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Tithonia diversifolia Mulch Stimulates the Growth of Plantain PIF Seedlings and Induces a Less Susceptibility to *Mycosphaerella fijiensis* in the Nursery

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Abstract

Plantain is an important crop that serves as a staple food and contributes significantly to income generation for millions of people in tropical and sub-Saharan Africa. Its cultivation faces the main constraint of seedlings unavailability in quantity and quality, essential for the creation of new plantations. The advent and popularization of the plantlets from stem bits (PIF) in the 2000s raised hopes for solving this problem. However, after about ten years, the PIF has shown some problems limiting its adoption and should be improved for more efficiency. The amendment of PIF substrate production with *Tithonia diversifolia* could be an alternative to seedlings' unavailability. This study aims to evaluate the potential stimulative effect of *T. diversifolia* mulch on plantain PIF seedlings growth and protection against black Sigatoka disease (BSD)caused by *Mycosphaerella fijiensis*. The parameters of vegetative growth stages and biomarkers accumulation were assessed in sterilized substrate and non-sterilized substrate conditions. *T. diversifolia* mulch treatment increases the germination rate, the number of shoots, the height and the diameter of shoots, the leaf area as well as the seedlings roots, but it also protects the seedlings against BSD up to about 81% compared to the control seedlings. It also enhances the accumulation of biomarkers such as proteins, polyphenols content and defense-related enzymes (peroxidase, polyphenol oxidase and glucanase). *T. diversifolia* mulch seems to act in PIF seedlings production as a vital stimulator. It can therefore be taken as a tool for a more sustainable and resilient agriculture, and for poverty alleviation of poor small holder farmers.

Keywords

Plantain, PIF Seedlings, Mulch, *Tithonia diversifolia*, *Mycosphaerella fijiensis*, Vital Stimulator

1. Introduction

Banana is a perennial monocotyledonous plant in the *Musaceae* family, with origin from South East Asia that grows in tropical and subtropical regions. The *Musaceae* family (*Musa* spp.) regroups diverse cultivars amongst which the plantains which are usually starchy even when ripped and need to be cooked before consumption [1]. Plantain with a high energy value and a rich mineral, dietary fiber and vitamin content, plays a vital role in contributing to food security in Central and West Africa, as well as income generation for millions of people in these regions [1]. Cameroon is ranked 3rd in the world (3.94 million tons per year) in terms of plantain production and the first in the Central African Economic and Monetary Community (CEMAC) zone [2]. Despite these performances, plantain production is very low to cover the large demand resulting in very high prices for the commodity on local, urban and transborder markets. To meet up with this demand, there is a need to create new plantations to improve the performance of this crop and meet up with the large demand despite the unavailability of seedlings in quantity and quality [1].

Plantains seedlings usually come from suckers extracted from farmlands and are usually disseminated with soil's pathogenic microorganisms. The use of vitroplants to mitigate this problem of seedlings availability is very expensive and not affordable to small holder farmers. An innovative macro-propagation technique called "PIF" (Plants Issus de Fragment de tiges) that is plantlets from stem bits which was developed by the Centre Africain de Recherches sur Bananiers et Plantains (CARBAP) is an alternative for small holders' farmers for its many advantages [1] [3]. However, seedlings produced by PIF technique face many problems during acclimatization, as well as contamination of the seedlings on farmlands that could be responsible for loses of about 60% during the establishment of new plantations, this limits the adoption of this technique that is now rejected by some farmers [1].

Plantain production is permanently threatened by many pathogens amongst which *M. fijiensis*, a virulent, an invasive and predominant pathogen that causes black Sigatoka disease (BSD), the most economically destructive disease of bananas, which accounts for loses in production estimated at about 50% [4]. Moreover, the use of chemical inputs like weed-killers, fertilizers, fungicides and pesticides in PIF seedlings production and on farmlands is harmful to human and the environment, and also responsible for the appearance of resistance in plant pathogens strains [5] and is not affordable to the small peasant farmers.

Tithonia diversifolia, a woody herb of 2 - 3 m tall in the family *Asteraceae,* is

highly rich in nutrients, averaging about 3.5% N, 0.37% P and 4.1% K and decomposes rapidly after its application to the soil thereby enriching the soil with N, P and K for the growth of crops [6]. With its anti-fungal properties, it plays an important role in phytopathological control and induces the crude synthesis of defense metabolites such as flavonoids, tannins, alkaloids, PR-proteins for plants defense [7]. Phytochemicals such as sesquiterpenoids, diterpenoids, alkaloids, flavonoids, chlorogenic acid derivatives, phenols, saponins, tannins, and terpenoids, are present in the leaves, the stems, and the roots of *T. diversifolia* [8] [9]. This confirms the *T. diversifolia* involvement in the growth promotion of plants and their protection against biotic and abiotic stresses.

Recent studies conducted in Cameroon have demonstrated that clam shell powder alone and its association with *T. diversifolia* have a strong influence on PIF plantain seedlings growth and susceptibility to BSD in nurseries by their dual role as a biofertilizer and as a biopesticide [1] [6]. The application of *T. diversifolia* mulch in the substrates of PIF seedlings could improve the performance of the PIF plantain seedlings produced. The aim of this study was to evaluate the stimulative effect of *T. diversifolia* mulch on the growth of plantain PIF seedlings and on their protection against *M. fijiensis* in nurseries.

2. Materials and Methods

2.1. Materials

Plantain (*Musa spp*., genome AAB) suckers of Big-Ebanga variety were obtained from Mbam and Kim division of the Centre region of Cameroon. They were selected for their short cycle of production and seedlings productivity.

T. diversifolia tissues were obtained from farmlands around the Biotechnology Centre of University of Yaoundé 1 located at Nkolbisson (Yaoundé-Cameroon), dried under the sun and then flaked with the hands.

M. fijiensis, the causal agent of black Sigatoka disease (BSD) was provided by CARBAP of Njombé in the Littoral region of Cameroon.

The sawdust, sand and black soil used to formulate the PIF substrates were collected around the Biotechnology Centre of the University of Yaoundé 1 and sterilized in an oven at different temperatures and time intervals as described by [1]. The substrate used for seedlings germination and emergence in the greenhouse was the sawdust while it was the sand and the black soil in proportions of 1/3 and 2/3 for plantlets growth in the shade.

2.2. Experimental Design

This study was conducted in the Centre Region of Cameroon (Yaoundé), located in the agroecological zone known as wet Rainforest with Bimodal Rainfall from a period of August 2016 to March 2017. The acclimatization phase for plantain seedlings in the shade was extended over the period of November 2016 to January 2017 marked by moderate temperatures (26˚C - 30˚C) and low rainfall (25 - 80 mm/month). The other phases of the study were conducted under controlled

conditions in the laboratory.

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.

1) Two blocks:

- Sterilized Substrate (SS)
- Non-Sterilized Substrate (NSS)

2) Five treatments (four *Tithonia diversifolia* mulch and one control):

- control only
- a mulch layer of 4 cm (M1)
- a mulch layer of 6 cm (M2)
- a mulch layer of 8 cm (M3)
- a mulch layer of 10 cm (M4)

Each treatment in each block was considered as an Experimental Unit (EU). The PIF explants were prepared following the method used by [1]. In each EU, three (03) explants were introduced and covered with a white transparent plastic paper in the greenhouse. The germination and emergence of seedlings in the greenhouse were favoured by watering.

2.3. *T. diversifolia* Mulch Evaluation Effect on PIF Seedlings Vegetative Growth Stages

The germination and emergence of seedlings parameters (the germination percentage and the number of shoots) of each treatment were assessed after every seven days starting from the second week of explants introduction in the greenhouse for a period of three successive weeks. In the shade, for each treatment, the vegetative growth parameters (the diameter, the height and the leaf area) were evaluated and after every fourteen days starting from the day, the seedlings were weaned and acclimatized for a period of six successive weeks. These vegetative growth stages evaluations were done for three selected explants and seedlings respectively in the greenhouse and the shade according to the method reported by [1]. The roots aspect of PIF seedlings was observed with the naked eye for each treatment and pictures made.

2.4. *T. diversifolia* Mulch Evaluation Effect on PIF Seedlings Susceptibility to BSD

The level of susceptibility of the seedlings was evaluated on the leaves of the same age *i.e.* about 12 weeks through artificial inoculation with *M. fijiensis* suspension (10^6 zoospores/mL). 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, while the ones to be inoculated were cleaned and kept for two hours at air temperature. The sporal solution of *Mycosphaerella fijiensis* strain was used for inoculations and the evaluation of the necrotic surface area of seedlings leaves was done as described in [1] [6] previous study on PIF seedlings.

2.5. *T. diversifolia* Mulch Evaluation Effect on PIF Seedlings Biomarkers Accumulation

The evaluation of total proteins, total polyphenols, peroxidase, polyphenol oxidase and glucanase accumulation was done at two stages (before inoculation and post-inoculation). 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 [10] [11] [12] [13] [14] 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 were measured in mg equivalent of gallic acid per g of fresh weight while that of the total proteins concentration were expressed in mg equivalent (Eq) of bovine serum albumin (BSA) per g of fresh weight (FW). The peroxidase and polyphenol oxidase concentration were expressed in UE/min/g FW, while the glucanase concentration was expressed in mg of glucose/g FW.

2.6. Statistical Analyses

T. diversifolia mulch effects on PIF seedlings vegetative growth stages, susceptibility to BSD and biomarkers accumulation were analysed by subjection of the variables (the percentage of germination, the number of shoots, the height of shoots and the diameter of shoots, the leaf area, the necrotic surface, total proteins, total polyphenols, peroxidase, polyphenol oxidase and glucanase) to mixed three-way ANOVA 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. *T. diversifolia* Mulch Effect on PIF Seedlings Vegetative Growth Stages

T. diversifolia mulch was found to significantly ($P < 0.0001$) influence the germination and emergence stage parameters notably, the germination percentage and the number of shoots (**Table 1** and **Figure 1**). The coefficient of determination (R^2) for both variables was close to a 100% (**Table 1**) indicating thus that *T. diversifolia* mulch model indicates a perfect fit, and is thus highly reliable. All the germination and emergence stage parameters evolve significantly in course of time and the most influential variable was the time.

For the germination and emergence stage, the variables treatment and day, as well as the interactions treatment and day were highly significant ($P < 0.0001$) while the other variables and interactions were non-significant (condition; condition and treatment; condition and day; condition, treatment and day) as shown in **Table 1**.

Overall, no significant difference was shown between the sterilized substrate

Table 1. Variance analysis of *Tithonia diversifolia* mulch effects on the PIF plantain seedlings growth (germination percentage, number of shoots, diameter of shoots, height of shoots, leaf area) and disease severity to BSD (necrotic surface of leaves) 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.

Source	DF	% Germination		Number of shoots			Diameter (cm)		Height (cm)		Leaf area (mm^2)			Necrotic surface (mm^2)	
		R^2 = 100%		R^2 = 100%			R^2 = 98%		R^2 = 92%		R^2 = 89%			R^2 = 100%	
		F	*P*	F	*P*	DF	F	*P*	F	*P*	F	*P*	DF	F	*P*
Condition	1	0.000	1.000	1.875	0.174	1	12.692	**0.001**	3.926	**0.051**	29.648	**<0.0001**	1	690.967	**<0.0001**
Treatment	4	4444.889	**<0.0001**	290.700	**<0.0001**	4	583.111	**<0.0001**	144.889	**<0.0001**	112.542	**<0.0001**	4	194,821.168	**<0.0001**
Day	4	229,446.556	**<0.0001**	5403.450	**<0.0001**	3	486.507	**<0.0001**	80.834	**<0.0001**	22.540	**<0.0001**	8	196,753.605	**<0.0001**
Condition * Treatment	4	0.000	1.000	1.125	0.349	4	2.175	**0.079**	1.181	0.326	1.321	0.269	4	98,421.763	**<0.0001**
Condition * Day	4	0.000	1.000	0.375	0.826	3	6.199	**0.001**	1.054	0.374	2.253	0.089	8	350.963	**<0.0001**
Treatment * Day	16	1666.833	**<0.0001**	65.934	**<0.0001**	12	3.730	**0.000**	2.194	**0.020**	1.357	0.204	32	5635.787	**<0.0001**
Condition * Treatment * Day	16	0.000	1.000	0.328	0.993	12	5.317	**<0.0001**	1.408	0.180	5.125	**<0.0001**	32	3494.100	**<0.0001**

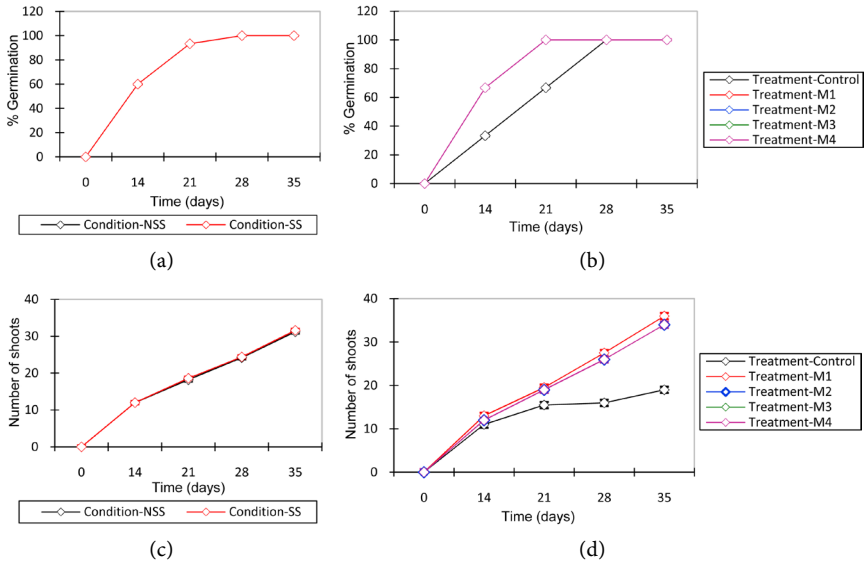

Figure 1. Effects of *T. diversifolia* mulch in the greenhouse on the germination percentage and the number of shoots of PIF plantain seedlings in course of time. Interaction plots of day and condition and of day and treatment respectively for germination percentage ((a); (b)) and for number of shoots ((c); (d)). Each point represents the average mean of three replicates with the standard deviation for each treatment.

(SS) condition and non-sterilized substrate (NSS) condition in terms of the germination percentage and the number of shoots (**Figure 1(a)** and **Figure 1(c)**). One statistical group was distinguished between the SS condition and the NSS condition for the germination percentage and the number of shoots (**Figure 2(a)**).

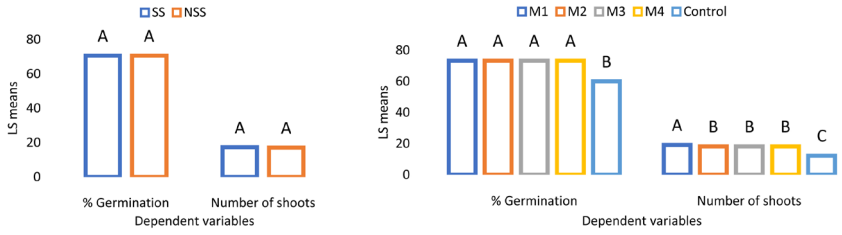

Figure 2. Least Squares (LS) means summary of the germination percentage and the number of shoots in the PIF plantain seedlings in the greenhouse after *T. diversifolia* mulch treatment: Condition (a); Treatment (b). Letters A, B and C represent different statistical groups defined by the Tukey test (5%).

The germination and emergence stage parameters were consistently higher in the treated seedlings compared to the control ones. A 100% germination was obtained 21 days after seeding (das) in the treated seedlings while it occurred 28 das in the control ones (**Figure 1(b)**). The number of shoots 35 das, was 36, 34, 34, 34 and 19 respectively for M1, M2, M3, M4 and the control (**Figure 1(d)**), showing thus that the treatment doubles the quantity of shoots generated.

There was no significant difference between the treatments (M1, M2, M3 and M4) in terms of the germination percentage while all of them were statistically different to the control, leading to two different statistical groups for this variable and three for the number of shoots (**Figure 2(b)**). Among the four treatments, the one that showed the best effect in terms of germination and emergence parameters is M1 (**Figure 2(b)**).

T. diversifolia mulch was found to significantly ($P < 0.0001$) influence the vegetative growth parameters notably the diameter of shoots, the height of shoots, the leaf area and the roots aspect (**Table 1** and **Figure 3**). The coefficient of determination (R^2) for the three variables was close to a 100% (**Table 1**) indicating thus that *T. diversifolia* mulch model is a good fit for the data. All the vegetative growth stage parameters evolve significantly in course of time and the most influential variable was the treatment.

All the variables and interactions were highly significant for the diameters of shoots, but only the variables treatment, day and the interactions treatment and day were highly significant for the height of shoots and the variables condition, treatment and day, and the interactions condition and treatment and day were highly significant for the leaf area (**Table 1**).

Overall, a significant difference was observed between the sterilized substrate (SS) condition and non-sterilized substrate (NSS) condition for the vegetative growth stage (**Figure 3(a)**, **Figure 3(c)** and **Figure 3(e)**). They were more important in the sterilized substrate (SS) condition compared to the non-sterilized substrate (NSS) condition, except for the height of shoots where it was slightly more important in the NSS condition than in the SS condition. The respective values were 1.98 cm and 1.9 cm for the diameter of shoots, 23.62 cm and 24.44 cm for the height of shoots and 1021.20 mm^2 and 830.37 mm^2 for the leaf area.

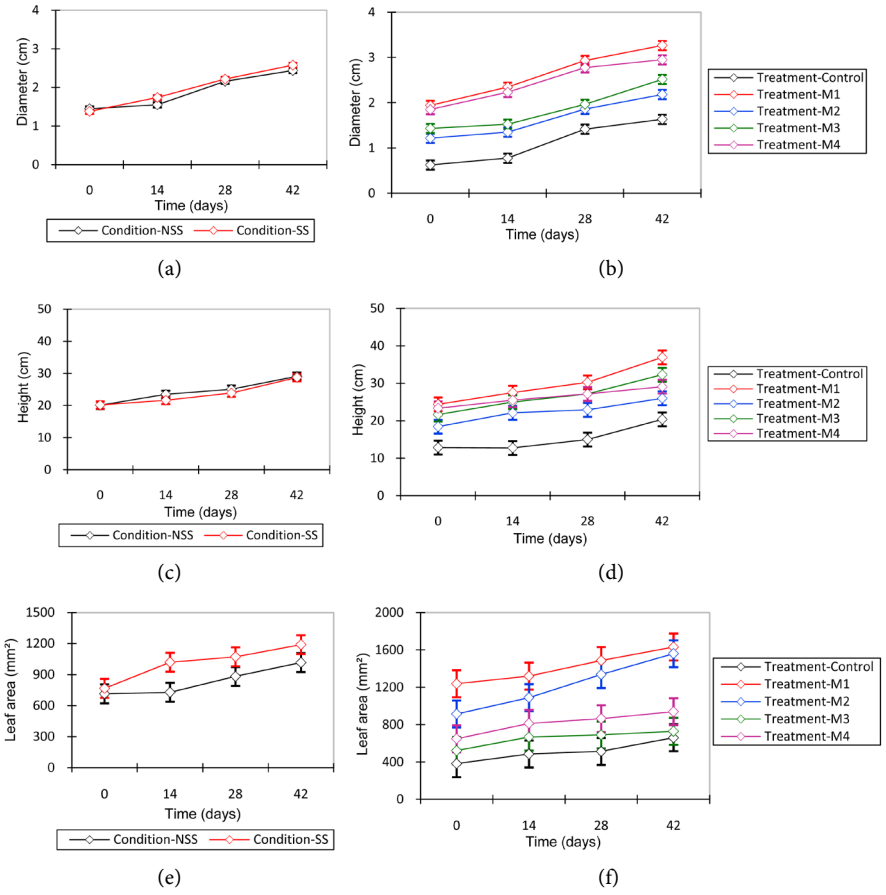

Figure 3. Effects of *T. diversifolia* mulch in the shade on the diameter, the height and the leaf area of PIF plantain seedlings in course of time. Interaction plots of day and condition and of day and treatment respectively for the diameter ((a); (b)), the height ((c); (d)) and the leaf area ((e); (f)). Each point represents the average mean of three replicates with the standard deviation for each treatment.

However, this significant difference was not showing a less or low effect in the non-sterilized substrate (NSS) condition because the effect in this condition was also significantly ($P < 0.0001$) efficient for seedlings development. Two different statistical groups were distinguished for the diameter of shoots and the leaf area, while only one statistical group was distinguished for the height of shoots (**Figure 4(a)**).

The vegetative growth parameters were consistently higher in the treated seedlings compared to the control seedlings. 42 das, the values of the diameter of shoots were 3.27 cm, 2.18 cm, 2.52 cm, 2.95 cm and 1.63 cm respectively (**Figure 3(b)**), the height of shoots were 36.92 cm, 26.00 cm, 32.28 cm, 29.08 cm and 20.37 cm respectively (**Figure 3(d)**) and those of the foliar surface of leaves were 1631.75 mm^2, 1560.01 mm^2, 728.86 mm^2, 939.26 mm^2, and 659.98 mm^2 respectively (**Figure 3(f)**). The roots morphology (length and emergence) were more developed for the treatments especially the M1compared to the control ones (**Figure 5**).

The effect of *T. diversifolia* mulch was clearly and significantly differentiated

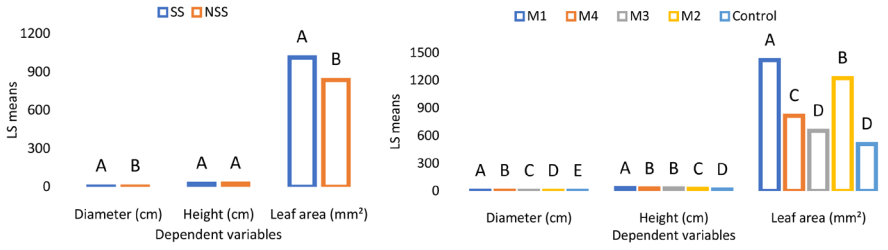

Figure 4. Least Squares (LS) means summary of the diameter, the height and the leaf area of shoots in the PIF plantain seedlings in the shade after *T. diversifolia* mulch treatment: Condition (a); Treatment (b). Letters A, B, C, D and E represent different statistical groups defined by the Tukey test (5%).

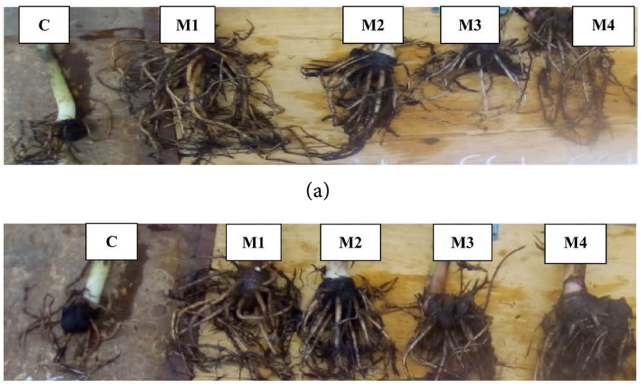

Figure 5. Effects of *T. diversifolia* mulch on the roots of PIF plantain seedlings at the age of 16 weeks in sterilized substrate (SS) condition (a) and non-sterilized substrate (NSS) condition (b); from the left to the right (c) control and treatments M1, M2, M3 and M4 respectively.

between the four treatments (M1, M2, M3 and M4) and the control in terms of the diameter of shoots, the height of shoots and the leaf area, leading to five, four and four different statistical groups respectively for these variables (**Figure 4(b)**). Among the four treatments, the one that showed the best effect in terms of growth promotion is M1 (**Figure 4(b)**).

3.2. *T. diversifolia* Mulch Effect on PIF Seedlings Susceptibility to BSD

The effect of *T. diversifolia* mulch was found to significantly ($P < 0.0001$) influence the severity of BSD, with very low necrotic development level after inoculation with a coefficient of determination (R^2) for necrotic surface equal to a 100% (**Table 1** and **Figure 6**). This indicated thus that *T. diversifolia* mulch model gives good response to necrosis development on the leaves and the most influential variable was time.

As indicated in **Table 1**, all the variables (condition, treatment and stage) were highly significant ($P < 0.0001$) as well as all the interactions (condition and treatment; condition and day; treatment and day; condition, treatment and day).

Overall, the BSD severity was more important in the non-sterilized substrate (NSS) condition compared to the sterilized substrate (SS) condition, with values of about 212.31 mm^2 and 206.23 mm^2 respectively (**Figure 6(a)**). However, this significant difference was not showing a less or low effect in the NSS, it was also efficient. Two different statistical groups were distinguished for necrotic surface between the sterilized substrate (SS) condition and the non-sterilized substrate (NSS) condition (**Figure 7(a)**).

The susceptibility to BSD was consistently higher in the control seedlings as compared to the treated seedlings. There was a significant difference between the four treatments (M1, M2, M3 and M4) and the control in terms of necrotic development with an efficient effect of the treatments on the less development of necrosis (149.65 mm^2, 144.01 mm^2, 217.18 mm^2 and 187.77 mm^2 respectively) as compared to the control (347.75 mm^2) (**Figure 6(b)**). The effect of *T. diversifolia* mulch was clearly and significantly differentiated between the four treatments and the control in terms of necrotic surface, leading to five different statistical groups for this variable (**Figure 7(b)**). The necrosis evolved in course of time as confirmed by the nine distinguished different statistical groups (**Figure 7(c)**). Among the four treatments, the one that showed the best effect in terms of less necrotic surface development is M2, followed by M1with a very light difference (**Figure 7(b)**).

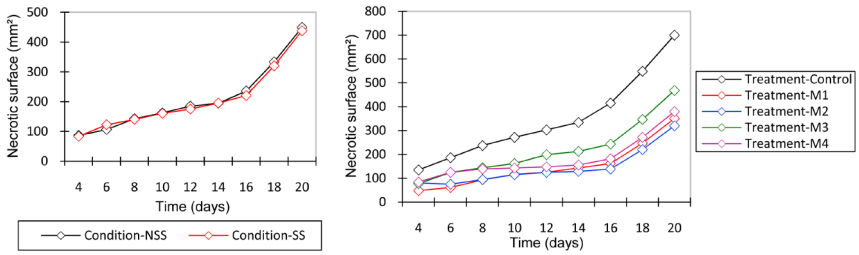

Figure 6. Effects of *T. diversifolia* mulch in the shade on the necrotic surface of PIF plantain seedlings in course of time. Interaction plots of day and condition (a) and of day and treatment (b) for necrotic surface. Each point represents the average mean of three replicates with the standard deviation for each treatment.

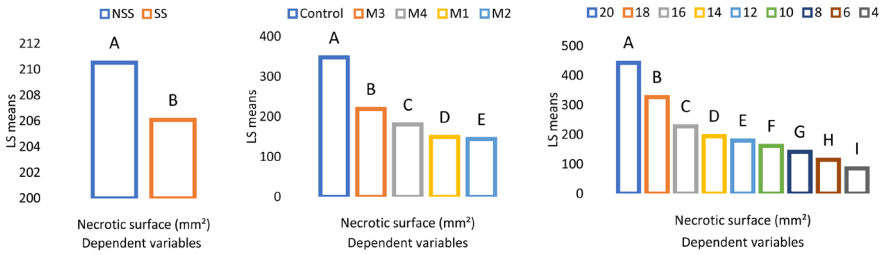

Figure 7. Least Squares (LS) means summary of the necrotic surface of PIF plantain seedlings in the shade after *T. diversifolia* mulch treatment: Condition (a); Treatment (b) and Time (c). Letters A, B, C, D, E, F, G, H, and I represent different statistical groups defined by the Tukey test (5%).

3.3. *T. diversifolia* Mulch Effect on PIF Seedlings Biomarkers Accumulation

The effect of *T. diversifolia* mulch was found to significantly ($P < 0.0001$) influence the biochemical markers notably the total phenolics, the total proteins, the peroxidase, the polyphenol oxidase and the glucanase (Table 2 and Figure 8). The coefficient of determination (R^2) for all these variables was close to a 100% (Table 2) indicating that the *T. diversifolia* mulch model was efficient. The most influential variable was the stage for the total phenolics and glucanase, while it was the treatment for total proteins, peroxidase and polyphenol oxidase.

As indicated in Table 2 for the total phenolics, all the variables (condition, treatment and stage), as well as the interactions condition and treatment; treatment and stage; and condition, treatment and stage were highly significant while only the interaction condition and stage were non-significant. For the total proteins, peroxidase and glucanase, all the previous variables were highly significant except for the polyphenol oxidase whereby the interaction condition, treatment and stage was non-significant.

Overall, there was a significant ($P < 0.0001$) difference between the PIF seedlings of the sterilized substrate (SS) condition and non-sterilized substrate (NSS) condition (Table 2). The peroxidase and glucanase contents were less important in the non-sterilized substrate (NSS) condition compared to the sterilized substrate (SS) condition and inversely for the total phenolics, total proteins and polyphenol oxidase (Figure 8(a), Figure 8(c), Figure 8(e), Figure 8(g) and Figure 8(i)). However, this significant difference was not showing a less or low effect since the *T. diversifolia* mulch was efficient in both conditions compared to the control seedlings. Two different statistical groups were distinguished between the sterilized substrate (SS) condition and the non-sterilized substrate (NSS) condition for all these biomarkers (Figure 9(a)).

Table 2. Variance analysis of *Tithonia diversifolia* mulch effects on the PIF plantain seedlings biochemical markers accumulation (total proteins, total phenolics, peroxidase, polyphenol oxidase and glucanase) at two stages (before inoculation and post-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.

Source	DF	Total Proteins (mg Eq BSA/g FW)		Total Phenolics (mg Eq Cat/g FW)		Peroxidase (UE/min/g FW)		Polyphenol oxidase (UE/min/g FW)		Glucanase (mg of glucose/g FW)	
		R^2 = 99%		R^2 = 97%		R^2 = 99%		R^2 = 97%		R^2 = 100%	
		F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Condition	1	33.195	**<0.0001**	17.753	**0.000**	1143.921	**<0.0001**	16.167	**0.000**	683.286	**<0.0001**
Treatment	4	720.978	**<0.0001**	146.703	**<0.0001**	740.967	**<0.0001**	242.215	**<0.0001**	885.360	**<0.0001**
Stage	1	712.984	**<0.0001**	561.597	**<0.0001**	824.652	**<0.0001**	328.404	**<0.0001**	3591.959	**<0.0001**
Condition * Treatment	4	10.935	**<0.0001**	10.802	**<0.0001**	90.322	**<0.0001**	14.448	**<0.0001**	53.396	**<0.0001**
Condition * Stage	1	17.902	**0.000**	1.280	0.265	178.321	**<0.0001**	12.029	**0.001**	96.889	**<0.0001**
Treatment * Stage	4	35.168	**<0.0001**	5.322	**0.002**	87.268	**<0.0001**	34.632	**<0.0001**	98.301	**<0.0001**
Condition * Treatment * Stage	4	35.351	**<0.0001**	4.381	**0.005**	7.400	**0.000**	1.163	0.342	69.847	**<0.0001**

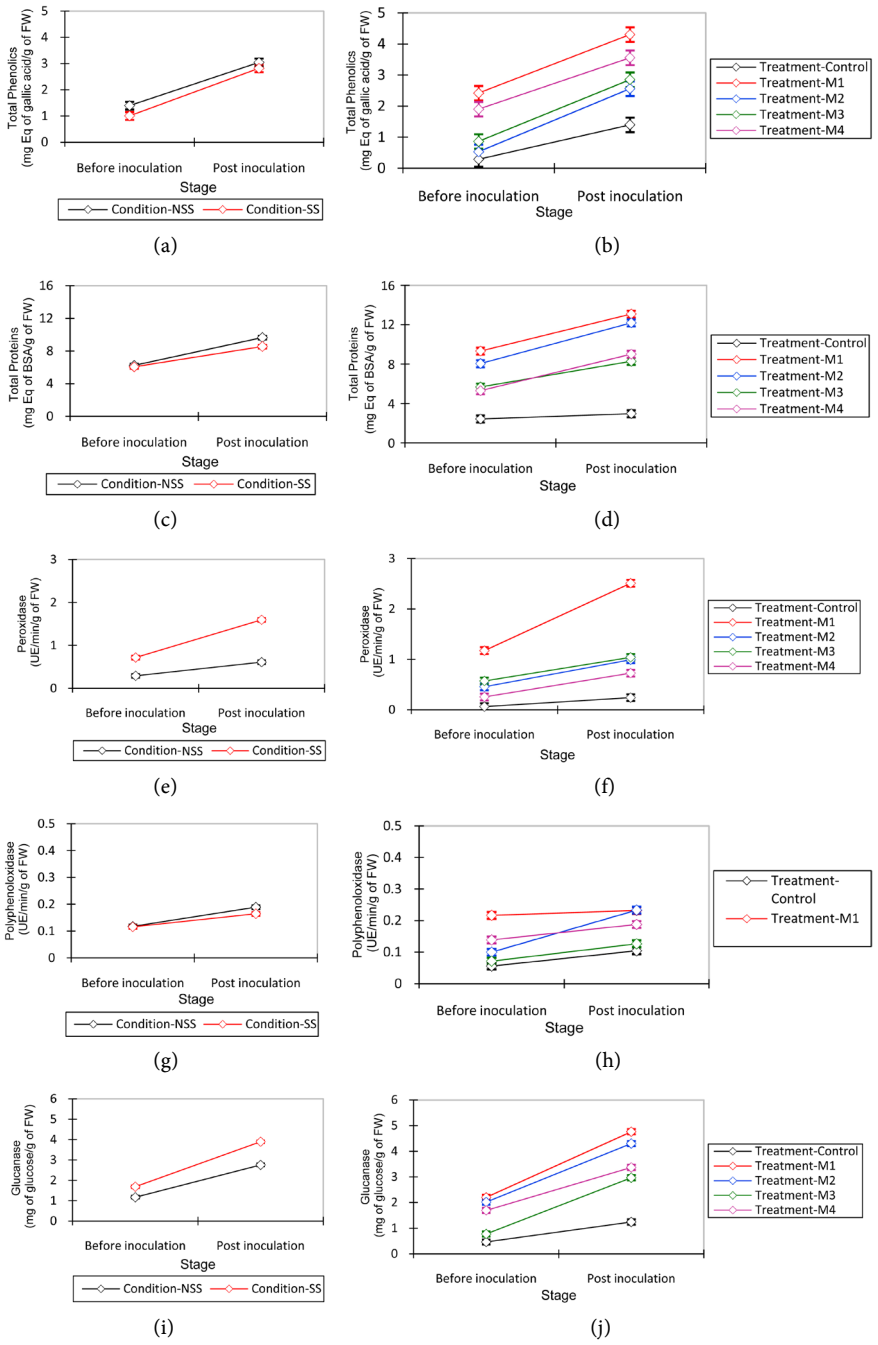

Figure 8. Effects of *T. diversifolia* mulch on the biochemical markers (total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase) accumulation before and post inoculation in the PIF plantain seedlings after treatment in the shade with *T. diversifolia* mulch. Interaction plots of 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.

These biomarkers were consistently higher in the treated seedlings compared to the control seedlings for total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase (**Figure 8(b)**, **Figure 8(d)**, **Figure 8(f)**, **Figure 8(h)**

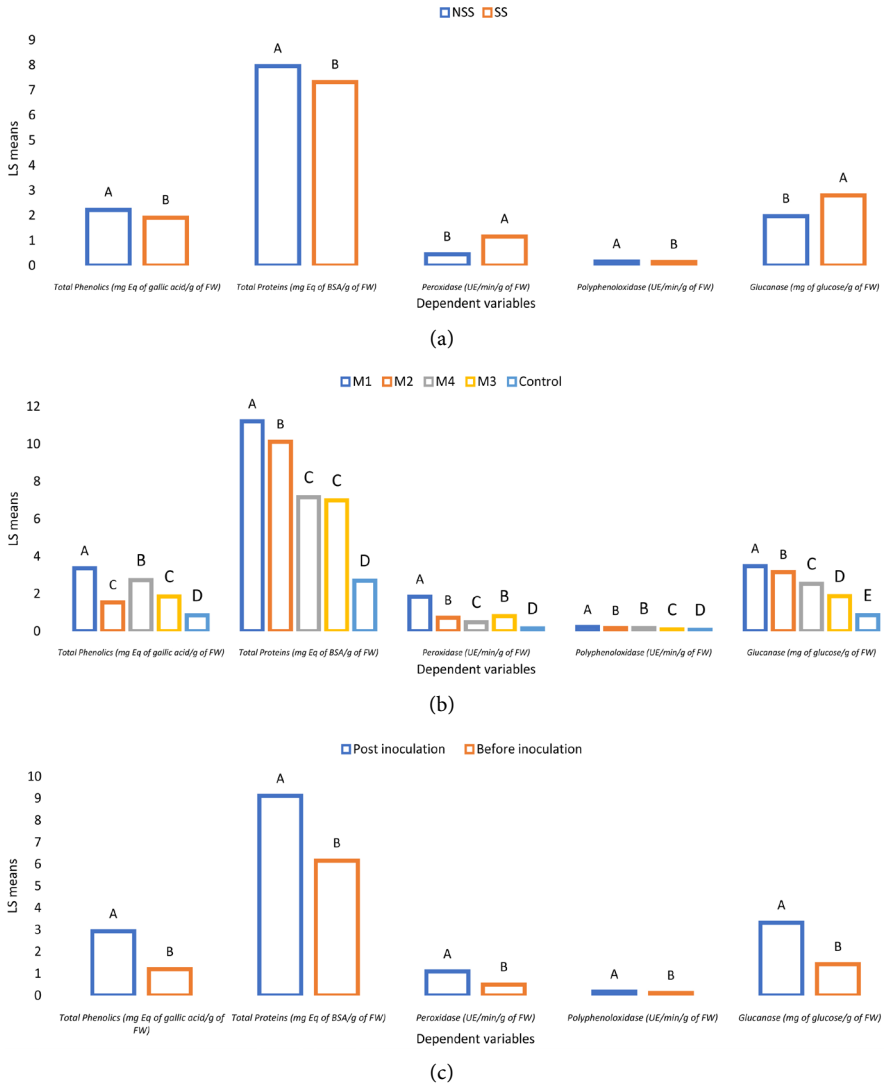

Figure 9. Least Squares (LS) means summary of biochemical markers (total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase) accumulation in the PIF plantain seedlings in the shade after *T. diversifolia* mulch treatment before and post-inoculation: Condition (a); Treatment (b) and stage (c). Letters A, B, C, D, E, F, G, H, and I represent different statistical groups defined by the Tukey test (5%).

and **Figure 8(j)** respectively). There was no difference between the treatments (M2 and M3 for total phenolics, M3 and M4 for total proteins, M2 and M3 for peroxidase and M2 and M4 for polyphenol oxidase) while all the four treatments (M1, M2, M3 and M4) were statistically different from the control, leading to four different statistical groups for the variables (total phenolics, total proteins, peroxidase and polyphenol oxidase) and five different statistical groups for the variable glucanase (**Figure 9(b)**). There was a significant difference between the stage before inoculation and the stage post-inoculation for all these biomarkers and their amount increases importantly after inoculation (**Figure 9(c)**). Among the four treatments, the one that showed the best effect in terms of biomarkers accumulation is M1 (**Figure 4(f)** and **Figure 9(b)**).

3.4. Principal Components Analysis (PCA) of All the Variables

The vegetative growth stages variables (the germination percentage, the number of shoots, the diameter of shoots, the height of shoots and the leaf area) were well correlated to one another (Figure 10). The biomarkers (total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase) were well correlated to one another (Figure 10). These defense-related variables were however weakly correlated with the vegetative growth stages variables.

The necrotic surface was negatively correlated with the vegetative growth parameters (the diameter of shoots and the height of shoots and the leaf area) and the biomarkers (total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase), and was however weakly correlated with the germination and emergence variables (the germination percentage and the number of shoots) (Figure 10).

4. Discussion

The generation of PIF seedlings that were having different morphological aspect while comparing the treated seedlings with the control was possible thanks to the experimental design that was put in place in the nursery. The kinetic evolution of the vegetative growth stages parameters was significantly different between the treated seedlings and the untreated ones with a positive effect of the treatment more pronounced for the M1 treatment. The predicted *T. diversifolia* mulch impact on PIF seedlings through an important growth promotion was confirmed by this study. This impact was observed on treated seedlings, especially for treatment M1 that had consistently increased all the vegetative growth parameters (the germination percentage, the number of shoots, the diameter of shoots and the height of shoots, the foliar surface and the roots aspect) compared to the control. Our results are in accordance with previous studies that have reported on the same type of growth promotion for plantain PIF seedlings [1] [6] and cocoa seedlings [15] [16].

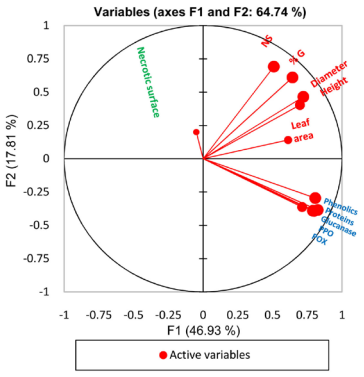

Figure 10. Principal componentsAnalysis (PCA) of all the variables: the germination percentage (% G), the number of shoots (NS), the diameter of shoots, the height of shoots, the leaf area, the necrotic surface of leaves, total proteins, total phenolics, peroxidise (POX), polyphenol oxidase (PPO) and glucanase. The PCA shows positive or negative correlation, but also the strength of the relationship between the variables.

Overall, *T. diversifolia* contains high level of essential nutrients for plant growth such as nitrogen, phosphorus, potassium and calcium and could therefore be considered as a seedling's biostimulator. Indeed, the major component of *T. diversifolia* tissues is the nitrogen, known as a constituent of chlorophyll and also involves in division and enlargement of cells in the apical meristem [17]. The rapid germination and emergence of treated PIF seedlings observed could be due to plants nutrients contained in *T. diversifolia* mulch, that have important functions in osmotic regulation, cellular permeability, and may act as structural components and essential metabolites [18]. Moreover, they could be involved in the improvement of soil physical, chemical and biological properties for soil fertility enhancement, the increase of microbial activity and the optimization of nutrients absorption by plants [17] [19]. These essential elements present in *T. diversifolia* mulch could be involved in the rapid PIF seedlings development at the different vegetative growth stages.

The non-sterilized substrate (NSS) condition has exhibited an overall slow growth rate compared to the sterilized substrate (SS) condition. Indeed, the nature of the substrate is making this big difference, since the PIF explants and seedlings are exposed to the microbiome present in the substrate of the non-sterilized condition during their vegetative growth stages in the nursery. This could either generate positive synergistic action and/or negative antagonistic action (stresses) in the PIF seedlings. Indeed, a recent study has exhibited a synergistic action in the non-sterilized substrate (NSS) condition revealed through the rapid rate of vegetative growth stage (the diameter of shoots and the height of shoots, the foliar surface) compare to the sterilized substrate (SS) condition [6]. The organic fertilizer used as substrate for soil microorganisms acts through a rapid rate of organic material decomposition and the release of nutrients for plant uptake [17]. Our results were slightly opposed to this previous study, probably because of a low level of the antagonistic relationship between the biotic factors presents in the substrate even if it was lower to slow down the seedlings development considerably. A lack in this study relies in the fact that the PIF seedling microbiome was not characterized.

Regardless of the condition, seedlings treated with *T. diversifolia* mulch germinates quickly, show good growth parameters probably through the strengthening of the seedlings pseudo-stems, the increase of photosynthesis rates, and the stimulation of seedlings roots. This positive effect could be explained by the presence of an element like phosphorus in *T. diversifolia* mulch that is part of molecular structure of nucleic acid, functions in storage, accelerate energy transfer processes within the plant and enhances roots development [18] [20]. This result confirms the previous observations suggesting that some natural products like *T. diversifolia* biomass [21], snail shells, oyster and the combination of clam shells and *T. diversifolia* have a potential effect on seedlings development in nursery [1] [6] [15] [16]. Indeed, these natural products are nutrients sources for plants, but they also influence the physicochemical and biological properties of the soil and the soil microbe's activity [22].

T. diversifolia mulch has clearly shown direct effects on seedlings physiology and probably indirect effects on PIF substrate in nursery. Indeed, organic amendment has been shown to contribute directly to seedlings growth and yield through nutrients supplementation and indirectly by modifying soil physical properties such as stability of aggregates and porosity that can improve the roots growth, rhizosphere and stimulate plant growth [23]. However, in excess they could slower the nutrients release, be stored for long in the soil and be toxic for seedlings through the development of stress as shown *in vitro* by the inhibitory and stimulatory effects on germination and growth of *Cleome gynandra* (spider plant) [24]. The stressful effect of *T. diversifolia* on PIF seedlings in nursery have been reported, but not yet assess.

T. diversifolia mulch has positively and significantly influenced the BSD development on the plantain PIF seedlings with less level of susceptible to *M. fijiensis* exhibited by the treated seedlings, especially M2 and M1. This level of susceptibility was lower as compared to the one obtained with an early study on clam shells [1] and almost the same for treated seedlings and condition with an early study on the combined effect of clam shells and *T. diversifolia* [6]. The lower susceptibility level to BSD in the PIF seedlings of treatments M2 and M1 could be explained by the fact that the treatments seem to act as a plant vaccine that trigger physiological changes resulting in an increase of assimilates availability during the growth, and lead to an enhanced growth promotion and biomarkers accumulation.

The *T. diversifolia* mulch equally positively and significantly influenced the accumulation of metabolites, especially defense-related enzymes such as peroxidase, polyphenol oxidase and glucanase. Indeed, it seems to promote natural defensive systems through the increase synthesis of nutrients and defensive metabolites [1] [6] [25] [26]. Nitrogen and potassium present in the *T. diversifolia* mulch could justify this increase accumulation of biomarkers. In one hand, nitrogen has the function of building up protoplasm, preparing macromolecules (amino acids, proteins, nucleic acids, nucleotides, hormones) and chlorophyll in the plant; while in the other hand, potassium serves as an enzyme activator used by the plant to activate different enzymes [18] [22]. Moreover, nitrogen induces cell division and initiates meristematic activity while potassium assists in the transport of assimilates from the leaf to the entire plant tissue being thus, a necessary element for overall metabolic and enzymatic activities, especially photosynthesis [18].

Our results are in accordance with previous report of nutrients and defensive metabolites accumulation enhancement, notably proteins and phenolic compounds as well as defense-related enzymes (peroxidase, polyphenol oxidase, glucanase, chitinase …) [1] [6] [13] [14] [27] [28]. Biomarkers of resistance have effectively been shown to be involved in the defensive mechanism of banana tissues [27]-[36] as well as different other plants tissues [37] [38]. The *T. diversifolia* mulch could be acting as an elicitor favouring plant immunization through the build up of response weapons such as defense-related enzymes be-

fore potential invaders attack [28]. Indeed, peroxidase are expressed to limit cellular spreading of infection through establishment of structural barriers or generation of highly toxic environments [33], while polyphenol oxidase plays a role in defense against plant pathogens [34], through immediate synthesis of antimicrobials weapons. In addition, chitinase and β-(1,3)-glucanase are direct defense enzyme of plants capable of attacking cell wall of fungal pathogens by degrading the pathogen cell wall [28].

T. diversifolia mulch influences the accumulation of large amount of phenolics, proteins and defense-related enzymes such as peroxidase, polyphenol oxidase and glucanase in both conditions, more important in the treated plantain PIF seedlings and at both stages. Our results are in accordance with a previous study that has clearly demonstrated that phenolics secondary metabolites (phenols and lignin) play an important role in the defence mechanisms of *Musa* to *R. similis* infection [27] [29] [31]. Moreover, phenolic acids are involved in the resistance against pathogen through phytoalexin accumulation, biosynthesis of lignin and formation of structural barrier [35]. These metabolites seem to act synergistically to inhibit fungal growth due to biotic stress, but also all other source of stresses such as abiotic stress. Indeed, the stresses seem to induce the accumulation of total phenolics, total proteins and polyphenol oxidase, which are known as biomarkers of resistance/tolerance to biotic stresses as well as abiotic stresses [27] [28] [39]. This difference in accumulation was more important for total phenolics, total proteins, peroxidase, polyphenol oxidase and glucanase probably because after inoculation, the plant has set down a mechanism to overcome the pathogenic attack through the use of preformed and *denovo* synthesis of biochemical markers [37] [38] [39] [40].

5. Conclusion

The aim of this work was to evaluate the vital stimulator effect of *T. diversifolia* mulch on the performance of PIF plantain seedlings in terms of growth promotion and disease susceptibility in nursery. Our results have highlighted a novel effect of *T. diversifolia* mulch on plantain PIF seedlings quality. Indeed, this study is the first reporting the use of *T. diversifolia* for mulching on plantain PIF seedlings in the nursery. However, the biochemical and molecular mechanism involved in the growth promotion and the less susceptibility to BSD severity are still unknown and need to be assessed. The use of *T. diversifolia* mulch should be considered by poor peasant farmers and nursery operators during the PIF seedlings production in order to put in the disposal of the population seedlings with good quality, easy for good cultivation practice essential in an approach of ecoagriculture. It will be important also to follow up this research on *T. diversifolia* mulch on the farm, since it seems to be a tool for a more sustainable and resilient agriculture. The use of *T. diversifolia* mulch in seedlings production could be a poverty alleviation tool alternative in the sub-Saharan countries for small holder farmers.

Conflicts of Interest

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

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