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Plantain Bananas PIF Seedlings Treatment with Liquid Extracts of *Tithonia diversifolia* Induces Resistance to Black Sigatoka Disease

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Abstract

Plantain bananas culture encounters the problems of seedlings quantity and quality unavailability. Black Sigatoka Disease (BSD) is one of the main pathological constraints of banana that can severely reduce the photosynthetic leaf area, leading to the losses of production of about 50% in banana plantation. The use of liquid extracts of *Tithonia diversifolia* could potentially induced the resistance of the PIF seedlings to BSD during the vegetative stages in the nursery. The aim of this work was to evaluate the effect of *Tithonia diversifolia* liquid extracts against the development of BSD on the PIF plantain bananas seedlings. The explants in the greenhouse and the seedlings in the shade were watered with liquid extracts of *T. diversifolia* during the process of PIF seedling production in sterile and non-sterile conditions. The treated seedlings show a more effective enhancement of seedlings quality parameters and also induce resistance against BSD. The vegetative stages parameters (the number of shoots, the diameter and the height of shoots, the foliar area, the length and weight roots) were improved in treated seedlings compared to controls ones. The treated seedlings showed maximum protection against BSD of up to 87% compared to controls. They also exhibited an increase in the accumulation of total proteins and total phenolics, as well as the activity of defense-related enzymes (peroxidase, polyphenol oxidase and glucanase). The treatment seems to acts as a vital stimulator and could therefore be a useful tool for small holder farmers favouring an eco-friendly agriculture using fewer synthetic inputs.

Keywords

Plantain Banana, PIF Seedlings, *Tithonia diversifolia*, Black Sigatoka Disease,

Induced Resistance, Vital Stimulator

1. Introduction

Banana (*Musa* spp.) is one of the most important fruits and food crops in the world with annual production of more than 100 Mt [1]. In the large family of *Musaceae*, there are many subgroups of hybrid triploid varieties: AAB and ABB (cooking banana) which result from the hybridization between wild ancestors AA (*Musa acuminata*) and BB (*Musa balbisiana*). Plantain bananas undergo various transformations and are used in the gastronomy due to their high energy value and rich mineral as well as dietary fiber and vitamin content. They, therefore, play a vital role in contributing to food security for the people in Central and West Africa.

In Cameroon, the production of plantain bananas is estimated at 3.94 million tons per year and it is ranked 3rd in the world and the first in the Central African Economic and Monetary Community (CEMAC) zone [1]. The Plantain bananas consumption is very high, therefore mobilizing more than 650,000 actors in the sector and they are at the heart of trade in the sub-Central African regions [2]. The per capita consumption of plantain bananas is 159 and 126 kg/person/year respectively in Gabon and Cameroon [3]. The production is managed by smallholder farmers in small scale which is characterized by low productivity and thus less creation of new plantations. Such low productivity is due to growing and emergent pests and diseases present in banana's phytobiome.

Plantain bananas suckers come from old plantations and are commonly extracted to produce seedlings for plants renewals in the traditional practice. In order to produce more plantain bananas seedlings, the vegetative multiplicative techniques like the micropropagation (tissue culture) and/or the macropropagation are required. The micropropagation technique allows rapid production of high quality, disease-free and true to type planting materials (vitro plants) within a short duration in limited space but the cost of production is however high for most of the developing countries [4]. However, the macropropagation technique of plants from stem bids (PIF) is an alternative to produces healthy and clean vivo plants [3].

PIF technique permits in a short period of time (2 to 3 months) at a low cost to generate quickly 20 to 100 plantain bananas seedlings from a single sucker depending on the variety and the technician's experience [3] [5]. However, PIF seedlings face the problems of acclimatization, contamination on farmlands and the position of the shoot on explants which influences the vigor of the plant [3] [6]. Indeed, this can lead to the loss of about 60% of the plants on farmlands (new plantation) and are sometimes rejected by some farmers.

The farmland milieu contains many pathogenic microorganisms which cause diseases on the plant amongst which *Mycospharella fijiensis*. It belongs to the

ascomycetous fungi family of *Mycospharella* leaf spot disease which generates leaf disease, and black Sigatoka disease (BSD) being the more virulent one since it is more invasive in banana growing and severely reduces the foliar synthetic area on plants [7]. Therefore, economically and physiologically, the BSD is the most destructive disease of plantain bananas and accounts for losses of 50% of bananas production [8]. Most cooking bananas and plantains (AAB and ABB) are moderately to highly resistant to black Sigatoka disease compared to bananas (AAA) generally highly susceptible [9]. In the nursery, the only control method for BSD is leaf removal (deleafing) [10], there is however a need to assess BSD effect on seedling in nursery.

Many chemical pesticides such as fungicides, bactericides, viricides, nematicides, insecticides … are often used to destroy pathogenic agents but they however cause problems to humans and the environment. Indeed, these chemical constituents used in the agricultural methods to solve contamination problems on the field cause the pollution of soil and water and are very harmful for human health and the environment [11]. Moreover, the rapid emergence of fungicide resistance is conducive to a significant increase in the cost of disease control but, above all, to increasing negative environmental effects [12]. Unfortunately, these current tools of diseases management are not efficient and do not respect the ecosystem. Therefore, it is of both biological and agricultural importance to set up an integrated pest management (IPM) approach precisely for resistance to *M. fijiensis* in the nursery and the farm.

The less development of BSD was observed on susceptible *Musa* spp. cultivars when they were cultivated on sites rich in organic matter [13]. Furthermore, recent studies carried out in Ivory Coast have demonstrated that aqueous extract of *T. diversifolia* has a strong influence on rice protection and resistance against termites on farmland by acting as an insecticide in the control of rice cultivation [14] and by acting as a fertilizer and fungicide in the control of another culture [15]. Nowadays, the potential impact of *T. diversifolia* in agriculture as biofertilizer and biopesticide is receiving more attention. The main components of *T. diversifolia* called wild sunflower or tree marigold are minerals like nitrogen (N), phosphorus (P) and potassium (K) averaging between (3% - 5%), (0.5% - 2.5%) and (4% - 6%) respectively and some secondary metabolites like Alkaloids (854 mg), Flavonoids (339 mg) and Terpenoids (65 mg) [16] [17] [18]. Indeed, it is known as a non-fixing nitrogen plant and has the ability to enrich the surrounding soil with nutrients during decomposition [19].

Besides, a recent research has shown that soil amendment with *Tithonia diversifolia* alone or *Tithonia diversifolia* combine to clam shells protects efficiently the PIF seedlings leaves against the development of *M. fijiensis*, and also improves growth promotion [6]. The use of liquid extracts of *Tithonia diversifolia* in the production of PIF seedlings could be a new approach to improve the PIF performance. This study is aimed to evaluate the effect of liquid extracts of *Tithonia diversifolia* on the induced resistance of PIF plantain bananas seedlings

against BSD. This work was conducted in Yaoundé, Cameroon from September 2016 to August 2017.

2. Materials and Methods

2.1. Materials

The study was carried out at the Biotechnology Center of the University of Yaoundé 1 in Cameroon (3˚52' North and 11˚25' East, 759 meters' altitude), located in the agroecological zone known as wet rainforest with Bimodal Rainfall.

Suckers of plantain (*Musa* spp., genome AAB) were obtained from Lékié division (Obala) of the Centre region of Cameroon and were the variety of Big-Ebanga, selected due to their short cycle of production and their capacity to produce great number of PIF seedlings.

M. fijiensis strain was provided by African Centre for Research on Bananas and Plantain of Njombé in the Littoral region of Cameroon.

The substrates used in the greenhouse (white sawdust) and in the shade (sand and black soil, 2/3:1/3) were collected in the locality of Yaoundé, sterilized in an oven at different temperatures and time intervals as described by [3].

2.2. Preparation of Extracts

T. diversifolia was collected around the Biotechnology Center of the University of Yaoundé 1. The leaves were washed in running tap water, cut and then mixed with water in the ratio of 1:5 (w/v) before fermentation in recipients for 10 and 15 days according to the principle of the cold maceration that is at dry and cold conditions at room temperature. At the end of the fermentation period, each liquid extract obtained was used directly and then diluted 1/2 in water to be used for explants and seedlings treatment while the ones treated with water served as control.

2.3. Experimental Design

This study was conducted under sterile condition and non-sterile (farmers) condition in the greenhouse and under shade following almost the same experimental design performed by [6], except for the treatments that were different.

The different treatments of the study were carried out in two completely randomized blocks with (05) treatments in each block in the greenhouse and in the shade.

- Two blocks:
  - Sterile Substrate (SS).
  - Non-Sterile Substrate (NSS).
- Five treatments (four liquid extracts of *Tithonia diversifolia* and one control):
  - Control only water;
  - A liquid extract-based of *Tithonia diversifolia* of 10 days (Extract 1);
  - A diluted liquid extract-based of *Tithonia diversifolia* of 10 days (Extract 2);
  - A liquid extract-based of *Tithonia diversifolia* of 15 days (Extract 3);

- A diluted liquid extract-based of *Tithonia diversifolia* of 15 days (Extract 4).

Each treatment in each block was considered as an Experimental Unit (EU). The PIF explants were prepared following the method used by [3]. In each EU, three (03) explants were introduced in the greenhouse. The seedlings germination and pre-emergence in the greenhouse was effective through explants watering with the different types of extracts.

2.4. Assessment of Seedlings Vegetative Stages Growth Parameters

The germination and pre-emergence parameter (cumulative number of seedling) was evaluation per EU after every seven days in the greenhouse for a period of five weeks.

After this period, the seedlings from the greenhouse were weaned in plastic planter bags at the stage of two or three small open leaves per seedling and for more than one root, then transferred under the shade following the same experimental design as in the greenhouse.

The effect of different liquid extract of *Tithonia diversifolia* on the vegetative growth stage were evaluated for each EU and three (03) seedlings were selected and labelled under the shade. The evaluation was done by measuring different agromorphological parameters:

- The height of the seedling;
- The diameter of the seedling pseudo-stems;
- The total foliar area of the seedling calculated with the formula reported by [3].

$$\text{TLS} = L \times W \times 0.8 \times \text{number of leaves} \times 0.662\ (\text{cm}^2)$$

This evaluation was done every fourteen days starting from the day that the seedling entered the shade for acclimatization and was done for a period of six (06) successive weeks.

2.5. Assessment of Seedlings Susceptibility to BSD

A suspension of *M. fijiensis* strain concentrated at 10^6 zoospores/mL was prepared as [3] and applied through artificial inoculation on the leaves of PIF plantain bananas seedlings almost of the same age. Three (03) seedlings leaves per treatment aged of about 12-weeks were selected and labelled under the shade. Before inoculation, a leaf of each plant was detached and conserved at −45˚C in a plastic sachet for biochemical analysis of the before inoculation stage. An amount of 50 µL droplet of inoculum suspension was then deposited midway through the exposed surface of the leaves. The symptoms were observed every two days, reported every four (04) days since the necrosis development on the treated leaves was very slow compared to the control. The necrotic surfaces area (NSA) was measured according to the method used by [3] [6]. After five weeks, a leaf of each plant was detached and conserved at −45˚C in a plastic sachet for biochemical analysis of biomarkers for the post-infection stage.

2.6. Assessment of Biomarker's Accumulation

Biochemical analyses were carried out following the disease assessment on the whole leaves. The samples involved were cut at 1 cm beyond the necrotic point or beyond the marked scar (sections with no symptoms) and each treatment was repeated trice. The evaluation of the accumulation was done at the before inoculation and post-inoculation stages. For each treatment, 0.5 g of fresh leaf was used for the sample's analyses. The extraction and quantification of samples were carried out according to the method reported by [20]-[24] modified respectively for total phenolic (760 nm), total protein (595 nm), peroxidase (470 nm), polyphenol oxidase (330 nm) and glucanase (540 nm). The total phenolics was measured in mg equivalent of gallic acid per g of fresh weight while that of the total proteins concentration was expressed in mg equivalent (Eq) of bovine serum albumin (BSA) per g of fresh weight (FW). The peroxidase and polyphenoloxidase concentration were expressed in UE/min/g FW, while the glucanase concentration was expressed in mg of glucose/g FW.

2.7. Statistical Analyses

The *T. diversifolia* liquid extracts effects on the PIF seedlings vegetative growth stages, the susceptibility to BSD and the biomarker's accumulation were analysed by subjection of the variables (the number of shoots, the height and the diameter of shoots, the foliar area, the necrotic surface, total proteins, total polyphenols, peroxidase, polyphenol oxidase and glucanase) to mixed three-way ANOVA performed with XLSTAT software. The length and weight of roots seedlings were analysed by subjection of both variables to mixed two-way ANOVA also performed with XLSTAT software. Each plant is being taken as experimental unit and condition or stage, treatment and day as factors. Multiple comparisons of the means were done by applying Tukey's test at 5% probability level. Principal components analysis (PCA) with Pearson correlation between the different variables was also performed with XLSTAT software.

3. Results

3.1. Effect of Seedlings Vegetative Stages Growth Parameters

Liquid extracts of *T. diversifolia* (Extract 1, Extract 2, Extract 3 and Extract 4) were found to significantly ($P < 0.0001$) influence the vegetative stages growth parameters of PIF plantain bananas seedlings notably the number of shoots, the diameter and the height of shoots, the foliar area, the roots length and weight of roots compared to control (**Table 1**, **Figure 1** and **Figure 2**). The coefficient of determination (R^2) for all these parameters was close to a 100% (**Table 1**) and showed thus that *T. diversifolia* model indicates a perfect fit. The most influential variable was the time for the number of shoots and foliar area, while it was the treatment for the diameter and the height of shoots, as well as the roots length and weight of roots. All these vegetative stages growth parameters evolve significantly in course of time.

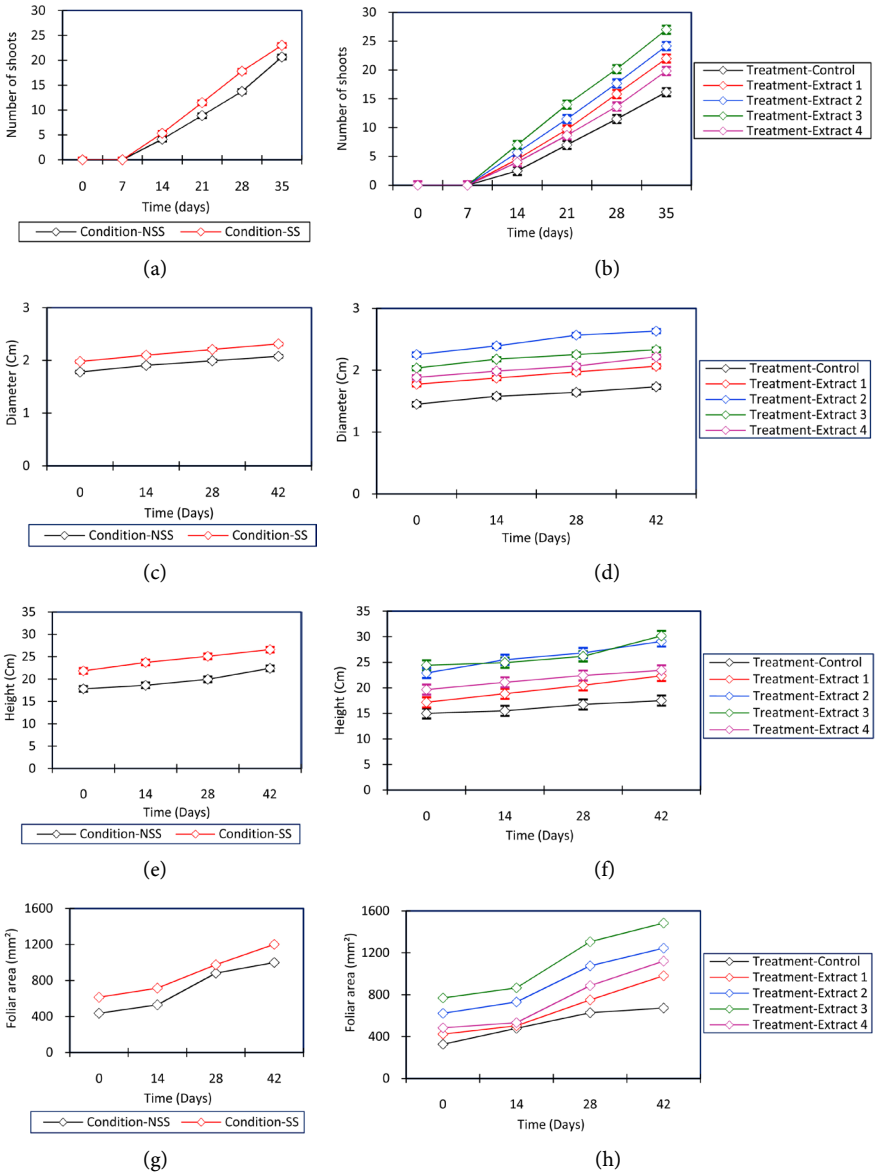


Figure 1. Effects of *T. diversifolia* liquid extracts in the greenhouse on the number of shoots, the diameter, height and foliar area of PIF plantain seedlings in course of time. Interaction plots of day and condition and of day and treatment respectively for the number of shoots ((a), (b)), the diameter ((c), (d)), the height ((e), (f)) and the foliar area ((g), (h)). Each point represents the average mean of three replicates with the standard deviation for each treatment.

For the number of shoots, the diameter of shoots, the height of shoots and the foliar area, all the variables and the interactions were highly significant ($P < 0.0001$) as shown in **Table 1** except for the non-significant interactions of the diameter and the height of shoots (condition and time) and of the height of shoots (condition, treatment and time). The condition, the treatment and the interaction condition treatment were highly significant for the root's length and weight of roots. The treatment influences positively the roots development and the pre-emergence as shown in **Figure 3**.

Table 1. Variance analysis of Extract based on *Tithonia diversifolia* effects on the PIF plantain seedlings agromorphological growth (number of shoots, diameter and height of shoots, foliar area, length and weight roots) and disease severity in the greenhouse and the shade. Values in bold correspond to tests where the null hypothesis is not accepted with a significance level alpha = 0.05. DF is the degree of freedom; *F* is the value of F test and *P* is the probability.

	Number of shoots			Diameter (cm)			Height (cm)		Foliar area (mm²)		Roots length (cm)			Roots weight (g)	
	R^2= 99%			R^2= 99%			R^2= 96%		R^2= 100%		R^2= 99%			R^2= 100%	
Source	DF	F	*P*	DF	F	*P*	F	*P*	F	*P*	DF	F	*P*	F	*P*
Condition	1	173.400	**<0.0001**	1	766.090	**<0.0001**	436.580	**<0.0001**	60,950.218	**<0.0001**	1	200.641	**<0.0001**	7359.818	**<0.0001**
Treatment	4	187.722	**<0.0001**	4	1429.833	**<0.0001**	308.658	**<0.0001**	91,072.287	**<0.0001**	4	481.893	**<0.0001**	5766.474	**<0.0001**
Time	7	3133.382	**<0.0001**	3	321.046	**<0.0001**	81.409	**<0.0001**	160,448.40	**<0.0001**	4	-	-	-	-
Condition * Treatment	4	2.752	**0.031**	4	123.465	**<0.0001**	22.551	**<0.0001**	8892.421	**<0.0001**	4	9.714	**0.000**	485.210	**<0.0001**
Condition * Time	7	25.684	**<0.0001**	3	1.614	0.193	1.857	0.144	1325.291	**<0.0001**	3	-	-	-	-
Treatment * Time	28	24.238	**<0.0001**	12	2.764	**0.003**	2.499	**0.008**	2872.525	**<0.0001**	12	-	-	-	-
Condition * Treatment * Time	28	1.881	**0.020**	12	5.087	**<0.0001**	1.327	0.220	553.150	**<0.0001**	12	-	-	-	-

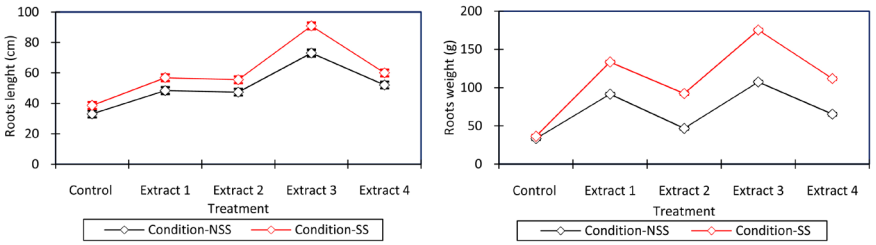


Figure 2. Effects of *T. diversifolia* liquid extracts on the root's length and weight of PIF plantain seedlings at the age of 16 weeks in course of time. Interaction plots of treatment and condition for the roots length (a), the roots weight (b). Each point represents the average mean of three replicates with the standard deviation for each treatment.

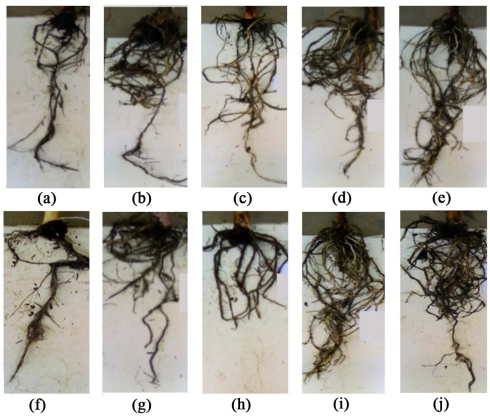


Figure 3. Effects of *T. diversifolia* liquid extracts on the roots of PIF plantain seedlings at the age of 16 weeks in sterilized substrate (SS) condition (**up**) and non-sterilized substrate (NSS) condition (**down**); from the left to the right (**C**) control and treatments **E1**, **E2**, **E3** and **E4** respectively.

A significant difference was observed between the seedlings of the sterile substrate (SS) condition and non-sterile substrate (NSS) condition for all the vegetative stages growth parameters (the number of shoots, the diameter and the height of shoots, the foliar area, the roots length and weight of roots) and confirmed by the two statistically distinct groups obtained. These parameters were more important in the sterile substrate condition (SS) compared to the non-sterile substrate (NSS) condition, that is respectively 23 and 20 for the number of shoots 35 days after weaning (daw), 2.3 and 2 cm for the diameter of shoots 42 daw, 26.6 and 22.4 cm for the height of shoots 42 daw and 1201.73 and 999.69 mm^2 for the foliar area 42 daw (**Figure 1(a)**, **Figure 1(c)**, **Figure 1(e)** and **Figure 1(g)**) and it was the same for the roots length and weight of roots (**Figure 2(a)** and **Figure 2(b)**). Despite the significant difference between the SS and NSS conditions, the *T. diversifolia* liquid extracts effect on the vegetative stages' growth was efficient in both conditions.

Regardless of the condition, the vegetative stages growth parameters (the number of shoots, the diameter and the height of shoots, the foliar surface, the roots length and the weight of roots) were consistently higher in the treated seedlings (Extract 1, 2, 3 and 4) compared to the control (untreated) ones as shown on **Figure 1(b)**, **Figure 1(d)**, **Figure 1(f)** and **Figure 1(h)** and **Figure 2(a)** and **Figure 2(b)**. Therefore, distinct statistical groups were obtained to be precisely five groups (05) for the number of shoots, the diameter of shoots, the foliar surface and the weight of roots, and four (04) groups for the height of shoots and the roots length. The treatments that showed the best effect in terms of growth promotion at the germination and pre-emergence stage as well as at the vegetative stage is Extract 3 (**Figure 1(b)**, **Figure 1(d)**, **Figure 1(f)** and **Figure 1(h)** and **Figure 2**).

3.2. Effect of Seedlings Susceptibility to BSD

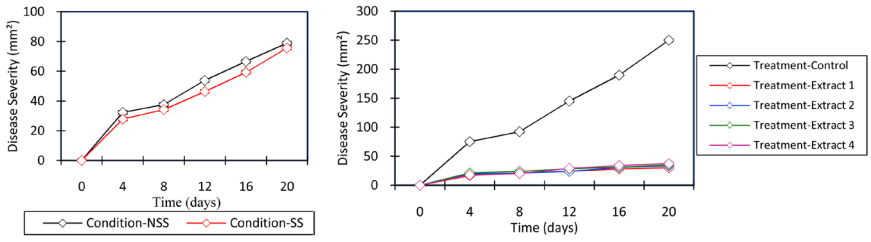
Liquid extracts of *T. diversifolia* were found to significantly ($P < 0.0001$) influence the susceptibility to BSD of PIF plantain bananas seedlings (**Table 2**, **Figure 4**). The R^2 value was equal to a 100% (**Table 2**) and the most influential variable was the treatment. The necrosis development evolves significantly and continuously in course of time. All the variables and the interactions were highly significant ($P < 0.0001$) for the BSD severity (**Table 2**).

A significant difference was observed between the seedlings of the sterile substrate (SS) condition and non-sterile substrate (NSS) condition confirmed by the two distinct statistical groups obtained. The BSD severity was less important in the sterile substrate condition (SS) compared to the non-sterile substrate (NSS) condition (**Figure 4(a)**). Despite the significant difference between the SS and NSS conditions, the *T. diversifolia* liquid extracts effect on BSD severity was efficient in both conditions.

Regardless of the condition, the BSD severity was consistently lower in the treated seedlings (Extract 1, 2, 3 and 4) compared to the control (untreated) ones

Table 2. Variance analysis of Extract based on *Tithonia diversifolia* effects on the PIF plantain seedlings biochemical markers accumulation (total proteins, total polyphenols, peroxidase, polyphenol oxidase and glucanase) at two stages (before inoculation and after inoculation). Values in bold correspond to tests where the null hypothesis is not accepted with a significance level alpha = 0.05. DF is the degree of freedom; *F* is the value of F test and *P* is the probability.

		Disease severity (mm^2)			Total Phenolics (mg Eq Cat/g FW)		Total Proteins (mg Eq BSA/g FW)		Peroxidase (UE/min/g FW)		Polyphenol oxidase (UE/min/g FW)		Glucanase (mg of glucose/g FW)	
		R^2= 100%			R^2= 100%		R^2= 97%		R^2= 97%		R^2= 93%		R^2= 89%	
Source	DF	F	*P*	DF	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Condition	1	311.955	**<0.0001**	1	3608.402	**<0.0001**	7.449	**0.009**	8.193	**0.006**	51.816	**<0.0001**	7.228	**0.010**
Treatment	4	28,812.060	**<0.0001**	4	1689.244	**<0.0001**	183.976	**<0.0001**	135.858	**<0.0001**	45.743	**<0.0001**	18.282	**<0.0001**
Stage	6	8272.020	**<0.0001**	1	2836.521	**<0.0001**	628.578	**<0.0001**	679.121	**<0.0001**	252.101	**<0.0001**	196.716	**<0.0001**
Condition * Treatment	4	58.958	**<0.0001**	4	233.226	**<0.0001**	34.732	**<0.0001**	31.085	**<0.0001**	5.079	**0.002**	10.235	**<0.0001**
Condition * Stage	6	21.987	**<0.0001**	1	198.410	**<0.0001**	40.879	**<0.0001**	4.081	**0.049**	7.856	**0.008**	2.970	0.092
Treatment * Stage	24	2693.513	**<0.0001**	4	112.794	**<0.0001**	24.198	**<0.0001**	16.232	**<0.0001**	15.870	**<0.0001**	9.052	**<0.0001**
Condition * Treatment * Stage	24	22.516	**<0.0001**	4	3608.402	**<0.0001**	7.449	**0.009**	8.193	**0.006**	51.816	**<0.0001**	7.228	**0.010**



(a) (b)

Figure 4. Effects of *T. diversifolia* liquid extracts in the shade on the necrotic surface of PIF plantain seedlings in course of time. Interaction plots of day and condition (a) and of day and treatment (b) for necrotic surface. Each point represents the average mean of three replicates with the standard deviation for each treatment.

(Figure 4(b)). The difference in terms of BSD severity was very significant between the treated (values between 30.17 and 37.42 mm^2) and the untreated PIF seedlings (250 mm^2) of plantain bananas (Figure 4(b)). However, distinct statistical groups were obtained between the treated seedlings leading to five groups (05) for the BSD severity. *T. diversifolia* liquid extracts (Extracts 1, 2, 3 and 4) showed the best effect in terms of protection against BSD (Figure 4(b)).

3.3. Effect of Biomarker's Accumulation

Liquid extracts of *T. diversifolia* were found to significantly ($P < 0.0001$) influence the biomarkers accumulation parameters in the PIF plantain bananas seedlings notably the content of the total proteins, the total polyphenols and the defense-related enzyme (peroxidase, polyphenol oxidase and glucanase) compared to control (Table 2, Figure 5). The R^2 value for all these parameters was

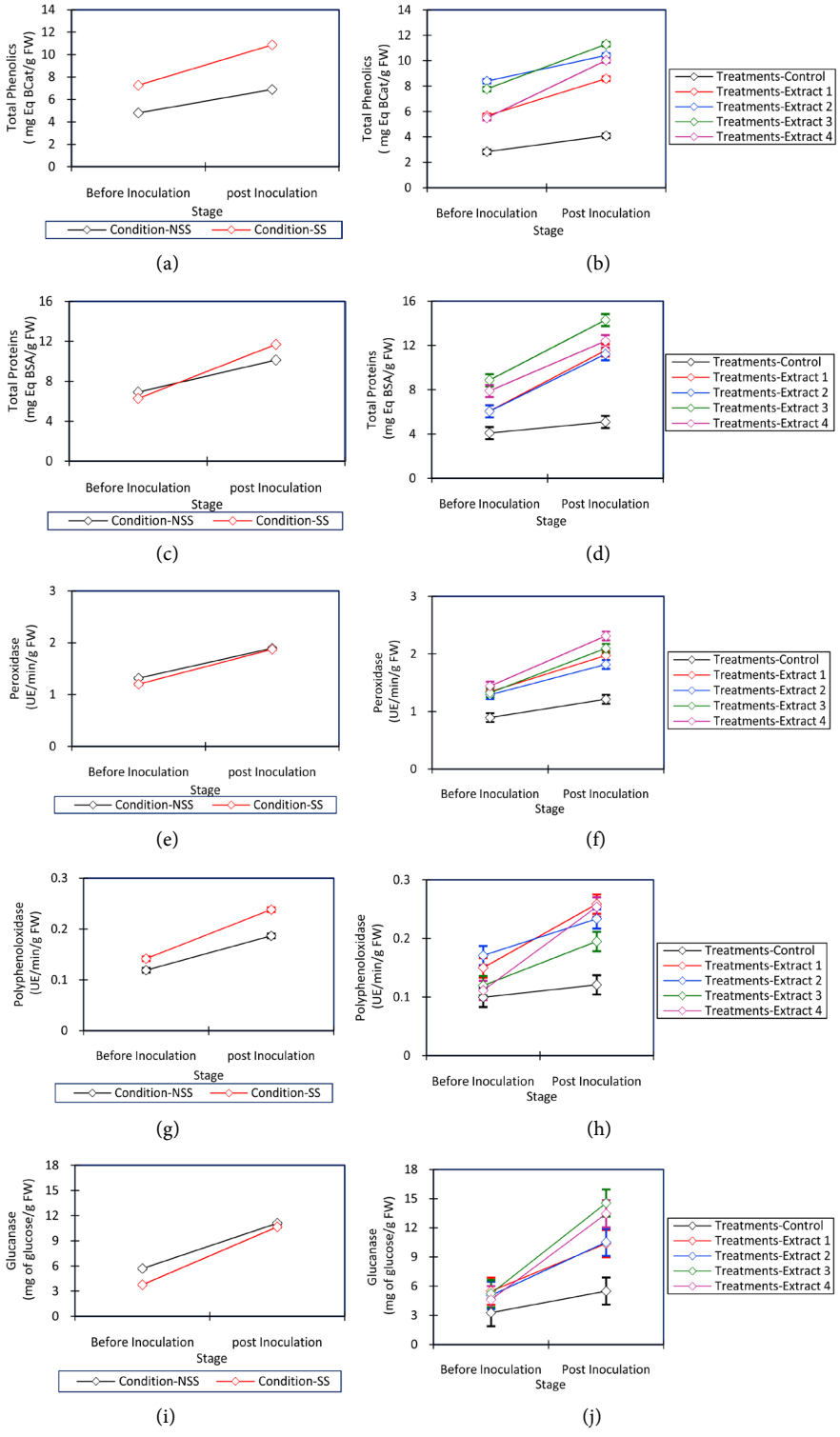


Figure 5. Effects of *T. diversifolia* liquid extracts on biochemical markers (total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase) accumulation before and after inoculation in the PIF plantain seedlings after treatment in the shade with *T. diversifolia* mulch. Interaction plots of stage and condition and of stage and treatment respectively for total phenolics ((a), (b)), total proteins ((c), (d)), peroxidase ((e), (f)), polyphenol oxidase ((g), (h)) and glucanase ((i), (j)). Each point represents the average mean of three replicates with the standard deviation for each treatment.

close to a 100% (Table 2) and the most influential variable was the treatment for the total proteins, while it was the stage for total polyphenols, peroxidase, polyphenol oxidase and glucanase.

For the biomarker's accumulation parameters (the total proteins, the total polyphenols, the peroxidase, the polyphenol oxidase and the glucanase), most of the variables (condition, treatment and stage) and the interactions (condition and treatment, condition and stage, treatment and stage, condition, treatment and stage) were highly significant ($P < 0.0001$) as shown in Table 2 except for the non-significant interaction of the glucanase accumulation (condition and stage).

A significant difference was observed between the seedlings of the sterile substrate (SS) condition and non-sterile substrate (NSS) condition for all these biomarkers accumulation parameters and confirmed by the two distinct statistical groups obtained. These parameters were more important in the sterile substrate condition (SS) compared to the non-sterile substrate (NSS) condition, that is respectively (Figure 5(a), Figure 5(c), Figure 5(e), Figure 5(g) and Figure 5(i)). Despite the significant difference between the SS and NSS conditions, the *T. diversifolia* liquid extracts effect on the biomarker's accumulation was efficient in both conditions (Figure 6(a)). There was a significant difference between the stage before inoculation and the stage after inoculation for all these biomarkers and their amount increases importantly after inoculation (Figure 6(b)).

Regardless of the condition, these biomarkers accumulation parameters were consistently higher in the treated seedlings (Extract 1, 2, 3 and 4) compared to the control (untreated) ones as shown on Figure 5(b), Figure 5(d), Figure 5(f), Figure 5(h) and Figure 5(j). Therefore, distinct statistical groups were obtained to be precisely four groups (04) for the total proteins, the total polyphenols and the peroxidase, and three (03) groups for the polyphenol oxidase and the glucanase. The treatments that showed the best effect in terms of biomarkers accumulation is Extract 3 (Figure 6(c)).

3.4. Principal Components Analysis (PCA)

The variables involved in the growth promotion (the number of shoots, the diameter and the height of shoots, the foliar surface, the roots length and the weight of roots, the total proteins and the total phenolics) were well correlated to one another, and better correlation ($P > 0.001$) were encountered for vegetative growth variables (diameter and height of shoots, foliar surface, length and weight of roots).

These vegetative growth stage variables were well correlated to one another, but weakly correlated with the total phenolics, the total proteins and the enzyme's activity (peroxidase, polyphenol oxidase and glucanase). The number of shoots was weekly correlated to all the variables involved in the growth promotion as well as the biomarkers accumulation variables (Figure 7).

The variables involved in the seedling's protection against diseases notably BSD (disease severity, total proteins, total phenolics, peroxidase, polyphenol oxidase and glucanase) were well correlated ($P > 0.001$) to one another, except

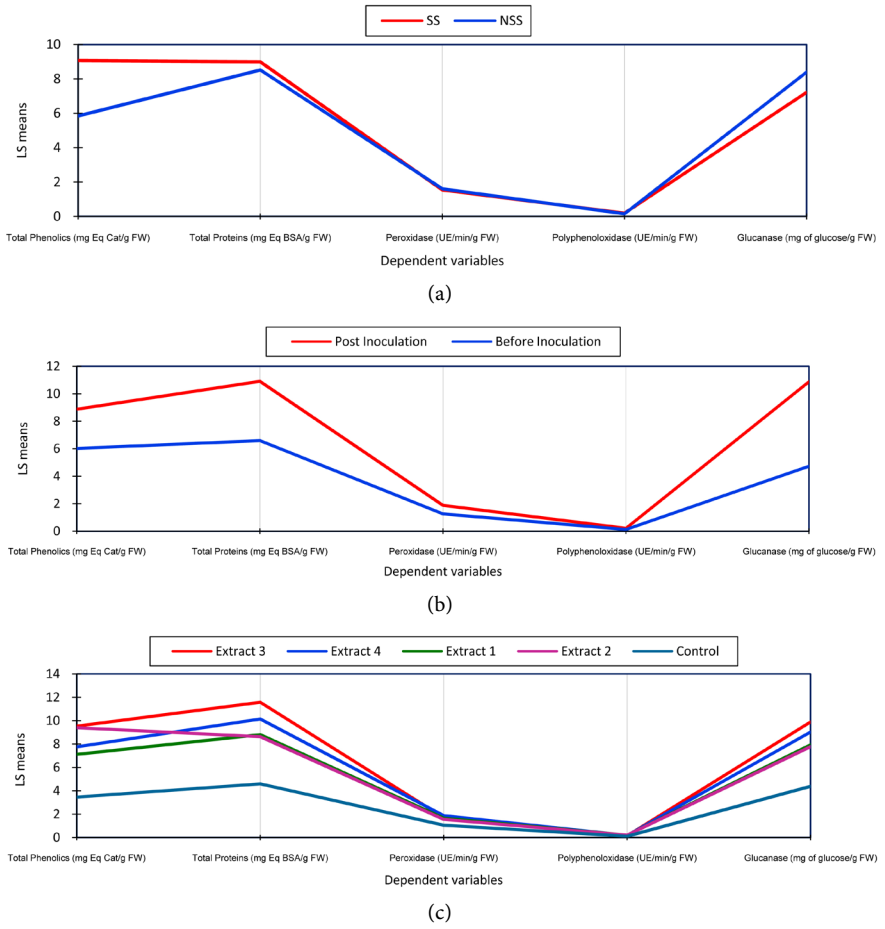


Figure 6. Least Squares (LS) means summary of biochemical markers (total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase) accumulation in the PIF plantain seedlings in the shade after *T. diversifolia* liquid extracts treatment before and after inoculation: Condition (a), Treatment (b) and stage (c). Letters A, B, C, D, E, F, G, H, and I represent different statistical groups defined by the Tukey test (5%).

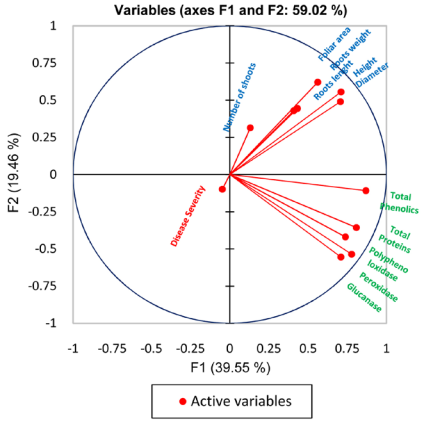


Figure 7. Principal components Analysis (PCA) of all the variables (number of shoots, diameter of shoots, height of shoots, foliar area, height and weight of shoots, necrotic surface of leaves, total proteins, total phenolics, peroxidase, polyphenol oxidase and glucanase). The PCA shows positive or negative correlation, but also the strength of the relationship between the variables.

for the BSD severity that showed a very weak negative correlation ($P > 0.05$) with all the others variables (**Figure 7**).

4. Discussion

The liquid extracts of *T. diversifolia* influences significantly the BSD disease incidence with reduced level of susceptibility in the treated PIF plantain bananas seedlings. The antifungal and repulsive activity of fermented extract of *T. diversifolia* has been demonstrated as well as its effective antimicrobial properties against pests and disease phytopathogens [14] [20] [25] [26]. The less susceptibility of treated seedling could be due to an induced resistance established against *M. fijiensis*. Several studies have also reported that *T. diversifolia* contribute to plant protection by modifying the microbial community and stimulating plant defense mechanisms [14]. Under both conditions, liquid extracts of *T. diversifolia* were efficient defense inducer in PIF seedlings compared to the untreated ones. The results are in accordance with previous research on PIF seedlings and cocoa seedlings in nursery that have shown the effect of shells alone or clam shells combine with *T. diversifolia* on the induction of plant defense [3] [6] [27] [28]. However, the *T. diversifolia* liquid extracts protection level against BSD is high compared to those obtained by [3] [6], with the PIF substrate amendment.

This induction of systemic resistance by *T. diversifolia* liquid extracts could be due to its composition, but also the capacity of the extract to stimulate plant defense mechanisms and increase the synthesis of plant defense metabolites such as phenolic compounds and pathogen-related proteins and defense-related enzymes. Indeed, the classes of phenolic compounds, proteins as markers of resistance defense-related enzymes were strongly involved in the host/parasite interaction [29] [30] [31]. The efficacy of *T. diversifolia* liquid extracts have been shown through the induced resistance against BSD in the PIF plantain bananas seedlings, the high accumulation of total proteins and total phenolics, as well as the increase activity of peroxidase, polyphenol oxidase and glucanase before and after inoculation. The total phenols act either as a defensive or primary barrier inhibiting growth by promoting or activating the mobilization of β-1,3-glucans but as a second blocking barrier invasion by fungi while protecting host plant tissue from phytotoxic substances [30] [31]. The PR proteins are indispensable component of innate immune response in plants under biotic and abiotic stress conditions; they protect plants from further infection by not only accumulating locally in the infected and surrounds tissues, but also in remote uninfected tissues and are also involved in hypersensitive (HR) or systemic acquired resistance against infection [32]. The peroxidase and polyphenol oxidase are associated with reduction in the rate of pathogen multiplication and spread through the oxidation of substrates leading to the accumulation of toxic compounds as well as their catalytic role in the formation of lignin which is a molecule involved in the reinforcement of the plant cell wall [33] [34]. β-1,3-glucanase is strongly expressed following an abiotic and/or biotic stress, since it exhibits a fungicidal ac-

tivity through the modification of the composition of the plant cell wall and involves in the destruction and destabilization of fungal wall structures [35] [36]. Our results are in line with previous studies that have clearly demonstrated that phenolics secondary metabolites play an important role in the defence mechanisms of *Musa* spp. [30] [37] [38]. The assessment of the early stage events post-inoculation in leaves treated with *T. diversifolia* liquid extracts could help to the elucidation of the interactions between the PIF seedling leaves and *M. fijiensis*.

The vegetative growth stages parameters evolve significantly in course of time and were significantly different between the treated seedlings and the untreated ones with a positive and clear treatment effect for Extract 3. The liquid extracts of *T. diversifolia* had the expected impact on PIF seedlings through an important growth promotion. This result is in line with formal findings which confirmed that aqueous extracts of *T. diversifolia* increased the germination percentage of maize [15], but also the germination and pre-emergence stage and vegetative growth stage of PIF plantain [3] [6] and of cocoa [27] [28] in nursery. The increase in the rate of germination and the vegetative growth stage of plantain bananas seedlings could be explained by nutrients release in the liquid extracts by *T. diversifolia* leaves such as nitrogen, phosphorus, potassium, magnesium well kwon for their physiological role in growth promotion and the improvement of soil properties [39] [40] as well as free secondary metabolites contained in the extracts and involved in physiological role of plant such as proteins and phenolic compounds (alcaoïds, Sesquiterpens, lactones and flavonoids) [41]. Beneficial effects of *T. diversifolia* liquid extracts start from the soil physicochemical and biological characteristic modification as microbiota which help plant roots to more and better assimilate uptakes [42]. This is in line with the roots pre-emergence and length development observed on the treated PIF plantain bananas seedlings suggesting a potential stimulatory role to *T. diversifolia* liquid extract that increases the assimilates availability during the growth and secondary metabolites accumulation.

5. Conclusion

The objective of this work was to evaluate the effect of liquid extracts of *T. diversifolia* on the susceptibility of PIF plantain bananas seedlings to black Sigatoka disease. Under both conditions of this assay, the liquid extract of *T. diversifolia* does not only improve the PIF seedlings susceptibility to BSD but also improves the vegetative growth stages of PIF plantain bananas seedlings, probably because it seems to acts as a biofungicide and a biofertilizer. Therefore, the liquid extracts of *T. diversifolia* could be acting as a vital stimulator improving the soil quality and the PIF seedlings vigor. It would be interesting to investigate the *T. diversifolia* liquid extracts microbiome, the bioactivity of these extracts as well as the biochemical and molecular mechanisms involved in plant defense and plant growth promotion. *T. diversifolia* liquid extracts could therefore be proposed to small holder farmers as a two in one solution for an eco-friendly agriculture in

the nursery. Hence, this research could later be extended to the field level and to other pathosystems.

Conflicts of Interest

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

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