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Performance of *Trichoderma harzianum* and *Bacillus amyloliquefaciens* Bioformulation on Germination and Expression of Bioprotective Molecules against Cercosporiosis in *Abelmoschus esculentus* in the Field

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Authors' contributions

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

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ABSTRACT

The present study aimed at assessing the performance of the formulation combining *Trichoderma harzianum* and *Bacillus amyloliquefaciens* on germination and induction of the synthesis of bioprotective molecules against pathogens in *Abelmoschus esculentus* causing cercosporiosis. A significant difference ($p = 0.05$) was revealed between the formulation (T1) and the control (T0) in the field on the seed germination rate of okra varieties *Clemson spineless* (V1) (83.3 vs 94.4%; 88.8 vs 100%; 77.7 vs 88.8%) and *Hire* (V2) (77.7 vs 94.4%; 88.8 vs 100%; 83.3 vs 88.8%) in agroecological areas V, III and IV respectively. The same effect observed on plant health with bioformulation was significant compared with the control in terms of protection against cercosporiosis pathogens. The protection ranges according to treatment and control for the two okra varieties in agroecological areas V, III and IV were 79.24 vs. 86.48%; 100 vs. 100%; 77.76 vs. 91.82% for variety V1 and 88.88 vs. 94.44%; 94.44 vs. 100%; 83.33 vs. 94.44% for variety V2. Gas chromatography combined with mass spectrometry revealed an overproduction of bioactive molecules in the hydro-methanolic extracts of okra leaves treated with the formulation, which are responsible for its bioprotection in the three agroecological areas. In conclusion, the use of the *Trichoderma harzianum* and *Bacillus amyloliquefaciens* formulation on okra crops significantly induced germination and systemic resistance of *Abelmoschus esculentus* plants against cercosporiosis agents.

Keywords: Cercosporiosis; Trichoderma harzianum, Bacillus amyloliquefaciens; Abelmoschus esculentus; germination; bioprotective molecules.

1. INTRODUCTION

Okra (*Abelmoschus esculentus* L.) is the fourth most important vegetable-fruit in Cameroon after tomatoes, chillies and eggplants [1], and has considerable economical potential for poor communities due to its potential for selling in rural and urban markets. Its fruits have a high nutritional value in bioactive molecules (nutrients and antioxidants). They are especially rich in calcium, iron, magnesium, vitamins A, C, E, K and lipids (oleic, linoleic and palmitic acids) [2].

Between 2013 and 2022, worldwide production of okra increased from 6.48 million tons to 10.5 million tons, with India, Pakistan and Iraq being major production centers [3]. In Africa, on the other hand, production has been declining and varied depending on the country during the same periods. It varied from 120000 to 90780 t/ha in Cameroon; from 100000 to 57721 t/ha in Egypt; from 71350 to 66360 t/ha in Ghana and 826170 to 1978256 t/ha in Nigeria [3]. Under optimal growing conditions, okra productivity can reach up to 30 - 40t/ha. However, there is a large gap between potential and effective yield [4]. The low yield of okra in Cameroon is mainly linked to disease severity in the soils of agroecological areas III, IV and V where its culture is favorable. Several diseases affect okra, including mildew, fusariosis, powdery mildew, cercosporiosis and mosaic.

Cercosporiosis leaf blight (CLB) is one of the main diseases of okra, and as it can severely affect foliage, it reduces photosynthetic activity, with a negative impact on okra yield. In order to effectively tackle this problem, it is important to understand the biology of the pathogen. Two different species of Cercospora (*C. malayensis & C. abelmoschi*) can cause the disease. The CLB has been reported from tropical and subtropical Asian countries and is commonly present in West and Central Africa where okra is grown, particularly during the warm rainy season [5]. Splashes of rainwater or sprinkler irrigation contribute to the spread of the pathogen in the form of conidia from one plant to another, creating a microclimate that contributes to dissemination within the field, while the wind spreads conidia from one field to another. Infected okra plants associated with animal movement can also easily spread the disease within the field and over long distances [6].

Biological plant protection technologies in small-scale agroecosystems, in combination with other agronomic management practices, would provide indispensable solutions to revitalize declining food production. The perfect crop resource, beneficial microorganisms, have thus been

brought to the forefront of quantitative and qualitative production [7]. Growth-promoting microorganisms (GPMs) are a group of microorganisms that not only promote plant growth but also act as biocontrol agents, specifically bacteria and fungi that provide essential agroecosystem services. They promote plant growth [8] and improve soil productivity [9]. PGPMs improve plant tolerance to biotic stresses (diseases and pests).

PGPMs such as the Bacillus species; mycorrhizal fungi; Trichoderma promote plant growth and productivity through a variety of direct and indirect mechanisms. Several direct mechanisms have been established by previous studies and can be classified as phytostimulant [10], biofertilizer, rhizomediator or stress regulator and biofertilizer [11]. Indirect mechanisms manifest themselves mainly in the form of biocontrol of phytopathogens through competition for nutrients, enzymatic lysis, antibiosis, secretion of volatile organic compounds (VOCs), triggering of the antioxidant defense mechanism [12], resistance systemic induced (RSI) response in the organism and systemic resistance (ISR) in the host plant.

Commercial microbial inoculants (commonly used as biofertilizers or biostimulants) containing single species or strains of Rhizobia, *Pseudomonas* sp., *Azotobacter* sp., *Bacillus* sp., *Trichoderma* sp., *Aspergillus* sp. and *Glomus* sp. have been widely used in small-scale agroecosystems for crop production [13]. Previous field research in different agroecosystems around the world has reported variable levels of success on the use of PGPMs to support crop performance quantitatively and qualitatively [14]. The use of PGPMs would therefore be adequate to reduce the incidence of diseases on the okra plant.

2. MATERIALS AND METHODS

2.1 Site Description

The study was carried out in agro-ecological areas III, IV and V. Area III is located in King-Palace Bandjoun in the Koung-Khi Division with altitude of 1,529 m, with geographic coordinates of 5°22'32" N and 10°24'48" E. Its climate is equatorial Cameroonian, with an average temperature of around 22°C and average annual rainfall of 916.6 mm. Area IV is located in Dome in the District of Kribi, Ocean Division. It has an average altitude of 18 m above sea level and geographical coordinates 2°55'48" N and 9°54'40" E. Its climate is equatorial Cameroonian, with an average temperature of 25.7°C and average annual rainfall of 2957 mm. Zone V is located in Mbele II, in Obala district, Lekie Division. This location is set at an altitude of 528 meters, with geographic coordinates of 4°10'0" N and 11°31'60" E. It has a humid tropical climate, with an average annual temperature of 24.7°C and rainfall of 1,638 mm [15].

The experiments were carried out in the Laboratory of Plant Protection and Valorization at the Biotechnology Centre, University of Yaounde I.

2.2 Plant and Microbial Materials

The plant material was made up of the seed of two okra varieties in relation to germination capacity and precocity, collected in the study areas. The *Clemson Spineless* variety, for its variable precocity between 40 and 50 days, and its widespread distribution. The *Hire* variety, for its precocity varying between 50 and 60 days, its good mucilage content, its average yield varying between 12 and 15 t/ha and its commercial potential in local markets. The microbial material used in this study was isolated and quantified on the crop soils. It was sent to China in Dora Agritech. laboratory to be characterize and identified. It included two strains of microorganisms, PGPR bacteria (*Bacillus amyloliquefaciens* 1x10^11 CFU/g) and fungi (*Trichoderma harzianum* 2x10^9 CFU/g). The main bacterial and fungi strains of this study were used because of their high potential biocontrol effects on plants already proved and their effect on plant biostimulation.

2.3 Compatibility Test

In vitro compatibility test by using the agar diffusion test based on the modified method of Irabor & Mmbaga [16] between *Trichoderma Harzianum* and *Bacillus amyloliquefaciens*. A 24-hour-old culture loop grown in LB agar (Luria-Bertani) was transferred to 4 glass test tubes containing 5 ml LB broth and incubated for 24 hours on an incubator shaker (New Brunswick Scientific CO., Inc, Edison, NJ 08817, USA) set at 200 rpm and 30°C. The concentration of the *Bacillus amyloliquefaciens* suspension was then quantified and adjusted to ~108 CFU/ml. Sterile 8 mm Whatman filter paper disks were aseptically immersed in the *Trichoderma*

Harzianum medium suspension and air-dried for 30 minutes. The first strain was buffered evenly onto the plate using a sterile cotton-tipped applicator. Four disks imbibed with the *Bacillus amyloliquefaciens* suspension were carefully pressed onto the *Trichoderma Harzianum*-inoculated agar medium surfaces at four equidistant positions using sterile forceps. Plates were incubated at 28 ± 2°C and observed over a 72h period. Incompatible strains were identified by a zone of inhibition between them.

2.4 Preparation of Bioformulation

The formulation with *Trichoderma Harzianum* and *Bacillus amyloliquefaciens* was developed by combining 500 g of microorganism strain in order to obtain 1 kg of microbial conidia. The "vehicle" used was rice bran following the modified method of Olivera et al. [17]. This involved mixing 1kg of microbial conidia with 1kg of brown sugar in 8l of non-chlorinated water. The mixture was fermented in a hermetically sealed bucket for 07days for the germination of conidia. The resulting product was then mixed with 50kg of rice bran and taken to a barrel for further fermentation for 7 days before application to the field.

2.5 Experimental Design

The experimental design was a split-plot with 10 plots spaced 0.5 m from each other and 2 m for each okra variety. The total plot area per variety was 229.5 m² (25.5 m x 9 m). All plots in a block represented the same treatment. Field applications of the microbial combination were 40 g per hole before sowing and the same concentration during vegetative growth 35 days after sowing.

2.6 Determination of Germination Rate

The seeds were sown in sterile petri dishes inoculated by bioformulation on four filter paper discs autoclaved at 105ºC for 30 min and moistened with sterile distilled water. Each was covered with a lid. To evaluate 100 seeds of each variety during the week, four Petri dishes were prepared for each okra variety, each containing 10 seeds. These dishes were used for each sowing once a week. New disks of filter paper were used in the petri dishes, which were washed and disinfected with 50° alcohol at each sowing to prevent the spread of pathogenic microorganisms. The emergence of the radicle, which marks the end of the germination process, was thus the main germination criterion [5]. Germination rate was assessed under the following conditions:

- petri dishes were placed on shelves
- room temperature (19°C and 25°C)
- lighting: daylight

The germination rate was calculated according to formula (2):

$$GR\ (\%) = \frac{NGS}{TNSS} * 100$$

GR = Germination rate,
NGS = Number of germinated seeds (unit),
TNSS= Total number of seeds sowed (unit)

2.7 Description of Symptoms

Observations on the leaves of seedlings in the field were made 35 days after sowing. Leaf symptoms were observed and described for each accession. The appearance, shape, size and coloration of symptoms were described by visual observation. Leaves from accessions showing different types of fungal attack symptoms were randomly sampled. Thus, five leaves with the same type of symptoms were collected from five plants per treatment for each variety in the different agro-ecological areas and placed in blotting paper, and then in a polyethylene plastic bag for laboratory analysis.

2.8 Identification and Characterization of Cercospora Sp. Strains

Cercospora sp. spores were identified through the symptoms observed on the plant using segments of infected leaves, which were washed in 200ml of water. This solution was filtered through 0.45µm millispore membranes. The gelatin/glycerine cube was used to recover spores from the filter surface. These were then transferred to PDA culture medium. Culture was carried out in an incubator at 28°C, alternating light and dark for 12 h/12 h. After 15 days incubation, the spore-laden surface was scraped to remove spores using a metal spatula and distilled water. The resulting suspension was filtered through muslin to separate the spores from the mycelium, and observed and photographed using a 40X magnification photonic microscope. Identification was carried out using the identification keys of Barnett and Botton et al. [18]. Observations focused on characteristics such as spore color and shape, partitioning and whether or not the mycelium was branched.

2.9 Plant Resistance to Cercosporiosis

Plant vigor on 18 plants per treatment at the three locations was determined on the 35th day after sowing. Disease severity was observed based on the size of symptomatic lesions (necrosis) caused by cercosporiosis agents on the leaves, by visual observation of the 18 plants in each treatment and morphological identification of the agents in the laboratory. The number of plants attacked per treatment was counted, and the percentage of plants resistant to attack was determined using the formula of Ibeawuchi et al. [19].

$$\%\text{infestation} = \text{total number of plants x } 100$$

% plant health = 100% - % infestation

2.10 Phytochemical Screening

Leaf extraction was carried out on soxhlet delipidated leaves (with methanol as solvent) according to the method of Oomah et al. [20] by reflux decoction of 50 g of powder in 500 ml of a hydro-methanolic mixture (20V/80V respectively) for 30 min, with the maximum aim of extracting polar compounds such as polyphenols. After filtration on Whatman No.1 paper, the filtrates obtained were evaporated using a rotary evaporator at 60°C. The filtrate residues were oven-dried for 48 h at 45°C to obtain the dry extracts. The methanolic extract was analyzed by capillary gas chromatography combined to mass spectrometry (GC/MS) using an Agilent 7890A GC coupled to an Agilent MSD 5975C inert mass spectrometer. The gas chromatograph was equipped with a VF-1MS capillary column (100% dimethylsiloxane, 20 m x 150 µm (internal diameter) x 0.15 µm (film thickness)) from Varian, liner internal diameter 4 mm. Carrier gas: helium (constant flow rate of 1 mL/min); oven programming: from 37°C (1 min) to 250°C at 5°C/min, then 11 min step-up to 250°C; source temperature: 230°C; transfer line temperature: 260°C; ionization energy: 70 eV. Electron ionization (EI) mass spectra were recorded between 40-400 u.

In mass spectrometry, detection is based on the mass-to-charge ratio (m/z) of an analyte. The analyte molecules in the vapor phase are bombarded with a high-energy electron beam. Positive ions are produced by bombardment in a magnetic field on the basis of their m/z ratio. The signal emitted by the ions from the spectrum is recorded and presented as a computer-generated graph. Constituent identification was based on comparison of mass spectra with commercial Wiley and in-house laboratory databases. Retention indices were calculated using the homologous alkane series (C7-C40) and compared with retention indices available in the NIST Webbook after Kondjoyan & Berdague[21]. Each analysis was performed in triplicate after experimental optimization.

2.11 Statistical Analyses

Data were first registered on Excel version 2016 and then analyzed using the Rcmd package of Rversion 3.6.3 softwares. Normality and homogeneity were tested using the Shapiro-Wilk and Bartlett's K-squared tests respectively. The Tukey test was used to compare the different means using one-way ANOVAs, in order to highlight any significant differences between them.

3. RESULTS AND DISCUSSION

3.1 Compatibility Test

Fig. 1 shows the compatibility test carried out between combinations of microbial strains to assess their ability to cohabit in the same bioformulation. The results illustrated in Fig. 1 show that *B. amyloliquefaciens* and *T. harzianum* have developed a capacity to cohabit in the same environment. In the case of organic matter management technologies, less attention is paid to microbiology, and physical, chemical and technical approaches predominate to ensure plant protection against phytopathogens while causing more environmental damage. All these factors need to be taken into consideration when producing bioformulations.

3.2 Effect of the Formulation on Germination

The variance of okra seed germination is represented by the number of seedlings in each site according to the two okra varieties 7 days after sowing and is analyzed according to Tukey's test at the probability threshold $p = 0.05$. For *C. spineless*, the difference was significant between treated and control plants ($p < 0.05$) at all three sites (Fig. 2). For the *Hire* variety, the difference was also statistically significant between T1 treatment and control at all three sites (Fig.3). Germination rates were higher in seeds treated with the *T. harzianum* and *B. amyloliquefaciens* formulation than in control

seeds at all three sites. This process is thought to be due to the action of *T. harzianum*, which secretes phytohormones such as gibberellin, capable of breaking seed dormancy, and auxin, which induces cell elongation for seedling growth.

The work of Rababe et al. [22] demonstrated the ability of *T. harzianum* on *Fragaria vesca* to stimulate germination via secreted phytohormones. It has also been shown that seed germination can be effective when seeds are pre-treated in a solution containing either *T. harzianum* or *B*. *amyloliquefaciens*, as the latter secrete phytohormones like gibberelin that break their dormancy [23]. A related study by Mohamad et al. [24] demonstrated the ability of *B. amyloliquefaciens* to produce indole acetic acid and gibberellic acid when used to treat and improve the malting of African Red Sorghum.

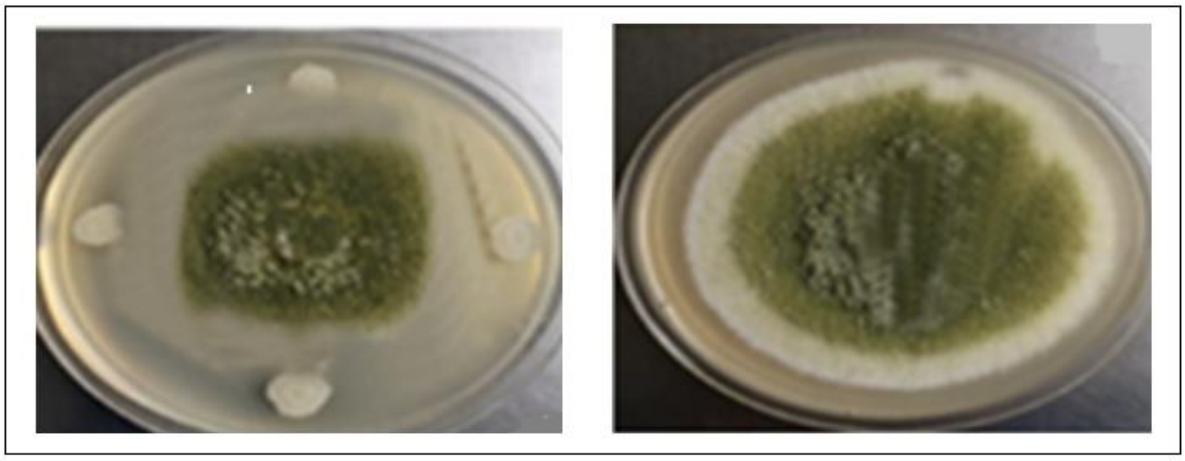

Fig. 1. Compatibility test between *Trichoderma harzianum* and *Bacillus amyloliquefaciens* strains (T1)

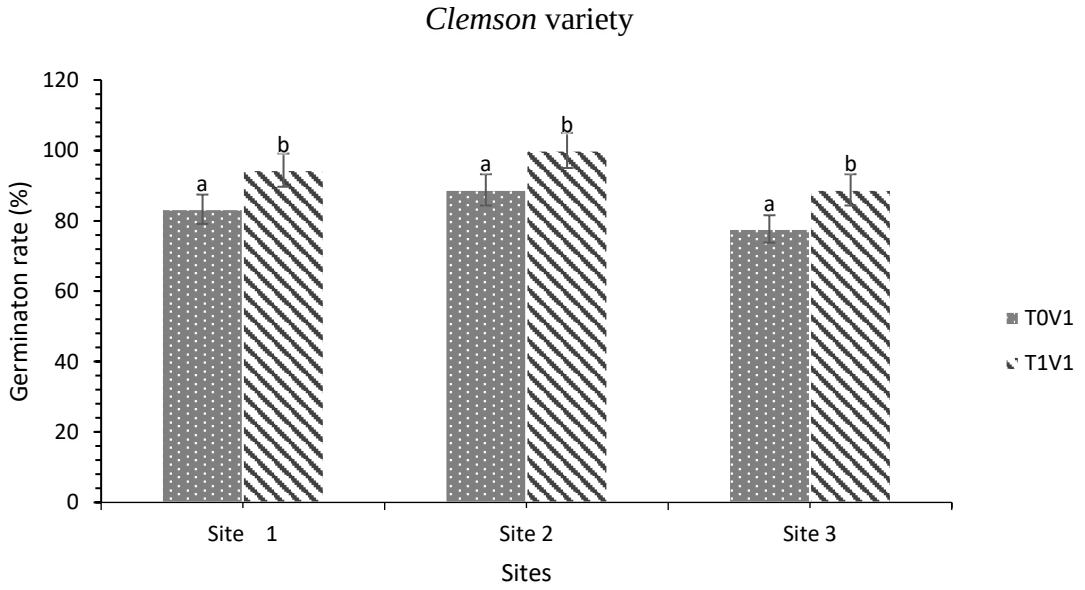

Fig. 2. Germination rate of the Clemson okra variety between treatments at each site at 7 after sowing (DAS)

** Treatments with identical letters in the same site are not significantly different according to Tukey's test P<0.05*

Fig. 3. Germination rate of the *Hire* okra variety between treatments at each site at 7 after sowing (DAS)

** Treatments with identical letters in the same site are not significantly different according to Tukey's test P<0.05*

3.3 Effect of Bioformulation against Cercosporiosis in Crops in the field

Fig. 4 shows the health status of plants through their leaves on the 35th day after sowing. It highlights the degree of attack caused by phytopathogens on plant leaves. Some plants treated with the formulation at all three locations (Fig. 4A) are healthy through their leaves and are characterized by good flowering, while other untreated plants (Fig. 4B) have been attacked and are showing disease symptoms. Attacks identified during okra growth at the different sites, in all varieties, were cercosporium blight caused by *Cercospora abelmoschi*.

The degree of severity of attack on plants in the various locations, according to treatments and okra variety, is shown in Figs. 5 and 6, and is characterized by the vigour of these plants in their resistance to pathogens causing cercosporiosis. The aim was to observe the

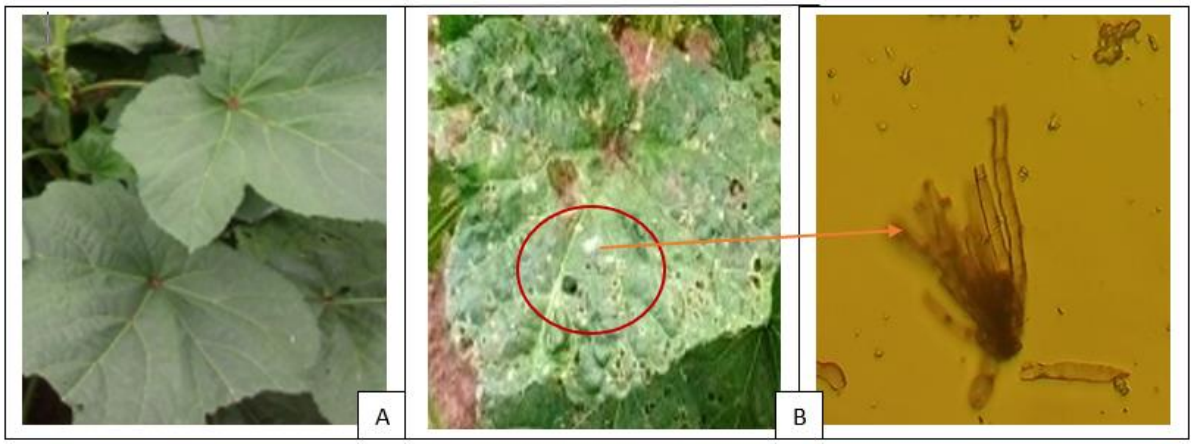

Fig. 4. Effect of plant pathogens on the health of okra plants. (A): Leaves from healthy plants; (B): Symptoms of cercosporiosis caused by *Cercospora abelmoschi* and its spores observed microscope (x40 magnification)

severity of attacks based on the size of symptomatic lesions, by counting the number of plants attacked, and identifying the agents responsible for these attacks. Analysis of variance on the V1 okra variety (*Clemson*) showed a significant effect ($p < 0.05$) of treatments compared to the control in sites 1 and 3. For the V2 variety (*Hire*), the difference was statistically significant between the T0 control and the treatment at all three sites (Table 1). In terms of the plants' ability to resist cercosporiosis agents, the results obtained placed the two plant varieties treated with the T1 formulation as those with the greatest resistance to infectious agents during the vegetative growth phase at each site, compared with the control.

The *Trichoderma harzianum* and *Bacillus amyloliquefaciens* strains in this bioformulation effectively reduced the incidence of attacks occurring during the development phase of okra. This could be justified by the activity of *T. harzianum* due to its ability to synthesize elicitors such as peptaïboles (trichorzianins A and B and trichorzins) and exo-chitinases that induced plant resistance by increasing immunity [25] Jian et al. [26] also demonstrated that *T. harzianum* had antagonistic activity against *Fusarium proliferatum* and *Fusarium verticillioides*, with mycelial inhibition rates of 68.38% and 60.64%, respectively. Culture filtrate suppression rates of *T. harzianum* strains showed antifungal activity against one strain of *F. verticillioides* (32.2%) that was stronger than the mycelium (23.50%). The efficacy of *T. harzianum* according to Abeer et al. [27] was demonstrated by its ability to reduce 35% to 50% respectively on Lactuca and white rot in *Allium cepa*. Zerihun et al. [28] demonstrated that the β-1,3 glucanase and chitinase enzymes secreted by *T. harzianum* are capable of hydrolyzing the cell walls of parasitic fungi. Harwoko et al. (2020) reported that *T. harzianum* eliminates phytopathogenic fungi by mycoparasitism and antibiosis.

This mycoparasitism is made possible by appressoria, which bind to the surface of parasitic fungi and secrete specific enzymes that disrupt the cell walls of these parasites. The volatile substances it secretes, such as gliotoxins, also act as antibiotics. This plant resistance is also linked to the activity of *Bacillus amyloliquefaciens*, which secretes the enzymes β-1,3 glucanase and chitinase, or bioactive molecules such as biosulfactants, plantozolicin, hydrogen cyanide, siderophores and polyketides having antifungal and bactericidal actions that enable the suppression of plant-damaging microorganisms [29]. This finding is also confirmed by the work of Ongena & Jacques [30], in which *Bacillus* sp. facilitated root colonization and interaction with host plant defense responses. In a related study, the use of *B. amyloliquefaciens* with other microorganisms was shown to enhance the biocontrol potential of the strain [31]. Field trials therefore demonstrated the ability of bioformulation to effectively protect plants of both okra varieties against cercosporiosis.

3.4 Phytochemical screening of Abelmoschus esculentus leaf extracts and tentative identification of bioactive molecules

The chromatograms below represent the phytochemical screening of methanolic extracts of okra from the T0 control (Fig. 7) and the T1 treatment with *Trichoderma harzianum* and *Bacillus amyloliquefaciens* (Fig. 8). Analysis of these extracts using gas chromatography-mass spectrometry (GC-MS) revealed several peaks determining the retention times of the molecules in the stationary phase, depending on their affinity with it. Retention times ranged from 3.788 min to 7.141 min for the control and from 3.587 min to 11.312 min for the T1 treatment.

Gas chromatography-mass spectrometry analysis of the okra leaf extract revealed the presence of several compounds obtained with different molecular weights at each peak in the okra leaf extract analysis. These molecules were specific to the control extract such as the sugar 2-O-Methyl-D-mannopyranose (Table 2) and specific to the T1 treatment such as decanoic, hexadecanoic and tetradecanoic acids (Table 3) and others common to both extracts such as nonadecatriene acids and cyclohexylamine (Table 4). As a result, this screening revealed an overexpression of secondary metabolites in okra leaves from the treated plot (Table 3), which are specific to it compared with the control plot (Table 2). Several molecules are also shared (Table 4), playing various roles in the plant. Exploration of the phytochemical potential of methanolic extracts from okra leaves in treatments T0 and T1 using gas chromatography-mass spectrometry revealed an overexpression of metabolites in plots treated with bioformulation T1 compared with the control plot at the different sites, for each okra variety. Treatment and control share certain molecules, while others are specific to them. This expression could be due to the action of certain bioactive

molecules secreted by *Trichoderma harzianum* such as 6-pentyl-a-pyrone and/or by *Bacillus amyloliquefaciens* such as iturin A or surfactin or fengycin, which stimulated the genes responsible for the induction of metabolic pathways [31,32], resulting in the expression of different types of metabolites in these okra plants.

Barakat et al. [29] reported that *Trichoderma harzianum* releases several volatile compounds such as benzene-ethanol, butanoic acid, propanoic acid, palmitoyl chloride, glycerol-1-palmitate and hexadecanoic acid. These metabolites have many functions in biological control (benzene-ethanol, butanoic acid, propanoic acid, tetradecanoic acid, benzotriazepine) and as phytoalexins (benzoic acid; scoparone; scopoletin). They have a significant inhibitory effect on the development of *Cercospora beticola*, the agent causing *cercosporiosis* of beet (Beta vulgaris), and *Sclerotinia sclerotiorum*, the agent responsible for white rot of carrot (*Daucus carota*) [33]. They also have inhibitory power on *Colletotrichum lagenarum*, agent of anthracnose of melon (*Cucumis melo*), and cucumber (*Cucumis sativus*) [34]. Benhamou & Picard [35] reported that there was a positive correlation between overall plant response and changes in the biochemistry and physiology of plant cells pre-inoculated by *Trichoderma* sp., which lead to structural modifications. These results thus highlight the performance of *Trichoderma harzianum* and *Bacillus amploliquefaciens* bioformulation on the expression of bioprotective molecules against cercosporiosis in okra.

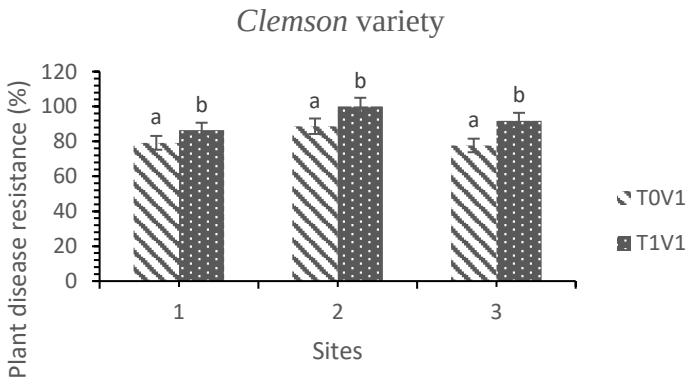

Fig. 5. Variation in plant vigor of the *Clemson spineless* okra variety between treatments at each site at 35 DAS

**Treatments with the same letters at the same site are not significantly different according to Tukey's test P<0.05*

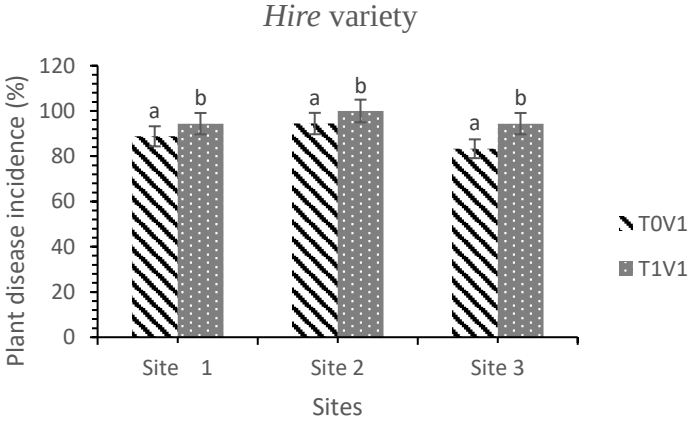

Fig. 6. Variation in plant vigor of the *Hire* okra variety between treatments at each site at 35 DAS

* Treatments with the same letters at each site are not significantly different according to Tukey's test *P<0.05*

Table 1. Effect of treatments on the plant vigor 35 days after sowing

	Sites	*Clemson spineless* variety (%)	*Hire* variety 2 (%)
Site 1	T0	79.24 ± 2.47 [a]	88,88 ± 0,91 [a]
	T1	86,48 ± 3,59 [b]	94.44 1.22 [b]
Site 2	T0	88,7 ± 0,00 [a]	94,44 ± 1,45 [a]
	T1	100,00 ± 0,00 [b]	100,00 ± 0,00 [b]
Site 3	T0	77,76 ± 0,85 [a]	83,33 ± 2,90 [a]
	T1	91,82 ± 6,97 [b]	94,44 ± 1,20 [b]
	F-value	19.05	17,5
	P-value	0,049*	0,040*

**Means with the same letters on the same site are not significantly different according to Tukey's test P<0.05*

Table 2. Specific molecules from okra leaves in the T0 control

Hypothetical molecules specific to T0	Retention time	Hypothetical formula	Molecular weight	Abundance (%)
Phthalic, 8-chlorooctyl isobutyl ester acid	3,78	$C_{20}H_{29}ClO_4$	230.3	14.14
2-O-Methyl-D-mannopyranose	3,90	$C_7H_{14}O_6$	194.18	76.22
Diisopropyl adipate	6,30	$C_{12}H_{22}O_4$	230.3	65.27
5.alpha.- Androstan-16-one,cyclic ethylene mercaptole	6,30	$C_{21}H_{34}S_2$	350.6	86.82
(Z)-Cyclohexylamine,	7,14		56.10	72.65
N-(2-chlorocyclopentylidene) -, N-oxide	7,14	$C_{18}H_{32}O_2$	280.4	53.40

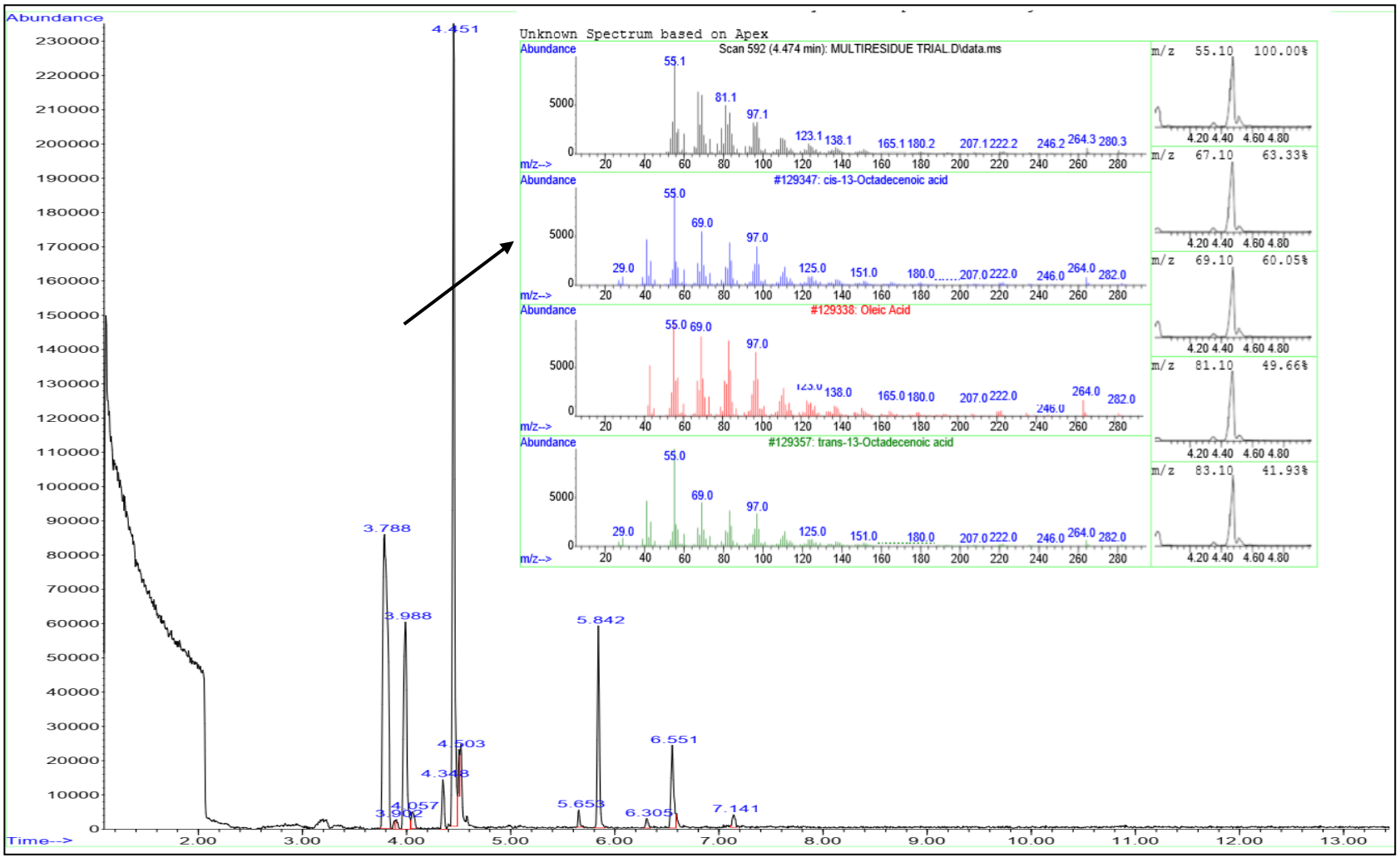

Fig. 7. Chromatogram of the methanolic extract of okra leaves from non-treated plants (T0)

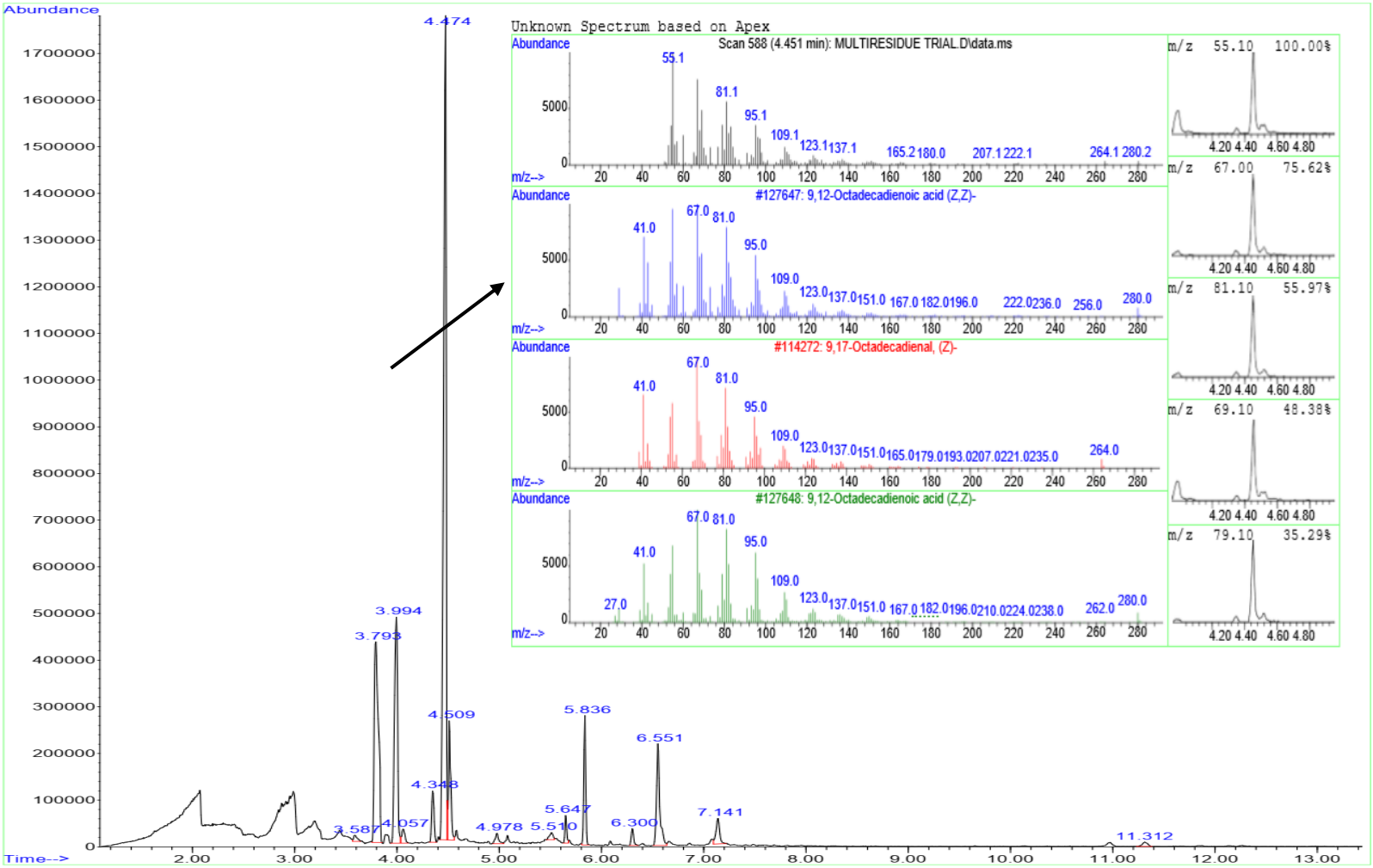

Fig. 8. Chromatogram of methanolic extract from okra leaves in the plot treated by bioformulation

Table 3. Specific compounds from okra leaves treated with T1 bioformulation

Hypothetical molecules specific to T1	Retention time	Hypothetical formula	Molecular weight	Abundance (%)
5-Chloro-2-pyridinol	6,3	C_5H_4ClNO	129.54	69.97
Succinic, ethyl 3-(2-methoxyethyl) nonyl ester acid	6,3		130	100
1-(2 Adamantylidene) semicarbazide	11,31	CH_5NO_3	92.1	100
1,3-Bis-(2-cyclopropyl,2-methylcyclopropyl)-but-2-en-1-one	11,31	$C_{16}H_{26}O$	258.4	73.20
7-Hydroxy-3-(1,1-dimethylprop-2-enyl) coumarin	5,51	$C_{14}H_{14}O_3$	230.26	100
trans-2-Methyl-.beta.-methyl-.beta.-nitrostyrene	5,51	$C_{10}H_{11}NO_2$	177.2	62.82
3H-1,3,4-Benzotriazepin-2-one,1,2-dihydro-3,5-dimethyl-	3,58	$C_{10}H_{11}N_3O$	189.2	100
1-Phthalazinecarboxamide,3,4-dihydro-4-oxo-	4,58	$C_{12}H_{13}N_3O_2$	279.29	27.6
Glycerol 1-palmitate	5,64	$C_{19}H_{38}O_4$	330.50	100
Palmitoyl chloride	5,64	$C_{16}H_{31}ClO$	274.9	78.49
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	3,79	$C_{16}H_{24}O_4$	278.3	100
4-Piperidinemethanol, alpha.,. alpha.- diphenyl-1-methyl-	4,97	$C_4H_6N_2O$	281.4	100
1H-Imidazole-2-methanol	4,97	$C_{19}H_{23}NO$	98.1	72.44
tetradecanoic acid	3,99	$C_{14}H_{28}O_2$	228.37	95.76

Table 4. Hypothetical compounds common to leaves from control and treated plants

Similar compounds to T0 and T1	Retention time	Hypothetical formula	Molecular weight
Phthalic Acid, hexylpropyl ester	5.84	$C_{22}H_{34}O_4$	362.5
n-hexadecanoic acid	T0 3.98 / 4.50	$C_{16}H_{32}O_2$	256.4
	T1 3.98		
octadecanoic acid	4.50	$C_{18}H_{36}O_2$	284.47
9,17-Octadecadienal, (Z)-	T0 4.45	$C_{18}H_{32}O$	264.44
	T1 4.34		
9,12-Octadecadiene	T0 4.45	$C_{18}H_{32}O$	264.44
	T1 6.55		
9-Octadecenal, (Z)-	T0 7.14	$C_{18}H_{34}O$	266.46
	T1 6.55		
9,12-Octadecadienoyl chloride, (Z,Z)-	T0 6.55	$C_{18}H_{31}ClO$	298.9
	T1 7.14		
1,3,12-Nonadecatriene	T0 6.55	$C_{19}H_{34}$	262.5
	T1 7.14		
(Z)-Cyclohexylamine	7.14		56.10

4. CONCLUSION

This study aimed at assessing the performance of bioformulation with *Trichoderma harzianum* and *Bacillus amyloliquefaciens* on germination and on the expression of bioprotective molecules in okra, able to control cercosporiosis agents. The results showed that this formulation effectively stimulated germination of okra seeds at a rate of over 88%. This bioformulation significantly influences metabolite production in the okra plant. These metabolites stimulate the plant's defense mechanisms against agents causing okra diseases such as cercosporiosis. This formulation could thus be useful for boosting okra production in agro-ecological zones through the bioprotection mechanisms that it develops in okra.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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