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Trichoderma Bio-inoculant Promotes the Growth and Yield of Pepper (*Capsicum annuum* L): An Open Field Trial

Lanvin Rochal Kepngop Kouokap[1], Pierre Eke[1, 4], Vanessa Nya Dinango[1], Diane Youmbi Yimta[1], Pierre Gilbert Ghomsi Tamghe[1], Germain Kansci[3], Louise Nana Wakam[1, 2, *]

[1]Antimicrobial & Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon

[2]Soil Microbiology Laboratory, Biotechnology Centre, Nkolbisson, Yaounde, Cameroon

[3]Laboratory of Food Science and Nutrition, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon

[4]Department of Crop Production and Technology, College of Technology, University of Bamenda, Bamenda, Cameroon

Email address:

Wnanalouise@yahoo.fr (Louise Nana Wakam)

[*]Corresponding author

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Abstract: *Trichoderma* is a ubiquitous fungal genus widely used in agriculture. Their ability to mininize fertiliser dosage, pesticide rationing, and increase crops yield have offered huge avenues for sustainable agriculture. Nonetheless, given the diversity of the world's agro-ecosystems, their widespread adoption has been restricted. This work aimed at investigating the impact of solid formulation of a consortium of two *Trichoderma* strains, *T. atroviridae* T2 and *T. Harzianum* T8, on the growth and yield of hot Pepper under open-field conditions. When compared to un-primed plants, *Trichoderma*-primed plants boosted Chla, Chlb, Chl (a+b) and Carotenoid by up to 122%, 11%, 113%, and 48%, respectively. Likewise, 108%, 220% and 76% more phosphorus, nitrogen and auxin were accumulated in *Trichoderma* inoculated tissues, leading to significant increases in almost all agro-morphological parameters, culminating at 23.5%, 54%, and 23.5%, respectively for production yield (PY), fruit diameter (FD), and Pepper fruits weight (PY) as compared to un-inoculated counterparts. There was no difference in specific activities of catalase (CAT) and peroxidase (POX), even though there were increases in phenolic (17%) and total proteins (15%). These findings suggest that *Trichoderma* fertilisation can redirected plant response depending of plant statue, which prioritised host fitness and associated biomolecules under our experimental settings with the absence/least-biotic stresses.

Keywords: *Trichoderma*-Consortium, Solid Fermentation, Nutrient Absorption, Photosynthetic Pigments, Pepper Yield

1. Introduction

Modern agriculture feeds about 7 billion people worldwide nowadays. Forecasts foresees an increase of 70% over current agricultural productivity to continue supplying food and fibre to the ever-growing demographics estimated to reach 9.6 billion by 2050 [1]. The task is likely more challenging given the current situation where 10% of arable land is being lost annually due to inappropriate agricultural practices, unbalanced agrochemical inputs, and erratic climatic conditions. Elsewhere, horticultural commodities such as fruits and vegetables are genuine tools towards sustainable food provision as they have always kept the promise of easy, sufficient, and safe food supply to the world needy.

Native of Central America, Pepper (*Capsicum* spp.) is the most cultivated and the second-most consumed vegetable worldwide [2]. 25% of the world's population consumes Pepper as spice, food colorant, or vegetable daily [3, 4]. Its fruits are abundant source of biochemicals and minerals of nutritional and medicinal significance. For instance, chili based foodstuffs contain vitamins (C and E), carotenoids, flavonoids, and capsaicinoids with antiseptic, antimetastatic, antifungal, antiviral, anti-inflammatory, and immunomodulatory properties [5]. The thus underlined

nutritional, economic and therapeutic attributes reveal the undeniable contribution of hot pepper to the global sustainability goals [6].

In 2018, approximately 2 million hectares were dedicated to Pepper cultivation around the world with 36.8 million tonnes of hot and sweet Pepper produced [7]. Nevertheless, in addition to the unbalanced supply and demand, the production of quality and quantity fruit depends entirely on chemical fertilizers. In comparison to other agricultural crops, pepper plants have extremely high needs for soil-available nutrients [8]. Farmers frequently use nitrogen fertilizers to afford those essential and yield-limiting nutrients. Nonetheless, it is widely recognized that application regimes frequently exceed crop demand, resulting in not only economic losses but also increasing environmental degradation, given that about 70% of N infiltrates in the ecosystem [9]. Tremendous efforts are currently deployed to establish a balance between nutrients inputs and/or native soil nutrients and plant's needs. In this regards, endophytes, or non-pathogenic plant inhabiting microbes, are among the microbial groups widely used as biological fertilizers. The formers bear ability to colonize crop inner tissues causing physiological and biochemical changes with subsequent benefits to the host crops. [1]. Cumulative studies report yield increases mediated by *Trichoderma*-based bio-fertilizers *vis-à-vis* many horticultural crops including tomato, cucumber, and many Leafy vegetables [10, 11]. *Trichoderma* endophytes colonize the host's tissues interior, resulting in altered root architecture [12], improved soil nutrients availability [13, 14], and fostered plant hormones biosynthesis and buffered tolerance to abiotic and biotic stressors [12]. In our earlier investigations, we identified an inoculum made up of two *Trichoderma* strains, *T. atroviridae* T2 and *T. Harzianum* T8, with potentials to promote pepper growth and dwarf off the occurrence of Pepper leaf curl virus under greenhouse conditions [15]. Therefore, the present work aimed at investigating the impact of solid formulation of the so-said consortium on the growth and yield of hot Pepper under open-field conditions.

2. Materials and Methods

2.1. Trichoderma Strains and Solid-Fermentation

Trichoderma strains, namely *T. Atroviridae* T2 and *T. harzianum* T8 were selected in accordance to their antagonistic properties against *F. solani*, *F oxysporum*, [13] and Pepper leaf curl virus [15]. Data pertaining to their identity, as well as the ability to solubilize inorganic phosphate and enhance N and P uptake are reported [13]. For the formulation, raw rice grains were sized and autoclaved (121°C, 15 min) twice [16]. *T. atroviridae* T2 and *T. harzianum* T8 were grown on potato dextrose agar (PDA, Himedia, India) medium for seven days (28 ± 2°C) and conidia were harvested by scraping off the mycelial mat with sterile glass steak and sterile distilled water (SDW). Cell loads of 10^8 conidia/ml from each biocontrol agent (BCA) were prepared in SDW. 10 mL of each suspension was poured into 500g sterilized rice, seeded in a sterile glass jar, and incubated for two weeks at room temperature and timely agitation [17]. Upon incubation, colonized rice grains were ground to powder and used for inoculation in farm assay (Figure 1).

2.2. Shelf Life Determination

The formulation was stored for 8 months on a laboratory bench with 12/12h photoperiod. Subsamples were collected at 0, 4, and 8 months and the number of colony-forming units (CFU) were estimated using the dilution plate technique [18]. Colonies of *Trichoderma* were quantified and expressed in terms of colony-forming units (CFU) per gram inoculant. Thereafter, hyphal tips emerging from each colony were seeded onto fresh PDA medium and their cultural and microscopic characteristics were compared to original inoculums [19].

2.3. Experimental Farm and Experimental Setup

The experiment was conducted at the experimental farm of the Faculty of Sciences of the University of Yaounde I (11°31'00" E; latitude 3°52'00" N). The site is located between 500 to 900 m in height from the sea level within the 5th agro-ecological zone of Cameroon. The site is characterized by a bimodal rainfall pattern (1500 mm) and a Sandy-clay-loam complex with Organic matter (3.62%), C/N ratio (20.5), cation exchange capacity (29 meq/100 g), and pH of 5.2 [20]. Weeds were cleared, and the land was plowed to a depth of 15–20 cm using a conventional manual hoe-plowing tillage technique.

Pre-germinated pepper plants (red variety, must consumed) were grown in the nursery for 45 days in trays, inoculated by adding 200mg of formulated *Trichoderma* or sterilise rice powder for control treatment, before transplantation. 60 cm and 40 cm distances were left between rows, and plants within each row, respectively. The overall treatments were set in a randomized complete block design with three replications per treatment watered every morning before sunrise. The plots were split into 4 rows. In each replication, the distance between plots was 1 m whereas the distance between neighbouring replicates was 2m. Each block was composed of control (C) and *Trichoderma*-treated seedlings (T2-T8). Plants were growth until production (3months) and Yield was calculated based on the weight of the marketable fruit (those free from damage associated with biotic or abiotic stress, from living plants) recorded weekly for 10 weeks. The individual weekly yields were summed to calculate total yield. Total yield was converted into kg per hectare using the following formula: Yield (kg per hectare) = {[total yield (kilograms)/[harvested area (square meters)/10,000 $m^2 \cdot ha^{-1}$]} [3].

2.4. Quantification of Some Biochemical Markers

2.4.1. Estimation of Nitrogen and Phosphorus Content

Young leaves were sampled, oven-dried (80°C) until

constant mass, and ground with Spex SamplePrep 2010 Geno/Grinder. Subsamples of dried powder were subjected to acid digestion [21] and the total P and N were estimated. The total P was determined as described by Watanabe FS, Olsen SR [22]. Briefly, an aliquot 3ml was transferred to a 15 ml volumetric flask and 4 ml of yellow solution (2.5 M H_2So_4: (NH4) 4%; $6Mo_7O_{24}$ $4H_2O$:10M $C_6H_8O_6$: 0.44M= 10:3:6:1) were added, followed by 5 ml SDW. The solutions were thoroughly mixed and incubated at room temperature for 15 min. Optical density (OD) readout at 710 nm using a Microtiter plate reader (Tecan Infinite M200). Total P content was then estimated using a standard curve of phosphoric acid (H_3PO_4) plotted in the concentration range of 10-100µg/mL (R^2 = 0.976). The N content was estimated as described by Hevia P, Cioccia A [23]. 3 ml of predigested pepper leaves was mixed up with phenol nitroprusside and alkaline hypochlorite, then incubated (20°C, 15 min). OD was measured at 625 nm and N content was estimated by extrapolation using a standard curve of pure NH_3 prepared at the concentration range of 10-100 µg/ml (R^2=0.98).

2.4.2. Determination of Indole Acetic Acid (IAA) Content

A slightly modified spectrophotometric technique described by Goswami et al [24] was used. In a 1:1 ratio, methanol extract was mixed with Salkowski reagent (50 ml, 35% perchloric acid, 1 ml 0.5 M FeCl3 solution). The concentration of IAA was estimated using a standard curve of pure IAA at concentration ranges of 10-100g/mL (R2=0.98) and OD readout at 530 nm.

2.4.3. Photosynthetic pigment measurement.

Photosynthetic pigments were extracted by maceration of leaflets in 10 ml acetone (80%) for 48h at 4°C. The homogenates were centrifuged (5000g; 5min) and ODs were measured at 470, 646, and 663nm. The pigments contents were calculated according to the formula described by Lichtenthaler Hk, Wellburn Ar [25] as follows:

$$\text{Chla (µg/ml)} = 12.21\ A_{663} - 2.81\ A_{646}$$

$$\text{Chlb (µg/mL)} = 20.13\ A_{646} - 5.03\ A_{663}$$

$$\text{229*Carot (µg/mL)} = 1000\ A_{470} - 3.27\ \text{Chla} - 104\ \text{Chlb}.$$

2.4.4. Assessment of Total Phenolic Content

Phenolic extracts were prepared as described by Tchameni et al [26] and Total phenolic content was determined using the Folin-ciocalteu methods as described by Siddhuraju et al [27]. 50 µL of the above-prepared extract at 0.5 g/mL in distilled was thoroughly mixed with 250 µL of Folin-ciocalteu's reagent (10%) and 750 µL 70% Sodium Carbonate. The mixture was incubated for 8 min at room temperature and 950 µL of SDW were added and incubated for 2 h at room temperature. Gallic acid was used as standard in the same experimental conditions. The absorbance was measured at 760 nm against a blank. The experiments were performed in triplicate and the results expressed as Gallic acid equivalents (mg of GAE/g sample) through a regression curve plotted from pure Gallic acid (r^2 = 0.99).

2.4.5. Total Proteins Content

For protein extraction, 0.5 g fresh root samples were crushed in 20 mL of 50 mM sodium phosphate buffer (pH 8.8), filtered, and centrifuged (13,000g, 4°C, 30 min), and the protein content was determined by Bradford MM [28]. 100µL of extracts and the standard (BSA; bovine serum albumin) were mixed with Bradford reagent, and the OD of the blue complex formed was measured at 595 nm. The experiment was repeated three times, and the findings were expressed as BSA equivalents (mg BSA/g sample) using a regression curve (r^2 = 0.99) plotted from pure BSA.

2.4.6. Assay of Phenylalanine Ammonia-Lyase Specific Activity.

In 20 mL of 50 mM sodium phosphate buffer, 0.5 g fresh leaf were crushed (pH 8.8) and centrifuged (13,000g; 4°C for 30 minutes). PAL activity was measured in the supernatant following by Whetten RW, Sederoff RR [29]. Briefly, an assay mixture, containing 100 µL of enzyme extract, 500 µL of 50 mM sodium phosphate buffer (pH 8.8), and 600µL of 1 mM Phenylalanine were allowed to stand for 1 h at room temperature followed by the addition of 2 mL of 2N Hydrochloric acids to stop the reaction. The absorbance of the released trans-cinnamic acid was measured at 290 nm and the result was expressed in terms of OD_{290}/min/mg protein.

2.4.7. Assay of Peroxidase Specific Activity

POX activity was assessed by measuring the increase in absorbance at 470 nm as a result of guaiacol oxidation into tetraguaiacol by the pro-oxidant (H_2O_2) Zheng X, van Huystee R [30]. The reaction mixture containing 0.1 mL 1% (v/v) guaiacol, 10 mM sodium phosphate PH 6.0, 0.1 mL of 0.3% (v/v) H_2O_2, and 0.3 mL enzyme extract. The enzyme activity was expressed in terms of mmol of substrate transformed per mg of enzyme extract per min (mmol/mg/min).

2.4.8. Assay of Catalase Specific Activity

The specific activity of the said enzyme was measured according to Aebi H [31]. The CAT activity was measured in a 3mL assay mixture consisting of 0.05 mL extract, 1.5 mL phosphate buffer (100 mM buffer, pH 7), 0.5 mL H_2O_2, and 0.95 mL distilled water. A drop in absorbance at 240 nm was recorded. The CAT activity was expressed in terms of µmol H_2O_2 oxidized per minute per gram fresh weight (µmol/min/g).

2.5. Statistical Analysis

The data from the overall experiments were normalized, and subjected to ANOVA. The difference between mean values was compared with the Least Significant Difference (LSD) post hoc test at the statistical threshold of 5% using the SIGMAPLOT 11.0 statistical software.

3. Results

3.1. Shelf-Life Determination

Formulated biofertilizer was concentrated at 25000 CFU

per gram of formulation. After storage at ambient and refrigerated conditions, the formulation shown a significant feeling of CFU/g of the formulation. In Refrigerator storage (23.4×10^3 cfu/g of formulation) CFU population reduced down to 6.4% and in ambient storage (9.6×10^3 cfu/g of formulation) the reduction is down by 61.6%.

(a) (b)

Figure 1. *Individual Trichoderma strains propagated on rice seeds in left Trichoderma harzianum and in right Trichoderma atroviridae (a) and powder formulation resulting from 1/1 (w/w) mixture of T. atriviridae T2 and T. harzianum T8 (b).*

3.2. Effect of Trichoderma-Based Biofertilizer on Growth and Yield of Chili Pepper

Relative to uninoculated controls, nearly all growth parameters assessed, such as leaf area (LA), fruit weight (FW), fruit diameter (FD), and production yield (PY), were significantly improved when seedlings were drenched with the biofertilizer ($P\leq0.05$). For instance, FW, FD, and PY increased by 23.5%, 54%, and 23.5%, respectively, as compared to un-inoculated counterparts. Except in LA, where no significant change was recorded (Table 1).

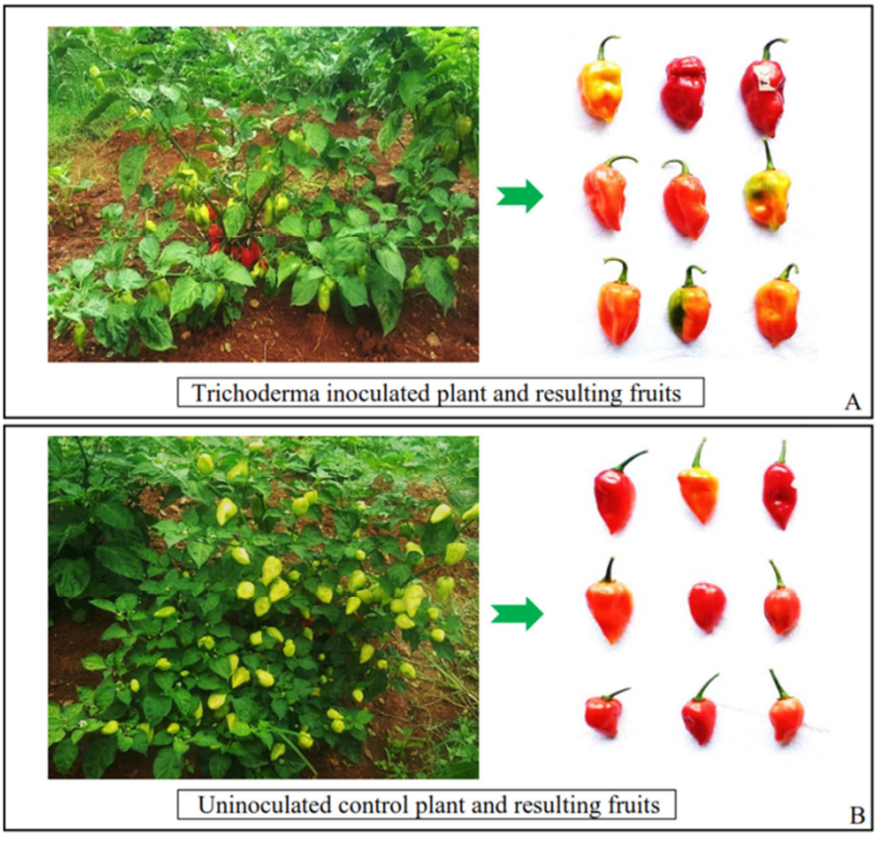

Figure 2. *Differential fruits bearing capacity and ripen chili pepper fruits size as affected (A) or not (B) by the tested Trichoderma-based bio-fertilizer.*

Table 1. *Differential effect of formulated biofertilizer on yield and yield components of pepper plants.*

Treatment	Leaf area (cm^2)	Fruit weight (g)	Fruit diameter (cm)	Yield (Kg/ha)
Control (C)	37.17 ± 9.39^a	$750.7 \pm 10,2^a$	8.66 ± 1.15^a	3753.5 ± 51^a
Biofertilizer (T)	71.25 ± 6.50^a	$926.9 \pm 6,7^b$	13.33 ± 0.58^b	4634.5 ± 33.5^b
Significance (P≤ 0.05)	Ns	**	*	**

Column bearing the same letter are not a significant difference at P<0.05; * or ** stands for the level of significance.

3.3. Effect of Trichoderma Fertilization on Phosphorus and Nitrogen Uptake

The phosphorus and nitrogen content increased considerably after biofertilizer application. Control plants accumulated 0.048 mg/g fresh matter total phosphorus whereas *Trichoderma* treated plantlets recorded 0.1 mg/g phosphorus per fresh weight, accounting for 108% more P than control plants (Figure 3a). Likewise, *Trichoderma*-primed pepper plants yielded 1.162 mg/g fresh weight, 220% greater than the untreated counterpart, which produced 0.363 mg/g dry weight. This reflects the formulation's ability to improve vital nutrient absorption.

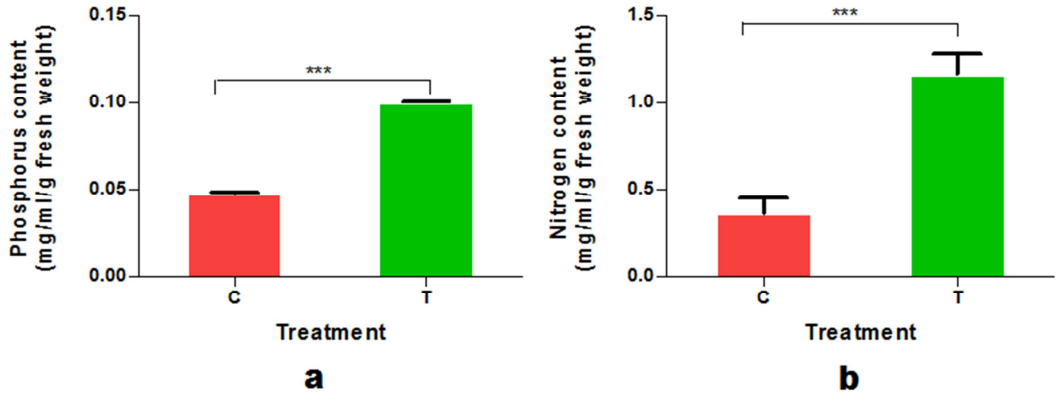

Figure 3. *Effect of Trichoderma fertilization on chili pepper Phosphorus (a) and Nitrogen (b) absorption. C: un-inoculated controls T: Trichoderma-treated plants.*

3.4. Effect of Bio-fertilization on Auxin and Photosynthetic Pigment Biosynthesis

IAA synthesis was significantly greater in *Trichoderma*-inoculated plants compared to un-inoculated plants (P≤0.05). For instance, 76.8% more IAA (42.47µg/g fresh weight) was produced upon Trichoderma bio-priming, relative to untreated controls (24.01µg/g fresh matter). Likewise, 122%, 11%, 113% and 48% more Chl_a, Chl_b, $Chl_{(a+b)}$ and carotenoids were biosynthesized by pepper plants primed by the *Trichoderma* consortium, with reference to the negative control. Moreover, the Chla/Chlb ratio was 99.9% greater in *Thichoderma*-inoculated plants than in un-primed plants (Table 2).

Table 2. *The impact of chilli pepper biofertilization with Trichoderma-based fertilizer on auxin and photosynthetic pigment content.*

Treatment	IAA (µg/ g FW)	Chl_a (µg/g FW)	Chl_b (µg/g FW)	$Chl_{(a+b)}$ (µg/g FW)	Chl_a/Chl_b	Carotenoids (µg/g FW)
Control (C)	24 ± 1.2^a	140.4 ± 1.4^a	12.1 ± 1^a	3.1 ± 0.04^a	11.7 ± 1^a	17.2 ± 2.9^a
Biofertilizer (T)	42.5 ± 1.0^b	311.5 ± 3.6^b	13.4 ± 0.9^b	6.5 ± 0.08^b	23.3 ± 1.4^b	25.4 ± 2.1^b
Significance	**	***	**	***	***	*

Column bearing the same letter is not a significant difference at P<0.05 and *, **, *** show a level of significance. At P<0.05.

3.5. Effect of Trichoderma Bio-fertilization on Some Pepper Defence Markers

Overall, *Trichoderma*-inoculated plants displayed significantly higher total soluble phenol contents than non-primed counterparts (P 0.05). 17% increase of the total soluble phenol content was recorded in *Trichoderma* primed plants (363.367 mg/g dry weight) when compared to the control group plants (310.467 mg/g dry weight) (Figure 4b). Equally, *Trichoderma*-inoculation (4.33 µg/mL per g fresh matter) induced 14.9% more protein biosynthesis with reference to negative controls (3.77 µg/mL per g fresh matter) (Figure 4a). Though not statistically different, experimental results revealed that bio-primed pepper plants modulated PAL specific activity comparative to non-inoculated counterparts. The same trend was observed for peroxidase and catalase activities, with controls and *Trichoderma* primed plants recording specific activities of 4.3 and 4.6 mmol/min/g proteins and 4.53 and 4.41 mmol/min/g proteins for POX and CAT, respectively, indicating no significant shift in the activities of the so-called enzymes (Figure 4e). Overall, the findings clearly demonstrated that *Trichoderma* inoculation caused alterations in chilli plant biochemical processes by

promoting more growth parameters.

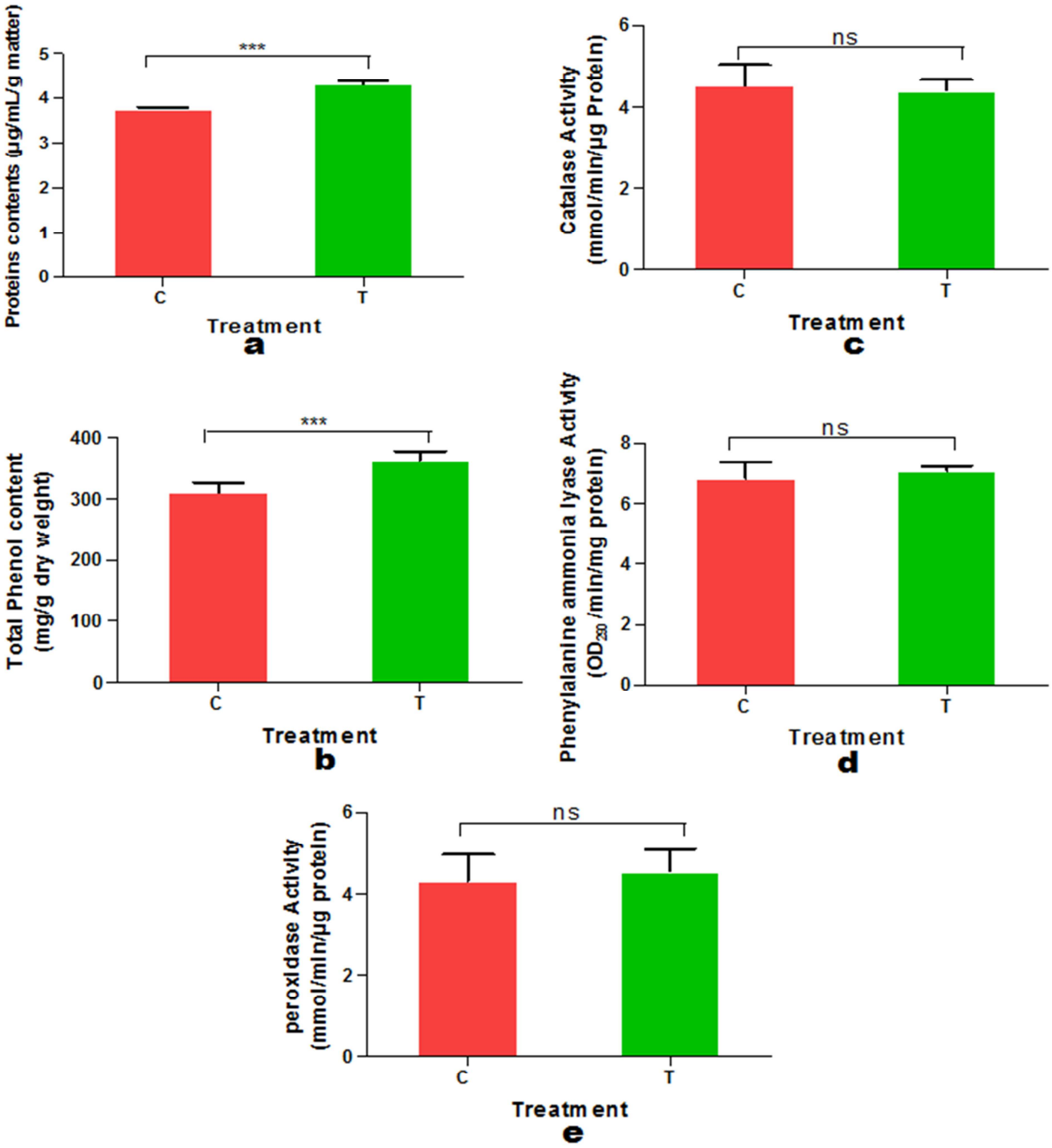

Figure 4. *Effect of bio-fertilization on Proteins (a), phenol content (b) and specific activities of CAT (c), PAL (d) and POX (e). C stands for control (un-primed) and T for Trichoderma-inoculated plants.*

4. Discussions

Plant-soil-microbe interactions are crucial for soil health, nitrogen cycling, and the suppression of soil-borne phytopathogens [32]. *Trichoderma* spp. are soil-borne fungi widely distributed in natural ecosystems and undergoing symbiotic association with majority of higher plants [33]. Despite the tremendous benefits associated with *Trichoderma* colonisation into the host, their efficacy in climatically fluctuating environments and different soil conditions has hampered their full integration into agricultural systems for greater sustainability [12, 34]. Furthermore, a single bioagent is more likely to fail in delivering beneficial features to crops than a consortium application, resulting in easier accommodation in the open field and higher efficiency [35]. Research evidences has extensively demonstrated the necessity of effective bioagents to withstand the repressive effects of autochthonous microflora prior to delivering beneficial traits to their hosts. Herein, a fungal inoculant developed from propagules of two *Trichoderma* sp.; *atriviridae* T2 and *harzianum* T8, both of which having

remarkable properties, were used. Interestingly, the propagules survived 8 months of storage at room temperature, with a 61.6% drop in conidial load per unit mass of the formulation. The conidial load of 9.6 x103 cfu/g of formulation recorded after 8 months storage was much higher than the density required by Singh et al. which showed that 10^{8} UFC/g are sufficient to successfully colonized and impart beneficial traits in pepper plants.[36].

Despite the challenges in extracting N and P from soil chemical complexes, *Trichoderma* fertilizer resulted in significant accumulation of these vital nutrients in pepper plant tissues [37]. While Li F, Huang C, Li Z, Zhou X demonstrated *Trichoderma'*s potential to improve tomato P absorption in P-deficient hydroponic culture via solubilization and chelation [38], many attempts have been made to demonstrate the ability of *Trichoderma* inoculants to adjust soil pH and release phosphatases that solubilize soil insoluble phosphorus [13]. Further, Fiorentino et al. indicated that *Trichoderma*-based inoculants were capable of enhancing N uptake in N-deficient land, favour its absorption in native soil conditions, and promote efficient use of this key nutrient in N-enriched soils. [10].

Indeed, N and P are essential building blocks for several key macromolecules, notably chlorophyll, proteins, DNA, and many others. Their eventual deficiency restricts the photosynthetic efficiency, the plant growth, and consequently the productivity.[39]. Photosynthesis is the primary determinant of crop yield, and the mean by which crops convert light into biomass over the growing season, leading to the expected yield. Numerous studies have demonstrated that genes, proteins, and pigments involved in photosynthesis in a variety of crops are up-regulated upon association with *Trichoderma* strains, enhancing therefore the rubisco accumulation and numerous components involved in the light and dark reactions (Calvin cycle) [40]. Our results indicated that the biosynthesis of IAA was increased by 76% in response to *Trichoderma* application. The so-said hormone is indeed well-known to control the meristematic cell maintenance and formation and cell enlargement and division, leading to vigorous plants [41, 42].

Slight increases in Phenolics (17%) and total proteins (15%) were also recorded. Though not significant, the specific activities of the destressing enzymes; CAT and POX were enhanced in bio-primed plants. These findings further shed light on the vaccine-like effect of BCAs, which trigger the biosynthesis and accumulation of defence-related compounds for a fast and accurate response upon future infections. The lack of significance in the activities of so-called defensive enzymes might potentially reflect a shift in host metabolism toward growth and development rather than defence processes. Interestingly, we recorded higher quantitative and qualitative fruit yield after land treatment with the *Trichoderma* consortium. As a result, De palma et al. and Mayo et al. highlighted *Trichoderma*'s potential to fine-tune host gene expression for efficient and strategic exploitation of the plant machinery to combat emerging threats [43, 44]. The lack of significant biotic stressor in our experiment setup might have shifted gene expression to efficient plant feeding, resulting in higher production.

5. Conclusion

Trichoderma inoculants recorded higher quantitative and qualitative fruit yield. These findings further shed light on the *Trichoderma'*s potential to improve P and N absorption. *Trichoderma* inoculants act like vaccine, which trigger the biosynthesis and accumulation of defence-related compounds for a fast and accurate response upon future infections. These findings suggest that *Trichoderma* fertilisation can redirected plant response depending of plant statue, which prioritised host fitness and associated biomolecules under our experimental settings with the absence/least-biotic stresses.

Declaration of Interest Statement

The authors declare no interest of conflict.

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