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Enhancement of *Theobroma cacao* Seedling Growth and Tolerance to *Phytophthora megakarya* by Heat-Treated Oyster Shell Powder

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Abstract

The aim of this study was to evaluate the ability of oyster shell powder soil amendment to enhance cocoa seedling growth and induce resistance against *Phytophthora megakarya* in nurseries. The results showed that heat-treated oyster shells powder at 1% (w/w) soil amendment significantly increased plant height, leaf number, leaf area, dry shoot and root weight more than chemical fungicide and control treatment after twelve weeks of growth. The results showed that heat-treated oyster shell powder raised soil pH significantly and reduced *P. megakarya* load of the soil suspension by 82%. Assessment of resistance stimulation by leaf inoculation showed the highest level of resistance recorded in plants treated either with heat-treated or non-treated oyster shell powder. Furthermore, total phenolic compounds contents, total soluble proteins contents, polyphenoloxidase, chitinase, peroxidase and β-1,3-glucanases activities increased in both healthy or infected leaves from cacao plants treated with oyster shell powder more than those treated with chemical fungicide. These findings demonstrated that heat-treated oyster shell powder could be used as biofertilizer and biofungicide to improve the quality of cocoa seedling production and protect the plant against *P. megakarya*.

Keywords

Cocoa, Seedling, *Phytophthora megakarya*, Oyster Shell, Biofungicide

1. Introduction

Cacao (*Theobroma cacao* L.), is an important economic crop in numerous developing countries. Cameroon is the fifth largest world cacao producer and its production represents about 30% of non-oil exports and generates revenue of over €152 million per year to more than 600,000 producers [1]. In Cameroon, cacao seedling and beans constitute an important source of revenue for many people. However, its cultivation is faced with numerous problems such as unavailability or insufficient healthy seedlings and parasitic constraints principally black pod disease. In Cameroon Black Pod Disease (BPD) is caused by *P. megakarya* [2].

Many *Phytophthora* species such as *P. megakarya* have a soil-borne phase in their natural life cycles even though disease expression often occurs on aerial plant parts such as cocoa seedling black pod disease [3]. Cocoa seedling production is a key step in the establishment of new cocoa plantations and generally requires forest soil as the production substrate. This soil used to produce the cocoa seedling is usually taken from areas already contaminated by *P. megakarya* [4]. In addition, the Cameroon forest soil generally has a pH of 5 to 6.5 favourable to fungal mycelia growth [5]. Primary inoculum of *P. megakarya* is the soil through which they act as vectors of infection of pods and young cocoa seedlings in nursery [3] [4]. In order to prevent this situation different strategies have been developed. The use of chemical products such as mancozeb or metalaxyl fungicides through soil applications has been reported to provide 50% of cocoa plant protection in some cases [5] [6]. However, soil porosity can influence water transport and thus fungicide movement [7]. Furthermore, the use of synthetic fungicide may be harmful to the environment and its repeated use could result in resistance in the pathogen population and its toxic residue could be accumulated in the plants. Biological control of soil, possible vector of fungal pathogens, is poorly investigated [5].

In this context, biological control using organic substances such as composts, snail shells and oyster shells constitute an alternative method with high efficiency and eco-friendly. Oyster shell is the waste oyster product and it main constituent are calcium carbonate and chitin [8] [9] [10]. Fresh or meal oysters shell is a very good liming material through increasing the soil pH and the modification of the biological properties of soil [11]. The sodium chloride concentration in oyster shell can be decreased by heating and composting. Xing *et al.*, 2013 [12] have reported the capacity of heat-treated oyster shell at 500 ppm to exhibit antifungal activities against plant pathogen more than non-treated oyster shell at 25,000 ppm. Moreover, heat-treated oyster shell has been widely used as liming material, growth stimulator and yield enhancer in many crops such as in soybean [13] and cabbage [14]. Recent studies have reported that oyster shell powder soil amendment reduces the occurrence of tobacco bacterial wilt in fields [15].

This investigation was carried out to assess the effects of the suppressive potential of oyster shell powder amendment and the capacity of this product to

enhance cocoa seedling growth by evaluation of plant agro-morphological characteristics, total phenolic compounds and peroxidase, chitinase, polyphenol oxidases, and β-1,3-glucanase activities.

2. Material and Methods

2.1. Soil

The soil used in this experiment was collected from Yaounde (Centre region, Cameroon) and is often used by farmers to sow young cocoa seedlings. The soil was air-dried and passed through a 4 mm sieve before mixing (3:1; v/v) with river sand. Chemical analysis (organic matter, nitrogen, calcium, magnesium, phosphorus contents and pH) of dry soil samples was carried out before the cultivation period. The contents of available nutrients in the soil were: organic matter, 3.40%; nitrogen, 1.23%; calcium, 6.48×10^{-3} meq·g^{-1} of soil; magnesium, 23.20×10^{-3} meq·g^{-1} of soil; phosphorus, 3.54 meq·g^{-1} of soil; and pH was 5.89.

2.2. Fungal Strains and Oysters Shell Powder Production

P. megakarya (strain PM5) used in this study was obtained from infected cacao pod from Yaounde (Central Region, Cameroon). Zoospore suspensions of *P. megakarya* isolate PM5 were obtained according to [16]. Oyster shell powder was obtained following the method of Xing *et al.*, 2013 [12]. The oyster shells from Mouanko (Littoral Region, Cameroon) were thoroughly washed using tap water and air-dried. They were then heated at 400˚C for 5 hours to facilitate the grinding. After this, they were ground in a grinding machine (MS 20B grinding machine), and then sieved using a 0.8 mm sieve to obtain the finest powder. The powder was separated in two parts, and then one part was heated in an oven at 1000˚C for 1h according to Xing *et al.*, 2013 [12]. Mancoxyl Plus 720 wp (with active compound mancozeb and metalaxyl) fungicide was purchased at the local market in Yaounde.

2.3. Evaluation of Agro-Morphological and Physiological Characters

The agro-morphological characters that were assessed include the dried weight of the plant root and shoot weight, height, leaf number, length, width and area of the leaf. These parameters were assessed every 4 weeks for a period of 12 weeks. To produce the plants, a single cocoa seed from mature cocoa pods (♀SNK64 × ♂UPA134) hybrids produced by manual pollination were collected from the SODECAO ("*Société de Développement du Cacao*") gene banks of Mengang Station (South Region, Cameroon). Cocoa seeds were extracted from the pods, washed with tap water. Seeds of similar weight and size were grouped and sown into each plastic pot, which contained treated and untreated soil. Each treatment was in duplicates of two hundred pots. All the pots were kept in the shade house (farmer condition seedling production) and watered with distilled water every two days for a period of 12 weeks. During assessment at the 4th week interval,

roots of harvested plants were washed to remove soil particles and plant height measured with a Vernier caliper. Length and width of leaves measured with a graduated ruler and the weight of shoots and roots of freshly harvested plants then measured separately. The experiment was a completely randomized design with four treatments of non-treated oyster shell powder at 5% w/w (S+OS), heat-treated oyster shell powder at 1% w/w (S+hOS) [12], chemical treatments (S+F) according to the SODECAO cocoa seedling production standard operation procedure and the control (C) (treatments without oyster shell formulation and chemical fungicide). Each treatment consisting of three replicates were repeated twice.

2.4. Induced Resistance Assessment

The tolerance of young cocoa plant seedling was performed as described by [17] with modification. Briefly, leaves from two-month-old cocoa plant were washed thoroughly with distilled water and sterilized with ethanol (70%) for 30 s. The leaf test was performed by deposition on abaxial surface of leaf, a 6 mm mycelium disc from pure culture of *P. megakarya* obtained after 7 days pure culture grown in PDA medium. The inoculated leaves were incubated in humid chamber at 25˚C ± 1˚C in total darkness. Control leaves were inoculated with sterile agar disc in the same conditions. The experimental design consisted of three replications of ten leaves per seedling. Disease expression was rated six days after, using the rating scale developed by [18]. This experiment was repeated twice, and the disease severity was determined for each treatment by calculating the ratio of the sum of individual scores to the total number of leaves used. The disease severity index used to express the resistance level [19] was as follows: Highly Resistant (HR: $0 < \text{index} \leq 1$); Resistant (R: $1 < \text{index} \leq 2$); Moderately Resistant (MR: $2 < \text{index} \leq 2.5$); Susceptible (S: $2.5 < \text{index} \leq 3.5$); and Highly Susceptible (HS: $3.5 < \text{index} \leq 5$).

2.5. Biochemical Analyses

Biochemical analyses were carried out following the assessment of infection on the whole leaves. The samples involved were cut at 1 cm beyond the necrosis point or beyond the marked scar. Samples from the same treatments were combined. The parts of the leaves from each treatment were combined. For biochemical analyses, each treatment was repeated twice.

2.6. Determination of the Content of Total Phenolic Compounds

The extraction and quantitative measurement of the content of total phenolic compounds were performed as described by [20] with modification. Total phenolic compounds were extracted twice using 80% methanol. One gram of fresh leaves was ground in 10 ml of 80% methanol at 4˚C. After 5 min of agitation, the ground material was centrifuged at 10,000 g for 5 min at 4˚C. The supernatant was collected, and the pellet was re-suspended in 5 ml of 80% methanol followed

by agitation for 5 min. After the second centrifugation at 4˚C, the supernatant was collected and mixed with the previously collected supernatant to constitute the phenolic extract. The concentration of phenolic compounds was determined spectrophotometrically at 725 nm according to the method of [21], using the Folin-Ciocalteu reagent. Total phenolic compound contents were expressed in mg equivalent of catechin per g of fresh weight.

2.7. Determination of the Content of Total Protein

For the determination of total native protein content, extraction was performed as described by [22]. One g of fresh tissue of inoculated and healthy leaves was ground separately in 10 ml of extraction buffer (Tris-HCl 10 mM pH 7.5, Triton X-100 1%) at 4˚C, stirred for 10 min and kept on ice. The samples were sonicated (8 pulses of 3 s each with 10 s intervals) with the setting at 70% output on an Ultrasonic processor (Gex 130, 130 W), and then centrifuged at 10,000 g for 25 min at 4˚C. The pellet was submitted to a second extraction. Both supernatants were mixed with 0.4 volume of n-butanol and 1/10 of 3 M NaAc pH 4.5. The samples were kept on ice for 30 min with agitation every 10 min, and then centrifuged at 10,000 g for 15 min at 4˚C. The supernatant containing total proteins was stored at 4˚C. The proteins were quantified using the [23] method. One ml of Bradford reagent was added to each ml of extract. The absorbance was measured at 595 nm using a UV-VIS 1605 Shimadzu spectrophotometer. BSA was used as the standard.

2.8. Determination of Enzymes Activities

Peroxidase activity was determined in the total native protein extracts according to the method of [24]. The enzyme activity was expressed in enzyme unit per g of fresh weight using spectrophotometry at 470 nm (A_{470}/min (EU)/g fresh weight). Polyphenoloxidase (PPO) activity was quantified in the total native protein extract as described by [15], using catechol as a substrate. The enzyme activity was expressed as "A_{330} nm/min (EU)/g fresh weight". β-1,3-glucanases activity was determined according to the protocol of [25] using laminarin as substrate. The amount of reducing sugars released was calculated from a standard curve prepared with glucose and the glucanase activity was expressed in µmol glucose equivalent/min (EU)/g fresh weight. The chitinase activity was determined by colorimetric assay according to the method of [26] using colloidal chitin as substrate. Chitinase activity is described by unit/g fresh matter/h. One-unit chitinase activity corresponds to an increased absorbance of 0.1 at 500 nm.

2.9. Evaluation of the Level of *P. megakarya* Inoculum Load in the Soil

The evaluation of the suppressive effect of heat-treated and non-treated oyster shell powder in the soil *P. megakarya* load after 12 weeks was done by infecting healthy cocoa pods with some suspension of the soil as described by [22] with

modification. 3-month-old healthy pods (SNK10, susceptible clone) were harvested, washed with tap water, sterilized with 70% ethanol (for 1 min), 10% (v/v) commercial sodium hypochlorite (for 5 min) and rinsed 3 times with sterilized distilled water. The inoculation was carried out by the deposition of 500 µl suspensions of untreated and treated soils collected after 12 weeks of experiment on the scar obtained with hand utensils. The scars are then closed with cotton that has been immersed in sterilized water. The soil suspension was obtained by mixing soil with sterilized distilled water. That is, 2 g of soil sample was mixed with 10 ml of sterilized distilled water, shaken and allowed to stand for 10 min. A control constituted of pods inoculated with only sterilized distilled water was realized. The entire inoculated pods were incubated in a dark room at 25˚C ± 1˚C in a humid chamber. The level of necrosis was quantitatively evaluated every two days for 12 days by measuring the necrosis length.

2.10. Statistical Analysis

Data analysis was performed using the Statistics software version 9.0. All results were expressed as means ± standard deviation and subjected to Analysis of Variance (ANOVA). Where significant differences were found, pairs of samples were compared by Tukey's test at $p \leq 0.05$.

3. Results

3.1. Agro-Morphological Characteristics

Twelve weeks after planting, plant height, leaf number, leaf area, root and shoot dry matter were variably affected (Table 1). Heat-treated (S+hOS) and non-treated oyster shell (S+OS) powder soil amendment significantly increased leaf number and plant height, compared to the chemical (S+F) and control (C) treatments (Table 1). The plant height was high in heat-treated oyster shell treatment compared to non-treated oyster shell powder treatment with 28 ± 1.1 and 24.96 ± 0.84 cm respectively. The difference in the mean of leaves of cocoa seedlings grown in sol treated with oyster shell powder was statistically significant between that of chemical and control (non-sterilized soil treatment). The leaf area was significantly different from non-treated treatment compared to treated treatment (Table 1). Plant dry matter increased in the presence of oyster shell powder (S+hOS and S+OS). The shoot dry matter was 9.44 ± 0.43 g/plant, 7.67 ± 1.03 g/plant, respectively for heat-treated oyster shell (S+hOS) and non-treated oyster shell (S+OS) treatment. This organic matter also increased root dry matter by 6.89 ± 0.41 and 4.15 ± 0.64 g/plant respectively for heat-treated oyster shell (S+hOS) and non-treated oyster shell (S+OS) treatment. Moreover, smaller changes in the agro-morphological characters were recorded for non-treated cocoa seedling.

3.2. Leaf Inoculation and Infection Intensity Indexing

Six days after leaf inoculation, necrotic lesions were observed on all the leaves inoculated with mycelium disc from pure culture of *P. megakarya* whilst no

Table 1. Effect of non-treated and heat-treated oyster shell powder on agro-morphological characteristics of cocoa seedling after twelve weeks of growth.

Treatment	Number of leaf/plant	Leaf area (cm^2)	Plant height (cm)	Dry shoot weight (g/plant)	Dry root weight (g/plant)
Control	5 ± 0.52^{c}	31.81 ± 0.28^{b}	15 ± 0.11^{d}	2.42 ± 0.50^{b}	1.18 ± 0.66^{c}
S+F	7 ± 0.52^{b}	36.96 ± 0.56^{b}	19.4 ± 0.93^{c}	4.12 ± 0.67^{b}	1.44 ± 0.45^{c}
S+OS	9 ± 0.51^{a}	42 ± 0.58^{ab}	24.96 ± 0.84^{b}	7.67 ± 1.03^{a}	4.15 ± 0.64^{b}
S+hOS	10 ± 0.51^{a}	64.81 ± 5.1^{a}	28 ± 1.1^{a}	9.44 ± 0.43^{a}	6.89 ± 0.41^{a}

Each treatment consisting of two hundred replicates was repeated twice. Means with the same letter within a column are not significantly different at $p < 0.05$. Control: none-sterilize Soil, S+F: Soil + fungicide, S+OS: non-sterilize Soil + non-treated oyster, S+hOS: shell non-sterilize Soil + heat-treated oyster shell.

symptom was seen on leaves inoculated with sterile agar disc. Analyses of variance showed that disease expression was significantly different among treatments ($p < 0.05$) (Figure 1). The highest level of disease severity index (lowest level of resistance) was observed with treatments without oyster shell powder and chemical treatment; these plants were therefore classified as highly susceptible (Figure 1). The lowest disease symptom was recorded in plants treated with both non-treated oyster shell powder and heat-treated oyster shell powder, showing a disease severity index of 1.0 for heat-treated oyster shell powder treatment and 1.4 for non-treated oyster shell powder. These plants were classified as resistant ($1 < \text{index} < 2$) (Figure 1). As far as, the chemical treatment showed the lowest disease severity index with 2.33, then these plants were classified as moderately resistant ($2 < \text{index} < 2.5$).

3.3. Phenols and Proteins Contents

The total phenolic compounds content in non-inoculated plants was lower than the inoculated ones. The inoculations of leaves had a significant effect on total phenolic contents in all the treatments. The treatment of plants with heat-treated and non-treated oyster shell powder before and after inoculation showed higher level of phenolic compounds than in the control plants (non-sterilized soil and chemical treatment). Heat-treated oyster shell powder treatment had a more significant effect as compared to chemical treatment (Figure 2). Heat-treated oyster shell powder showed an increase of phenolic compound after inoculation, with an increase of 53.43% compared to 25.57% of chemical treatment.

The amount of proteins was lower in the plants grown in chemical and non-sterilize soil treatments before and after inoculation. The treatment with heat-treated and non-treated oyster shell powder increased the protein level in healthy and inoculated plants (Figure 3). The inoculation induced a significant accumulation of proteins in the cocoa plants treated with oyster shell powder with an average of 7.9 mg Equivalent of BSA/g fresh weight, and this amount significantly rose to 28% in all the plant leaves after inoculation. The protein accumulation after inoculation was higher in plants treated with heat-treated oyster shell than those treated with chemical fungicide (Figure 3).

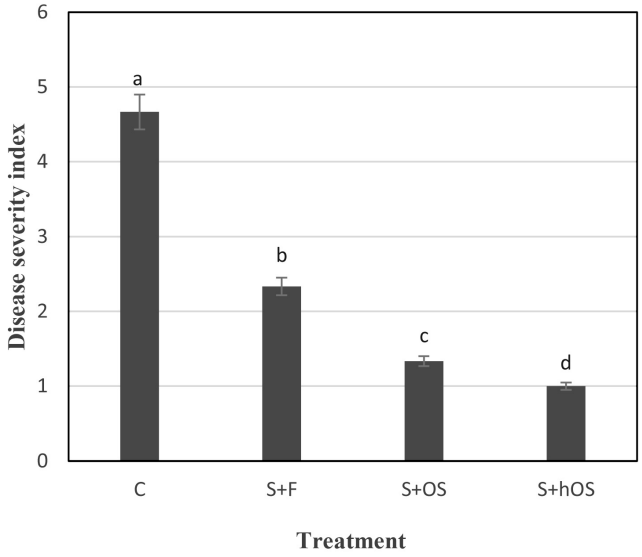

Figure 1. Disease severity of plants treated and non-treated with oyster shell powder six days after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

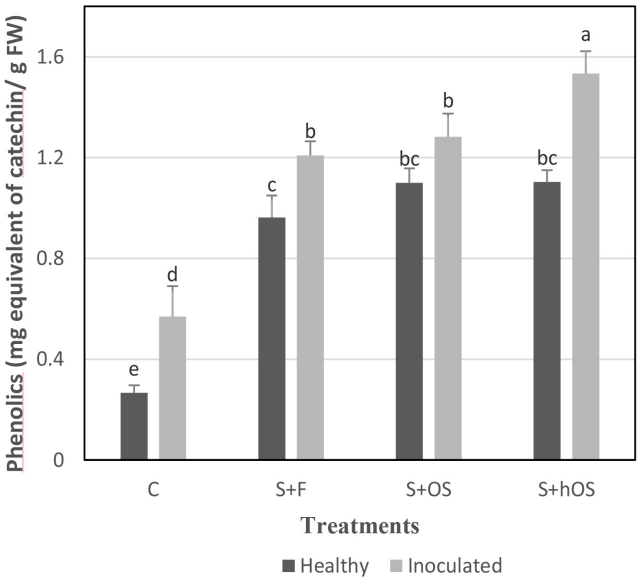

Figure 2. Variation of total phenolic content in plant treated and non-treated with oyster shell powder before and after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

3.4. Enzymatic Activities

The peroxidase (POX) and polyphenoloxidase (PPO) activities in the protein extract varies in function of the health status of the plant (**Figure 4** and **Figure 5**). The peroxidase accumulation was higher in the plant treated with oyster shell

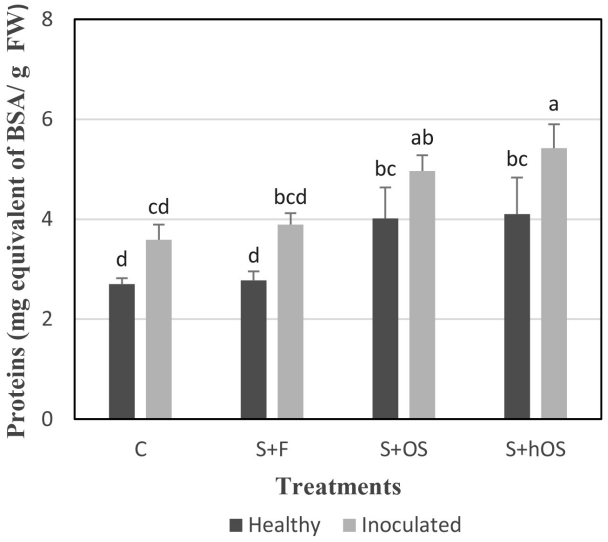

Figure 3. Variation of total proteins content in plant treated and untreated with oyster shell powder before and after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

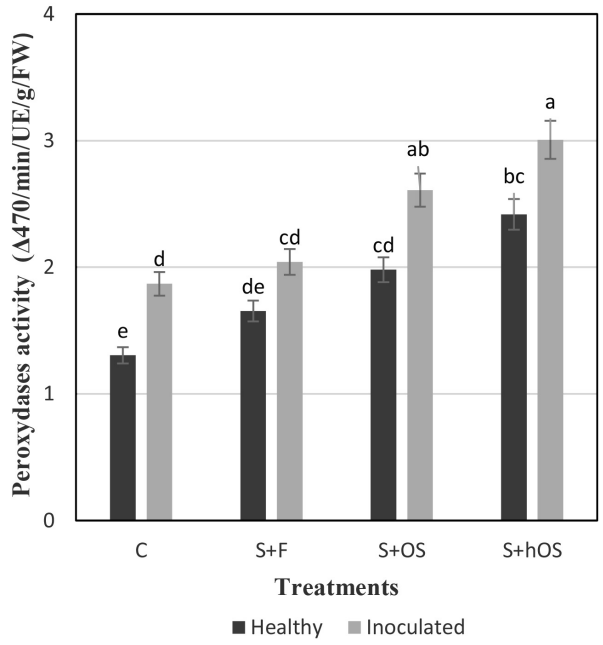

Figure 4. Variation of total peroxidases activities in plant treated and untreated with oyster shell powder before and after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

powder with average of 2.81 UE/g of fresh weight compare to the chemical treatment, and this amount significantly rose to 40 % in all oyster shell treatment after inoculation (S+OS and S+hOS). Furthermore, there was a significant

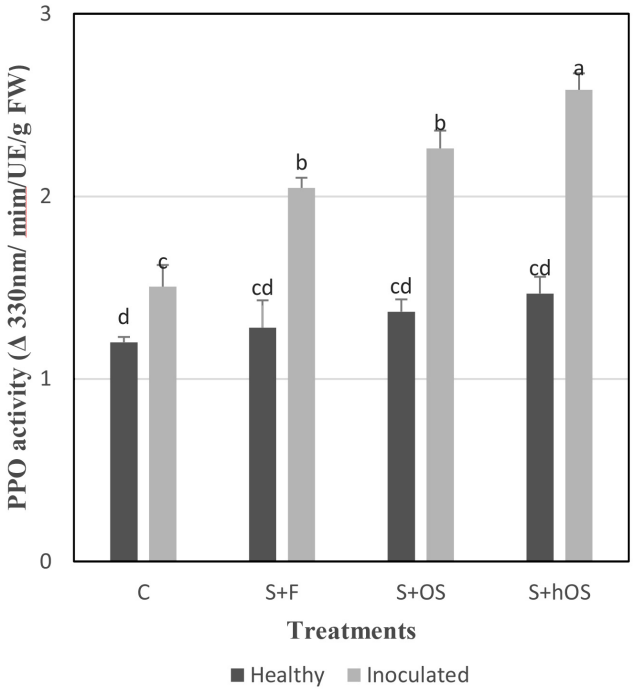

Figure 5. Variation of total polyphenoloxidases activities in plant treated and untreated with oyster shell powder before and after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: Control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

difference in peroxidase accumulation between the leaves of plant treated with chemical fungicide and those without any treatment (**Figure 4**). The activity of polyphenoloxidase was much higher in plant grown in soil treated with heat-treated oyster shell powder after infection compare to the other treatments (**Figure 5**). The polyphenoloxidase accumulation level was increased in leaves from plant treated with heat-treated oyster shell powder to 74% after inoculation compared to the chemical treatment which increased to 59%. There was no significant difference in the polyphenoloxidase level between the non-treated oyster shell powder and heat-treated oyster shell powder treatment before infection (**Figure 5**).

The β-1,3-glucanase and chitinase activity of plant leaves grown in heated oyster shell powder soil treatment before and after inoculation was significantly different from that of the treatments C and S+F respectively (**Figure 6** and **Figure 7**). In plants treated with oyster shell powder, the chitinase was higher in the non-inoculated plants with an average of 7.76 UE/g of fresh weight, and this amount significantly increases by 80% in all plant leaves after inoculation (S+OS and S+hOS). Therefore, there was a significant difference in chitinase accumulation after inoculation between the leaves of plants treated with chemical fungicide and those without any treatment (**Figure 6**). The β-1,3-glucanase accumulation level is highest in leaves from plant treated with heat-treated oyster shell

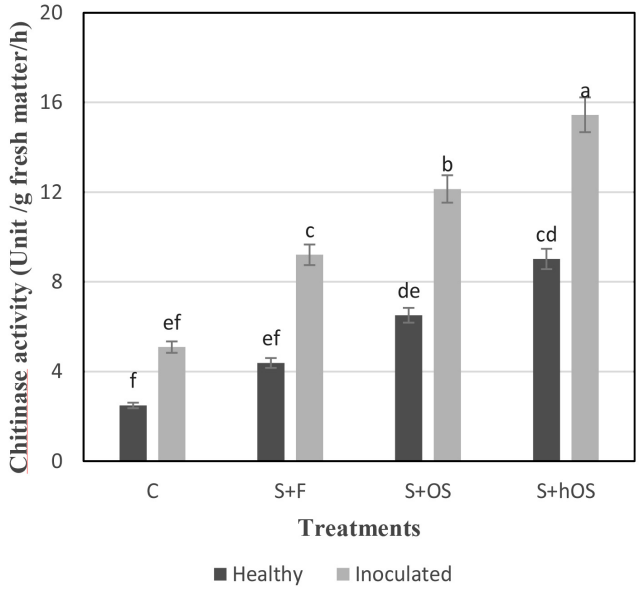

Figure 6. Variation of total chitinases activities in plant treated and untreated with oyster shell powder before and after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

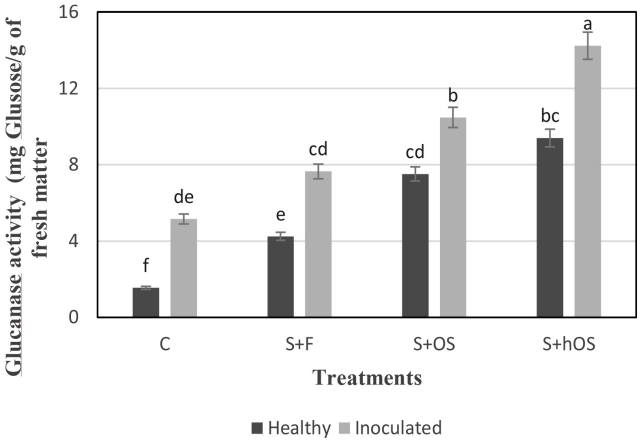

Figure 7. Variation of total β-1,3-glucanases activities in plant treated and untreated with oyster shell powder before and after inoculation. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

powder treatment before and after inoculation as compare to non-treated oyster shell powder and chemical treatment. In effect, the inoculation enhances the β-1,3-glucanase accumulation level to 51 % in S+hOS treatment and 39 % in S+OS treatment. Furthermore, there was a significant difference in β-1,3-glucanase accumulation between the leaves from plants treated with chemical fungicide and those without any treatment (Figure 7).

3.5. Evaluation of *P. megakarya* Soil Inoculum Load and Soil pH

The results obtained after 12 days of inoculation of cacao pods with soil suspension from various batches showed a weak degree of necrosis on pods inoculated with soil suspension treated with heat-treated and non-treated oyster shell powder. The cocoa pods inoculated with non-treated soil suspension presented greater levels of necrosis (Figure 8). There was absence of necrosis in treatments with heat-treated and non-treated oyster shell powder for the first four days, whereas by the second day, the control (C) had developed necrotic lengths of 0.8 cm. By the 12th day, necrotic lengths had been developed in all treatments at different levels (Figure 8). Necrotic levels in sample treated with chemical fungicide were a bit higher than with a sample treated with both heat-treated oyster shell and non-treated oyster shell powder by a difference factor of 2.8. Generally, adding oyster shell powder in soil reduced the fungal load averagely by 82 % as compared to non-sterilize soil treatment while addition of chemical fungicide reduced fungal load by 40% on average.

The original pH of the soil determined before treatment and planting of the plant was gotten as 5.89; close to the acidic pH. After the substrate was treated with oyster shell and the plant grown for 12 weeks, the pH increased in every treatment at different rates (Figure 9). The pH of substrate treated with oyster shell increased more significantly than in the rest of the treatments with an average percentage of 32%.

4. Discussion

The results from nursery pot experiments showed that the heat-treated oyster shell powder significantly increased the growth parameters of cocoa seedling more than non-treated oyster shell powder and chemical fungicide treatment. This finding agrees with [13] report, who found that heat-treated oyster shell

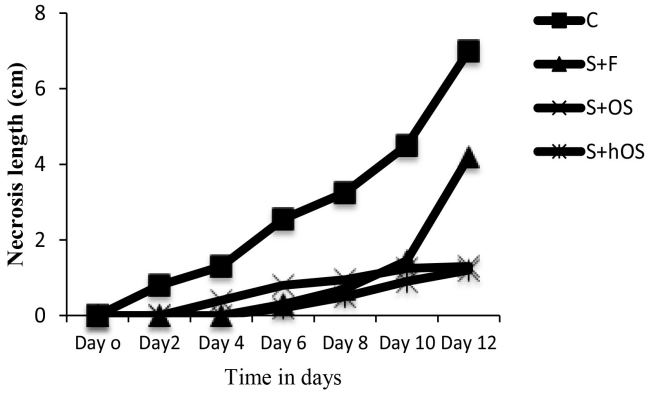

Figure 8. Variation of necrosis length on cocoa pod inoculated with soil treated and untreated with oyster shell powder after twelve weeks of growth. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

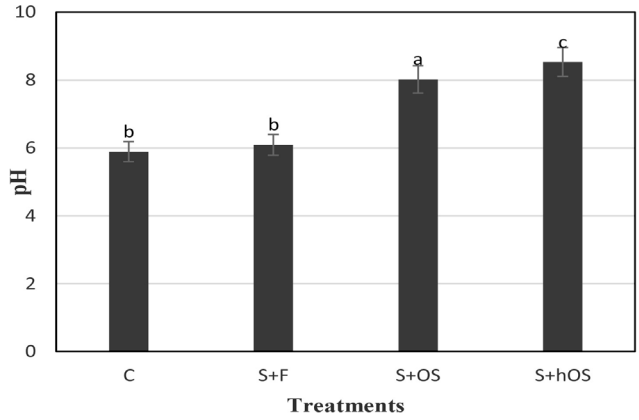

Figure 9. Variation of soil pH in soil after twelve weeks of growth. Each treatment consisting in three replicates were repeated twice. Means with the same letter are not significantly different at $p < 0.05$. C: control, S+F: chemical treatments with fungicides, S+OS: non-treated oyster shell, S+hOS: heat-treated oyster shell.

powder increased root and shoot fresh weight in soybean and cabbage respectively more than non-treated oyster shell powder. The plant growth promoting effects of heat-treated oyster shell powder observed in this study can be due to their chemical composition. In effect, many reports have shown that the heat-treated oyster shell powder is mainly composed of calcium oxide and chitin which have the properties to stimulate plant growth [12] [27]. However, [4] showed that the addition of heat-treated oyster shell powder in soil increased the organic matter available, exchange cation concentrations and soil pH. Recent study has shown that the increased of the morphophysiological parameters translates an improvement in the health status of the plant and the healthier sanitary condition of nursery soil [22] [28].

The low level of necrosis observed in pods inoculated with soil samples treated with non-treated and heat-treated oyster shell powder after twelve days of inoculations could be related to the reduction of the *P. megakarya* load in soil. This decrease could be due to modification of soil microbial flora and nutrients which lead to the healthier condition observed in plant treated with heat-treated oyster shell powder compared to the control plants. This result agrees with [29] who found that oyster shell powder soil amendment of tobacco field decrease tobacco bacterial wilt incidence by 43.33% and modify soil bacteria flora such as actinobacteria. Furthermore, higher pH and higher calcium concentration are important for plant disease control [13]. Our result showed that soil samples treated with oyster shell increased in pH by 32%. These results corroborate with the finding of [30] who showed that addition of oyster shell in soil increased the pH by a factor of 0.4 to 0.5. The application of oyster shell powder can raise soil pH, promote the metabolic diversity of soil microorganisms and the stability of soil micro-ecology environment, and further achieve better control efficiency on tobacco bacterial wilt [29].

Pathogenicity tests showed that leaf disease symptoms were significantly re-

duced in plants treated with oyster shell powder. This decrease of leaf disease symptoms could be correlated to the higher levels of resistance which might be due to the stimulation of cocoa seedling defense mechanisms by heat-treat oyster shell powder component such as chitin and calcium. [12] and [31] argued that organic chitinous amendment exhibited antifungal activities and stimulated plant defense mechanisms. Results from these studies suggested that the mechanism of disease suppression could be the induction of systemic resistance since there was no direct contact between *P. megakarya* and oyster shell powder within the plant. This occurrence was established in our study by the significant higher accumulation of a phenolic compounds, total soluble protein content and higher polyphenoloxidases, peroxidases, chitinases and glucanase activity in the leaves of cacao seedlings following oyster shell powder treatment in comparison to the control plants. These enzymes are well known as molecules involved in numerous plant functions, among which the defense mechanism of plant against pathogenic agents.

5. Conclusion

The present study clearly demonstrated that the nursery soil treatment with heat-treated oyster shell powder increases cocoa seedling growth parameters. This came along with the reduction of *P. megakarya* soil load and enhancement of soil pH. Moreover, this treatment induced disease tolerance in cocoa (*T. cacao*) seedlings against infection with *P. megakarya.* Therefore, further study is needed to evaluate their effect on plant defense related gene and apoplastic proteins expression. The heat-treated oyster shell powder treatment could be an environmentally safe approach in controlling *P. megakarya* damage in nursery. As a result, the heated-treated oyster shell powder used in this study can be formulated and used by farmers as biofertilizers as well as biofungicide in the production of organic cocoa seedling and several economically important crops in the country.

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Conflicts of Interests

The authors have not declared any conflict of interests.

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