mixture was then incubated at 37˚C for 3 hours. The reaction was stopped with 200 µL of 2 M HCl. The samples obtained were cooled for 15 min to eliminate unhydrolyzed substrate and then centrifuged at 10,000 g for 20 min to remove non-degraded substrate. The spectrophotometric analysis of the supernatant was done at 500 nm. Chitinase activity is described by units per g of fresh matter/h. One-unit chitinase activity corresponds to an increased absorbance of 0.1. For each sample, three independent repetitions were used.

2.8. Evaluation of the Effect of SCaB on the Gene's Expression Level

This evaluation was done through a real-time PCR and analysis of PR protein gene expression in response to infection with *P. megakarya* and SCaB treatment.

2.8.1. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 100 mg of fresh infected and uninfected cocoa plant leaves using the AccuPrep® Universal RNA Extraction Kit (K-3140, BIONEER, Republic of Korea) according to the manufacturer's protocol. The extracted RNA samples were treated with RNase-free DNase to remove any residual genomic contamination. The purity and concentration of the total RNA extract were determined spectrophotometrically (NanoDrop ONE^{C}; Thermo Fisher Scientific, USA). cDNA was synthesized from RNA using the AccuPower RT PreMix (Bioneer, Korea). The template Dnase-treated RNA (1 µg) was mixed with 1 µL (25 pmol) of oligo dT reverse primer in a sterile tube and incubated at 70˚C for 5 min, then placed on ice. The mixture was transferred into the AccuPower® RT PreMix tube, and the total reaction volume was filled up to 20 µL with DEPC-DW (BIONEER). cDNA synthesis was carried out at 42˚C for 60 min and then RTase inactivation at 94˚C for 5 min using the Applied Biosystems® 7500 fast real-time PCR system (Thermo Fisher Scientific, USA).

2.8.2. Quantitative Real-Time PCR (rt-PCR) Assays

The relative levels of expression of the TcPer-1, TcGlu-1, TcChiB, and TcMYBPA genes were assessed by qPCR. The qPCR reaction was performed in a 50 µL reaction mixture containing 100 ng of cDNA sample, 25 µL of the AccuPower® 2X GreenStar™ qPCR MasterMix (Bioneer, Korea), 2 µL each of the forward and reverse primers, 5µL of ROX dye, and the volume made up to 50 µL with DEPC-DW (Bioneer). The sequences of primers used for RT-PCR analysis are presented in Table 1. The qPCR reactions were carried out in the Applied Biosystems® 7500 fast real-time PCR system (Thermo Fisher Scientific, US) using the following PCR cycling conditions: 1 cycle of pre-denaturation at 95˚C for 2 minutes, followed by 45 cycles of denaturation at 95˚C each at 5 seconds, and annealing extension at 60˚C at 5 seconds each. This was followed by an extension at 72˚C for 30 seconds. The Arabidopsis *β*-Actin gene was used as the housekeeping gene for normalizing the expression of the target genes. The comparative gene expression analysis of target genes (TcChiB, TcGlu-1, TcPer-1, and TcMYBPA genes) was normalized to the *β*-Actin (internal control) relative to control experiments (uninfected) according to the $2^{-\Delta\Delta CT}$ method [28]. Data was analyzed using Sequence Detection System software (version 2.2, Applied Biosystems). The cycle at which an increase in the fluorescence level above the background was statistically significant (cycle threshold (Ct), was chosen in the exponential phase of the amplification. All the relative expression analysis was carried out in triplicate for every condition in the experiment.

2.9. Statistical Analysis

The experimental designs were randomized complete blocks, and each value reported is the average of multiple repeats. The raw data was imported into Microsoft Excel 2010 for calculations and graphic representation was done using GraphPad Prism 8.0.1. IBM SPPS software version 28.0.1.1 (15) was used for the analysis of the data. Quantitative changes in parameters were evaluated through analysis of variance (one-way ANOVA) with Turkey multiple comparison tests at $P \leq 0.05$ to find significant differences among treatments. All results are presented as the means ± standard deviation (SD).

Table 1. List of primers used to assess the relative level of expression of the TcChiB, TcGlu-1, TcPer-1, and TcMYBPA genes.

Reference gene (*β*-actin)	**Forward Primer** *5′……3′*	Reverse Primer *5′……3′*
β-Actin	GTGGGCCGCTCTAGGCACCAA	TCATACTCTGCCTTAGCAATCC
Target gene		
TcChiB	GTGGCTTTGCTTGTGAATCTC	CACTGCTTCTCACCCTTATGT
TcGlu-1	GCTATGATTCCCTTCCCTCTTC	CAGGCCAAGTGCTAGGATAAG
TcPer-1	TGCGCTGATATTCTCGCTATT	CTGTGAACCCATCCCTTCTT
TcMYBPA	GATGGGAAGGGCTCCTTGTTG	ATCTCGTTATCGGTTGGACCAG

β-Actin a housekeeping gene whose expression was unchanged for all exposure conditions was used as internal control.

3. Results

3.1. Effect of SCaB on Growth of Cocoa Seedlings

Cocoa seedling treatment with SCaB showed significant increases ($P \leq 0.05$) in the agronomic metrics of growth compared to the untreated seedlings (Table 2). Thus, after treating the seedlings with SCaB increases were seen in the number of leaves, stem length, leaf surface area, shoot fresh weight, and root fresh weight of 8%, 12%, 4%, 15%, and 39%, respectively compared to the untreated seedlings.

3.2. Effect of SCaB on Disease Severity

Necrotic lesions appeared on leaf disc samples from all treatments six days after inoculation. Leaf discs inoculated with sterilized distilled water showed no necrotic lesions across all treatments (Figure 1). Following *P. megakarya* inoculation, plants treated with SCaB had the lowest disease severity index compared to chemically treated and untreated plants. In effect, the lowest disease severity index of 0.83 (very resistant) was observed for SCaB-treated seedlings compared to 1.83 (resistant) for the chemically treated seedlings and 2.3 (moderately resistant) for the untreated seedlings. Indeed, a 64% reduction in disease severity following treatment with SCaB was observed compared to the untreated seedlings.

3.3. Effect of SCaB on Induction of Biochemical Markers

3.3.1. Effect of SCaB on Total Phenolic Compounds and Flavonoids

Treatment of cocoa seedlings with SCaB significantly induced the synthesis of total polyphenols, and flavonoids, both before and after infection of cocoa leaves with *P. megakarya* (Figure 2a and Figure 2b). Production of these compounds increased significantly ($P \leq 0.05$) across all treatments when leaves were infected with *P. megakarya*. In fact, after treatment with our bioformulation, percentage increases of 35% and 28% were seen for total polyphenols, and flavonoids, before infection and 40% and 47% after infection compared to the untreated seedlings (Figure 2).

3.3.2. Effect of SCaB on Total Protein Content and Enzymatic Activities of Peroxidases, β-1,3-Glucanases, and Chitinases

Cocoa seedlings treated with SCaB showed an increase in the content of total proteins and the enzymatic activities of peroxidases (PR-9), β-1,3-glucanases (PR-2) and chitinases (PR-3), both before and after infection compared to the chemically treated and untreated seedlings (Figure 3). Inoculation of leaves with *P. megakarya* is characterized by an increase in total protein content and the activity of these enzymes across all treatments. When compared to untreated seedlings, total protein content by 21% for seedlings treated with SCaB before infection and by 30% after infection with *P. megakarya* (Figure 3a) The enzymatic activities of peroxidases, β-1,3-glucanases and chitinases increased for seedlings treated with SCaB by 39%, 40% and 25% respectively before infection and by 58%, 59% and 33% after infection (Figures 3b-d).

Table 2. Effect of SCaB on agronomic parameters of cocoa seedlings in nursery after 30 days of growth.

Treatment	Leave number/ plant	Stem length (Plant/cm)	Leaf Surface area (Plant/cm^2)	Shoot Fresh weight (g/plant)	Root Fresh weight (g/plant)
NT	4.0 ± 0^a	17 ± 1^b	30.31 ± 2.16^a	4.64 ± 0.18^b	1.56 ± 0.07^b
C	4.3 ± 0.58^a	19 ± 1^a	31.77 ± 1.44^a	4.91 ± 0.33^b	1.73 ± 0.07^b
T	4.3 ± 0.58^a	19 ± 1^a	34.62 ± 1.64^a	5.34 ± 0.22^a	2.17 ± 0.11^a

*Each value represents the mean ± SD (n = 60). Values with the same superscript letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66WP); T: Treated (with SCaB).

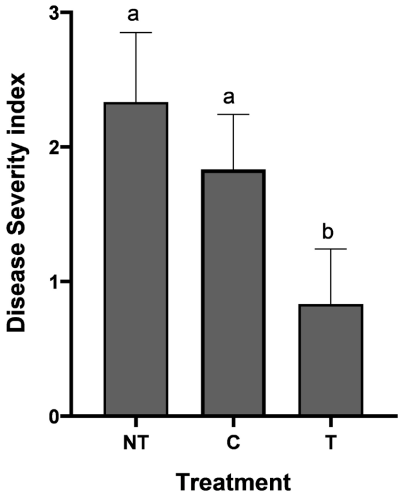

Figure 1. Disease Severity of cocoa plants treated with SCaB six days after inoculation. Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66 WP); T: Treated (with SCaB).

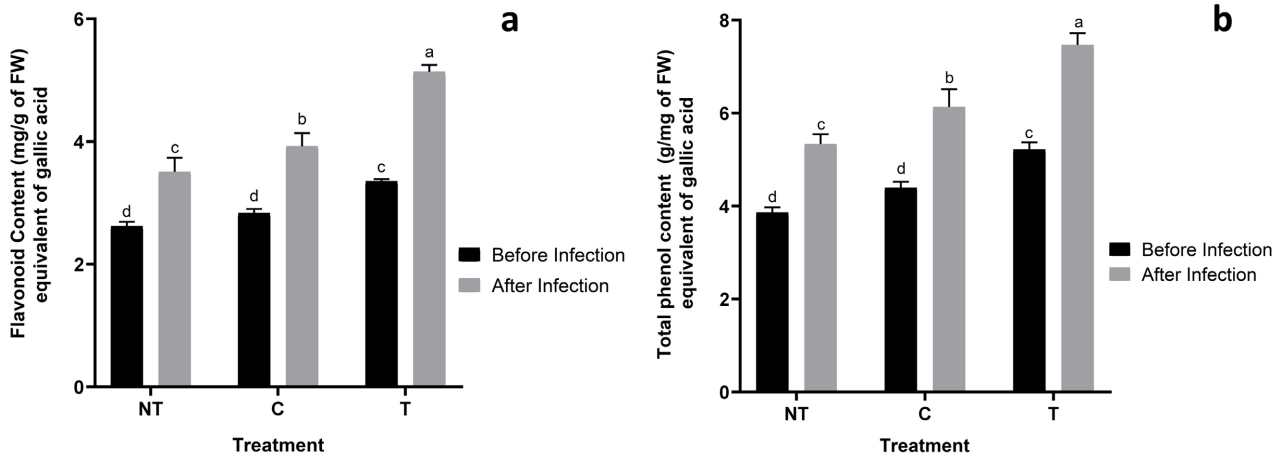

Figure 2. Effect of SCaB on (a) Total phenolic content (b) flavonoid content in 30 days cocoa leaves challenged with *P. megakarya*. Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66 WP); T: Treated (with SCaB).

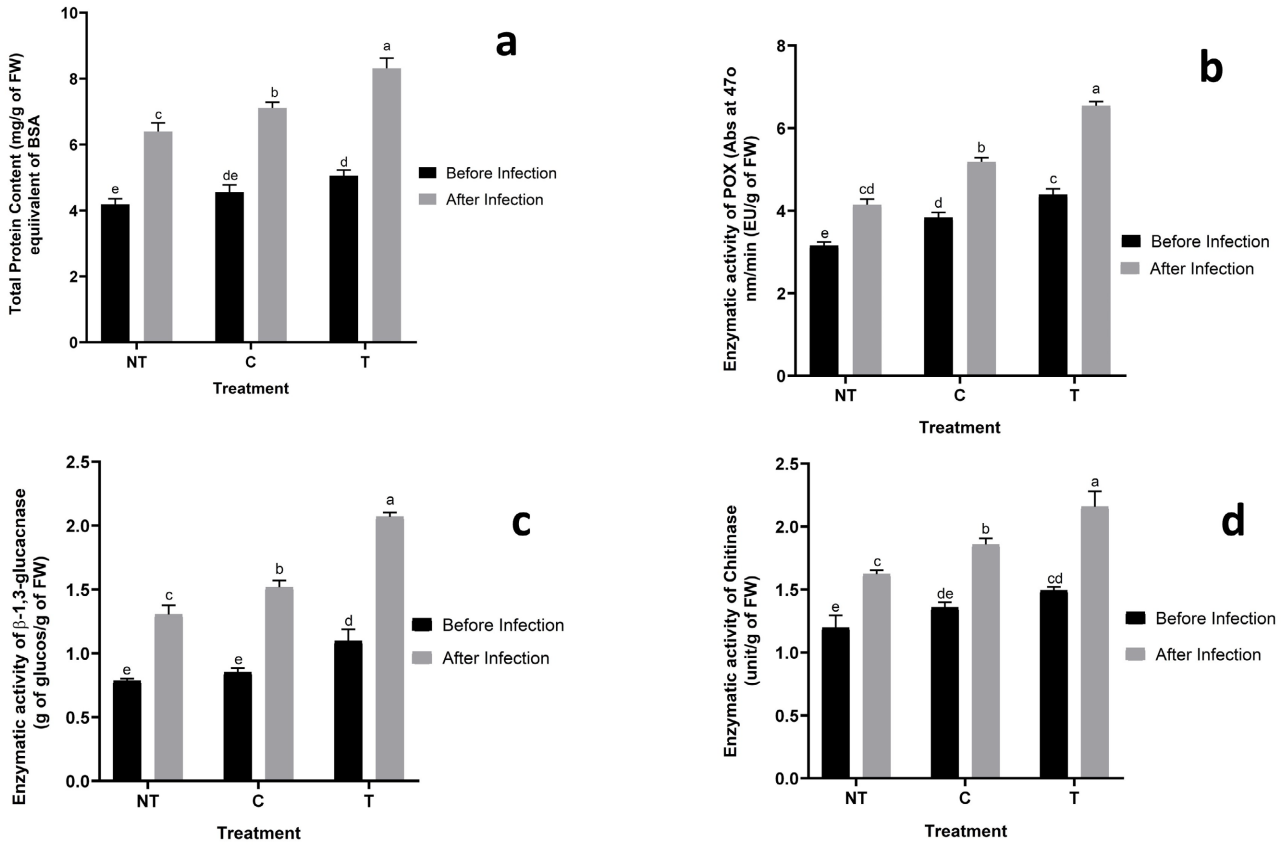

Figure 3. Total protein content (b) Total phenolic content and the enzymatic activities of (b) peroxidase, (c) β-1,3-glucanase and, (d) Chitinase in leaves of 30 days old cocoa seedlings challenged with *P. megakarya*. Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66WP); T: Treated (with SCaB).

3.4. Effect of SCaB on the Level of Gene Expression

Treatment of cocoa seedlings with SCaB significantly induced an increased expression of defense-related genes that code for peroxidase (TcPer-1), glucanase (TcGlu-1), chitinase (TcChiB), and the transcription factor involved in the regulation of phenolic compounds (TcMYBPA) in cocoa leaves both before and after infection with *P. megakarya* when compared to the chemically treated and untreated seedlings (Figure 4). Seedlings treated with SCaB showed a relative fold expression of 2.07, 2.33, 1.33, and 1.35, respectively, for TcPer-1, TcGlu-1, TcChiB and TcMYBPA genes, as opposed to a relative fold expression of 0.79, 1.04, 1.05, and 0.79 for untreated seedlings. In fact, when compared to untreated seedlings, treatment with SCaB increased the expression of the TcPer-1, TcGlu-1, TcChiB, and TcMYBPA genes by 122%, 128%, 29%, and 70% respectively (Figure 4). Infections with the pathogenic agent *P. megakarya* triggered an upregulation in the expression of these genes across all treatments. As a result of *P. megakarya* infection, we see a 74%, 191%, 67%, and 143% increase in the gene fold expression for the TcPer-1, TcGlu-1, TcChiB, and TcMYBPA genes across all treatments.

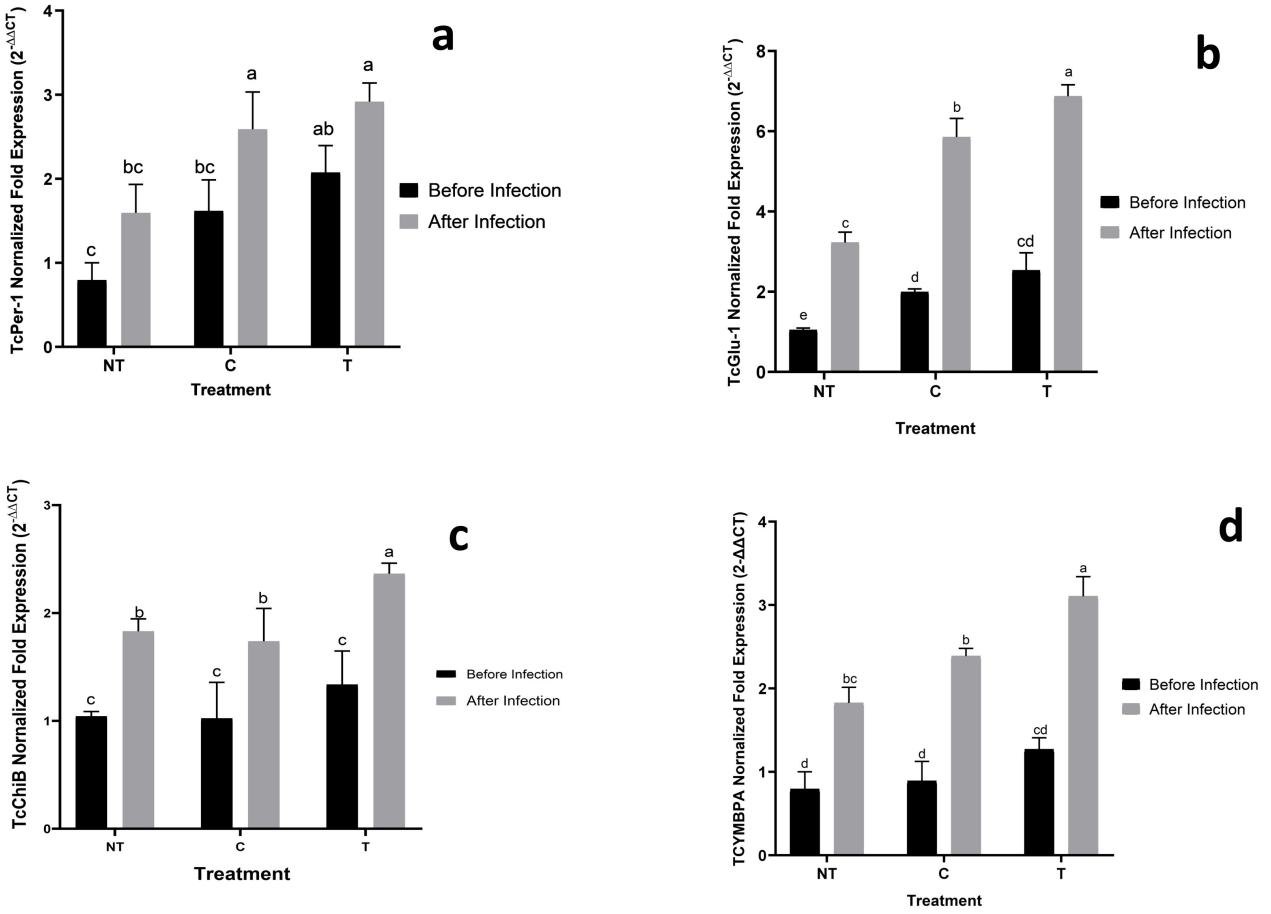

Figure 4. Quantitative reverse transcriptase-PCR analysis of the expression (a) Peroxidase (TcPer-1) (b) glucanase (TcGlu-1) (c), Chitinase (TcChiB), and (d) anthocyanidin transcription factor (TcMYBPA) defense-related genes of cocoa seedling leaves treated SCaB challenged with *P. megakarya.* Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66WP); T: Treated (with SCaB).

4. Discussion

Biological control of plant diseases and promotion of growth using beneficial microorganisms have been proven in numerous studies aimed at preserving plant health and enhancing crop yield. These beneficial microorganisms can be optimized for the development of novel biofertilizers that are better suited for agricultural systems, usage, transportation, storage, and durability [2] [6]. The mechanisms by which these novel bio formulations interact with the plant and the pathogenic agent have not been fully discussed. Our present study has shown that a bioformulation based on *Streptomyces cameroonensis* (SCaB) can improve growth, resistance and the expression of genes associated with defense in cocoa seedlings. Given that the SCaB is applied as seed treatment by enrobing cocoa grains when the treated seeds are sown, the bacteria spores will establish well into the seed surface and colonize the roots after germination [2]. The presence of the *S. cameroonensis* spores in the root system of the plant and soil is thus expected to account for the changes in the growth and disease-resistant

properties of the cocoa seedlings [7]. The SCaB treatment significantly increased the agronomic parameters by 18% and reduced the disease severity by 64% after a 30-day period when the cocoa seedlings were challenged with *P. megakarya*, thus raising the health status of the seedlings from tolerant to highly resistant. The improved agronomic characteristics and reduced disease severity at this early stage of growth can be attributed to the priming or pre-immunization of the seedlings with SCaB, following the activation of microbe-associated molecular patterns (MAMPSs) or Pathogen-associated molecular patterns (PAMPs) upon interaction of the plant with spores of *S. cameroonensis* and/or *P. megakarya*. This reduction in disease severity index is translated by an increased synthesis of primed defense biomolecules like phenolic compounds, flavonoids, oxidative enzymes, and PR-proteins that are highly implicated in the cocoa plant defense system against *P. megakarya.* A Previous study [2] has shown that the growth-promoting and disease-resistant effects of SCaB are directly linked to the presence of *S. cameroonensis* spores in the bioformulation.

Beneficial bacteria, such as *Streptomyces*, have been demonstrated to be reliant on the ethylene, salicylic acid, and jasmonic acid (JA) pathways in *Arabidopsis*. These pathways may be used to induce systemic resistance [29] [30]. which underlines this priming mechanism by SCaB in cocoa. This primed state is accompanied by important fitness benefits associated with an accumulation of defense-related molecules [8]. This priming mechanism occurs in two major phases, namely, the post-challenged primed state and the priming phase [5]. Firstly, the recognition of the microbe or pathogen by the host cells occurs when a pattern of elicitors from the microbe (MAMPs) is recognized by a pattern of plant receptors. This recognition stimulates the transduction of the signal into the plant cells, which consequently activates the transcription of defense genes [31]. In our study, the primed state is accompanied by changes in the agro-morphological, transcriptional, and metabolic states of the plant. An increase in the expression of target transcription factors and defense-related genes was observed. In this study, plants treated with SCaB showed higher defense-related gene expression for the TcChiB, TcGlu-1, and TcPer-1 genes, which code for chitinases, glucanases, and peroxidases, and the Tc-MYBPA gene, which encodes a transcription factor involved in the regulation of phenolic compounds involved in plant defense systems like proanthocyanidin synthesis in cocoa [32]. The TcPer-1 gene is highly expressed in young leaves of cocoa upon mechanical wounding and treatment with ethylene [12]. These results correspond with ours, where an up-regulation of the TcPer-1 gene in 30 days, detached cocoa leaves treated with SCaB and infected with a sporal solution of *P. megakarya* was observed. The high expression of the TcPer-1 gene in young leaves is related to the high expectations of expansion and lignification at this early stage of growth. The TcGlu-1 gene was transiently primed by treatment with SCaB. This gene shows a high degree of homology to genes encoding for β-1,4-glucanases in cocoa. Endo-1,4-glucanases are believed to be involved in cell wall loosening during reorganization or degradation processes [12]. The

β-1,3-glucanases of the glycosyl hydrolase family are made up of diverse groups with distinct patterns of expression that are modulated and induced by a variety of stimuli, such as inducers of plant defense [33]. The TcChiB gene was overexpressed in leaves treated with SCaB and infected with *P. megakarya*. The TcChiB gene is associated with several putative chitinases from *Gossypium hirsutum*, including a salicylic acid-activated class VII chitinase [34]. Our results are consistent with previous research that has demonstrated the ability of cocoa plants, in response to attack by the fungus *Phytophthora capsici,* to stimulate the expression of the TcChiB gene, leading to an increase in the synthesis of chitinases (PR3) that degrade the wall of this fungal pathogen and inhibit its spread [31]. Thus, the high expression of these genes because of induction by SCaB will likely destabilize the invading *P. megakarya*, providing both a protective and an inducing effect. Expression of the TcMYBPA gene was observed to be significantly upregulated in cocoa plants treated with SCaB and infected with *P. megakarya*. TcMYBPA encodes an R2R3-MYB transcription factor involved in regulating the biosynthesis of cocoa proanthocyanidin (PA), a derivative of flavonoids that contributes to plant defense mechanisms against biotic and abiotic stress [32] [35]. This transcription factor highlights the initial steps of the transduction pathway leading to cocoa resistance against *P. megakarya*. The development of cocoa and flavonoid (mainly anthocyanin) synthesis has been described previously [36]. The increase in the expression of TcMYBPA genes in cocoa following infection with *P. megakarya* and treatment with SCaB is consistent with previous research, which has demonstrated that in *Arabidopsis*, several genes encoding MYB transcription factors are upregulated after infection by *Pseudomonas syringae* and act as a positive regulator of the hypersensitive response and in response to bacterial pathogens [37]. The generation of reactive oxygen species and activation of the mitogen-activated protein (MAP) kinase pathways may also be some of the early responses elicited in the plant as a result of priming [29]. In the second stage of priming, pathogen signaling is relayed by phytohormones, which operate the mechanisms of systemic acquired resistance and systemic induced resistance [38]. The interaction between plants and beneficial microbes such as *Streptomyces Ssp*, *Trichoderma Ssp*, *Bacillus Ssp have* been linked to these pathways [2] [6] [17] [39]. This suggests that SCaB containing *S. cameroonensis* spores initiates these responses linked to these pathways. This primed response can also be because the cocoa plant may initially perceive *S. cameroonensis* as a potential invader, resulting in a rapid activation of the plant immune system [40]. SCaB can be seen to elicit such early and robust responses from the plant, leading to an up-regulation of these target genes.

5. Conclusion

The treatment of cocoa seedlings with SCaB led to an 18% increase in the growth parameters of our cocoa seedlings in the nursery and an average reduction of about 64% in the disease severity index when challenged with the pathogen *P. megakarya* at an early stage of growth. This reduction is characterized by in-

creased synthesis of total proteins, total polyphenols, and flavonoids, increased enzymatic activity of peroxidases, β-1-3-glucanases and chitinases, and an up-regulation of defense-related genes like TcPer-1, TcGlu-1, TcChiB, and the TcMYBPA gene. This knowledge gain provides a basis for understanding the priming mechanisms involved in the interaction between formulations derived from biocontrol agents, the cocoa plant, and the pathogenic agent *P. megakarya*. It also lays the framework for the optimization of beneficial microbes to induce an early alert state of defense responses in cocoa seedlings against potential pathogen invasion, reducing dependence on chemical pesticides along the way. In future studies, the changes observed in the expression of these defense-related genes and transcription factors may also serve as molecular markers for the response of cocoa to formulations of beneficial microorganisms and infection by *P. megakarya*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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