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VERTICAL LAYERS OF *TITHONIA DIVERSIFOLIA* FLAKES AMENDMENT IMPROVES PLANTAIN SEEDLING PERFORMANCE

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ABSTRACT

Plantain seedlings availability in sub-Saharan countries are very weak to support a high productivity level required. The plantain cultivation faces problem of the seedling's unavailability in quantity and quality for intensification of this crop. The amendment of PIF substrate production with natural products could be an alternative to this problem. This study objective is to evaluate the effect of vertical layers of *Tithonia diversifolia* flakes on the performance of PIF plantain seedlings in terms of vegetative growth and susceptibility to black Sigatoka disease (BSD) in nursery. The vertical layer of T. *diversifolia* flakes amendment was introduced in the propagator in the presence of the control without amendment in controlled and uncontrolled conditions for shoots, then seedlings generation, followed by the evaluation of the vegetative growth parameters, the inoculation of the leaves with *Mycosphaerella fijiensis* and the pool of biomarkers evaluation. The treatment increases the germination rate, the number of shoots, the height and the diameter of shoots, the area of leaves as well as the seedlings roots, but also protects the seedlings against BSD up to about 56% compared to the controls. It also enhances the accumulation of proteins, polyphenols content and enzymes such as peroxidase, polyphenoloxidase and glucanase. The dual role of the vertical layer of T. *diversifolia* flakes in seedling production as a biofertilizer and as a biopesticide was revealed in this research. Taking into account this bad herbs flakes as a tool for sustainable and green agriculture is an open door to poor peasants for their empowerment.

Keywords: Plantain (Musa spp.), PIF seedlings, *Tithonia diversifolia*, biofertilizer, biopesticide, black Sigatoka disease (BSD), biomarkers.

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1. Introduction

The availability of banana and plantain seedlings, especially the healthy ones is the major challenges for the establishment and expansion of banana plantations in sub Saharan Africa. Industrial plantation of banana in this region are using vitroplants that are imported and very expensive for poor famers. There is a lack of formal systems for producing and distributing quality planting material, thereby forcing farmers to depend on natural regeneration of plants for their supply [1]. Indeed, farmers are used to plant one sucker to obtain one banana plant as a traditional way of creating banana plants in their plantation and this practice is usually subjected to many diseases. However, suckers used by farmers are often infested with pest and diseases as their only source of planting material [2]. Moreover, bananas do not produce planting material readily, the field regeneration is indeed a very slow process with low productivity of viable suckers. Tissue culture is one way of producing high quality, clean plantlets and uniform planting material rapidly but it requires expensive laboratory equipment's, electricity and technical skills, which are not affordable for small poor farmers in sub-Saharan Africa. An alternative is the 'plantlet from stem bits' (PIF), a horticultural propagation method developed by the Centre Africain de Recherches sur Bananiers et Plantains (CARBAP) located in Njombé-Cameroon [2]. This technique generates plants from stem fragments, easy to implement and is obviously one of the possible solutions to get healthy banana seedlings at low cost. PIF technique allows massive production of banana seedlings in just two to three months, in a sanitized environment.

PIF technique is a method for massive production of healthy banana seedlings. In rural communities, producer groups may constitute propagation units to meet up the need of seeds availability. Hence, the application of healthy seedlings of banana is measured in tens of thousands in rural regions of Cameroon. Seeds production by this technique is undoubtedly a great opportunity to contribute to the improvement of the sector specially to earn extra income, and the fight against poverty. Indeed, the PIF technique allows the *in vivo* induction of an active bud proliferation on banana stem fragments under particular conditions of temperature and hygrometry and without hormone in soil-less culture conditions [3]. However, PIF seedlings are facing many acclimatization problems responsible for plants mortality of about 60% during the establishment of new plantations and are now rejected by some farmers [4]. Some of these problems are the declining soil fertility [2], difference observed in term of vigour between the PIF shoots of the same explants (the one develops in the center being more vigorous compared to the peripheric ones), pest and disease pressure (black Sigatoka disease, banana nematodes and weevil). There is therefore, a need to improve the quality of PIF seedlings in term of growth promotion and disease susceptibility against black Sigatoka disease (BSD) responsible for about 50% of production loses. The smallholder poor farmers that are practicing extensive agriculture could not buy chemical inputs to improve the performance of the PIF seedlings. Moreover, these expensive synthetic products are harmful to human and the environment.

It has been recently demonstrated that clam shells powder, the combination of clam's shells and *Tithonia diversifolia* powder have a strong influence on PIF plantain seedlings growth and susceptibility to BSD in nurseries by its dual role as a biofertilizer and as a biofungicide [4, 5]. However, little is known about the effect of *Tithonia diversifolia* on PIF plantain seedlings growth and susceptibility to BSD in nurseries. This woody herb is commonly known as a bad herb, however very rich in nutrients (nitrogen, phosphorus and potassium), thus good for the crop's growth and in pesticidal properties excellent for plants defense [6]. The pesticidal effect of *T. diversifolia* relies on the presence of phytochemicals in its tissues (leaves, stems, and roots) such as sesquiterpenoids, diterpenoids, alkaloids, flavonoids, chlorogenic acid derivatives, phenols, saponins, tannins, and terpenoids [7, 8].

The assessment of *T. diversifolia* flakes effects on PIF plantain seedlings growth and susceptibility to diseases could be a new approach to improve the quality of plantain seedlings in sub-Saharan Africa and above. Although *T. diversifolia* is known as a bad herb, present everywhere in the region and therefore affordable to the poor small holder farmer, it could be a good tool for poverty alleviation through a green and sustainable agriculture, enhancement of the bioavailability of improved PIF plantain seedlings for creation of new plantation. The aim of this work therefore is to examine the effect *T. diversifolia* flakes vertical layers on the performance of PIF plantain seedlings in term of vegetative growth and disease susceptibility in nursery.

2. Materials and Methods

2.1. Plant materials

Plantain banana suckers (*Musa* spp., genome AAB, Big-Ebanga variety) were collected from Ntui farms in Cameroun. They were selected based on their height and their healthy nature at an age of about four to five months. *T. diversifolia* tissues were obtained from farmlands around the Biotechnology Centre of University of Yaoundé 1 located at Nkolbisson (Yaoundé-Cameroon). The causal agent of black Sigatoka disease (BSD) was provided by the African Centre for Research on Bananas and Plantains (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 University of Yaoundé 1 and sterilized in an oven at different temperatures and time intervals as described by a published article[4]. The greenhouse PIF substrate was the sawdust while the sand and the black soil respectively 1/3 and 2/3 were used in the shade.

2.2. Experimental design

This experiment was conducted in Yaoundé (Centre Region, Cameroon), an agroecological zone favourable to BSD from July 2016 to March 2017 following the design presented in completely randomized block device for vegetative growth analysis of Figure 1. The PIF technique method used for plantain propagation followed two steps: (1) the germination phase of the explants in the greenhouse and (2) the acclimatization phase of the seedlings under shade. During this second step (November 2015 to January 2016), the average temperature and the mean monthly rainfall of the locality were respectively 28 °C and 53 mm.

The suckers were prepared through trimming, shelling and the trauma of the shoot apical meristem following the method used in a published article [4]. The different modalities were classified in the greenhouse and watered for seedlings germination.

2.3. Effect of treatment on PIF seedlings vegetative growth and disease severity to BSD

The germination parameters (germination percentage and the number of shoots) of each modality was assessed after every seven days starting from the second week of explants introduction in the greenhouse for a period of three successive weeks (Figure 1). In the shade, for each modality the agromorphological parameters (diameter, height and foliar surface) were evaluated and after every fourteen days starting from the day the seedlings were wean and acclimatized for a period of six successive weeks (Figure 1). These evaluations were done for three selected explants and seedlings according to the method reported [4]. The roots aspect was observed with the naked eye.

Mycosphaerella fijiensis strain was used for artificial inoculations of the seedling leaves as described in previous research on PIF seedlings [4, 5] and following the three steps presented in disease severity evaluation of Figure 1.

2.4. Effect of treatment on PIF seedlings biomarkers pool

The evaluation of the biomarker's pool (total proteins, total polyphenols, peroxidase, polyphenoloxidase and glucanase) was carried out in the whole leaves for each modality in two stages: before inoculation and after inoculation (Figure 1).

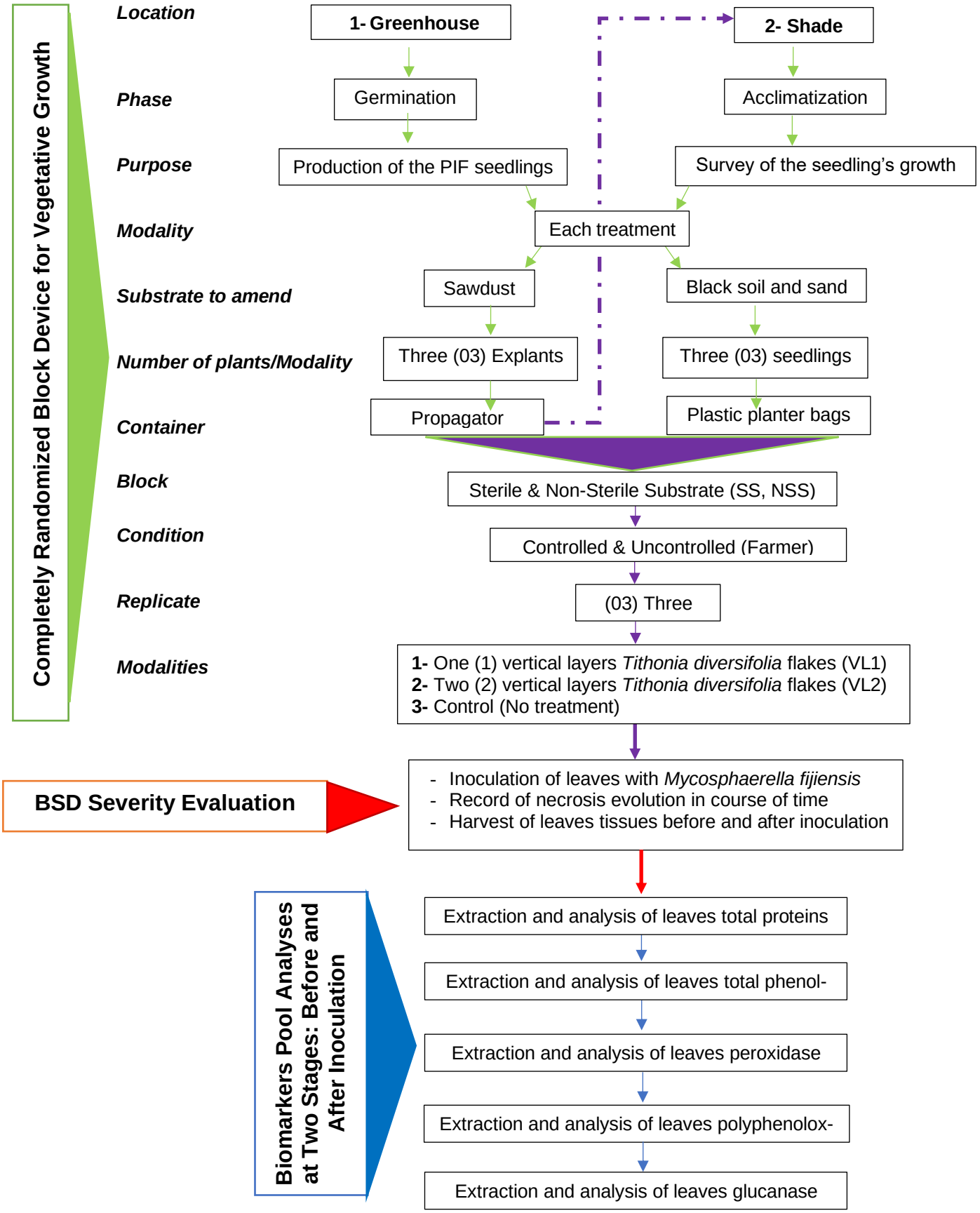


Figure 1: Different phases of the study of the influence of vertical layers *Tithonia diversifolia* flakes on vegetative growth of plantain PIF seedlings and their susceptibility to *Mycosphaerella* leaf spot disease.

0,5 g of fresh leaf was used for the samples analyses and each modality was repeated thrice. Extraction samples followed by quantification were carried out according to the method reported by [9-13] modified respectively for total protein (595nm), total phenolic (760nm), peroxidase (470nm), polyphenoloxidase (330nm) and glucanase (540nm). The total proteins concentration was expressed in mg equivalent (Eq) of bovine serum albumin (BSA) per g of fresh weight (FW) while that of the total phenolics was measured in mg equivalent of gallic acid per g of fresh weight. The peroxidase and polyphenoloxidase concentration were expressed in UE/min/g FW, while the glucanase concentration was expressed in mg of glucose/g FW.

2.5. Statistical analyses

The effects of vertical layers *Tithonia diversifolia* flakes on vegetative growth of plantain PIF seedlings and their susceptibility to BSD was analysed by subjection of the variable (percentage of germination, number of shoots, height and diameter of shoots, foliar surface, necrotic surface, total proteins, total polyphenols, peroxidase, polyphenoloxidase and glucanase to mixed three-way ANOVA performed with XLSTAT software. Each plant being taken as experimental unit and condition, modality and day as factors. Multiple comparisons of the means were done by applying Tukey's test at 5% probability level. Pearson correlation analysis between the different variables was also performed with XLSTAT software.

3. Results

3.1. Vertical layers *T. diversifolia* flakes effects on the PIF plantain seedlings vegetative growth

The vertical layer of *T. diversifolia* flakes was found to significantly (P< 0,0001) influence the vegetative growth parameter notably germination percentage, number of shoots, diameter and height of shoots, foliar surface and roots aspect (Table 1 and Figure 2). The coefficient of determination (R^2) for all the vegetative variables is close to a 100% (Table 1) indicating thus that the vertical layer of *T. diversifolia* flakes amendment model explains all the variability response data around its mean. The most influential variable was the time for germination percentage and number of shoots, while it was the modality for the diameter of shoots, the height of shoots and the foliar surface. All the vegetative growth parameter evolves significantly in course of time.

As shown in Table 1 for germination percentage and number of shoots, the variables modality and day, as well as the interactions modality and day were highly significative (P< 0,0001) while the others variables and interactions were non-significative (condition, condition and modality, condition and day, condition, modality and day). All these variables were highly significant for the diameters of shoots while for the height of shoots and the foliar surface area only the variables condition, modality and day, and the interactions condition and modality, modality and day were highly significant.

Figures 3C, 4A, 4C and 4E, and Table 1 show a significant difference between the seedlings of the sterile substrate (SS) condition and non-sterile substrate (NSS) condition except for the germination percentage (Figure 3A). These vegetative growth parameters were more important in the sterile substrate condition (SS) compared to the non-sterile substrate (NSS) condition, excepted for the germination; that is respectively 24,6 and 24 for the number of shoots, 1,77 cm and 1,65 cm for the diameter of shoots, 23,76 cm and 22,71 cm for the height of shoots and 1601,81 mm^2 and 1328,48 mm^2 for the foliar surface leaves. However, this significant difference was not showing a less or low effect in the non-sterile condition; the effect in this condition was also significantly (P< 0,0001) efficient for seedlings development. Two statistically different group were distinguished between the sterile condition (SS) and the non-sterile substrate (NSS) condition for all the vegetative variables (number of shoots, diameter of shoots, height of shoots and foliar surface), except the germination percentage that had only one statistical group (Figure 3A).

Table 1: Variance analysis of vertical layers *Tithonia diversifolia* flakes effects on the PIF plantain seedlings vegetative growth (germination percentage, number of shoots, diameter and height of shoots, foliar surface) 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.

	% Germination		Number of shoots		Diameter (cm)		Height (cm)		Foliar surface (mm^2)		Necrotic surface (mm^2)		
	R^2= 100		R^2= 99		R^2= 100		R^2= 99		R^2= 100		R^2= 100		
Source	DF	F	*P*	F	*P*	DF	F	*P*	F	*P*	F	*P*	DF	F	*P*
Condition	1	0,000	1,000	3,993	0,050	1	455,730	**< 0,0001**	75,303	**< 0,0001**	1223,737	**< 0,0001**	2	69069,352	**< 0,0001**
Modality	2	6503,338	**< 0,0001**	285,172	**< 0,0001**	2	3897,895	**< 0,0001**	1753,585	**< 0,0001**	23548,411	**< 0,0001**	8	122155,141	**< 0,0001**
Day	4	120228,710	**< 0,0001**	1966,775	**< 0,0001**	3	261,766	**< 0,0001**	226,554	**< 0,0001**	573,387	**< 0,0001**	2	59117,269	**< 0,0001**
Condition*Modality	2	0,000	1,000	1,498	0,232	2	248,696	**< 0,0001**	17,238	**< 0,0001**	424,130	**< 0,0001**	8	34115,390	**< 0,0001**
Condition*Day	4	0,000	1,000	0,672	0,614	3	28,817	**< 0,0001**	1,490	0,229	3,218	**0,031**	16	1846,594	**< 0,0001**
Modality*Day	8	2431,476	**< 0,0001**	64,393	**< 0,0001**	6	4,841	**0,001**	38,488	**< 0,0001**	48,317	**< 0,0001**	16	3293,052	**< 0,0001**
Condition*Modal-ity*Day	8	0,000	1,000	0,523	0,835	6	8,795	**< 0,0001**	1,047	0,407	1,334	0,261	1	1551,918	**< 0,0001**

Figure 2: Vertical layers of *T. diversifolia* flakes effects on the roots of the PIF plantain banana seedlings in sterilized substrate (SS) condition: (a) control and (b) modalities VL1 (left) and VL2 (right); and non-sterilized substrate (NSS) condition: (c) control and (d) modalities VL1 (left) and VL2 (right).

These vegetative growth parameters were consistently higher in the treated seedlings compared to the control ones. For germination percentage, 100% germination was obtained 21 das in the treated modalities and 28 das in the control one (Figure 3B) while for the number of shoots 35 das, it was 29, 28 and 16 respectively for VL1, VL2 and the control modalities (Figure 3D). 42 das, the values of diameter of shoots were 2,69 cm, 1,33 cm and 1,11 cm (Figure 4B), the ones of height of shoots were 32,73 cm, 21,42 cm and 15,54 cm (Figure 4D) and those of the foliar surface of leaves were 2559,54 mm^2, 1221,02 mm^2 and 614,87 mm^2 (Figure 4F) respectively for VL1, VL2 and the control modalities. The roots aspect was more developed for the treated modalities compared to the control one (Figure 2). No difference was found between the two modalities (VL1 and VL2) in term of germination percentage while both were different statistically to the untreated one, leading to two statistically different groups for this variable.

Indeed, the treatment activate the germination of shoots compare to the control ones. The effect of vertical layer of *T. diversifolia* flakes was clearly and significantly differentiated between the two modalities (VL1 and VL2) and the control in term of number of shoots, diameter of shoots, height of shoots and foliar surface, leading to three statistically different groups for these variables (Figures 3D and 4B, 4D and 4F), except for germination that had two statistically different groups (Figures 3B). Between the two treated modalities, the one showing the best effect in term of growth promotion was VL1 (Figures 3D and 4B, 4D and 4F).

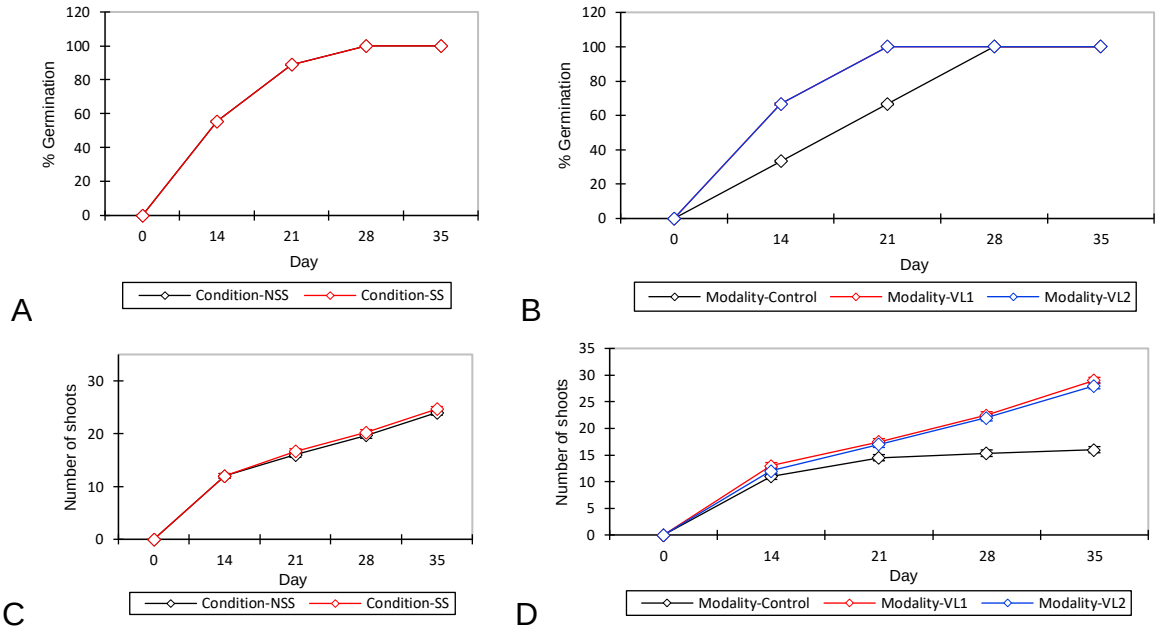


Figure 3: Vertical layers of *T. diversifolia* flakes effects in the greenhouse, on the PIF plantain seedlings germination in course of time. Interaction plots of day and condition (day*condition) for germination parameters (A) and number of shoots (C), and of day and modality (day*modality) for germination parameters (B) and number of shoots (D). Each point represents the average mean with the standard deviation of three replicates for each modality.

3.2. Vertical layers *T. diversifolia* flakes effects on the BSD severity

The vertical layer of *T. diversifolia* flakes was found to significantly (P< 0,0001) influence the severity of BSD, with very low level of necrosis development after inoculation (Table 1). The coefficient of determination (R^2) for necrotic surface was equal to a 100% (Table 1) indicating thus that the vertical layer of *T. diversifolia* flakes amendment model give good response to necrosis development on the leaves and the most influential variable was the time.

As shown in Table 1, all the variables were highly significant (P< 0,0001) (condition, modality and day) as well as the interactions (condition and modality, condition and day, modality and day, and condition, modality and day).

The severity of the BSD was more important in the non-sterile substrate (NSS) condition compared to the sterile condition (SS) condition, about 527,7 mm^2 and 428,2 mm^2 respectively. The necrotic surface was less important in the sterile substrate condition (SS) compared to the non-sterile substrate (NSS) condition. However, this significant difference was not showing a less or low effect in the non-sterile condition; it was also efficient. Two statistically different groups were distinguished for necrotic surface between the sterile condition (SS) and the non-sterile substrate (NSS) condition (Figure 4G).

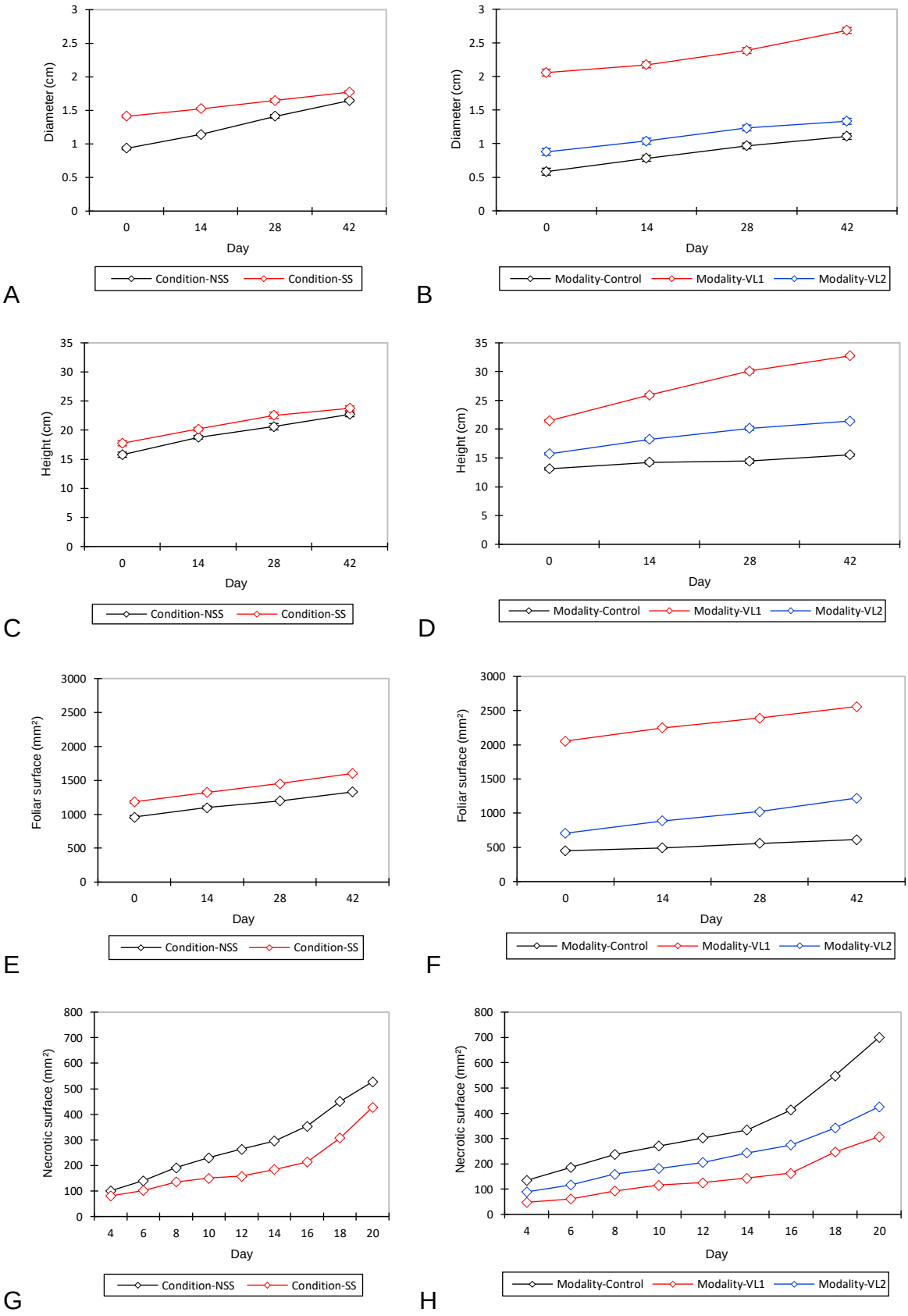


Figure 4: Vertical layers of *T. diversifolia* flakes effects in the shade, on the PIF plantain seedlings growth in course of time. Interaction plots of day and condition (day*condition) for the diameter (A), the height (C), the surface area (E) and the necrotic surface of leaves (G), and of day and modality (day*modality) for the diameter (B), the height (D), the surface area (F) and the necrotic surface of leaves (H). Each point represents the average mean with the standard deviation of three replicates for each modality.

BSD susceptibility was consistently higher in the control seedlings, followed by the VL2 modality and the VL1 modality (Figure 4H). There was a difference between the three modalities (VL1, VL2 and control) in term of necrosis development with an efficient effect of the treated modalities on the less development of necrosis (307,8 mm^2, 426,04 mm^2 and 700 mm^2 respectively). The effect of vertical layer of *T. diversifolia* flakes was clearly and significantly differentiated between the two modalities (VL1 and VL2) and the control in terms of necrotic surface, leading to three statistically different groups for this variable (Figure 4H). Between the two treated modalities, the one showing the best effect in terms of less necrotic surface development was VL1 (Figure 4H).

3.3. Vertical layers *T. diversifolia* flakes effects on the pool of biomarkers

The vertical layer of *T. diversifolia* flakes was found to significantly (*P*< 0,0001) influence the pool of biomarkers notably the total proteins, the total phenolics, the peroxidase, the polyphenoloxidase and the glucanase (Table 2 and Figure 5). The coefficient of determination (R^2) for all these variables was close to a 100% (Table 2) indicating thus that the vertical layer of *T. diversifolia* flakes amendment model was efficient. The most influential variable was the modality for the total proteins, the total phenolics and the peroxidase while it was the stage for the polyphenoloxidase and the glucanase.

As shown in Table 2 for the total proteins, the variables condition, modality and stage, as well as the interactions condition and modality, condition and stage, modality and stage were highly significant while only the interaction condition, modality and stage were non-significant. For the total phenolics, all the previous variables were highly significant except the interaction modality and stage. All these variables were highly significant for the peroxidase while for the polyphenoloxidase and the glucanase only the variables condition, modality and stage, and the interaction condition and modality were highly significant.

Figures 5A, 5C, 5E, 5G and 5I show a significant (*P*< 0,0001) difference between the seedlings of the sterile substrate (SS) condition and non-sterile substrate (NSS) condition. The total proteins and polyphenoloxidase content were more important in the non-sterile substrate condition (NSS) compared to the sterile substrate (SS) condition and inversely for total phenolics, peroxidase and glucanase. However, this significant difference was not showing a less or low effect since the vertical layer of *T. diversifolia* flakes amendment was efficient in both conditions compare to the control one. Two statistically different groups were distinguished between the sterile condition (SS) and the non-sterile substrate (NSS) condition for all the biomarkers (total proteins, total phenolics, peroxidase, polyphenoloxidase and glucanase).

This pool of biomarkers was consistently higher in the treated seedlings compared to the control ones for total proteins (Figure 5B), total phenolics (Figure 5D), peroxidase (Figure 5F), polyphenoloxidase (Figure 5H), glucanase (Figure 5I). There was no difference between the two modalities (VL1 and VL2) in term of total proteins and glucanase, while both treated modalities were different statistically to the control one, leading to two statistically different groups for these variables (Figures 5B and 5J). The effect of vertical layer of *T. diversifolia* flakes was clearly and significantly (*P*< 0,0001) differentiated between the two modalities (VL1 and VL2) and the control in terms of total phenolics, peroxidase and polyphenoloxidase, leading to three statistically different groups for these variables (Figures 5D, 5F, 4D and 5H). Between the two treated modalities, the one showing the best effect in terms of biomarkers accumulation was VL1 (Figure 5).

3.4. Relationship between all the variables in term of growth promotion and disease resistance

The variables involve in the growth promotion (germination percentage, number of shoots, diameter and height of shoots, foliar surface, total proteins and the total phenolics) were analysed and they were well correlated to one another,

and better correlation (*P*> 0,001) were encountered for vegetative growth parameters in the sterile substrate (SS) condition and non-sterile substrate (NSS) condition (Table 3). These vegetative growth parameters were also weakly correlated with the enzyme's contents (peroxidase, polyphenoloxidase and glucanase) in both conditions.

The variables imply in the disease susceptibility (necrotic surface, total proteins, the total phenolics, peroxidase, polyphenoloxidase and glucanase) were analysed and they were well correlated to one another, and better correlation (*P*> 0,001) were encountered for vegetative growth parameters in the sterile substrate (SS) condition and non-sterile substrate (NSS) condition (Table 3). A weak correlation was found between the necrotic surface and all the variables in the sterile substrate (SS) condition, as well with all the biomarkers in the sterile substrate (SS) condition (Table 3). The necrotic surface was weakly correlated with the germination percentage and number of shoots in both conditions. However, the necrotic surface was negatively correlated (*P*> 0,05) with the biochemical markers parameters as well as with the diameter and height of shoots, and the foliar surface in the non-sterile substrate (NSS) condition (Table 3).

Table 2: Variance analysis of vertical layers *Tithonia diversifolia* flakes effects on the PIF plantain seedlings biochemical markers accumulation (total proteins, total polyphenols, peroxidase, polyphenoloxidase and glucanase) at two stage (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.

		Total Proteins (mg Eq BSA/g FW)		Total Phenolics (mg Eq Cat/g FW)		Peroxidase (UE/min/g FW)		Polyphenoloxidase (UE/min/g FW)		Glucanase (mg of glucose/g FW)	
		R^2= 99		R^2= 99		R^2= 99		R^2= 97		R^2= 98	
Source	DF	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Condition	1	406,690	**< 0,0001**	39,682	**< 0,0001**	163,445	**< 0,0001**	5,755	**0,025**	6,577	**0,017**
Modality	2	715,381	**< 0,0001**	745,936	**< 0,0001**	319,698	**< 0,0001**	113,612	**< 0,0001**	176,963	**< 0,0001**
Stage	1	318,551	**< 0,0001**	251,524	**< 0,0001**	384,962	**< 0,0001**	446,319	**< 0,0001**	352,037	**< 0,0001**
Condition*Modality	2	218,284	**< 0,0001**	215,425	**< 0,0001**	129,284	**< 0,0001**	19,176	**< 0,0001**	72,040	**< 0,0001**
Condition* Stage	1	13,512	**0,001**	4,787	**0,039**	21,917	**0,000**	0,609	0,443	0,501	0,486
Modality* Stage	2	23,590	**< 0,0001**	0,445	0,646	6,585	**0,005**	2,127	0,142	5,664	**0,010**
Condition*Modality* Stage	2	2,710	0,088	0,246	0,784	7,698	**0,003**	0,050	0,951	2,269	0,126

4. Discussion

The experimental design put in place in the nursery has permitted to generate PIF seedlings that were having different morphological aspect while comparing the treated ones with the control.

The kinetic of vegetative growth parameters evolution was significantly different between the treated seedlings and the untreated ones with a positive effect of the treatment more pronounced for the VL1 modality. The vertical layer of *T. diversifolia* flakes amendment had the expected impact on PIF seedlings through an important vegetative growth promotion. This vegetative growth promotion was observed for treated seedlings, especially modality VL1 that had at least double all the vegetative growth parameters (germination percentage, number of

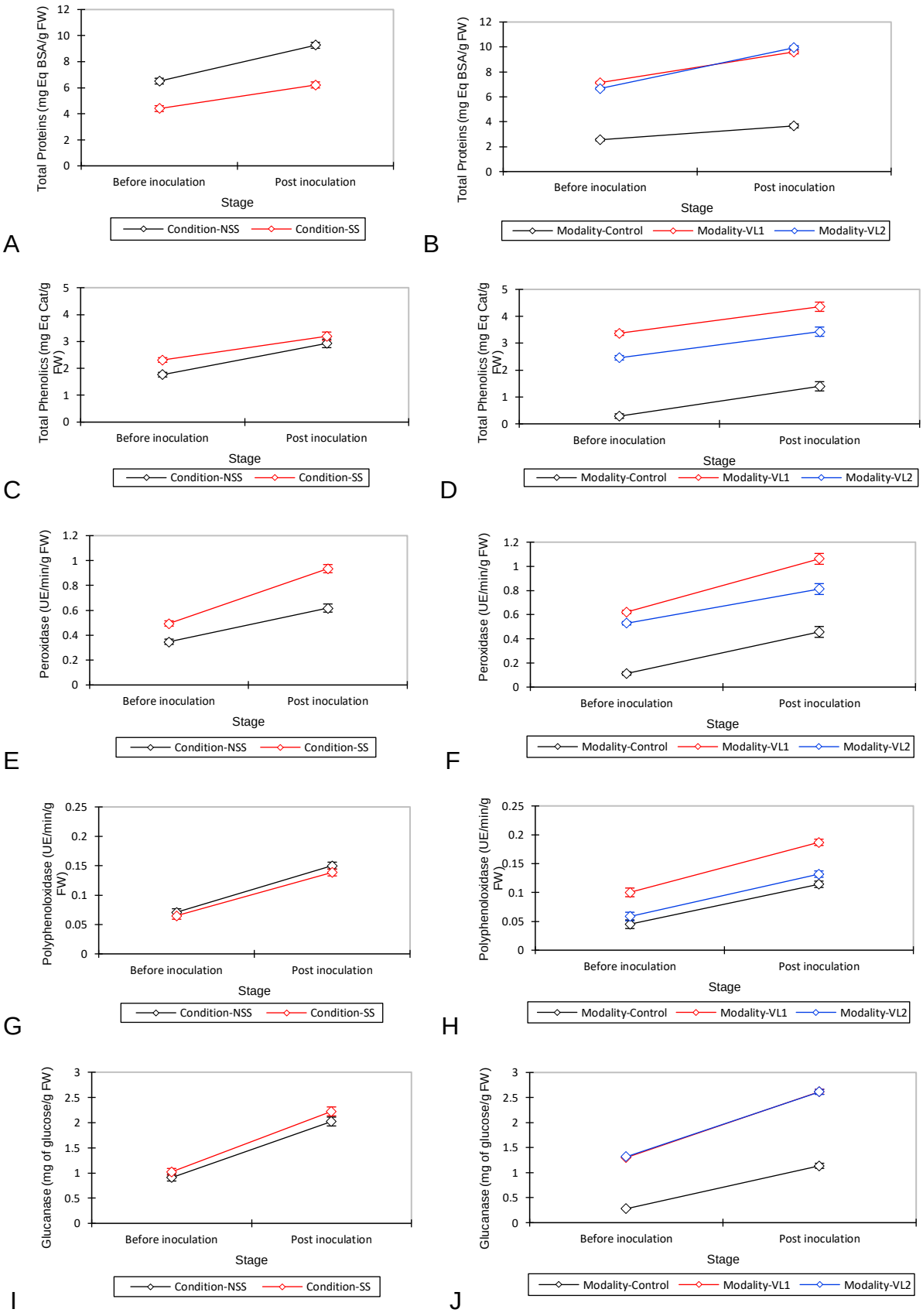


Figure 5: Vertical layers of *T. diversifolia* flakes effects on the PIF plantain seedlings accumulation of bio-chemical markers. Interaction plots of stage and condition (stage*condition) for the total proteins (A), the total phenolics (C), the peroxidase (E), the polyphenoloxidase (G) and glucanase (I), and of stage and modality (stage*modality) for the total proteins (B), the total phenolics (D), the peroxidase (F), the polyphenoloxidase (H) and glucanase (J). Each point represents the average mean with the standard deviation of three replicates for each modality.

Table 3: Analysis of correlation between all the variables in terms of implication in growth promotion (germination percentage, number of shoots, diameter and height of shoots, foliar surface, total proteins and total polyphenols), and in BSD severity (necrotic surface of leaves, total proteins, total polyphenols, peroxidase, polyphenoloxidase and glucanase) after vertical layers of *T. diversifolia* flakes treatment. The correlation matrix of Pearson (n) shows positive or negative correlation, but also the strength of the relationship (**bold**) for the sterile condition (SS, above diagonal) and the non-sterile condition (NSS, below diagonal). Values in bold are different from 0 with a significance level alpha= 0,05.

Variables	Germination (%)	Number of shoots	Diameter (cm)	Height (cm)	Foliar surface (mm²)	Necrotic surface (mm²)	Total Proteins (mg Eq BSA/g FW)	Total Phenolics (mg Eq BCat/g FW)	Peroxidase (UE/min/g FW)	Polyphenoloxidase (UE/min/g FW)	Glucanase (mg of glucose/g FW)
Germination (%)	**1**	**0,897**	**0,248**	**0,430**	**0,270**	0,154	**0,291**	**0,244**	**0,325**	**0,441**	**0,376**
Number of shoots	**0,900**	**1**	0,217	**0,377**	**0,242**	0,114	0,177	0,141	**0,239**	**0,383**	**0,278**
Diameter (cm)	**0,500**	**0,432**	**1**	**0,927**	**0,988**	-0,127	0,114	**0,372**	**0,594**	**0,326**	0,118
Height (cm)	**0,502**	**0,444**	**0,961**	**1**	**0,957**	-0,109	0,166	**0,315**	**0,474**	**0,316**	0,180
Foliar surface (mm²)	**0,279**	**0,245**	**0,936**	**0,928**	**1**	-0,136	0,126	**0,350**	**0,559**	**0,311**	0,126
Necrotic surface (mm²)	0,158	0,120	-0,123	-0,155	-0,205	**1**	-0,014	-0,040	-0,045	0,008	0,002
Total Proteins (mg Eq BSA/g FW)	**0,290**	0,192	**0,464**	**0,480**	**0,552**	-0,135	**1**	**0,884**	**0,542**	**0,434**	**0,924**
Total Phenolics (mg Eq BCat/g FW)	**0,250**	0,193	**0,530**	**0,494**	**0,634**	-0,124	**0,903**	**1**	**0,759**	**0,486**	**0,841**
Peroxidase (UE/min/g FW)	**0,382**	**0,312**	**0,223**	**0,305**	0,213	-0,051	**0,764**	**0,561**	**1**	**0,798**	**0,615**
Polyphenoloxidase (UE/min/g FW)	**0,376**	**0,318**	**0,456**	**0,454**	**0,515**	-0,078	**0,843**	**0,907**	**0,669**	**1**	**0,642**
Glucanase (mg of glucose/g FW)	**0,378**	**0,306**	**0,456**	**0,467**	**0,515**	-0,092	**0,910**	**0,910**	**0,761**	**0,954**	**1**

shoots, diameter and height of shoots, foliar surface and roots aspect) compared to the control one. This growth promotion of seedlings has been widely reported in previous studies on cocoa [14, 15] and plantain banana [4, 5].

T. diversifolia flakes contain high level of nitrogen (N), phosphorus (P) and potassium (K), it could be therefore considered as a seedling vegetative booster. Indeed, nutrients in plants have important functions in osmotic regulation, cellular permeability, and may act as structural components and essential metabolites, being therefore critical for proper growth and development [16]. *T. diversifolia* flakes seem to improved soil physical, chemical and biological properties, leading to enhance soil fertility, to increase microbial activity and to optimized nutrients absorption by plant [17, 18]. Plant growth rates are influenced by elements like N, P and K contained in *T. diversifolia* flakes, with N being a constituent of chlorophyll, but it is also involved in division and enlargement of cells in the apical meristem [17]. PIF regeneration is related to the activity of the apical meristem generation leading to many shoots' growth on the suckers, to the growth improvement especially the height increase.

As expected, the growth promotion was more important in the sterile substrate (SS) condition compare to the non-sterile (NSS) condition. Indeed, the non-sterile condition exposes the seedlings during their growth in nursery to the microbiome present in the PIF substrate and this could either generate positive synergistic action and/or negative antagonistic action (stresses) during their development. Therefore, morphological differences in the diameter and height of VL2 shoots, more pronounced in the foliar surface were observed as a result of possible antagonistic interactions due to the presence of different microorganisms in the substrate, that could avoid the seedlings feedings in a good manner. Indeed, the existence of antagonistic relationship between the biotic factors in the substrate could be in the contrary of organic fertilizer used as substrate for soil microorganisms, slow down the rate of organic material decomposing and release of nutrient for plant uptake [17]. However, this level of stress induced by the non-sterile substrate condition was lower to induce a slow development of the seedlings.

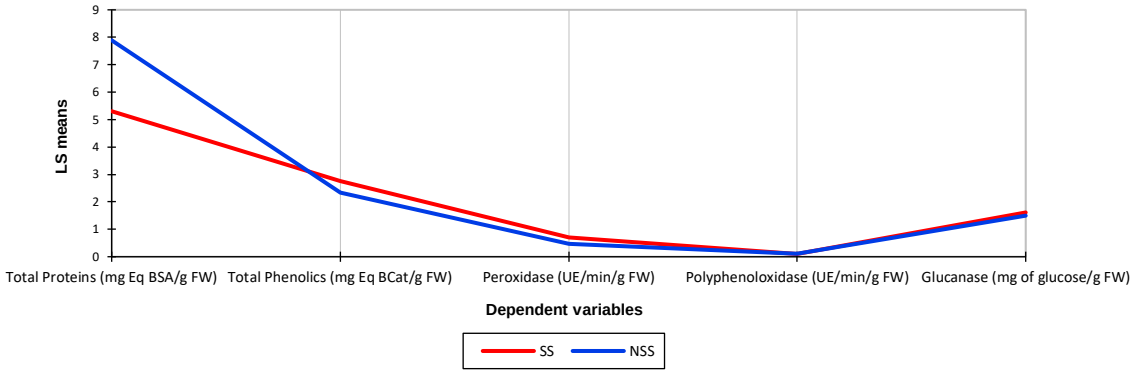
Despite the fact that the organic fertilizer has positive effects in maintaining the soil properties, applied in excess they could act as chemical fertilizers. Indeed, high dose chemical fertilizers are associated with reduction of soil properties and crop yields over time, thus less beneficial input to get higher crop productivity [19]. Since the presence of excessive chemical inputs during cultivation generates stresses during the seedling's development, a stressful impact seems to be observed for the seedlings of modality 2VL. Indeed, compared to the VL1 modality, the agromorphological parameters (diameter and height of shoots, foliar surface and roots aspect) were reduced, except for the germination rate and the number of shoots. It is known that most plants nutrients are taken from the soil and their total amount in the soil is not the only one that is responsible for optimal growth of plants, since physico-chemical-biological properties of soil such as: soil texture, organic matter, cation exchange capacity, pH, electrical conductivity and activity of soil microbes are also involved [20]. Therefore, an excess of nutrients like nitrogen, phosphorus and potassium during amendment could lead to a stress in the plant development. Moreover, organic fertilizer effects on plants are slower in term of release of nutrients which are store thus for long in the soil and could therefore be toxic for seedlings. Stimulatory and inhibitory effects *T. diversifolia* have be shown *in vitro* on *Cleome gynandra* (spider plant) germination and growth [21].

Our results confirm the vertical layer of *T. diversifolia* flakes direct effects on seedlings physiology in nursery. Indeed, *T. diversifolia* flakes seems to contribute directly to seedlings growth and yield eventually by supplying nutrients, and indirectly by modifying soil physical properties such as stability of aggregates and porosity that can improve the root growth, rhizosphere and stimulate plant growth [22]. As expected, in both conditions, treatment with *T. diversifolia* flakes germinates quickly and present good vegetative growth parameters. Moreover, the treatment seems to strengthen seedlings pseudo-stems, increase photosynthesis rates and stimulates seedlings roots. Indeed, phosphorus present in *T. diversifolia* flakes could be involved in this response to the treatment since it is part of molecular structure of nucleic acid, functions as a storage, accelerate energy transfer processes within the plant and enhanced root development [16, 23]. This result confirms previous observations suggesting that some natural products such as *T. diversifolia* biomass 24, as snail, oyster and clam shells, *T. diversifolia* have a potential effect on seedlings development in nursery [4-5, 14-15].

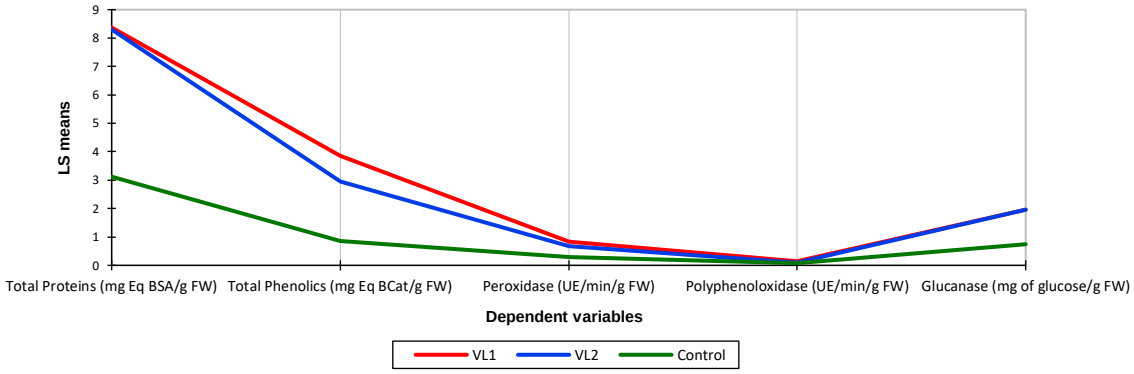
The presence of biotic stress in the substrate during the seedling's development induced a reduction in the vegetative growth parameters notably for VL2 in NSS condition. Moreover, *T. diversifolia* tissues are very rich in nutrients and phytochemicals [6, 7, 8] that could induce stresses if present in large amounts in the substrate. The interaction between the substrate, the plant and the plant and/or the substrate microbiome could lead to antagonistic interactions that slow down the seedling's growth. We can therefore hypothesize that such a reduction of vegetative growth parameters is coupled with a reduction of assimilates accumulation and could account for an increase susceptibility to diseases. Indeed, the reduction of the seedling's morphology was more important in the modalities with potential stress (NSS and VL2), and this could account for the high increase in seedlings susceptibility to BSD severity.

A significant effect of *T. diversifolia* flakes was found in BSD development on PIF seedlings. Indeed, the treated seedlings were less susceptible to *M. fijiensis* than the control ones. Also noteworthy is the fact that the level of necrotic surface measured in the seedlings leaves of the non-sterile condition and the VL2 modality were very important compared to the ones of the VL1 modality. This level of susceptibility was lower as compared to the one obtains with an early study on clam shells [4] and almost the same for treated modalities and condition with an early study on the combined effect of clam shells and *T. diversifolia* [5]. The lesser susceptibility of seedlings of

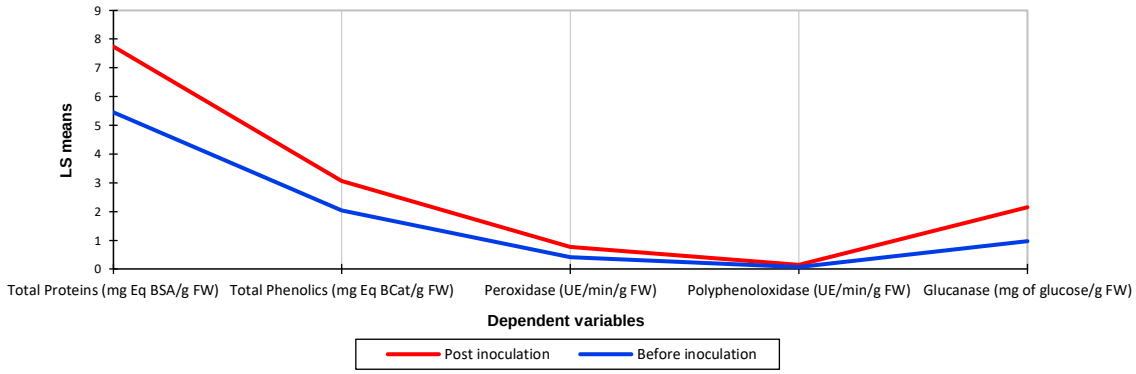
VL1 modality to BSD could be explained by the fact that the treatment seems to trigger physiological changes on PIF seedlings resulting in an increase of assimilates availability during the growth, leading to an enhanced growth promotion and biomarkers accumulation.



A



B



C

Figure 6: LS means summary of biochemical markers (total proteins, the total phenolics, the peroxidase, the polyphenoloxidase and glucanase) accumulation in the PIF plantain seedlings in the shade after treatment with the vertical layers of *T. diversifolia* flakes: Condition (A), Modality (B), Stage (C).

T. diversifolia flakes seems to activated the vegetative growth promotion and natural defense systems through the increased synthesis of nutrients and defensive metabolites [18, 25-26]. This could be due to the presence of nitrogen and potassium in the *T. diversifolia* flakes. Indeed, nitrogen has the function of building up protoplasm, preparing amino acids, proteins, nucleic acids, nucleotides, hormones and chlorophyll in the plant, while potassium serves as an enzyme activator use by the plant to activate many (over 60) different enzymes [16, 23]. Moreover, nitrogen induce cell division and initiate 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 [16]. This enhancement of nutrients

and defensive metabolites could justified the increase in the formation of proteins, phenolic compounds and enzymes (peroxidase, polyphenoloxidase, glucanase, chitinase) as shown recently [4-5, 14-15]. Such biomarkers have effectively been shown to be involved in the defense mechanism of banana tissues [27-30] as well as different others plant tissues [31-32]. If *T. diversifolia* effect on other plant is well documented, this is one of the first report of *T. diversifolia* effect on plantain PIF seedlings biomarkers accumulation.

T. diversifolia flakes effectively influence the larger amount of proteins, phenolics and enzymes activity such as peroxidase, polyphenoloxidase and glucanase accumulated in both conditions, more importantly in the treated modality and at both stages. These biomarkers are abundantly accumulated in the sterile substrate (SS) condition compared to the non-sterile (NSS) condition except for the total proteins and polyphenoloxidase which are more accumulated in the non-sterile substrate (NSS) condition (Figure 6A), probably because of the stress's effects of the NSS condition. The biomarkers accumulation was more important in the seedlings leaves of the VL1 modality, followed by the VL2 modality and very low in the control modality one; except for the total proteins and polyphenoloxidase which are accumulated at the same level in VL1 and VL2 treated modalities probably because of the stress's effects of VL2 modality (Figure 6B). Indeed, the stresses seems to induced the accumulation of total proteins and polyphenoloxidase, which are known as biomarkers of resistance/tolerance to biotic stresses as well as abiotic stresses [33]. The accumulation was more important in the treated seedlings compare to the control's ones (Figure 6B). The amount of these biomarkers was found to be more important at the stage after inoculation compared at the stage before inoculation in PIF seedlings leaves (Figure 6C). This difference in accumulation was more important for proteins, phenolics, glucanase, peroxidase and polyphenoloxidase probably because after inoculation, the plant have set down a mechanism to overcome the pathogenic attack through the use of preformed and the *novo* synthesis of biomarkers [31-34].

5. Conclusion

In summary, the aim of this work was to examine the effect *T. diversifolia* flakes vertical layer on the performance of PIF plantain seedlings in term of vegetative growth and disease susceptibility in nursery. Our results have highlighted a new effect of *T. diversifolia* flakes vertical layer on plantain banana PIF seedlings quality. However, the physiological and molecular mechanism involved in the growth promotion and the less susceptibility to BSD severity are still unknown. The use of vertical layer of *T. diversifolia* flakes should be considered by poor peasants 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 and the preservation of our environment at low cost. It will be important to access the effect of this *T. diversifolia* flakes in the farm, since it seems to be a tool for an agriculture more sustainable and resilient. Vertical layers of *T. diversifolia* flakes in PIF seedlings productivity could be a poverty alleviation tools for small holders' farmer, small-scale and medium-scale enterprises in sub-Saharan countries.

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