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Stimulatory Effects of Flakes and Compost Amendment Based on *Tithonia diversifolia* on the Quality of PIF Plantain Seedlings Growth and Tolerance to *Mycosphaerella fijiensis*

Meshuneké Arouna[1], Dakolé Daboy Charles[1], Wassom Damien Fabrice[1], Fotsing Silatsa Landry[1], Elock Mbang Gaston[1], Kengoum Djam Marie Paule[1], Ché William Asah[1], Kom Timma Jospin Walter[1], Niemenak Nicolas[2, 3], Boudjeko Thaddée[1, 4], Ewané Cécile Annie[1, 3, 4, *]

[1]Department of Biochemistry, Faculty of Science, University of Yaoundé 1, Yaoundé, Cameroon

[2]Department of Biological Science, Higher Teacher's Training College, Yaoundé, Cameroon

[3]Laboratory of Biochemistry and Plant Physiology of the Higher Teachers' Training College, University of Yaoundé 1, Yaoundé, Cameroon

[4]Laboratory of Phytoprotection and Valorization of Genetic Resources, Biotechnology Centre, University of Yaoundé 1, Yaoundé, Cameroon

Email address:

cecile-annie.ewane@facsciences-uy1.cm (Ewané Cécile Annie)

[*]Corresponding author

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Abstract: Plantain (*Musa* AAB) in the banana family of *Musaceae*, contribute to food security for sub-Saharan African population due to its high energy value (128.6 kcal/100 g), minerals (potassium, magnesium, calcium, phosphorus), dietary fiber and vitamins (A, B and C) as well as poverty alleviation for millions of people in these regions. However, plantain production in Africa and Cameroon in particular still encounters numerous problems despite these performances. Some of these problems include the decline of soil, ineffective control methods, parasitic constraints and principally, the unavailability of quantity and quality seedlings. The use of micropropagation technique as an alternative of seedlings unavailability in quantity and quality has been explored, yet, it requires expensive laboratory equipment's and technical skills and is not affordable by small scale farmers. Chemical control remains the principal method which consists in the use of pesticides, fungicides and herbicides that is reasonably efficient, yet the use of these chemical products shows some limits such as toxicity to the environment and human, costly to small scale farmers, pathogen resistance in plants and destruction of non-targeted species. The production of plantlets from stem bits (PIF) plantain seedlings in substrates with organic inputs like *Tithonia diversifolia* flakes and compost could be an alternative to the problem of growing healthy and good quality plantain plants. The objective of this research is to evaluate the stimulatory effects of flakes and compost amendment based on *Tithonia diversifolia* on the quality of PIF plantain seedlings growth and tolerance to *Mycosphaerella fijiensis* in nursery. The vegetative growth parameters, susceptibility to black Sigatoka disease and accumulation of biomarkers were assessed in the sterilized and non-sterilized substrate state. Flakes and compost amendment based on *T. diversifolia* significantly increase the height and the diameter of pseudo-stems, the total leaf area but also protect the seedlings against BSD up to about 89% compared to the control ones. They enhance the accumulation of biomarkers such as chlorophyll, sugars, amino acids, polyphenols, proteins, content and defense-related enzymes (peroxidase, polyphenoloxidase, phenylalanine ammonia lyase and ascorbate peroxidase). Flakes and compost amendment based on *T. diversifolia* seem to act as vital stimulators. They can therefore be seen as a tool for more sustainable and resilient agriculture, and poverty reduction for poor small farmers.

Keywords: PIF Plantain, *Tithonia diversifolia*, Clams, Flakes and Compost Amendment, *Mycosphaerella fijiensis*, Induced Tolerance, Vital Stimulator

1. Introduction

Plantain (*Musa* AAB) in the banana family of *Musaceae*, is grown in tropical and subtropical regions in well-drained soil that has pH between 6.0 and 7.5 and in temperatures between 18 and 32 degree Celsius [1]. The cycle of production ranges between 9 to 12 months for a plantain tree to make fruit (plantain) depending on the variety [2]. Plantain plays an essential role in contribution to food safety for the Central and West African population due to its high energy value (128.6 kcal/100g), minerals (potassium, magnesium, calcium, phosphorus), dietary fiber and vitamins (A, B and C) [3], as well as income generation for millions of people in these regions [3]. Thanks to its organoleptic properties, it is subjected to numerous transformations by the small and medium size agro-food enterprise for the principal production of fries, chips, sweet or non-sweet flour [4]. The world production of plantain is estimated to more than 20 million tons per year and Cameroon is ranked 3rd in the world (3.94 million tons per year) and the first in the CEMAC zone [5].

However, plantain production in Africa and Cameroon in particular still encounters numerous problems despite these performances. Some of these problems include the decline of soil, ineffective control methods, parasitic constraints and unavailability of quantity and quality seedlings. Amongst these problems, the major being the unavailability of quantity and quality seedlings and parasitic constraints, principally black Sigatoka disease (BSD) caused by *Mycosphaerella fijiensis*. It is the most economically destructive disease of plantain, which represents plantain production losses estimated at approximately 30 to 50% [6]. These problems have caused low plantain productivity and reduction in the creation of new plantations hence leading to high demand and consequently high product prices in local, urban and cross-border markets [7].

The use of micropropagation technique as an alternative of seedlings unavailability in quantity and quality has been explored, yet, it requires expensive laboratory equipment's and technical skills and is not affordable by small scale farmers. A new 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 Recherche sur Bananiers et Plantains (CARBAP) is an alternative for small scale farmers for its benefits [7, 8]. This technique allows the massive production of plants in quantity in a very short time (2 to 3 months) and at a lower cost [7, 8]. However, seedlings produced by PIF technique encounter many problems like acclimatization, as well as contamination of agricultural land in a soil that often contains pathogenic microorganisms, causing their attack by diseases such as black Sigatoka disease leading to plants mortality during the establishment of new plantations [7].

Various methods have been employed to mitigate the problem of BSD of plantain, principally, cultural control, genetic control, chemical control, biological control and integrated control methods. Chemical control remains the principal method which consists in the use of pesticides, fungicides and herbicides that is reasonably efficient, yet the use of these chemical products shows some limits such as toxicity to the environment and human, costly to small scale farmers, pathogen resistance in plants and destruction of non-targeted species [3]. Hence, the importance to explore the bio-agricultural control method using organic inputs (*Tithonia diversifolia*, compost and clam shells) that are environmental viable and cost effective.

Recent studies carried out in Cameroon have shown that the clam shells powder alone and its association with *T. diversifolia* have a strong influence on the growth of PIF plantain seedlings and a lower sensitivity to BSD in the nursery due to their dual role of biofertilizer and biopesticide [7, 9]. Additionally, the author [3] also demonstrated that mulching of *Tithonia diversifolia* stimulates the growth of PIF plantain seedlings and induces less susceptibility to *Mycosphaerella fijiensis* in the nursery. Moreover, the use of dried and ground chicken manure and plantain peels in compost induced the growth of banana-plantain seedlings [10]. Confirming thus the importance of these organic inputs compared to chemical inputs in the growth promotion of plants and their protection against biotic and abiotic stresses.

Application of *T. diversifolia* flakes and compost in the substrates of PIF plantain seedlings could improve their performance. The aim of this research is to evaluate the stimulating effects of *Tithonia diversifolia* flakes and compost on the growth quality of PIF plantain seedlings and tolerance to *Mycosphaerella fijiensis* in nursery.

2. Materials and Methods

2.1. Materials

Plantain suckers (*Musa* spp., genome AAB) of the Big-Ebanga variety selected for their short production cycle and seedlings productivity were obtained from the Agricultural Research Institute for Development situated at Nkolbisson in the Yaoundé VII district of Mfoundi division.

T. diversifolia leaves and stems were obtained from agricultural land around the Higher Teachers' Training College of the University of Yaoundé 1 and were sun-dried and then crumbled by hands.

Plantain by-products (peels, trunks and leaves) used in the formulation of compost were obtained from a small plantain field located in Melen in the Yaoundé VI district of Mfoundi division.

Clam shells powder which was used in combination with *T. diversifolia* flakes and compost was obtained from the Laboratory of Biochemistry and Plant Physiology of the Higher Teachers' Training College of the University of Yaoundé 1.

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 [7].

Sawdust was used for germination and emergence of seedlings in the greenhouse while sand and black soil were used in proportions of 1/3 and 2/3 for the growth of seedlings in the shade.

NPK (20-10-10) chemical fertilizer and chemical fungicide (Terazeb) were used in the positive control treatment in the greenhouse and under the shade.

2.2. Experimental Design

This research was conducted in the Centre Region of Cameroon (Yaoundé), located in the agroecological zone known as the Bimodal Rainforest Humid Forest for a period from August 2020 to January 2021. The acclimatization phase for plantain vivoplants under the shade was extended over the period from September 2020 to November 2020 marked by moderate temperatures (26-30°C) and low rainfall (25-80 mm/month). The other phases of the research were carried out under controlled laboratory conditions.

The different treatments of the study were carried out in two totally randomized blocks with (06) treatments in each block in the greenhouse and under the shade house.

Two blocks:

Sterilized Substrate (SS) State

Non-Sterilized Substrate (NSS) State

Six treatments (two *Tithonia diversifolia* combinations, two compost combinations and two controls):

Substrate only (negative control): T^-

Substrate + chemical fertilizer and fungicide (positive control): T^+

Substrate + *T. diversifolia* + Clams powder as Amendment: Td+Cl_A

Substrate + *T. diversifolia* + Clams powder as Mulch: Td+Cl_M

Substrate + Compost + Clams powder as Amendment: TCo+Cl_A

Substrate + Compost + Clams powder as Mulch: TCo+Cl_M

Each treatment in each block was considered as an Experimental Unit (EU). The PIF explants were prepared according to the method used [7]. In each EU, three (03) explants were introduced and covered with white transparent plastic paper in the greenhouse. Germination and emergence of seedlings in the greenhouse were favored by watering.

2.3. Evaluation of Flakes and Compost Based on T. diversifolia Amendments Effect on PIF Seedlings Vegetative Growth Stages

The second week after the introduction of the explants in the greenhouse, the germination and emergence of seedlings were observed and complete after four weeks. Agronomic parameters (diameter and height of pseudo-stems, and total leaf surface) were assessed after every 7 days of the sixth week to the fourteenth week of acclimatization of PIF seedlings in the shade for each treatment. These evaluations were done for five selected seedlings in the shade house according to the method reported [7]. The total leaf surface (TLS) of each seedling was calculated using the formula described by [2]:

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

0.8 and 0.662 are constants.

L is the length of the widest leaf of each treatment.

W is the width of the widest leaf of each treatment.

2.4. Evaluation of Flakes and Compost Based on T. diversifolia Amendments Effect on PIF Seedlings Susceptibility to BSD

The susceptibility level of PIF seedlings was assessed on leaves of the same age for approximately 42 days (6 weeks) by artificial inoculation of the leaves with a suspension of (106 zoospores/mL). Before inoculation, a leaf of each plant was stored at -45°C in a plastic bag for biochemical analysis of the before inoculation stage (BI), while those to be inoculated were cleaned and left for two hours at room temperature before inoculation. Sporal solution of *Mycosphaerella fijiensis* strain was used for inoculations and the assessment of necrotic leaf area of seedlings performed every 3 days on PIF seedlings as previously described by [7, 9] to obtain the leaves for the biochemical analysis of post inoculation stage (PI).

2.5. Evaluation of Flakes and Compost Based on T. diversifolia Amendments Effect on PIF Seedlings Biomarker's Accumulation

The evaluation of total chlorophyll, total soluble sugars, total proteins, total polyphenols, total free amino acids and specific activities of enzymes like phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and ascorbic peroxidase (POX Asc.) was carried out in two stages (before inoculation and post inoculation) in the leaves of PIF seedlings while only the post inoculation analysis was done in the roots of PIF seedlings. Total chlorophyll and total soluble sugars were not accessed in the roots. For each treatment, 0.5 g of fresh leaf and root was used for the analyses of the sample. The extraction and the quantification of samples were carried out according to the method reported by [11, 12] modified respectively for total chlorophyll and total soluble sugars, the research by [13-19] modified respectively for total proteins (595 nm), total phenolics (760 nm), PAL (290 nm), PPO (330 nm), POX (470 nm), POX Asc. (290 nm) and total free amino acids (570 nm). The total chlorophyll was measured in mg per gram of fresh weight (mg/g FW), total soluble sugars in mg per gram of fresh weight (mg/g FW), total free amino acids in mg per gram of fresh weight (mg/g FW), total phenolics in mg equivalent (Eq) of gallic acid (GA) per gram of fresh weight (FW) while the one of the total proteins concentration was expressed in mg equivalent (Eq) of bovine serum albumin (BSA) per gram of fresh weight. Enzymes specific activities were expressed in enzymatic unit per minutes per gram of fresh weight (EU/min/g FW).

2.6. Statistical Analyses

Flakes and compost based on *T. diversifolia* amendments

effects on vegetative growth stages of PIF seedlings, BSD susceptibility and biomarker's accumulation were analyzed by subjection of the variables (height and diameter of pseudo-stems, total leaf surface, necrotic surface, total chlorophyll, total soluble sugars, total free amino acids, total proteins, total phenolics, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and ascorbic peroxidase) to mixed three-way ANOVA performed with XLSTAT software version 2021. Each plant being taken as experimental unit and condition [the sterilized substrate (SS) state and the non-sterilized substrate (NSS) state] or stage [before inoculation (BI) and post inoculation (PI)], treatment and day as factors. Multiple comparisons of means were performed by applying Tukey's test at a probability level of 5%.

3. Results

3.1. Effects of Flakes and Compost Based on T. diversifolia Amendments on PIF Seedlings Vegetative Growth Stages

Flakes and compost based on *T. diversifolia* amendments (Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A) were found to significantly ($P< 0.0001$) influence the vegetative growth parameters of PIF plantain seedlings, specifically the height and the diameter of pseudo-stems as well as the total leaf surface (Table 1). The coefficients of determination (R^2) for the three variables were 80.5%, 79.6% and 76.5% respectively (Table 1), close to a 100% indicating that *T. diversifolia* flakes and compost treatments model are good fit for the data, are reliable and reproducible. All the variables and interactions were highly significant for the height and diameter of pseudo stems, as well as total leaf surface (Table 1). All the parameters of the vegetative growth stage evolve significantly over time and the most influential variable was the treatment.

Overall, a significant difference was observed between the sterilized substrate (SS) state and the non-sterilized substrate (NSS) state only for the height of seedlings (Table 1). A significant difference was observed between the treated seedlings and non-treated seedlings in the two conditions (Figures 1A, B and C). Two distinct statistical groups were distinguished for the height of pseudo-stems while only one was distinguished for the diameter of pseudo-stems and total leaf surface (Figure 2A).

Table 1. *Analysis of variance (ANOVA) of the flakes and compost based on T. diversifolia amendments on the PIF plantain seedlings growth [(height and diameter of pseudo-stem and total leaf surface (TLS)].*

	Height (cm)	**Diameter (cm)**	**TLS (cm^2)**
R^2 (%)	80.5	79.6	76.5
F	15.914	15.059	12.571
P	**<0.0001**	**<0.0001**	**<0.0001**
Condition	26.212	3.196	0.997
	<0.0001	0.075	0.319
Treatment	278.480	273.902	158.157
	<0.0001	**<0.0001**	**<0.0001**
Week	20.268	18.410	40.419
	<0.0001	**<0.0001**	**<0.0001**
Repetition	9.775	7.795	10.714
	<0.0001	**<0.0001**	**<0.0001**
Condition*Treatment	6.423	13.220	8.899
	<0.0001	**<0.0001**	**<0.0001**

Values in bold correspond to tests where the null hypothesis is not accepted with a significance level alpha= 0.05.
F is the value of F test and *P* is the probability.

Flakes and compost based on *T. diversifolia* amendments effects were clearly and significantly differentiated between the four treatments (Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A) and the positive (T+) and negative (T-) control treatments in terms of the height and diameter of pseudo-stems, as well as the total leaf surface. The vegetative growth parameters were consistently higher in the treated seedlings compared to non-treated seedlings. They were greater for the PIF seedlings treated as mulch (Td+Cl_M and TCo+Cl_M) compared to ones treated as amendment (Td+Cl_A and TCo+Cl_A) and highly greater than those of the positive and negative control treatments (T+ and T-) both in the sterilized substrate (SS) state and in the non-sterilized substrate (NSS) state (Figures 1A, B and C). The positive control treatment (T+) having received the chemical fertilizer showed greater agro-morphological parameters compare to the negative control one (T-) that have not received any treatment.

Indeed, the mean height of pseudo stems for PIF seedlings treated as mulch was 28.86 ± 5.41 cm and 25.22 ± 4.95 cm respectively for Td+Cl_M and TCo+Cl_M in the sterilized substrate (SS) state. It was 28.03 ± 6.23 cm and 21.80 ± 2.81 cm for Td+Cl_M and TCo+Cl_M in non-sterilized substrate (NSS) state. The height was lower in the control treatments compared to the treated ones with respective mean values of 17.28 ± 1.93 cm and 18.73 ± 2.69 cm for positive control seedlings (T+), and 14.16 ± 1.99 cm and 13.01± 2.52 cm for negative control one (T-) grown respectively in the sterilized substrate (SS) state and in the non-sterilized substrate (NSS) state (Figure 1A).

The mean diameter of pseudo stems for PIF seedlings treated as mulch was 2.75 ± 0.41 cm, and 2.51 ± 0.4 cm

respectively for Td+Cl_M and TCo+Cl_M in the sterilized substrate (SS) state. It was 2.91 ± 0.61 cm and 2.15 ± 0.33 cm for Td+Cl_M and TCo+Cl_M in non-sterilized substrate (NSS) state. The mean diameter was lower in the control treatments compared to the treated ones with respective mean values of 1.41 ± 0.14 cm and 1.63 ± 0.25 cm for positive control seedlings (T+), and 1.47 ± 0.34 cm and 1.31± 0.19 cm for negative control one (T-) grown respectively in the sterilized substrate (SS) state and in the non-sterilized substrate (NSS) state (Figure 1B).

The mean total leaf surface of PIF seedlings treated as mulch was 1374.51 ± 760.12 cm^2, 1840.49 ± 786.70 cm^2 respectively for Td+Cl_M and TCo+Cl_M in the sterilized substrate (SS) state. It was 934.44 ± 848.80 cm^2 and 902.53 ± 408.48 cm^2 respectively for Td+Cl_M and TCo+Cl_M in non-sterilized substrate (NSS) condition. The mean total leaf surface was lower in the control treatments compared to the treated ones with respective mean values of 628.29 ± 270.85 cm^2 and 575.14 ± 295.60 cm^2 for positive control seedlings (T+), and 334.68 ± 163.48 cm^2 and 359.90 ± 198.56 cm^2 for negative control one (T-) grown respectively in sterilized substrate (SS) state and non-sterilized substrate (NSS) state (Figure 1C).

Five distinct statistical groups were obtained for each of these variables (Figure 2B). Among the four treatments, the one that showed the best effect in terms of growth promotion was (Td+Cl_M), mulch of *T. diversifolia* flakes and clams (Figure 2B).

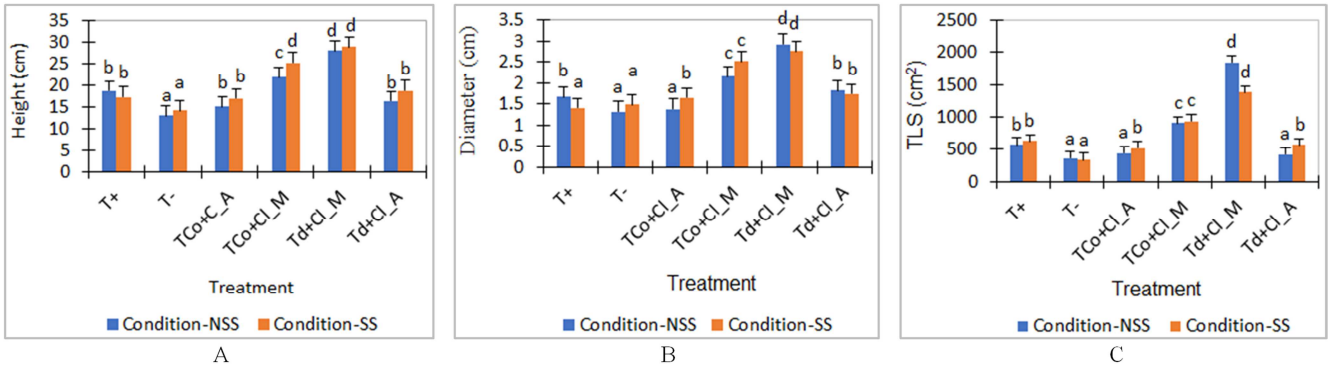


Figure 1. *Effects of flakes and compost based on T. diversifolia amendments on the height and diameter of pseudo-stems, and total leaf surface of PIF plantain seedlings through interaction plots of condition and treatment for the height (A), the diameter (B) and the total leaf surface (C). Each point represents the average mean of three replicates with the standard deviation for each treatment.*

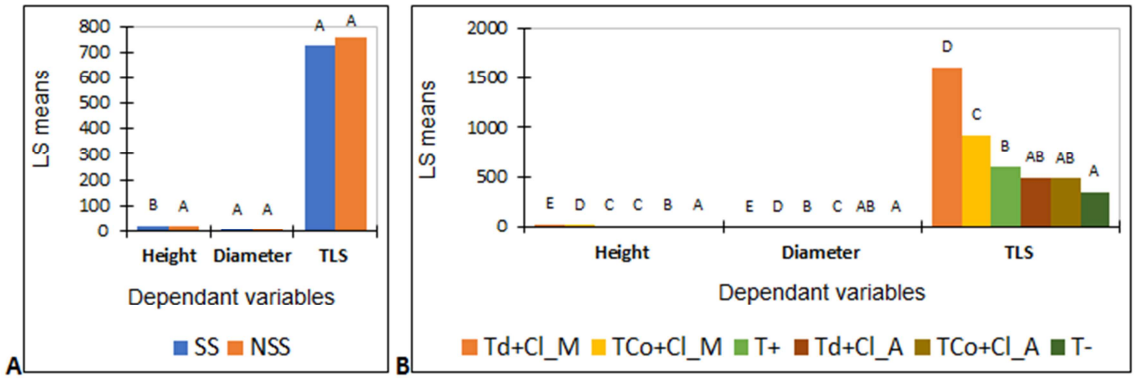


Figure 2. *Least Squares (LS) means analysis of the flakes and compost based on T. diversifolia amendments on the height, diameter and total leaf surface of PIF plantain seedlings for condition (A) and treatment (B). Letters A, B, C, D and E represent the different statistical groups defined by the Tukey test (5%).*

3.2. Effect of Flakes and Compost Based on T. diversifolia Amendments on PIF Seedlings Susceptibility to BSD

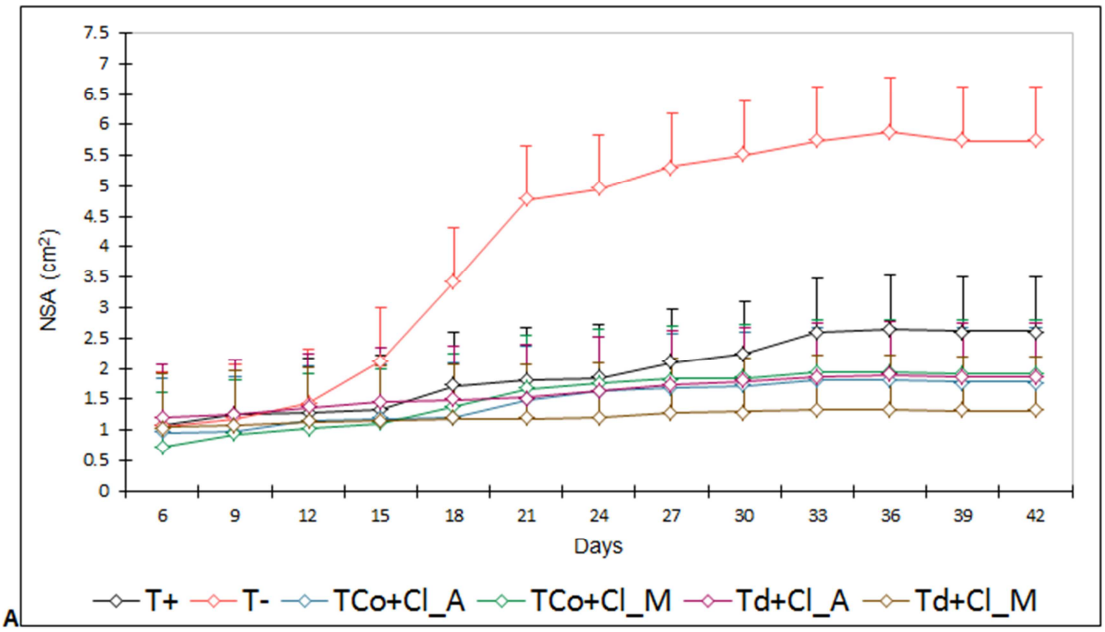
Flakes and compost based on *T. diversifolia* amendments (Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A) were found to significantly ($P< 0.0001$) influence the severity of BSD, with a low level of necrosis development after inoculation of treated seedlings (Figure 3). Suggesting thus that the necrosis development on the leaves is mostly influenced by the treatment.

Overall, there was no significant difference in the severity of BSD between the non-sterilized substrate (NSS) state and the sterilized substrate (SS) state, with values around 1.99 cm^2 and 1.96 cm^2 respectively (Figure 4A).

One statistical group was obtained for necrotic surface area between the sterilized substrate (SS) state and the non-sterilized substrate (NSS) state.

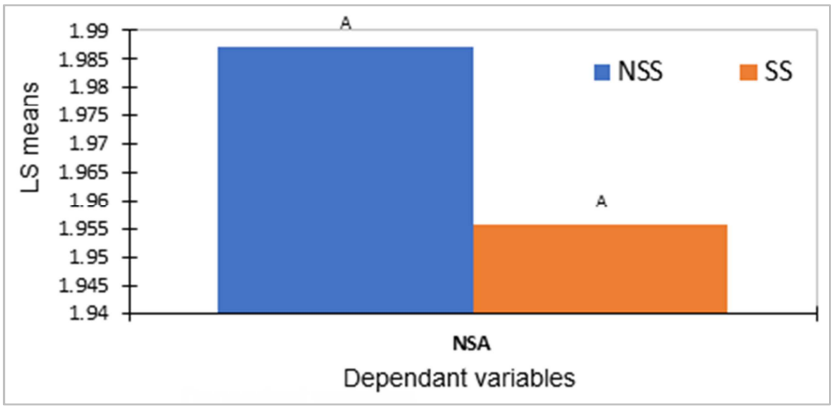
Sensitivity to BSD was consistently lower in the treated seedlings than in the non-treated ones. However, sensitivity to BSD was higher in the positive control seedlings treated with chemical fungicide (T+) compared to the ones treated with *T. diversifolia* flakes and compost (Figure 3). There was a difference between the four treatments (Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A) and the controls (T+

and T-) in terms of necrosis development in course of time. Indeed, an effective effect of treatments at the slightest development of necrosis was observed with mean values of 1.21 ± 0.19 cm^2, 1.60 ± 0.38 cm^2, 1.52 ± 0.50 cm^2 and 1.45 ± 0.51 cm^2 respectively for Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A as compared to the controls one T+ and T- with mean values of 1.92 ± 0.87 cm^2 and 4.03 ± 2.82 cm^2 respectively (Figure 3).

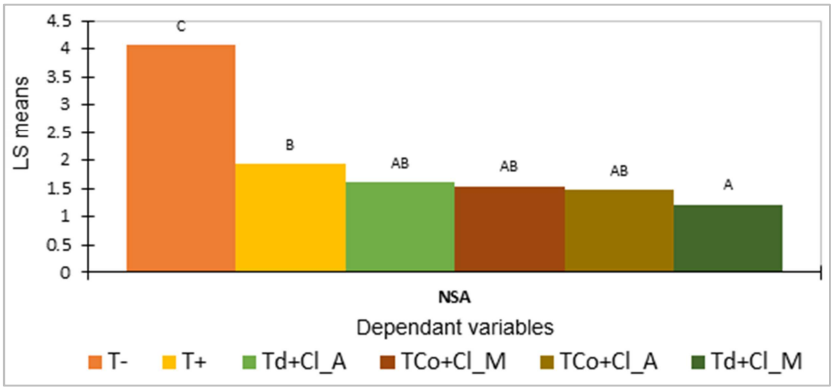


A

Figure 3. *Effects of flakes and compost based on T. diversifolia amendments on the necrotic surface area of PIF plantain seedlings in course of time through interaction plot of day and treatment for necrotic surface area. Each point represents the average mean of three replicates with the standard deviation for each treatment.*



A



B

Figure 4. *Least Squares (LS) means analysis of the flakes and compost based on T. diversifolia amendments on the necrotic surface area (NSA) of PIF plantain for Condition (A); Treatment (B). Letters A, B and C represent the different statistical groups defined by the Tukey test (5%).*

The effects of *T. diversifolia* flakes and compost, precisely the effects of clams on the development of BSD were clearly and significantly differentiated between the four treatments and the controls. Four distinct statistical groups were obtained for the necrotic surface area (Figure 4B). Thirty-six (36) days after inoculation with *Mycosphaerella fijiensis*, the plant tissues were senescent as shown by the beginning of decrease in necrotic surface area, especially for the negative control (T-). Among the four treatments, the one that showed the best effect in terms of less necrotic surface development was mulch of *T. diversifolia* flakes and clams (Td+Cl_M), followed by amendment of *T. diversifolia* compost and clams (TCo+Cl_A) with a very light difference (Figure 3).

3.3. Effects of Flakes and Compost Based on T. diversifolia Amendments on PIF Seedlings Biomarker's Accumulation

Flakes and compost based on *T. diversifolia* amendments (Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A) were found to significantly ($P< 0.0001$) influence the accumulation of biomarker's notably the total chlorophyll, total soluble sugars, total phenolics, total free amino acids, total proteins, polyphenol oxidase (PPO), peroxidase (POX), ascorbic peroxidase (POX Asc.) and phenylalanine ammonia lyase (PAL) content in the leaves and roots of treated PIF seedling (Tables 2 and 3). The coefficient of determination (R^2) for all these variables was close to a 100% indicating that *T. diversifolia* flakes and compost treatments models were efficient (Tables 2 and 3). The most influential variable in the leaves was stage for total chlorophyll, total phenolics, PPO and PAL, while it was treatment for total soluble sugars, total free amino acids, total proteins, POX and POX Asc. (Figure 5). In the roots, the most influential variable was treatment for all the biomarker's (Figure 6).

Overall, in the leaves, there was a significant ($P< 0.0001$) difference between the sterilized substrate (SS) state and non-sterilized substrate (NSS) state PIF seedlings for total chlorophyll, total soluble sugars, total phenolics, PPO and PAL (Figure 7A). In the roots, this significant difference was observed between both condition for total soluble sugars and POX (Figure 7B). Two distinct statistical groups were obtained in the leaves and the roots of the PIF plantain seedlings (Figures 7A and B).

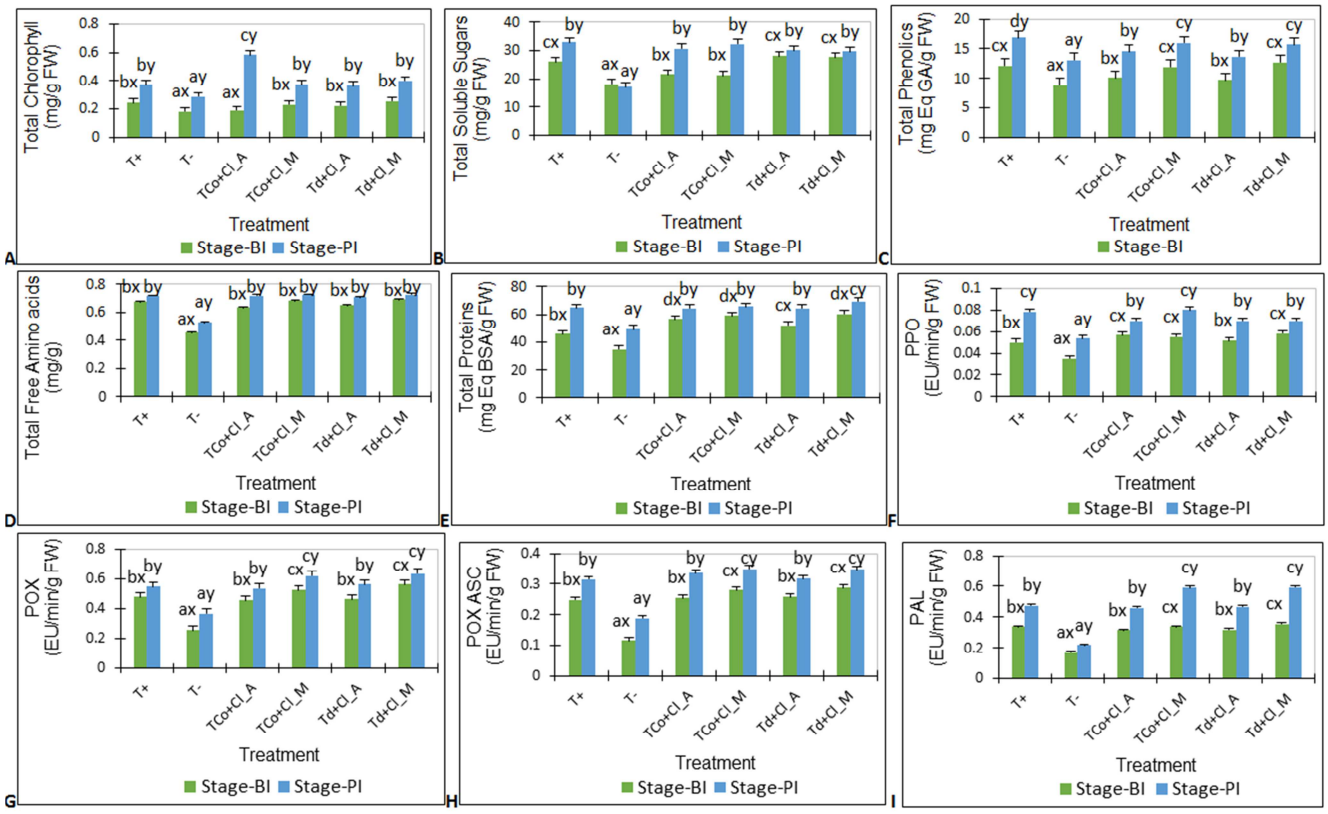


Figure 5. *Effects of flakes and compost based on T. diversifolia amendments on the accumulation of biomarker's (total chlorophyll, total soluble sugars, total phenolics, total proteins, peroxidase, peroxidase ascorbate, phenylalanine ammonia lyase, polyphenoloxidase and amino acids) in the leaves of PIF plantain seedlings before and post inoculation. Each point represents the average mean of three replicates with the standard deviation for each treatment. Letters a, b, c and d on the bars represent statistical groups defined by the Tukey test (5%) and letters x and y represent the statistical groups for the two stages.*

Accumulation of before-inoculation (BI) and post-inoculation (PI) biomarkers in leaves was consistently higher in treated seedlings than in non-treated seedlings (T-) (Figure 5). In the roots following post inoculation, there was a consistent increase in biomarkers of treated seedlings compared to non-treated (T-) one (Figure 6). In general, the

accumulation of biomarkers in seedlings treated as mulch (Td+Cl_M and TCo+Cl_M) was higher than in those treated as amendment (Td+Cl_A and TCo+Cl_A) both in the leaves and roots. This increase of biomarkers was more important for total phenolics, POX, POX Asc. and PAL in the leaves (Figures 5C, E, F and G), while it was more important for total soluble sugars, total phenolics, POX, POX Asc. and PAL in the roots (Figures 6A, B, D and E). However, the accumulation of biomarkers was also important in positive control seedlings treated with chemical fertilizer and fungicide (T+) compared to non-treated one (T-) both in the leaves and roots.

The four treatments (Td+Cl_M, Td+Cl_A, TCo+Cl_M and TCo+Cl_A) were statistically distinct from the controls both in the leaves and the roots. In the leaves, three distinct statistical groups were obtained for the PPO, PAL, POX Asc and total free amino acids, five for the POX and two for total proteins (Figure 8A). In the roots, two distinct statistical groups were obtained for the PAL, POX, PPO and total free amino acids, three for the total soluble sugars, total proteins and POX Asc. and four for the total phenolics (Figure 8B). Among the four treatments, those that showed the best effect in terms of biomarker's accumulation in the leaves and roots were the ones applied as mulch and both containing clams (Td+Cl_M and TCo+Cl_M) i.e. *T. diversifolia* flakes and clams as well as *T. diversifolia* compost and clams (Figures 8A and B).

Table 2. *Analysis of variance (ANOVA) of the flakes and compost based on T. diversifolia amendments on the biomarker's accumulation (total chlorophyll, total soluble sugars, total phenolics, total proteins, polyphenoloxidase, peroxidase, ascorbate peroxidase, phenylalanine ammonia lyase and total free amino acids) on the PIF plantain seedlings leaves at two stages (before inoculation and post inoculation).*

	Total Chlorophyll (mg/g of FW)	**Total Sugars (mg/g of FW)**	**Total Phenolics (mg Eq of GA/g of FW)**	**Total Amino acids (mg/g of FW)**	**Total Proteins (mg Eq of BSA/g of FW)**
R^2 (%)	95.3	95.6	87.3	99.5	93.6
F	37.207	39.600	12.691	371.453	27.071
P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Condition	26.255	219.588	32.752	0.233	0.382
	<0.0001	**<0.0001**	**<0.0001**	0.631	0.540
Treatment	23.627	62.370	14.384	1576.618	76.516
	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Repetition	3.272	0.475	3.150	1.271	0.101
	0.047	0.625	0.052	0.290	0.904
Stage	449.400	118.315	163.502	1154.434	232.998
	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Condition*Treatment	22.258	10.654	6.176	12.680	2.003
	<0.0001	**<0.0001**	**0.000**	**<0.0001**	0.096
Condition*Stage	38.386	72.804	0.004	0.026	3.425
	<0.0001	**<0.0001**	0.951	0.873	0.071
Treatment*Stage	27.173	17.606	0.623	22.678	6.256
	<0.0001	**<0.0001**	0.683	**<0.0001**	**0.000**
Condition*Treatment	8.860	25.040	1.760	13.841	3.179
*Stage	**<0.0001**	**<0.0001**	0.140	**<0.0001**	**0.015**

Table 2. *Continued.*

	PPO (EU/min/g of FW)	**POX (EU/min/g of FW)**	**POX ASC (EU/min/g of FW)**	**PAL (EU/min/g of FW)**
R^2 (%)	95.7	92.8	97.8	99.5
F	40.697	23.636	80.439	365.146
P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Condition	9.754	0.207	1.473	216.561
	0.003	0.652	0.231	**<0.0001**
Treatment	72.950	92.621	291.341	899.141
	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**
Repetition	0.120	0.260	2.519	0.571
	0.887	0.772	0.092	0.569
Stage	573.314	106.646	531.719	3429.706
	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**
Condition*Treatment	2.240	2.340	0.302	12.044
	0.066	0.056	0.909	**<0.0001**
Condition*Stage	1.084	0.827	3.844	253.361
	0.303	0.368	0.056	**<0.0001**
Treatment*Stage	10.910	0.582	1.306	121.578
	<0.0001	0.713	0.278	**<0.0001**
Condition*Treatment*Stage	0.506	0.996	0.831	12.814
	0.770	0.431	0.534	**<0.0001**

Values in bold correspond to tests where the null hypothesis is not accepted with a significance level alpha= 0.05. *F* is the value of F test and *P* is the probability.

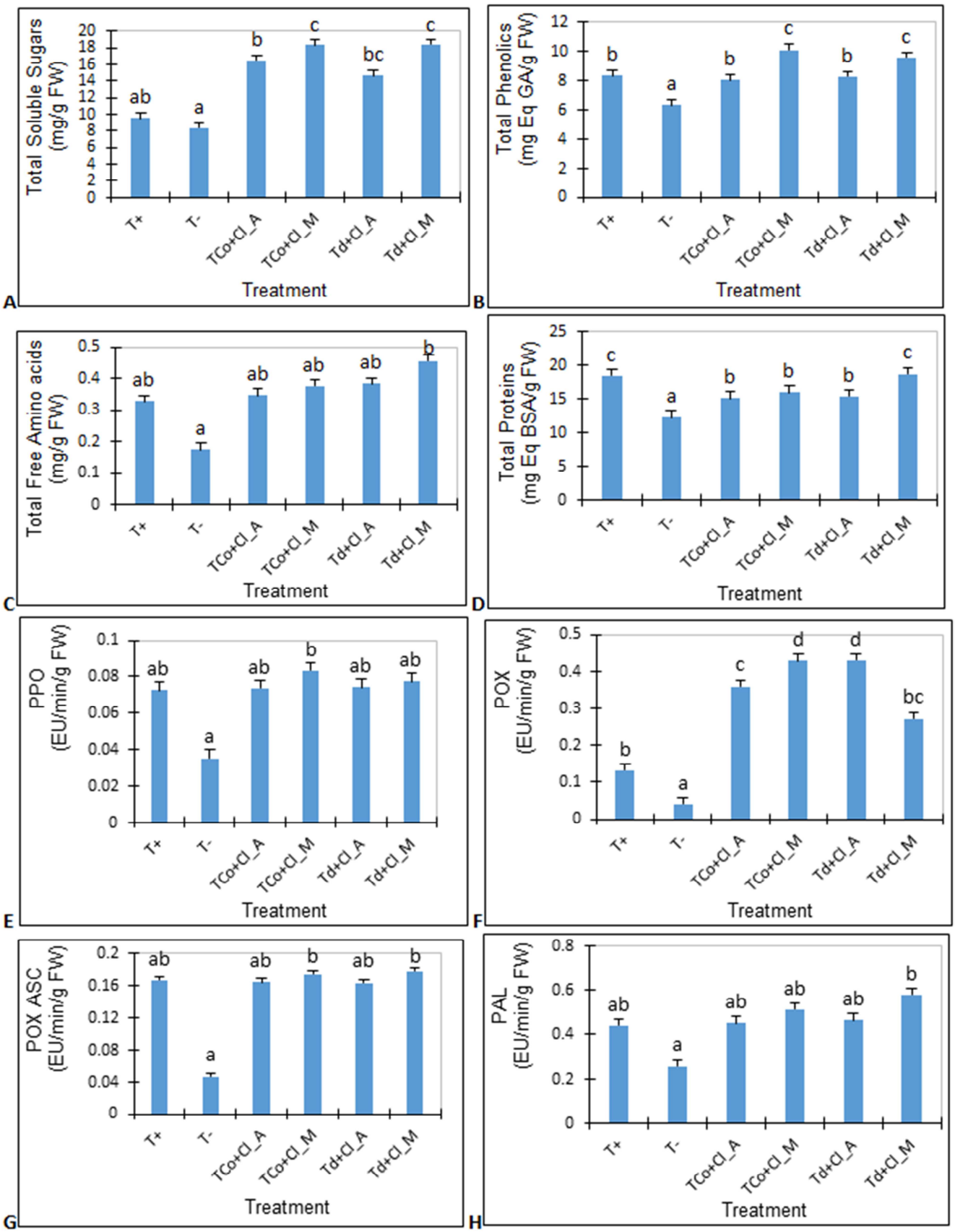


Figure 6. *Effects of flakes and compost based on T. diversifolia amendments on the accumulation of biomarker's (total soluble sugars, total phenolics, total proteins, peroxidase, peroxidase ascorbate, phenylalanine ammonia lyase, polyphenoloxidase and amino acids) in the roots of PIF plantain seedlings post inoculation. Each point represents the average mean of three replicates with the standard deviation for each treatment. Letters a, b, c and d on the bars represent the different statistical groups defined by the Tukey test (5%).*

Table 3. *Analysis of variance (ANOVA) of the flakes and compost based on T. diversifolia amendments on the biomarker's accumulation (total soluble sugars, total phenolics, total proteins, polyphenoloxidase, peroxidase, ascorbate peroxidase, phenylalanine ammonia lyase and total free amino acids) on the PIF plantain seedlings roots at one stage (post inoculation).*

	Total Sugars (mg/g FW)	**Total Phenolics (mg Eq of GA/g of FW)**	**Total Amino acids (mg/g of FW)**	**Total Proteins (mg Eq of BSA/g of FW)**
R^2 (%)	98.2	94.1	95.5	85.7
F	93.192	26.996	36.161	10.102
P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Condition	9.801	3.496	0.188	0.334
	0.005	0.075	0.669	0.569
Treatment	233.662	58.316	92.703	25.310
	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**
Repetition	0.777	0.115	0.117	2.078
	0.472	0.892	0.890	0.149
Condition*Treatment	6.366	11.129	1.231	0.056
	0.001	**<0.0001**	0.328	0.998

Table 3. *Continued.*

	PPO (EU/min/g of FW)	**POX (EU/min/g of FW)**	**POX ASC (EU/min/g of FW)**	**PAL (EU/min/g of FW)**
R^2 (%)	93.6	98.9	99.4	93.9
F	24.779	146.307	259.677	26.183
P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Condition	3.824	27.665	0.643	0.559
	0.063	**<0.0001**	0.431	0.463
Treatment	61.405	327.294	667.375	66.423
	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**
Repetition	1.117	0.779	5.442	0.382
	0.345	0.471	**0.012**	0.687
Condition*Treatment	1.810	47.260	5.481	1.388
	0.152	**<0.0001**	**0.002**	0.267

Values in bold correspond to tests where the null hypothesis is not accepted with a significance level alpha= 0.05. *F* is the value of F test and *P* is the probability

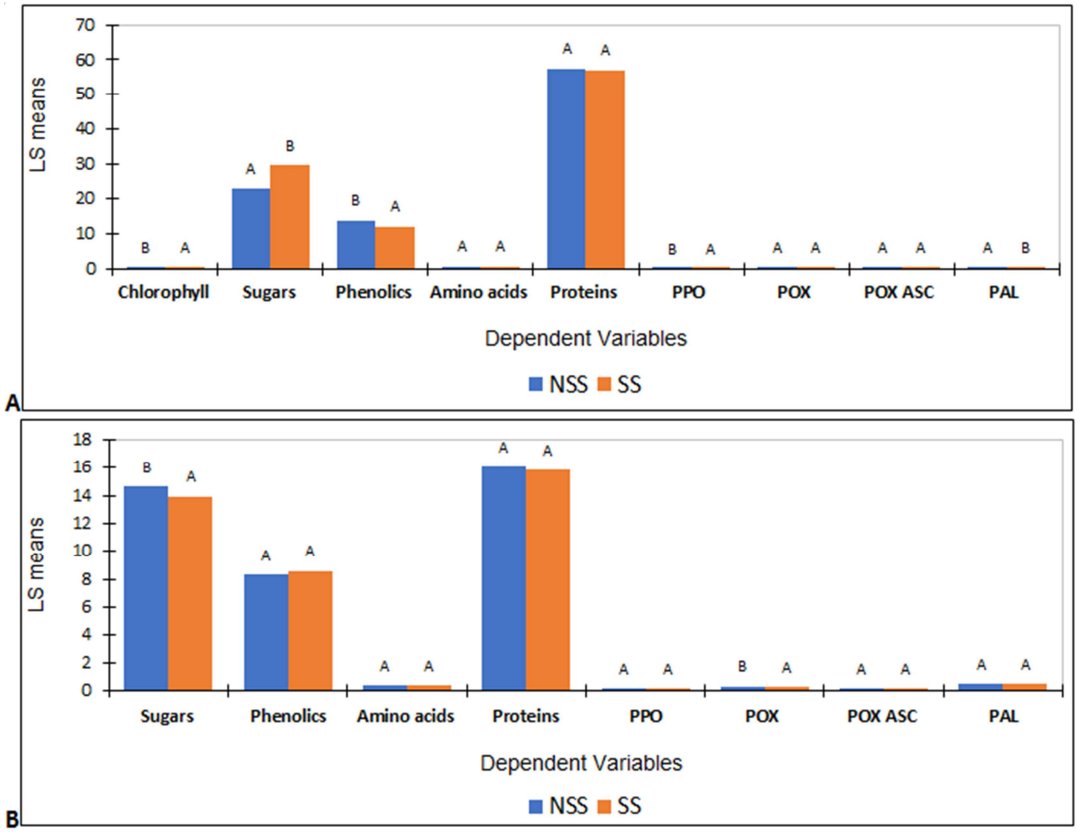


Figure 7. *Least Squares (LS) means analysis of the flakes and compost based on T. diversifolia amendments on biomarker's accumulation in the leaves (A) and in the roots (B) of PIF plantain seedlings for the sterilized and non-sterilized substrates conditions and regardless of the treatments as well as the inoculation stages. Letters A and B represent the different statistical groups defined by the Tukey test (5%).*

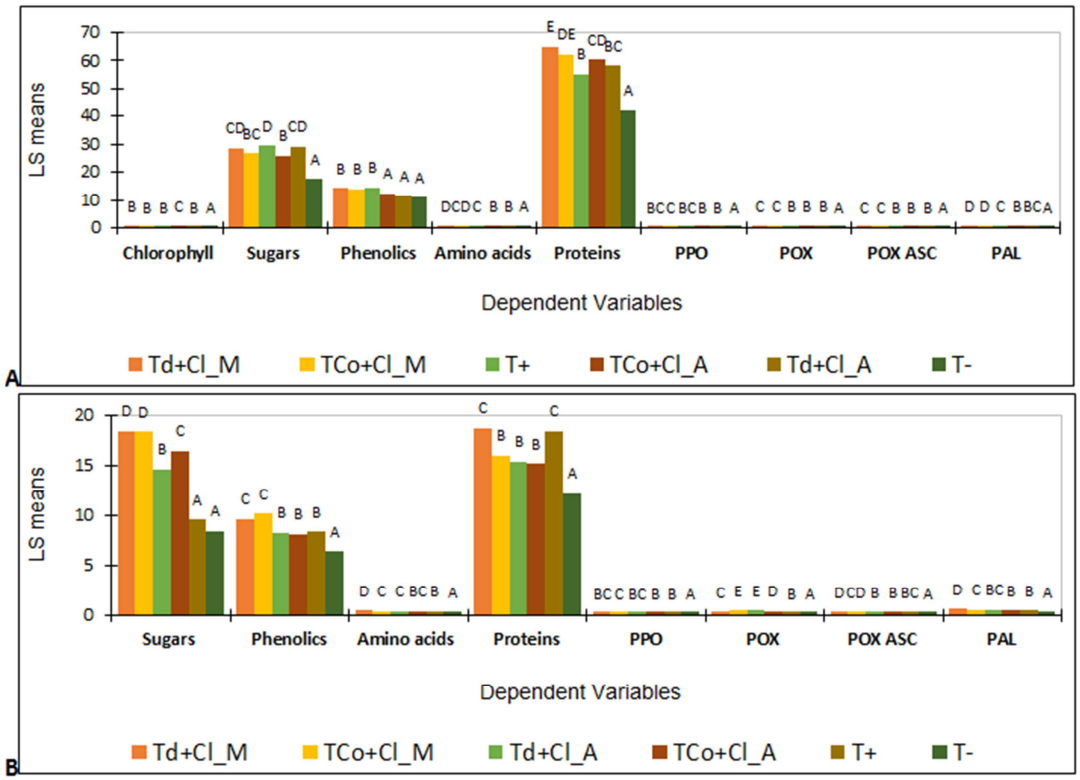


Figure 8. *Least Squares (LS) means analysis of the flakes and compost based on T. diversifolia amendments on biomarker's accumulation in the leaves (A) and in the roots (B) of PIF plantain seedlings for the treatments and regardless of the inoculation stages as well as the substrate conditions. Letters A, B, C, D and E represent the different statistical groups defined by the Tukey test (5%).*

4. Discussion

The aim of this research was to evaluate the stimulating effects of flakes and compost based on *T. diversifolia* amendments on the quality of PIF plantain seedlings growth and tolerance to *Mycosphaerella fijiensis* in nursery. The monitoring of the PIF seedlings which presented different morphological aspects while comparing the treated seedlings with the controls was possible thanks to the experimental device set up in the nursery. The kinetic evolution of vegetative stage parameters was significantly different between treated and non-treated seedlings with a more pronounced positive treatment effect on mulch treatments (Td+Cl_M, TCo+Cl_M). The predicted impact of *T. diversifolia* flakes and compost on PIF seedlings through significant growth promotion was confirmed by this research. This impact was observed on the treated seedlings, in particular for the mulch treatments which systematically increased all the vegetative growth parameters (height and diameter of the pseudo-stems, as well as the total leaf area) compared to the controls. Our results are consistent with previous studies that reported the same type of growth promotion for PIF plantain plants [7, 9, 3] and cocoa trees [20, 21].

Overall, *T. diversifolia* flakes and compost contain a high level of nutrients essential for plant growth such as nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg) as well as calcium (Ca), and could therefore be considered as a seedlings biostimulator. Indeed, the major component of *T. diversifolia* tissues is nitrogen, known as a constituent of chlorophyll and also involved in apical meristem cell division and enlargement [22]. Moreover, these essential nutrients (N, P, K, Mg and Ca) could be involved in improving the physical, chemical and biological properties of the soil involved in improving fertility, increasing microbial activity and optimizing plant nutrient uptake [22, 24].

Whatever the condition, seedlings treated with *T. diversifolia* flakes and compost show good growth parameters probably due to the strengthening of seedling pseudo-stems, increased photosynthesis rates and root stimulation of sowing. This positive effect could be explained by the presence of an element like phosphorus in the flakes and compost of *T. diversifolia* which is part of the molecular structure of the nucleic acid, functions in storage, accelerates the transfer processes of energy within the plant and improves root development [23, 25]. This result confirms the previous observations suggesting that certain natural products like *T. diversifolia* biomass [26], snail and oyster shells, compost and the combination of clam shells and *T. diversifolia* have a potential effect on the development of seedlings in the nursery [7, 9, 20, 10, 21].

The non-sterilized substrate (NSS) state showed a slightly slow growth rate of pseudo-stem height compared to the sterilized substrate (SS) state. Indeed, the nature of the substrate could make this slight difference, since the PIF seedlings are exposed to the microbiome present in the

substrate from the non-sterilized state during their vegetative growth phases in the nursery. A shortcoming of this research is that the microbiome of PIF seedlings has not been characterized.

T. diversifolia flakes and compost treatments positively and significantly influenced the development of BSD on PIF plantain seedlings with a lower level of *M. fijiensis* exhibited by the treated seedlings, especially Td+Cl_M and TCo+Cl_A. This level of sensitivity was lower than that obtained with a first study on clam shells [7] and almost the same for seedlings and substrate state treated with an earlier study on the combined effect of clam shells and *T. diversifolia* [9]. The lower level of BSD sensitivity in PIF seedlings from these treatments could be explained by the fact that after infection, the treatments appear to act as a plant vaccine that stimulates the plants to establish a mechanism to overcome the pathogen attack through the use of preformed and *de novo* synthesis of biochemical resistance markers.

T. diversifolia flakes and compost also positively and significantly influenced the accumulation of metabolites, particularly defense-related enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and ascorbic peroxidase. Indeed, it seems to promote natural defensive systems by increasing the synthesis of nutrients and defensive metabolites [7, 9, 27, 28]. The nitrogen and potassium present in the flakes and compost of *T. diversifolia* could explain this increase accumulation of biomarkers. On the one hand, the function of nitrogen is to constitute the protoplasm, to prepare macromolecules (amino acids, proteins, nucleic acids, nucleotides, hormones) and chlorophyll of the plant. Indeed, an increased production of chlorophyll is assimilated to a better growth of plants because it allows, thanks to photosynthesis, the production of the various basic molecules (sugars, proteins, etc.) [29]. On the other hand, potassium serves as an enzyme activator used by the plant to activate different enzymes [23, 30]. Our results are consistent with previous reports that showed the improved accumulation of nutrients and defensive metabolites including proteins and phenolic compounds as well as defense-related enzymes (peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, glucanase, chitinase …) [3, 7, 9, 18, 31, 32]. It has indeed been shown that resistance biomarkers are involved in the defense mechanism of banana tissues (leaves and roots) [31, 33, 34], as well as various other plant tissues [35, 36].

T. diversifolia flakes and compost treatments could act as an elicitor promoting plant immunization through the accumulation of response weapons such as defense-related enzymes prior to the attack of potential invaders [32]. Indeed, peroxidase is expressed to limit cellular spread of infection by establishing structural barriers or generating highly toxic environments [37], polyphenol oxidase plays a role in defense against plant pathogens [38], by the immediate synthesis of antimicrobials weapons while phenylalanine ammonia lyase is involved in the phenylpropanoid pathway (polyphenols synthesis). In addition, chitinase and β-(1, 3)-glucanase are plants direct defense enzyme capable of attacking cell wall of fungal pathogens by degrading the cell wall of the pathogen [32]. Our results are in agreement with a previous study which clearly demonstrated that phenolic secondary metabolites (phenols and lignin) play an important role in the defense mechanisms of *Musa* to *Radopholus similis* infection [31, 33, 39].

In addition, phenolic acids are involved in pathogen resistance through the accumulation of phytoalexin, the biosynthesis of lignin and the formation of structural barrier [40]. These metabolites seem to act to inhibit fungal growth due to biotic stress, but also to any other source of stress 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 [31, 32, 41]. This accumulation was greater for the treated seedlings probably because after inoculation, the plant has put in place a mechanism to overcome the pathogenic attack through the use of preformed and *de novo* synthesis of biochemical markers [35, 36, 41, 42].

5. Conclusion

The aim of this research was to evaluate the stimulatory effects of *T. diversifolia* flakes and compost on the quality of PIF plantain seedlings growth and tolerance to *Mycosphaerella fijiensis* in nursery. Our results have highlighted new effects of *T. diversifolia* flakes and compost on the quality of plantain PIF seedlings. Indeed, this study is the first reporting the stimulatory effects of *T. diversifolia* flakes and compost on the quality of plantain PIF seedlings in the nursery. However, the molecular mechanism involved in promoting growth and decreasing susceptibility to BSD severity has not yet been assessed.

The use of *T. diversifolia* flakes and compost should be considered by poor peasant farmers and nurseries when producing PIF seedlings to provide the population with good quality seedlings and easy-to-follow good cultural practices essential in an eco-agriculture approach. It will also be important to continue this research on *T. diversifolia* flakes and compost effects on the farm, as they appear to be a tool for a more sustainable and resilient agriculture. The use of *T. diversifolia* flakes and compost formulations in seedlings production could be an alternative tool for poverty alleviation and less chemical inputs in the sub-Saharan countries for smallholder farmers.

Conflicts of Interest

All the authors do not have any possible conflicts of interest.

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