THE UNIVERSITY OF YAOUNDE I

CENTRE FOR RESEARCH AND TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES

RESEARCH AND DOCTORATE TRAINING UNIT IN LIFE SCIENCES

UNIVERSITE DE YAOUNDE I

CENTRE DE RECHERCHE ET DE FORMATION DOCRORALE EN SCIENCES DE LA VIE, SANTE ET ENVIRONNEMENT

UNITE DE RECHERCHE ET DE FORMATION DOCTORALE EN SCIENCES DE LA VIE

DEPARTMENT OF BIOCHEMISTRY
DEPARTEMENT DE BIOCHIMIE

LABORATORY OF PHYTOBIOCHEMISTRY AND MEDICINAL PLANT STUDY
LABORATOIRE DE PHYTOBIOCHIMIE ET D'ETUDE DES PLANTES MEDICINALES

EFFECT OF SOME *TRICHODERMA sp.* AGAINST LEAF CURL VIRUS Disease AND ON CHILI FRUIT QUALITY (*CAPSICUM ANNUUM* L.)

Thesis presented for partial fulfilment of the requirements for the awards of a Doctorat/PhD in Biochemistry

Speciality: **Phytobiochemistry and Phytopathology**

by

KEPNGOP KOUOKAP LANVIN ROCHAL

Registration Number: 08R0378
Master in Biochemistry

Directed by

NANA WAKAM LOUISE
Associate Professor, UY1

KANSCI GERMAIN
Professor, UY1

Academic year : 2024/2025

THE UNIVERSITY OF YAOUNDE I

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DEPARTMENT OF BIOCHEMISTRY

CERTIFICATE OF CORRECTION OF THE PH. D THESIS

ATTESTATION DE CORRECTION DE LA THESE DE DOCTORAT PH. D

The undersigned members of jury involved in the Ph.D thesis in Biochemistry entitled : **« Effect of some *Trichoderma* sp. against leaf curl virus disease and on chili fruit quality (*Capsicum annuum* L.)»** defended on Monday, 3rd of February 2025 at 10:00 a.m. in the S01/S02 room of the faculty of Sciences by **M. KEPNGOP KOUOKAP Lanvin Rochal (registration number 08R0378)**, are hereby certifying that the candidate has effected the corrections of the above mentioned thesis as requested by the examiners.

Therefore, they are satisfied with the corrections made and are recommending the doctorate/ph./D degree to be awarded to the candidate.

Yaounde, the 0 3 MARS 2025

pervisors

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Docteur de l'Univesité de Liège
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Examiners

Pr FOKOU.

Pr EFFA ONOMO
Pierre

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Le Chef de Département
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Moundipa Fewou
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ACADEMIC YEAR 2023/2024

(By Department and by Grade)

LAST UPDATE 04 June 2024

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9.	EMADAK Alphonse	Associate Professor	O n duty
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12.	KENNE DEDZO GUSTAVE	Associate Professor	On duty
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25	MVOGO ALAIN	Associate Professor	On duty
26	NDOP Joseph	Associate Professor	On duty
27	SIEWE SIEWE Martin	Associate Professor	On duty
28	VONDOU Derbetini Appolinaire	Associate Professor	On duty
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32	AYISSI EYEBE Guy François Valérie	Senior Lecturer	On duty
33	CHAMANI Roméo	Senior Lecturer	On duty
34	DJIOTANG TCHOTCHOU Lucie Angennes	Senior Lecturer	On duty
35	EDONGUE HERVAIS	Senior Lecturer	On duty
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Distribution of permanent lecturers in the faculty of science according to departments

NUMBER OF LECTURERS					
Department	**Professors**	**Associate Professors**	**Senior Lecturers**	**Assist. Lecturers**	**Total**
BCH	8 (01)	20 (12)	9 (04)	6 (05)	**43 (22)**
BPA	11 (01)	19 (09)	16 (05)	3 (02)	**49 (17)**
BPV	6 (01)	10 (02)	14 (08)	2 (00)	**32 (11)**
CI	7 (01)	14 (04)	5 (01)	1 (00)	**27 (06)**
CO	7 (01)	15 (05)	11 (05)	1 (00)	**33 (11)**
RE	1(00)	/	/	/	**1(0)**
IN	2 (00)	2 (00)	14 (01)	4 (00)	**22 (01)**
MAT	1 (00)	8 (00)	19 (02)	5 (01)	**33 (03)**
MIB	3 (01)	7 (03)	9 (05)	5 (02)	**24 (11)**
PHY	18 (01)	12 (04)	11 (01)	1 (00)	**42 (06)**
ST	10 (00)	17 (03)	13 (03)	3 (01)	**43 (07)**
Total	**74 (07)**	**124 (42)**	**121 (35)**	**31 (11)**	**350 (95)**

A total of……………………**349 (95)**, with :

Professors……………………………**73 (07)**

Associate Professors……………………….**124 (42)**

Senior Lecturers………………….**121 (35)**

Assistant Lecturers……………..**31 (11)**

( ) = Number of women……….**95**

DEDICATION

I dedicate this thesis to:
My late Mother KOMEGNI Margueritte,
and my Father KOUOKAP Marc

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ABSTRACT

Peppers leaf curl virus disease (PeLCVD) is a cosmopolitan plant viral disease hindering a broad range of important crops. Chemicals are preconized to curb the trend regardless of their setbacks. Herein, *Trichoderma* is a ubiquitous fungal genus widely used in agriculture. Its ability to minimize biotic and abiotic plant attacks, and increase crop yield have offered huge avenues for sustainable agriculture. This work aimed to assess the biological control capacity of some *Trichoderma* spp. on Pepper leaf curl virus disease and fruit quality of chili. The sequencing of the ITS region confirmed the name of our *Trichoderma* as *Trichoderma polysporum* T1, *T. atroviridae* T2, and *T. harzianum* T8. After colonisation of pepper plant in greenhouse and infection with pepper leaf curl virus through *Bemisia tabaci* MED previously identified by sequencing, some parameters of defense were recorded and the best consortia selected for field culture. We evaluated nutrition status of the plant and fruits were used to evaluate their quality after storage in cold and ambient condition during 20 days. *In planta*, these *Trichoderma* were able to endophytically colonize pepper and increase the shoots, roots dry matters, with the best colonization recording by a consortium of *T. atroviridae* T2 and *T. hazianum* T8 (T2T8). This dry matter can be due to the increasing IAA content produced by all bioagents, during in vitro and in vivo production, up to 100 µg/mL recorded by T2T8 consortium. Likewise, *Trichoderma*-induced innate host immunity priming and stress tolerance mediated by the triggered phenylpropanoid biosynthesis pathway culminated at as high as 183% more total phenolic content ($P \leq 0.05$). Interestingly, no difference was recorded in the specific activity of the depressing enzymes 40 days post-infection and can be due to early ROS accumulation at virus infection sites leading to rapid host reactions without any oxidative stress showed by the PeLCVD suppression in *Trichoderma*-primed seedling up to 50%. The consortium T2T8 were used for their impact on yield and fruit quality. When compared to un-primed plants, *Trichoderma*-primed plants boosted all growth and nutrition parameters such as total Chlorophyll (122%), Carotenoid (48%), Phosphorus (108%), and nitrogen (220%) uptake and IAA (76%) leading to significant increases in almost all agro-morphological parameters. Biostimulation enhanced all evaluated biochemical and physiological parameters lead to fruit quality ($P \leq 0.05$), like physiological weight loss, pH, carotenoid content, capsaicin, ascorbic acids, and phenol; this effect was confirmed by principal component analysis (PCA), which showed that carotenoids, capsaicin, phenol and PWL were most correlated together. Based on PCA, *Trichoderma*-treated fruits store at ambient condition have the best quality after 10 to 20 days.

Keys words: *Capsicum annuum* L, viral disease, *Trichoderma*, biological control, fruit quality.

RÉSUMÉ

La maladie du virus de l'enroulement des feuilles du piment (PeLCVD) est une maladie virale cosmopolite des plantes qui attaque un grand nombre de cultures. Les produits chimiques sont utilisés malgré leurs effets sur la santé et l’environnement. *Trichoderma* est un genre fongique largement utilisé en agriculture et sa capacité à protéger les plantes des attaques biotiques et abiotiques et à augmenter le rendement des cultures offre d'énormes possibilités pour une agriculture durable. Ainsi, ce travail vise à évaluer les capacités biocontrôle de trois souches de *Trichoderma* contre le virus de l'enroulement des feuilles et dans l’amélioration de la qualité des fruits de piments. Pour ce faire, une caractérisation moléculaire des souches et leurs effets contre la maladie de l’enroulement des feuilles et sur la qualité des fruits après conservation pendant 20 jours à température ambiante et réfrigéré ont été effectués, en évaluant quelques paramètres biochimiques, physiologique et agronomique. Le séquençage de la région ITS a confirmé l’identité des *Trichoderma* comme étant *Trichoderma polysporum* T1, *Trichoderma atroviridae* T2, and *Trichoderma harzianum* T8. *In planta*, ces *Trichoderma* ont pu coloniser les tissus internes du piment et augmenter les biomasses sèches aériennes et racinaires avec la meilleure colonisation enregistrée par le consortium de *T. atroviridae* T2 et *T. hazianum* T8 (T2T8). Cette augmentation des matières sèches serait due à stimulation de la teneur en IAA induite par tous les agents biologiques *in vitro* et *in vivo* avec la meilleure enregistré par T2T8. De même, la résistance innée de l'hôte et la tolérance au stress ont été induites par *Trichoderma* et médiés par l’activation de la voie de biosynthèse des phénylpropanoïdes ($P \leq 0.05$). Toutefois, aucune différence significative n'a été enregistrée pour ce qui est de l'activité spécifique des enzymes dépressives 40 jours après l'infection entre le control et les traitements. Ceci serait probablement due à une accumulation précoce des ROS sur les sites d'infection virale conduisant à des réactions rapides de l'hôte (*Trichoderma* réduit maladie 50%). En comparaison aux plantes non stimulées, les plantes stimulées par T2T8 ont présenté les meilleurs paramètres de croissance et de nutrition ($P \leq 0.05$), confirmé par la teneur en IAA. La biostimulation a amélioré tous les paramètres biochimiques et physiologiques évalués conduisant à la qualité. Ceci s’est confirmé, par l'analyse en composantes principales (ACP) qui a montré que les caroténoïdes, les capsaïcinoides, l'acide ascorbique et les phénols ont montré une forte corrélation. D’après l’ACP les fruits traités par *Trichoderma* et stockés en condition ambiante ont présenté la meilleure qualité après 20 jours de conservation. Dès lors l’utilisation des agents biologique tel que *Trichoderma* sera un atout pour la production biologique, idéale dans la santé humaine et environnementale.

Mots clés : *Capsicum annuum* L, Maladie virale, *Trichoderma*, biocontrôle, qualité des fruits

LIST OF ABBREVIATIONS

Symbol	Full name
BCA	Biological Control Agent
DEPC	Deionized diethyl pyrocarbonate
FD	Fruit diameter
FW	Fruit Weight
GI	Germination Index
GMT	Germination Mean Time
GP	Germination Percentage
IAA	Indole acetic acid
ISR	Induced Systemic Resistant
ITS	Internal Transcribe spacer
KASP	kompetitive allele specific PCR
MEAM	Middle East Asia Minor 1
MED	Mediterranean
PAL	Phenylalanine-ammonia Lyase
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PeLCD	Pepper Leaf curl Disease
PeLCV	Pepper Leaf curl Virus
PWL	Physiological weight loss
PY	Production Yield
RDA	Recommended Dietary Allowance
RDI	Recommended Dietary Intake
ROS	Reactive oxygen species

SA	Salicylic Acid
SAR	Systemic acquired Resistance
TSS	Total Soluble Solid

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INTRODUCTION

INTRODUCTION

Modern agriculture feeds around 7 billion people worldwide nowadays **(Eke *et al.*, 2020)**. Forecasts expect a 70% increase in present agricultural productivity to keep up by providing food and fiber to ever-increasing demography estimated to reach 9.6 billion by 2050 **(Adedeji *et al.*, 2020)**. In the current scenario where 10% of arable land is degraded annually, owing to inappropriate farming practices, unbalanced agrochemicals inputs, and usage of heavy machinery, keeping up with the demands for food require a steady shift of paradigm **(Fanelli and Romagnoli, 2019)**. Accordingly, horticultural commodities such as fruits and vegetables (pepper) have held the promise of providing easy, sufficient, and safe food to the needy world population.

Originating from Central America, Pepper (*Capsicum* spp.) is the most cultivated and the second-most consumed vegetable worldwide **(Mateos *et al*., 2013)**. Twenty-five percent of the world's population consumes Pepper as a spice, food colorant, or vegetable daily **(Barchenger *et al.*, 2020)**. Chili contains abundant biochemical and mineral components of nutritional and medicinal importance. Chili-based foodstuffs contain vitamins (C and E), carotenoids, flavonoids (phenolic acids, quercetin, and luteolin), and capsaicinoids **(de Sá Mendes and Branco de Andrade Gonçalves, 2020)** bearing antiseptic, antimetastatic, antifungal, antiviral, anti-inflammatory, and immunomodulatory properties **(Batiha *et al.*, 2020)**. The underlined nutritional, economic, and therapeutic attributes unveil the undeniable contribution of chili pepper to families' livelihood and quantitative and qualitative food provision, making it a key contributor to global sustainable goals **(Valenzuela *et al.*, 2017)**.

Since 2010, world production has increased gradually from 29.7 million tonnes to 38 million tonnes in 2019 **(FAO, 2020)**. In Cameroon, chili peppers' production doubled from 29910 tonnes to 66856 tonnes between 2010 and 2019 **(FAO, 2020)**. The global vegetable production trend reveals a steady decline, which is attributed to a set of constraints topped by diseases, among which viral infestations are of paramount importance globally **(Tolin and Fayad, 2016)**. Geminiviruses transmitted by whiteflies (*B. tabaci*) cause Pepper Leaves Curling virus (PeLCV), considered one of the most economically significant plant viral diseases causing up to a billion USD in annual loss worldwide **(Gangwar and Gangwar, 2018; Harrison and Robinson, 1999)**. A recent survey conducted in Western and Central Africa has thrown further insights into the prevalence and destructive character of Germinivirus towards major indigenous staples **(Leke *et al.*, 2011)**.

In Cameroon, for instance, an outbreak of a highly deadly viral infection was reported, in several commercial chili pepper farms **(Achiangia *et al.*, 2013)**. Given their virulence, broad host range, and dissemination, such diseases require special attention since they have the propensity to become epidemic, harming local and global food security. In endemic regions, many chemically diverse synthetic pesticides, such as organophosphate, organochlorine, and others, are now used to target whiteflies as the primary vector **(Oliveira *et al.*, 2013)**. Nonetheless, the intrinsic carcinogenic, mutagenic, and teratogenic hazards of such synthetic pesticides, as well as the emergence of resistant whiteflies **(Naveen *et al.*, 2017)**, have motivated the quest for a more environmentally friendly alternative **(Faoro and Gozzo, 2015)**.

As a result, small RNAs, recognized as key regulators of host plant resistance gene, expression to dwarf viral-induced plant diseases, hold a lot of promise **(Djami-Tchatchou and Dubery, 2015)**. However, in addition to their transiency, fine monitoring of miRNA activities is necessary before implementation in modern farming to avoid “off-type” outcomes **(Faoro and Gozzo, 2015)**. Similarly, attempts are being made to harness endophytes, which share similar functions and work in tandem with their hosts to improve crop fitness under adverse conditions **(Eke *et al.*, 2016a; Niu *et al.*, 2017)**. Endophytes, or plant-inhabiting microorganisms, are believed to detect physiological changes in stressed plants and adjust plant gene expression accordingly **(De Palma *et al.*, 2019; Liu *et al.*, 2017)**.

Mayo *et al.* (2016) revealed suppression of circumstantial irrelevant genes in favor of defense-related counterparts in common bean (*P. vulgaris* L) primed with *Trichoderma velutinum* under *Rhizoctonia solani* assault. In addition, **Srijana *et al.* (2017)** showed that *Trichoderma* fungus can help tomato plant to fight against *Fusarium solani*, *Rhizoctonia solani* et *Sclerotium rolfsi*. Also, **Borges *et al.* (2015**) showed that *Trichoderma* can stimulate the growth and productivity of the rice through the solubilisation of inorganic phosphorus. Meanwhile, the diversity of the earth's ecosystems, as well as the notion of coevolution emphasize the significance of exploring *Trichoderma* from geographically diverse regions. Moreover, a consortium of biological control agents (BCA) is presently strongly advocated over a single BCA since they are likely to be more effective and transposable in an open field **(Eke *et al.*, 2016a)**.

Hypothesis

Hence, we hypothesized that endophytic *Trichoderma* reduce viral diseases and improve the fruit quality of Capsicum annuum L.

Objectives

This work aimed to evaluate the Effect of some *Trichoderma* sp. against pepper leaf curl virus disease and on chili fruit quality (*Capsicum annuum* L.)

More specifically, this work aimed to:

(1) Evaluate the biological control capacities of three strains of *Trichoderma*

(2) Evaluate the effect of *Trichoderma* against Pepper Leaf Curl Virus Disease

(3) Determine the effect of *Trichoderma* on production yield and fruit quality of *Capsicum annuum* L.

Chapter I:
REVIEW OF LITTERATURE

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1.1 THE HOST PLANT (*CAPSICUM ANNUUM* L.)

1.1.1 Taxonomy of pepper

The genus Capsicum with several universal English names which include hot pepper, chili pepper, bell pepper, and sweet pepper belongs to the family Solanaceae; sometimes, the plant is just called pepper (Figure 1). This genus consists of approximately five domesticated species and twenty-two (22) wild species. Capsicum species can be sorted into different classes based on pod or fruit features such as shape, size, pungency, flavor, and color **(Habtie *et al.*, 2017; García-Gaytán *et al.*, 2017)**. Despite their enormous trait variations, the commercially cultivated cultivars of peppers globally are *C. annuum* species **(Bosland and Votava, 2012)**. Capsicum spp. are diploids, mostly having 24 chromosomes (n = x = 12), and numerous wild species consisting of 26 chromosomes (n = x = 13). The domesticated species belong to the first group **(OCDE, 2010)**. Like most other plants, peppers have a preference for well-drained moisture-holding loamy soil having optimal growth and production temperatures ranging from 18 to 30°C. The optimal temperatures for seed germination range from 25 to 30 °C **(Grubben & El Tahir, 2004)**.

Pepper (*Capsicum* spp.) is among the oldest cultivated and most employed crops. The crop is classified in the phylum of Tracheophyta, Class of Magnoliopsida, Order of Solanales, Family of Solanaceae, and Genus Capsicum. The varieties and cultivars of *Capsicum annuum* are classified based on their fruit shapes. Just like other plants in the family Solanaceae such as potatoes and tomatoes, peppers have increasingly become central components of various cuisines in the world as seen in the large acres of land dedicated to their cultivation in countries.

Figure 1: Pepper plant (Capsicum annuum) bearing mature fruit in field. (Kepngop, 2022 in this study).

1.1.2 Origin, distribution and description of Capsicum annuum

The Capsicum genus is endemic to the Americas, and for several millennia, it has been an important dietary component throughout the New World **(Zhigila *et al.*, 2014)**. Thirty-eight Capsicum species are currently recognized in United State Department of Agriculture's Genetic Resources Information Network (GRIN), of which five are domesticated: *C. annuum* L., *C. chinense* Jacq., *C. frutescens* L., *C. baccatum* L., and *C. pubescens* Ruiz & Pav **(OCDE, 2010)**. Domesticated Capsicum species show wide morphological variability, including traits, that are useful for discriminating among the species, such as plant pubescence, number of flowers per node, calyx constriction, corolla color and spots, and seed color **(Zhigila *et al.*, 2014)**. The red (Safi) and yellow (Big sun) pepper varieties are the most widely grown varieties in Cameroon, with the red variety having more than 3 to 5 times more provitamin A.

Capsicum annuum L. includes a vast number of horticultural varieties (sweet and hot) and is by far the most important pepper economically. It is an important and popular vegetable in agriculture because of its economic importance and the combination of taste, color, and nutritional values of the fruit **(Al-Snafi, 2015)** *Capsicum annuum* comprises a range of bioactive compounds and essential nutrients that display various bioactivities such as antioxidant, antiviral, antimicrobial, anticancer, and anti-inflammatory activities **(Khan *et al.*, 2014)**. This specie originates from Central and South America, and presently, it is extensively cultivated on above 1.5 million hectares in various countries, especially throughout the tropical, subtropical, and temperate regions of Mexico, tropical Africa, East Africa, Europe, the Southern US, India, Turkey, China, and Ghana **(FAO, 2007)**. *Capsicum annuum* grows in tropical climates, because of it requires a warm, humid climate to survive. It is a small herb which grows up to 1 m in height. The leaves are ovate, oblong-ovate, or ovate-lanceolate, 4–13 cm by 1.5–4 cm with entire margin, while the flowers are small, tinged purple, or white **(Al-Snafi, 2015)**. Most *Capsicum* fruits are red but several other types with green, orange, and yellow colors exist. The seeds have a pale-yellow color, reniform, or discoid and are about 3–5 mm in size **(Li, 2014)**. *Capsicum annuum* such as several plants require well-drained, moisture-holding loam soil that contains organic matter **(Li, 2014)**. The optimal temperature requirement for growth and fruit production is in the range of 18–30 °C, while the optimum temperature for seed germination is 25–30 °C. Delayed flowering is encountered if daytime temperature is <25 °C, and at night temperatures above 32°C, flower buds are aborted. The viability of the pollen is considerably reduced when temperatures are higher than 30 °C and <15 °C, while night temperatures down to 15 °C favor the setting of fruits.

1.1.3 Importance of peppers

1.1.3.1 Economic importance

Capsicum species, most notably *C. annuum*, quickly spread around the world, becoming a part of local cuisines and a source of income for farmers worldwide **(Thampi, 2003)**. Capsicum exports alone generated nearly US$ 5.7 billion in 2011, making it one of the most important vegetables and spices in international trade. In several countries, native Capsicum diversity is gaining importance to develop processed food products for niche markets **(Reifschneider *et al.*, 2009)**. Pepper occupies a good rank among diversification crops for agricultural exports in Cameroon **(Segnou *et al.*, 2013)**. Prices are more and more attractive all year round, particularly for out-of-season production (between February and April). The crop, therefore, offers small farmers interesting incomes, thereby increasing their living standards.

1.1.3.2 Nutritional importance

Studies by nutritionists have shown that the only sustainable approach that will enhance micronutrient status in humans is by the integration of foods high in micronutrients in the diet, and vegetables are an effective source of micronutrients based on the production cost and unit of land occupied. Vegetables also have advantages over legumes and cereals in providing vitamins C and A as these are unavailable in these two sources **(Ali and Tsou, 1997)**. The fruits of pepper are rich sources of capsaicinoids, carotenoids (with some of them having provitamin A activity), flavonoids, tocopherols (vitamin E), and ascorbic acid (vitamin C). The consumption of chili is rising, and this may represent an essential source of vitamins for the world's populace. Several antioxidants, vitamins C, E, and provitamin A are sufficiently available in high concentrations in several pepper types. Peppers apart from carotenoids are also good sources of xanthophylls and may contain high amounts of vitamins B, (riboflavin) B2, (thiamine) B1, (niacin) B3, and P (citrin), and are richer sources of vitamins A and C than the regularly recommended food sources **(Bosland and Votava, 2012)**.

Values of provitamin A are expressed as retinol activity equivalents (RAE), in which 1 RAE is equal to 12 μg of β-carotene, 24 μg of α-carotene, and 24 μg of β-cryptoxanthin **(Wahyuni *et al.*, 2013)**. For men and women, recommended daily intake (RDI) values are 900 and 700 μg RAE/day, respectively. The levels of provitamin A per 100 g fresh weight of fruits are threefold to fivefold more in the red-, orange-, and brown-fruited cultivars of chili than in yellow-, purple, and green-fruited cultivars and amount to 5%–10% RDI/100 g fresh weight edible portion **(Wahyuni *et al.*, 2013)**. The daily vitamin A requirement of an adult can be met by the consumption of just 3–4 g (about half a tablespoonful) of ground red pepper **(Lantz, 1946)**. Also, several epidemiological studies have revealed that increased consumption of vitamin A or carotene can reduce cancer risk **(Ziegler *et al.*, 1986)**.

Vitamin C also referred to as ascorbic acid, a water-soluble vitamin is an important antioxidant and a cofactor for enzymes that partake in the metabolism of humans **(Wahyuni *et al.*, 2013)**. The fruits of Capsicum as well as several other vegetables, such as cucumber, tomatoes, carrots, lettuce, and broccoli, provide high contents of vitamin C. Vitamin C contents in pepper genotypes vary between 43 and 247 mg/100 g of fresh fruits. This may increasingly supply about 50% to over 100% RDI (Recommended Dietary Intake). The recent RDI for vitamin C in men and women is 90 and 75 mg/day, respectively **(Wahyuni *et al.*, 2013)**. A pepper fruit can contain six times as much vitamin C as an orange. Pepper fruits, from the green to the succulent red stage, each contain sufficient vitamin C that meets or exceeds the adult RDA (Recommended Dietary Allowance).

Besides some other vegetables in the human diet such as broccoli, asparagus, eggplant, and cabbage, peppers are also on the list of the best sources of natural vitamin E. On a dry weight basis, dry red pepper powder has α-tocopherol levels that are similar to those of spinach and asparagus and fourfold higher than that of tomatoes. The recommended daily intake of vitamin E is 15 mg/day of α-tocopherol for both women and men. Pepper fruits can supply above 100% α-tocopherol RDI per 100 g serving depending on the cultivar **(Wahyuni *et al.*, 2013)**.

Chilies are also an excellent source of minerals such as molybdenum, manganese, foliate, potassium, and copper (Table 1). **Ali and Tsou (1997)** in their attempt to indicate that vegetables can supply more protein and iron in certain instances per unit of land per day than cereals, standard micronutrients per 100 g of edible portion from various cereals and vegetables were multiplied with the various average per hectare per day yield of these crops grown under Taiwan conditions. In their report, pepper, when compared with cereals, produced double the amount of iron from a given piece of land daily.

Table 1: Nutrition values for 100 g of chili peppers.

COMPOUND	AMOUNT	% RECOMMENDED DAILY ALLOWANCE BY USDA
Vitamin A	428 IU	32%
Vitamin B-6	0.2 mg	39%
Vitamin C	143.7 mg	240%
Vitamin K	6.4 µg	-
Niacin	0.6 mg	-
Pantothenic Acid	0.1 mg	-
Iron	0.5 mg	13%
Copper	0.1 mg	14%
Potassium	145 mg	7%
Magnesium	10.4 mg	5%
Calcium	6.3 mg	-
Phosphorus	19.4 mg	-
Total Fat	0.4 g	-
Total Carbohydrate	9 g	3%
Dietary Fiber	1.5 g	6%
Sugar	5 g	-
Protein	1.9 g	3%
Lutein	319 µg	-

1.1.4 Pepper cultivation practices

Hot pepper is a major vegetable crop and an important constituent of local dishes in Africa **(Norman, 1992)**. Pepper is grown as a perennial fruit vegetable crop in the tropics, but it is also grown as an annual in the sub-tropics **(Swiader *et al.*, 1992)**. The optimum growth and yield of pepper depend on good-quality seeds and seedlings. According to **Norman (1992)**, no agronomic practices can compensate for poor quality seedlings in the nursery. Seedling quality depends on its ability to produce new roots, increase the speed of roots anchorage, and have a balanced shoot-to-root ratio **(Jaenicke, 1999)**. To survive the harsh environmental conditions in the field, looking good seedlings must develop very healthy and strong root systems. It is only a strong and healthy root system that can withstand some adverse arid conditions such as moisture stress, flooding, salinity, and nutrient deficiency **(Jaenicke, 1999)**. Seedling cultivation, transplanting, and harvesting are the three key periods of chili pepper production. Chili pepper seedling lasts one month, and the cycle of production after transplanting varies from 2.5 to 4 months **(Orobiyi *et al.*, 2016)**.

1.1.5 Cultivated area and yield performance

The total land area for the cultivation of pepper worldwide is estimated to be four million hectares with an average annual increase of 5% (**Weiss, 2002)**. However low production of hot pepper is attributed to poor varieties, poor cultural practices, and the prevalence of fungal, bacterial, and viral diseases **(Marame and Gelmesa, 2006)**. It can be grown entirely under irrigation, due to its sensitivity to an abundance of moisture and excessive temperatures **(Almuktar *et al.*, 2015)** and can also be grown in a well-drained, fertile loam soil. However, its maximum growth occurs at a temperature range between 21°C and 29°C (**Nikels, 2012)** and is sensitive to high levels of salinity **(FAO, 2003)**. Peppers normally germinate between 5 to 14 days in moist and nutrient-rich soil in a warm climate with temperatures ranging between 18 to 27°C during the day and between 15°C to 18°C at night **(Almuktar *et al.*, 2015; Campiglia *et al.*, 2010)**. The plant grows well in light and well-drained soil that is rich in organic matter such as sandy loam or loams with a pH value between 6.5 and 7.5. The maturity period is between 60 to 90 days **(Baudion and FAO, 2013)**.

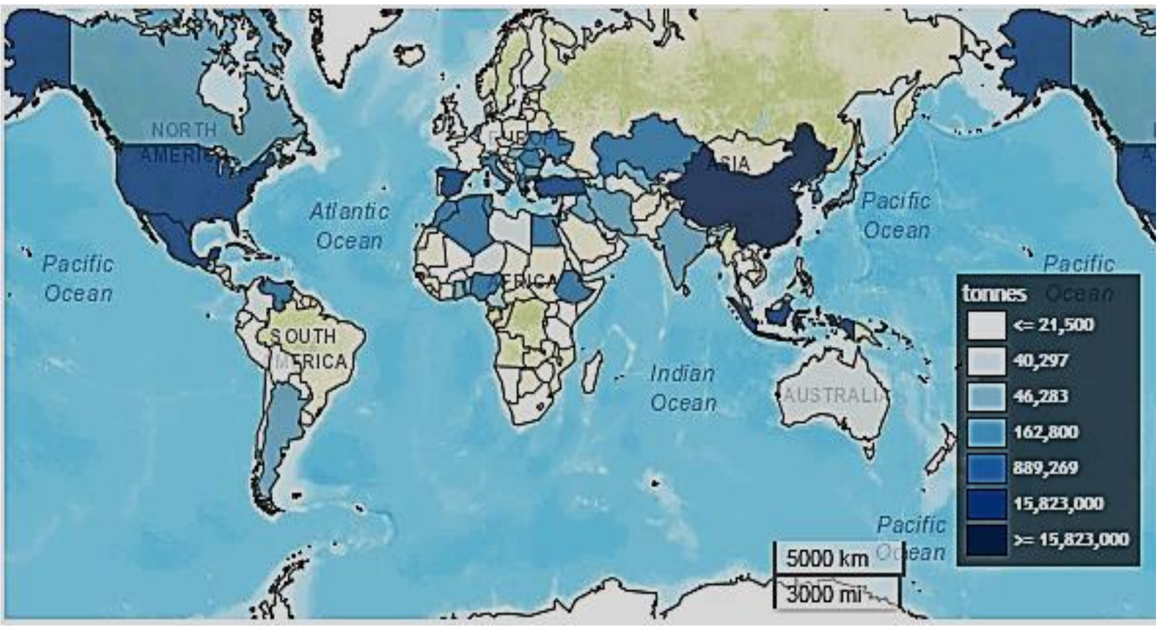


Figure 2: Pepper's production area in world (Faostat, 2014).

1.1.6 Pepper production in Cameroon

In central Africa, Cameroon is the main Peppers producer and the principal provider of the neighbouring countries. Between 2015 and 2017, an increase in the production of chili peppers of about 10 000 t. was recorded showing the capacities of Cameroon for the production of chili peppers **(FAO, 2020)**. This increase in production is thought to have resulted in numerous collaborative research efforts between the World Vegetable Center (AVRDC) and the Institute of Agricultural Research for Development (IRAD). The latter has led to key achievements like improved breeding technologies, leading to the release of high-yielding and disease-tolerant chili peppers seeds varieties to farmers. However, the current supply fails to satisfy the national and neighboring countries demands. In Cameroon, there is many varieties growth in all part of the country among them Safi variety (big red fruit) and big sun variety (big yellow fruit) are the most cultivated. Pepper is growth in all agro-ecological zone in Cameroon with an average yield of 2.1 tons/ha.

1.1.7 Pepper production constraints

1.1.7.1 Abiotic constraint

Several constraints are currently limiting the production of pepper among which abiotic and biotic are most represented. Abiotic constraints pertaining to the climate (drought, flooding, strong winds, extreme temperature and sunlight), and to the soil (moisture and nutrients content) may add up to biotic constraints and lead plants to stress leading to anatomical and physiological disorders that reduce yield **(Orobiyi *et al.*, 2016)**. One of the most common physiological disorders of pepper is blossom-end rot, a calcium deficiency disorder that appears only at the blossom end of the fruit **(Hochmuth and Hochmuth, 2009)**. The threat of abiotic constraints is getting increasingly alarming as a result of population growth and climate change with expected greater adverse effects in vulnerable regions such as semi-arid West and Central Africa **(Raza *et al.*, 2019)**.

1.1.7.2 Biotic constraint

Peppers are usually considered a robust crop compared to tomatoes. However, peppers are susceptible to several pests and pathogens causing considerable economic losses **(Gangwar and Gangwar, 2018)**. The most economically important pests to peppers in West Africa are thrips (*Frankliniella* sp) feeding on the leaves, flowers, or fruits; aphids (*Myzus persicae Sulzer, Aphis sp,* and *Macrosiphum euphorbiae Thomas***)** feeding on young leaves and shoots; root-knot nematodes (*Meloidogyne* spp) feeding on the roots; the Mediterranean fruit fly [*Ceratitis capitate* Wiedemann)] feeding on the fruit flesh; red spider mites (*Tetranychus* sp) feeding on the leaves; fruit borers (*Lepidopterae* sp) and whitefly (*Bemisia tabaci* Gennadius) feeding on the leaves **(Lawal *et al.*, 2015)**. In addition to damages caused to the plants by direct feeding, some pests such as nematodes, whiteflies, aphids and thrips are vectors of viruses. About 35 viruses are known to infect peppers worldwide **(Green and Kim, 1991)**. Among viruses, 11 were reported in Africa out of which Potyviruses and Germiniviruses are the most economically important and the most widespread in the Western and Eastern Africa sub-region **(Waweru *et al.*, 2019)**. The latter is restricted to semi-arid and arid zones.

In Cameroon, numerous constraints are reported to affect Chilli Pepper: (i) fruit fall off before maturity, due to attacks of fruit flies (*Ceratitis* spp., *Bactrocera* spp., etc.), (ii) *Fusarium* attacks, causing a fungal disease characterized by senescence followed by the sudden death of entire pepper plants, and (iii) a viral disease, transmitted by whiteflies (*Bemisia tabaci*) and characterized by leaf discolorations culminating in severe distortions of leaves or irreversible plant stunting **(Segnou *et al.*, 2013)**. These constraints are present in all pepper commercial production basins in Cameroon, and are responsible for significant marketable fruit yield losses.

1.2 VIRAL DISEASES OF PEPPER

Viruses are the major constraints in chili production worldwide because, at present, nearly 75 viruses are known to infect chili, of which 37 are the International Committee on Taxonomy of Viruses (ICTV) recognized species and six are tentative species **(Kenyon *et al* 2014).** As obligatory intracellular parasites, viruses depend on the host machinery to multiply and invade their hosts. Because of the plant's natural physical barriers (cuticle, cell wall), viruses are delivered into plant cells through wounds or through the action of vectors (insects, nematodes, fungi) that feed on or infect the plants **(Legg *et al.*, 2014)**. Following the entry into a host cell and genome decapsidation, the infectious cycle includes translation and replication of the viral genome, assembly of progeny virus particles, generalized invasion of the host, and finally, transmission to new hosts by vectors **(Czosnek *et al.*, 2002)**. Plant infection by viruses causes diseases of economic and agronomic significance in many crops. In 2012, the International Committee on Taxonomy of Viruses reported 92 genera of plant viruses **(King *et al.*, 2012)**, and geminiviruses were cited among the most economically impactful **(Harrison and Robinson, 1999; Kumar *et al.*, 2006)**. The former harms a wide range of hosts including but not limited to common beans (*Phaseolus vulgaris L.*), cassava (*Manihot esculenta*), cotton (*Gossypium spp.),* squash (*Cucurbita spp.*), Okra (*Abelmoschus esculentus*), tomato (*Solanum lycopersicum*) and chilli peppers (*Capsicum annuum L.*) **(Stansly and Naranjo, 2010)**.

1.2.1 Begomovirus

The whitefly; *Bemisia tabaci* sibling species complex is the whitefly vector for all viral species in the genus Begomovirus, family Geminiviridae **(Brown *et al.*, 2012)**. Begomoviruses are among the most important, emergent plant viral pathogens to food and fiber crops, causing considerable damage in subtropical–tropical, and mild temperate habitats (Figure 3) **(Navas-Castillo *et al.*, 2011)**. Begomoviruses have either a monopartite or bipartite genome that consists of single-stranded circular DNA of approximately 2.8 or 5.2 kilobases (kb), respectively **(Brown *et al.*, 2012)**. One type, previously called DNA-β, is now referred to as beta satellite and it is often required for wild-type symptom development in the respective naturally-infected host plant **(Briddon *et al.*, 2001; Zhou *et al.*, 2003)**. They require a helper begomovirus to support their replication, systemic infection, and whitefly vector-mediated transmission. Certain beta satellites have been shown to modulate symptom severity **(Briddon *et al.*, 2001)** and suppress gene silencing **(Cui *et al.*, 2004)**. Beta satellites contain one predicted ORF (Open Reading Frame) (>10 kDa) that encodes a protein, referred to as βC1, which has been shown to assist in helper virus accumulation by suppressing host-mediated silencing **(Cui *et al.*, 2004)**. In Africa, Okra leaf curl disease (OLCD) has been reported in Burkina Faso, Chad, Ghana, Mali, Nigeria, and the Ivory Coast **(N'Guessan *et al.*, 1992; Swanson and Harrison, 1993; Kon *et al.*, 2009)**. Throughout western Africa, from where okra (*Abelmoschus esculentus*) is believed to have originated, OLCD is the most damaging viral disease of okra. *Cotton leaf curl Gezira virus* (CLCuGeV) has been associated with OLCD in Burkina Faso, Niger, and Sudan **(Shih *et al.*, 2009; Tiendrébéogo *et al.*, 2010; Venkataravanappa *et al.*, 2011)**, and *okra yellow crinkle virus (*OYCrV) has been associated with the disease in Mali **(Kon *et al.*, 2009)**.

In Cameroon, typical symptoms of begomovirus infection and whitefly infestations have been observed in economically important crops such as cassava **(Fondong *et al.*, 2000)**, tomato **(Leke *et al.*, 2011)** and okra **(Leke *et al.*, 2007)**. Symptoms in okra can be quite variable but they usually manifest as leaf curling and distortion, a green or yellow foliar mosaic, stunting, and reduced yield.

1.2.2 Epidemiology

Pepper Leaf Curl Virus (PeLCV) infection is distributed in both the tropical and sub-tropical regions around the globe where different hosts are available **(Senanayake *et al.*, 2007)**. The climatic conditions like a sudden rise in temperature, rainfall and relative humidity, intensification of cropping systems and presence of alternate hosts enhance the whitefly population which leads to the rapid spread of begomoviruses and disease incidence **(Kenyon *et al.*, 2014)**. Warm weather conditions favor whitefly multiplication on host plants and there is a rapid increase in whitefly population even in the presence of natural enemies. Initially, under field conditions about 15–25% of chili plants were observed with typical leaf curl symptoms and whitefly transmitted PeLCV rapidly to reach about 50–100% infection in the field **(Senanayake *et al.*, 2007)**. The PeLCD (pepper leaf curl disease) incidence is directly correlated with the abundance of the whitefly population. *B. tabaci* is polyphagous, and it shows a high frequency of reproduction and quicker dispersal ability thus, facilitating its existence in large groups of populations with different agro-ecological zones. Even single whitefly has the ability to transmit the virus, whereas the presence of eight or more whiteflies on a plant may result in a 100% chance of transmission **(Senanayake *et al.*, 2012)**. In an epidemic spread, several isolates of PeLCV can be effectually transmitted by whiteflies, all of which can produce mild to severe characteristic symptoms in chili **(Senanayake *et al.*, 2012)**. *B. tabaci* can complete around 12–15 overlapping generations in a year. The young and adult flies suck the sap and colonize the ventral surface of the leaves. If activities like quick acquisition, fast transmission into the host, and faster lifecycles are maximized at a single geographical location then it may lead to the abrupt development of a new Begomovirus strain. *B. tabaci* transmits begomoviruses in a persistent manner which enables the spread of the virus through the juvenile, pupal, or adult stages of the whitefly. Adults of *B. tabaci* cannot fly efficiently however, can be transported for quite a long distance by the wind. Begomoviruses have the ability to manipulate the behavior of their vectors and this may improve their rate of transmission, consequently, these viruses affect the longevity and fertility of whitefly. However, the feeding habits and behavior of whiteflies can affect the population's genetics, behavior, and virus evolution **(Holkar *et al.*, 2017; Saxena and Tiwari, 2017)**. Recently, the various transmission parameters of PeLCV were estimated for the development of the simulation of PeLCD dynamics for devising management strategies. Subsequently, **Roy *et al.* (2021)** developed the spatiotemporal distribution of whitefly population.

1.2.3 Transmission

For transmission of begomoviruses, whitefly requires a minimum of 90 min of acquisition and 120 min of inoculation feeding periods **(Czosnek *et al.*, 2002)**. *B. tabaci* are the lonely mediator that transmit begomoviruses in a circulative-persistent manner. Upon entry of the virus by ingestion, it gets translocated via the digestive system to hemolymph, the salivary glands, and then expelled in the phloem of the plant. For the transmission of begomoviruses, the vector possesses many proteins which facilitate efficient viral transmission. During the infection process, the insect vector delivers viral particles presumably in the uncoated form to the plant and the viral genome is transported into the host cell nucleus. The viral genome encodes only a few proteins therefore, for their DNA replication they rely largely on the cellular DNA replication proteins. Begomovirus particles of 22 × 38 nm in size have two (geminate) incomplete T = 1 icosahedral capsid particle containing viral CP and each CP is bound by seven bases of viral ssDNA **(Hesketh *et al.*, 2018)**. As CP is the only structural protein in the geminiviral capsid, it is necessary for viral capsid assembly. In addition, CP also plays a crucial role in viral DNA transportation by interacting with cellular transporters as exemplified by mono-partite begomoviruses **(Sharma and Ikegami, 2009)**. The CP of several monopartite begomoviruses possess a nuclear localization signal and a leucine-rich nuclear export signal and are thus localized in the nucleus (especially nucleolus), cytoplasm, and cell periphery **(Unseld *et al.*, 2001)**. A rich variety of interactions has evolved between viral proteins and host factors to develop the virus replicative cycle. In the first stage, once the viral genome is released from the virus capsid, it enters into the cytoplasm of the host cell and subsequently enters the nucleus, where it undergoes a rolling circle and recombination-dependent replication process **(Gutierrez, 1999)**. These newly replicated viral ssDNAs can be (1) converted into dsDNA which can act as a template for another round of replication or transcription, (2) wrapped by viral movement proteins for transportation from the infected cell to adjacent cells through plasmodesmata, or (3) encapsidated into infectious virions for long-distance virus movement **(Gutierrez, 1999; Hanley-Bowdoin *et al.*, 2013)**. The complex of viral DNA movement in begomoviruses has the ability to bind ssDNA or dsDNA and can transport the viral DNA to neighboring cells. Furthermore, begomoviruses possess a limited coding potential and thus rely heavily on host proteins to complete their infection cycle. Begomoviruses depend on host enzymes for their replication and transcription processes, coordinate with several cellular mechanisms to modulate cell division, cell cycle, and manipulate host components at different cellular levels **(Hanley-Bowdoin *et al.*, 2013)**. Transmission of a monopartite genome alone can lead to infection, but the presence of DNA A or DNA B helper genomes plays an important role in symptom development. Begomovirus proteins have a significant impact on a variety of host cell pathways which include changes in plasmodesmata structure and function, host cell defense mechanisms, and changes in gene expression in the infected cell **(Peele *et al.*, 2001)**.

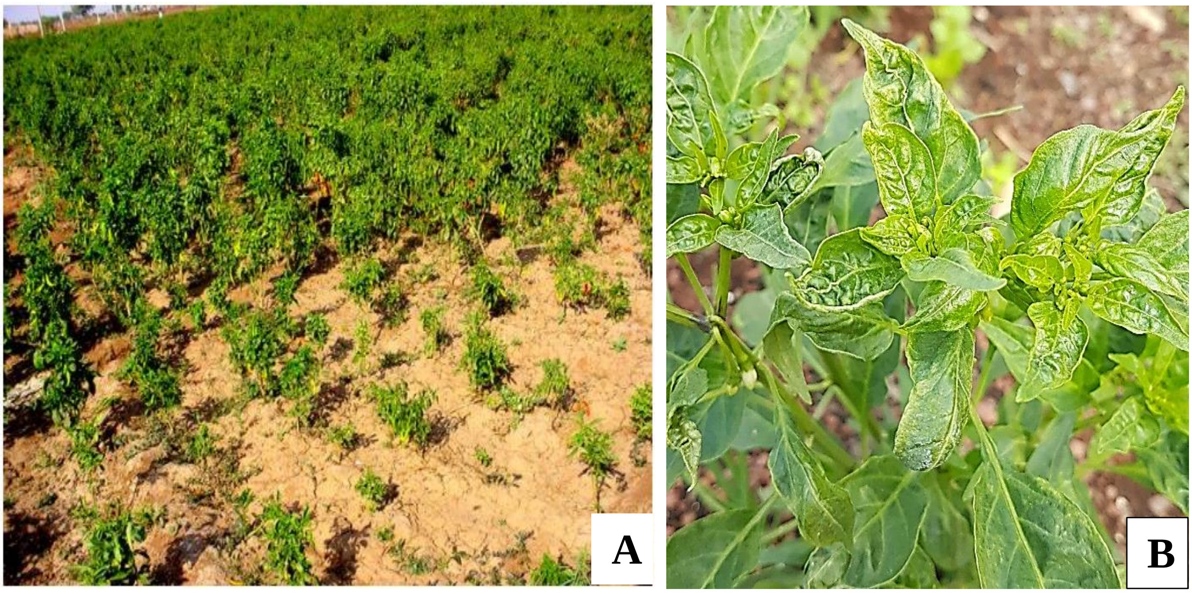


Figure 3: Symptoms of leaf curl virus in field. (A) severe filed affected, (B) plant showing begomovirus symptoms (leaf curling and plant stunting) (Senanayake et al., 2012).

1.3 Whitefly *Bemisia tabaci*: vector of pepper leaf curl virus

1.3.1 Taxonomy, Origin, and Distribution of Whitefly *B. tabaci*

Bemisia tabaci was first reported and named Aleyrodes tabaci by Gennadius in 1889, as a pest of tobacco in Greece. Currently, it is distributed worldwide inhabiting every continent of the world except Antarctica **(Kanakala and Ghanim, 2019)**. In contrast, some European countries, such as Finland, Sweden, the Republic of Ireland, and the United Kingdom, are still yet to report the existence of *B. tabaci* **(Gangwar and Gangwar, 2018)**. Whitefly taxonomy is based exclusively on puparial characteristics; however, very little taxonomic information can be found on non-puparial life stages **(Hodges and Evans, 2005)**. Sexual dimorphism in these puparia contributes to existing knowledge of the whitefly's taxonomic tools of identification, development, reproductive potential, and management **(Baig *et al.*, 2016)**. The family Aleyrodidae is divided into three subfamilies: Udamoselinae, Aleyrodinae, and Aleurodicinae **(Liu *et al.*, 2015)**. In recent years, important progress has been made at the taxonomic level based on the analysis of the mitochondrial cytochrome oxidase subunit I (mtCOI) gene, with at least 43 species complexes of *B. tabaci* identified **(Wosula *et al.*, 2020)**. The Middle East–Asia Minor 1 (MEAM1) and Mediterranean (MED) complexes (previously known as B biotype and B. argentifolii, and Q biotype, respectively) are considered the most invasive species with a broad host range of plants **(Casinga *et al.*, 2022)**. The nature of their broad host range and the global trade of *B. tabaci* host plants may have contributed to their worldwide distribution **(Wosula *et al.*, 2017)**.

1.3.2 Biology of Whitefly *B. tabaci*

Whiteflies have a characteristic life cycle of six stages: the egg, four immature stages (nymphal instars), and the adult stage **(Sani *et al.*, 2020)**. Temperature, relative humidity, and host plants are the main factors that greatly influence the life cycle of whitefly species **(Li *et al.*, 2017)**. *Bemisia tabaci* deposit eggs on the upper and lower leaf surfaces of plants (Figure 4), and the number of eggs deposited is significantly affected by temperature, with 28°C being the most favorable for *B. tabaci* production **(Li *et al.*, 2017)**. Eggs laid are pear-shaped (approximately 0.2 mm long), with a gleaming white color that darkens over time, and usually incubate for about 5–9 days depending on the host species, temperature, and humidity **(Gangwar and Gangwar, 2018)**. Soon after hatching, the first instar (crawler) travels to a short distance until it successfully probes the leaf to feed on the phloem sap before undergoing three more nymphal instar stages (second, third, and fourth) **(Sani *et al.*, 2020)**.

During the second instar stage, the whitish-yellow nymphs turn yellowish and dome-shaped after feeding. The pale yellow freshly molted third instar nymphs, however, gradually turn dark yellow and more flattened in shape after feeding **(Sani *et al.*, 2020)**. The fourth instar nymphs have a yellowish-white color with large eyes visible through the integument; this stage is also known as the “pupal” stage or “red-eye nymph” **(Sani *et al.*, 2020)**.

Fully developed adults of *B. tabaci* emerge from the dorsal surface of the pupal case through an inverted “T”-shaped slit **(Sani *et al.*, 2020)**. An adult is yellow-bodied with a pair of white wings that form an inverted V-shape covering the thorax and abdomen. The abdomen of a *B. tabaci* female is large and round-shaped, while that of the male is pointed **(Henneberry *et al.*, 2007**). The entire life cycle of *B. tabaci* from egg to adult takes approximately 16 to 31 days, with some differences between the duration of each stage depending on the host plants used to rear them **(Lindquist, 1991)**.

1.3.3 Damage and Losses Caused by *B. tabaci*

Bemisia tabaci can cause significant economic losses to crops by causing damage to the host plants during feeding through secretion of honeydew and transmission of plant viruses (Figure 4) **(Czosnek *et al.*, 2002)**. Both nymphs and adults of *B. tabaci* cause damage by inserting their mouthparts into the plants during feeding and by transmitting a large number of viruses that can severely damage susceptible plants species **(Gangwar and Gangwar, 2018)**.

1.3.3.1 Feeding Damage

It has been found that *B. tabaci* nymphs can inject enzymes that cause changes in plant physiology, leading to irregular ripening of fruit and retarded internal coloration **(Sani *et al.*, 2020)**. The honeydew excreted by *B. tabaci* provides a medium for the growth of sooty mold on the leaves and fruits, thus reducing photosynthetic activities, which could negatively affect the quality of farm produce **(Legg *et al.*, 2014)**. In addition, the feeding of *B. tabaci* on leaves can cause yellowing and crumpling, which subsequently results in stunted plant growth and deformed fruits **(Sani *et al.*, 2020)**.

1.3.3.2 *Bemisia tabaci* as a Virus Vector

More than 200 plant viruses are able to be transmitted by *B. tabaci*, with the majority of these viruses belonging to the genera Begomovirus, Carlavirus, Crinivirus, Ipomovirus, and Torradovirus **(Lu *et al.*, 2019)**.

Some of the most vulnerable crops to these viruses are cassava, cotton, cowpea, cucurbits, crucifers, eggplants, tobacco, tomato, potato, soybean, sweet potato, okra, lettuce, pea, bean, poinsettia, chrysanthemum and pepper **(Sani *et al.*, 2020)**. Of all the viruses transmitted by *B. tabaci*, Begomoviruses are well known as the leading cause of yield losses in crops, ranging from 20–100% and losses worth millions of dollars **(Gangwar and Gangwar, 2018)**.

The method of transmission provides knowledge about the periods of virus acquisition and inoculation, which can be used to develop effective management strategies **(Czosnek *et al.*, 2002)**. Plant viruses, such as Criniviruses, Carlaviruses, Ipomoviruses, and Torradoviruses, are transmitted in a semi-persistent manner, while the Begomoviruses are transmitted in a persistent circulative manner **(Navas-Castillo *et al.*, 2011)**.

Semi-persistent transmission of viruses usually requires at least 15 min of acquisition access with a retention time of up to days in the foregut **(Czosnek e*t al.*, 2017)**. In contrast, several hours are required for acquisition access in the persistent transmission, with retention time in the hemolymph of up to the entire life of the vector **(Navas-Castillo *et al.*, 2011)**. In the persistent transmission mode, virus moves from the foregut and into the hemolymph through the midgut of *B. tabaci* before being transported into salivary glands to be egested with saliva into the plant tissues **(Czosnek *et al.*, 2017)**.

1.3.4 Control and Management of Whitefly

IPM is an internationally recognized approach to pest control and is intended to reduce ecological and health damage caused by chemical pesticides. The IPM program for *B. tabaci* includes biological control, crop plant resistance, physical and mechanical methods, and using selective chemical pesticides when necessary **(Horowitz *et al.*, 2011)**. Host plant resistance to whiteflies has been successfully developed due to the growing concern over the increasing use of synthetic chemical pesticides. Cultivars from different varieties of cotton, tomato, pepper and other field crops have been screened against *B. tabaci* and many other sap-sucking insects **(Sani *et al.*, 2020)**. However, the selection and development of resistant cultivars against whitefly-transmitted viruses in breeding programs are quite challenging, because there is a need to screen and inoculate large numbers of plants to select genotypes with resistance genes **(Senanayake *et al.*, 2012)**.

Physical and mechanical methods are techniques emphasizing the creation of unfavourable environments for pests, which include the removal of pest breeding sites and the use of healthy seedlings and resistant varieties **(Sani *et al.*, 2020)**. Cultural methods such as crop rotation could increase host periods or reduce intercrop migrations through careful consideration of the types and special arrangement of planted crops, thus, ultimately leading to the control of the *B. tabaci* population **(Stansly and Naranjo, 2010)**. The application of an electric field screen to greenhouse windows can prevent the entry of whitefly, but requires the presence of a guard at the greenhouse entrance area **(Takikawa *et al*., 2015)**.

Based on the principle of IPM, pesticides should be the last choice for farmers to use when other options are not successful against the infestation of pests in crops **(Taquet *et al.*, 2020)**. The effect of chemical pesticides on the non-target organism, environmental contamination, and resistance of insect pests have led to research on biological control agents as alternative control measures. *B. tabaci* can be effectively controlled by integrating multiple biological control agents such as parasitoids, predators, and entomopathogen fungi **(Sani *et al.*, 2020)**.

Figure 4: Whitefly Bemisia tabaci on leaves surface of chili plant at different magnification (Kepngop, 2022 in this study).

1.4 Management of viral disease in peppers

Being large genera of the Geminiviridae family, begomoviruses alone, are accountable for the enormous devastation of many economically important field crops **(Gangwar and Gangwar, 2018)**. The epidemiology caused by these viruses is a global concern, and its management is a prime necessity. There are three main means of managing virus diseases in pepper crops: (1) preventing or reducing the rate at which the virus reaches the crop, (2) preventing or reducing the rate at which the virus spreads once it reaches the crop, and (3) reducing the severity of the disease caused by the virus and thus reduce the economic impact on yield and quality of the product **(Legg *et al.*, 1999)**. These can be achieved using different management strategies (Figure 5A) though the relative efficacy or importance of each measure will differ depending on the type of virus and how it is transmitted.

1.4.1 Cultural practices

There are many cultural measures that can be used to help address the first two routes. Most pepper production starts with sowing the seed in a nursery bed, pots, or plugs, and the young seedlings are subsequently transplanted to the net house/glass house/polytunnel (protected cultivation) or open field **(Kenyon *et al.*, 2014)**. Virus disease management has to start with seedling production and continue for most of the vegetative duration of the crop, with a slightly different set of practices for protected and open-field cultivation. Seedling production areas should be located away from field production areas and be kept free of weeds, volunteer pepper plants, and other plants that can serve as overwintering or alternative hosts to viruses and/or vectors **(Arogundade *et al.*, 2020)**. Because the seedling stage is generally very susceptible to virus infection, in most locations it is advantageous to grow the seedlings in a fine-mesh net cage or net house that excludes flying or crawling virus vectors such as aphids, whiteflies, and thrips **(Legg *et al.*, 2014)**. Alternatively, if the hedge around the crop is dense enough it may act as a physical barrier, preventing or delaying the vectors from reaching the crop. Using reflective plastic mulch may deter some vectors, such as whiteflies or aphids, from alighting on the pepper plants, feeding, and transmitting the virus **(Arogundade *et al.*, 2020)**. Close and regular monitoring of the crop and removing diseased plants from the field as soon as virus symptoms are noticed will remove the virus inoculum and reduce the spread within the field. Care should also be taken to avoid spreading viruses from plant to plant during the usual crop management operations in the field: work in areas where the disease has been or is known to be last present, after working in unaffected areas; and regularly disinfect tools and equipment by soaking in bleach solutions or washing in strong detergent solutions **(Kenyon *et al.*, 2014)**.

1.4.2 Chemical control

Control measures for the high infestation of *B. tabaci* mostly rely on the application of a wide variety of insecticides (Figure 5B). This technique is highly reliable because of its efficiency and convenience **(Horowitz *et al.*, 2011)**. Conventionally practiced insecticides belong to the class of organochlorines, organophosphates, carbamates, and pyrethroids. Diafenthiuron aromatic ether is one of the most widely used insecticides **(Thakur *et al.*, 2018)**. However, the profuse and intensive application of insecticides (with similar repeated chemical compounds) leads to the development of a resistance mechanism in whiteflies against particular insecticide classes. Thus, this has exacerbated the chemical control measure tactics. Subsequently, these insecticides have been replaced with a novel group of insecticides that are more specific to target and effective at low concentrations. Neonicotinoids (imidacloprid and thiamethoxam), the Ketoenols (spiromesifen and spirotetramat), Diamides (flubendiamide, chlorantraniliprole, cyclaniliprole, tetraniliprole, and cyantraniliprole), and insect growth regulators such as buprofezin, pyriproxyfen are among the novel classes of insecticides. Moreover, some researchers have reported the resistance development in *B. tabaci* against these new classes of insecticides too **(Horowitz *et al.*, 2020)**. Alone and continuous usage of toxic and harmful pesticides has adversely disturbed the environment and mankind. Conventionally, ChiLCV management has mainly relied on the application of insecticides to control the vector whitefly. The use of pesticides is less effective, expensive for farmers, and causes hazards to the environment, consumers, as well as farmers **(Borah and Dasgupta, 2012)**. Furthermore, the presence of pesticide residues limits the export of chili. Besides, the uses of insecticides to control the vector whitefly are mostly unproductive as they are usually applied after the appearance of symptoms wherein the virus might have already been transmitted **(Kenyon *et al.*, 2014)**. The usage of these toxic pesticides can be reduced and the efficiency can be elevated by implementing alternative integrated control strategies which involve the use of a combination of pesticides and other management tactics **(Naranjo and Ellsworth, 2009)**. The efficiency can also be enhanced by the use of a mixture of insecticides however, these insecticides must be used in label recommendations. Besides these synthetic insecticides, the use of natural plant product extracts or biopesticides has been mentioned in some contexts. The biopesticides, neem seed kernel extract, and neem oil are effective in the reduction of disease incidence. Sapindus trifoliatus, and Solanum trilobatum seed extracts, the leaf extract of Clerodendrum aculeatum and the bark extract of Terminalia arjuna have been found to be effective biocontrol agents **(Chaubey *et al.*, 2017).** Although there are several challenges in ChiLCV resistance breeding in chili, efforts on identification and introgression of resistant traits through resistance breeding is a very important option for reducing the input cost to get good returns to the chili growers worldwide and that would help to avoid virus-vector epidemics in future.

1.4.3 Biotechnological Control

- **Pathogen-derived resistance**

Classical methods of controlling virus infection have been proven to be unsuccessful against PeLCD. The breeding approaches provide alternative methods to produce resistant sources (Figure 5C, page 26); however, they have their limitations, lack of resistant germplasm to Begomoviruses in chili. Pathogen-derived resistance (PDR) is a more effective and fast approach for developing resistance against plant viruses in various crops **(Powell *et al.*, 1990)**. PDR strategy relies upon the post-transcriptional gene silencing (PTGS) mechanism for the successful utilization of virus-derived genes to get rid of viruses **(Patil *et al.*, 2011)**. Amongst PTGS, RNAi is one of the efficient technologies to induce resistance against viral pathogens. RNAi is a native defense antiviral system mediated through double-stranded RNA (dsRNA) which involves the degradation of sequence-specific viral RNA. Dicer-like proteins process the dsRNA into small 21–24 nts RNAs which interferes with the viral RNA **(Sharma *et al.*, 2015)**. In a study by **Sharma *et al.* (2015)**, a demonstration of the ability of RNA silencing approaches to limit PeLCV infection was attempted. Effectiveness was found through over-expression of AC1/AC2/βC1 specific dsRNA targeting various PeLCV species. The PTGS strategy of RNAi through dsRNA results in the biogenesis of target-specific 21-24 nt small RNAs which governs begomovirus resistance in transgenic chili plants. **Mishra *et al.* (2020)** identified chili miRNAs specific to essential genes of the PeLCV through computational methods. They have predicted chili-encoded miRNAs targeting CP (V1) and Rep (C1) genes that could be used for silencing against PeLCV infection. RNAi method will have to overcome some limitations like the ability to withstand natural field conditions and heavy loads of viruses, rapidly evolving PeLCV variants that escaped sequence-specific recognition in RNAi mechanism, and also uncertainties about the release of genetically modified (GM) crops. Recently, a novel strategy of application of a cocktail of dsRNAs in Nicotiana benthamiana was attempted to prevent PeLCV infection through RNAi **(Singh *et al.* 2022)**. In this study, under in vivo conditions, three separate dsRNA molecules were prepared using E. coli strain HT115 with the three suppressor genes, C2, V2, and C4 of PeLCV. Further, an equal concentration of three dsRNAs (dsC2, dsV2, and dsC4) with 0.1% celite was used to prepare a cocktail of dsRNAs. A single spray of the cocktail of dsRNAs reduced PeLCV incidence up to 66.7% in N. benthamiana plants for 2 weeks.

- **Transgenic approach**

Biopesticides are potent microbial pesticides and biochemicals obtained from microbes and other natural resources. Advancement in plant genetic transformation technology has made it easy to overexpress the potent biomolecules into crop plants thereby conferring resistance against insect pests. Successful application of biopesticides depends on exact information of feeding habits and life cycle of the insect and the mechanism of action of the potent biopesticides **(Arogundade *et al.*, 2020)**. This facilitates the determination of the precise time and stage of biopesticide application for efficient results. To date, δ-endotoxin from *Bacillus thuringiensis* (Bt) is the most commonly used commercial biopesticide. The advantage of Bt biopesticides is their high specificity to target insects (*Lepidoptera, Coleoptera,* and *Diptera*). Nevertheless, the sap-sucking homopteran whiteflies remain insensitive to Bt toxin. **Shukla *et al.* (2016)** reported the Tma12 gene from an edible fern (*Tectaria macrodonta*) has insecticidal activity against whitefly. Tma12 overexpressing cotton transgenic lines were resistant to whitefly infestation and cotton leaf curl viral disease, with no detectable yield penalty. There is scope to identify such insecticidal proteins that are lethal to whiteflies. These biopesticide molecules need to be expressed in chili to overcome the whitefly-borne PeLCV.

- **CRISPR/Cas system (Clustered regularly interspaced short palindromic repeat/RNA-guided Cas9 nuclease)**

In the last decade, the CRISPR/Cas (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) system has successfully emerged as one of the promising and precise genome-editing methods **(Kale *et al.*, 2021)**. The CRISPR/Cas system was reported for the first time in plants in the year 2013. Recently, gene editing by using the CRISPR/Cas system has rapidly introduced genetic manipulations to achieve resistance against plant viral diseases **(Ali *et al.*, 2016; Tashkandi *et al.*, 2018; Yin *et al.*, 2019)**. Generally, two different mechanisms are present in the CRISPR-Cas system governing antiviral activities viz., to recognize, interfere, and cleave the virus genome so as to inhibit of multiplication of invasive viruses and to manage host susceptibility factors essential for virus replication and ultimately improve plant immunity and block the viral attack. Severely affecting members of the family of Geminiviridae consist of around 485 ssDNA species. Multiple strategies have been initiated to destroy and get rid of the genomic DNA of begomoviruses through gene editing methods. Amongst the gene-editing methods, CRISPR/Cas system is more convenient for its simplicity in construction and design over the use of transcription activator-like effector nucleases (TALENs) and Zinc finger nucleases (ZFN). Therefore, this method has become more dominant and found promising for developing antiviral engineering in plants. In the transgenic model plant system, N. benthamiana CRISPR/Cas9 single guide RNA (sgRNAs) constructs specific to viral Rep, or IR region, showed effective DNA intrusion which thereby conferred resistance against *Cotton leaf Curl Multan Virus* (CLCuMuV) **(Yin *et al*., 2019)**. One more study reported CP or Rep specific sgRNA mediated effective interference of *Tomato Yellow Leaf Curl Virus* (TYLCV) genome in both the transgenic tomato and N. benthamiana lines **(Tashkandi *et al*., 2018)**. Interestingly, **Ali *et al*. (2016)** reported that the stem-loop specific sgRNAs exhibit more efficient interference of various begomoviruses (Cotton leaf curl Kokhran virus, Merremia mosaic virus, and TYLCV) than the sgRNAs targeting CP and Rep regions of the viral genome **(Ali *et al*., 2016)**. CRISPR/Cas-mediated virus resistance in plants can also be established by targeting host factors. For plant virus protein translation, the recruitment of host-specific translation factors to facilitate their infection processes is required. Such host-specific translational factors are recognized and targeted as pro-viral factors. Further, in the recent past, numerous other pro-viral host factors, such as movement, replication, and metabolism-associated regions were characterized and used to limit plant virus diseases. eIF4E, eIF4G, and their isoforms are effective host translation initiation factors, which are exploited to achieve defense against various sub-species of viruses. **Chandrasekaran *et al*. (2016)** reported the eukaryotic initiation factor, eIF4Eknockout in cucumber through CRISPR/Cas9, which exhibits complete resistance to viruses involved in papaya ringspot, zucchini yellow mosaic, and cucumber vein yellowing diseases. Recent advancements in CRISPR/Cas9 technologies have resulted in increasing reports of efficient plant DNA virus resistance. As an example, this CRISPR/Cas9 method targeting MP or CP region established resistance to TYLCV (Tashkandi et al., 2018). **Roy *et al*. (2019)** designed multiplexed sgRNA targeting PeLCV genome and their approach was effective in removing the *Pepper Leaf Curl Virus* (PeLCV) genome. Thus, CRISPR/Cas system becomes a potent tool for plant defenses against viruses and offers new avenues for genetic modification of chili against devastating Pepper leaf curl virus disease (PeLCD). Although these advanced methods like RNAi and CRISPR/Cas systems hold the promise of achieving robust resistance, care has to be taken on various potential drawbacks associated with these methods. Like host and virus, vectors can also be tapped for this novel technique, since, *Bemisia tabaci* is the only vector of begomoviruses and a serious pest of agricultural and horticultural crop plants. Recently, **Heu *et al*. (2020)** have developed a CRISPRCas9 gene editing technique for silver leaf whitefly based on the vitellogenic adult females instead of embryos. The ovary-targeting peptide ligands were attached to Cas9 and inserted into adult females of *B. tabaci.* The offsprings were found to have a heritable edited genome. Thus, the development of a gene-editing procedure for *B. tabaci* will be helpful for the researchers to use the application of reverse genetics and will lead to the management of this serious insect vector.

- **Resistant germplasms**

Over the years since the 1970s, many monogenic and polygenic virus resistance quantitative trait loci (QTL) have been detected, and many have been identified and mapped in *Capsicum* species. But most provide only partial resistance controlled quantitatively by multiple genes. The action of these QTLs is either to restrict virus entry and uncoating (installation) in the host cells **(Caranta *et al.*, 1997)**, restrict virus multiplication, or restrict the long-distance movement of the virus. Despite the increasingly devastating effect of whitefly-transmitted leaf curl viruses (begomoviruses) of pepper in much of Asia, Central America, and West Africa, there are as yet (as far as we are aware) no commercial pepper cultivars resistant to any of the viruses in this large group because integrated genes are transiency and they need fine monitoring to avoid "off-type" results prior to the implementation into modern farming (Figure 5C, Page 26) **(Faoro and Gozzo, 2015; Walters *et al.*, 2013)**.

1.4.4 Biological control

As aforesaid, the profuse and uncontrolled use of chemicals has resulted in insecticide resistance in the population of whitefly and also impacted the environment. Thereby, alternative practice such as biological means of control management (The using of biological tools such a microorganisms) has been put into practice to combat unwanted effects of insecticides/pesticides on crops, the environment, abiding pest resistance, and resurgence **(Legg *et al.*, 2014)**. The potential biological control agents constitute the use of natural enemies against *B. tabaci*, which primarily include predators, parasitoids, and entomopathogenic fungi **(Ou *et al.*, 2019)**. Some of the effective predators include *Amblyseius swirskii*, *Neoseiulus cucumeris, Amblyseius tamatavensis, Coccinella septempunctata, Chrysoperla carnea, Clitostethus arcuatus, Orius spp, Chrysopa sp., Sinea confuses*, etc. **(Soleymani *et al.*, 2016; Cavalcante *et al.*, 2017;)**. The ladybird beetle, *Serangium japonicum* is also considered an important predator of whiteflies **(Tian *et al.*, 2017)**. Parasitoids of *Encarisa* sp. and *Eretmocerus* sp. have been reported as efficient biocontrol agents in whitefly management. The biological control practice of using solitary natural enemies might be difficult in suppressing the whitefly population. But, the effectiveness of biological control agents can be elevated by using a combination of any two types of natural enemies. In this way, the use of insect parasitoids *Eretmocerus hayati* in combination with entomopathogenic fungi *Cordyceps javanica* resulted in higher efficiency against *B. tabaci* **(Ou *et al*. 2019)**. In integrated pest management approaches, the efficiency of the biological agents might get reduced by the application of insecticides. However, a proper assessment of the lethal effect of the use of insecticides on biocontrol agents must be optimized to increase their efficiency **(Tize *et al.*, 2023)**.

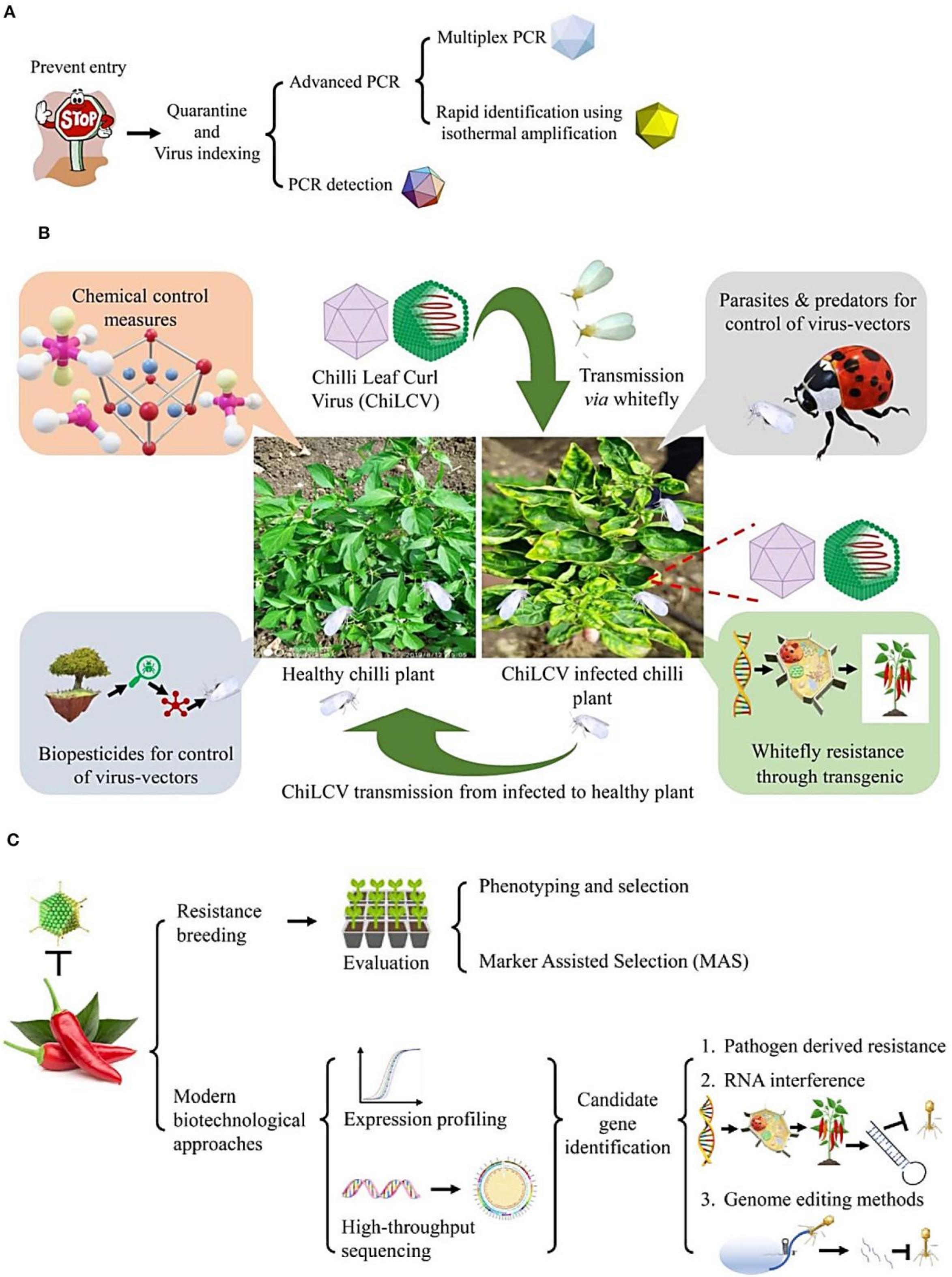


Figure 5: Conventional and non-conventional strategies for management of pepper leaf curl disease (PeLCD) in chili: (A) Strategies for pepper leaf curl virus (PeLCV) management, (B) strategies for management of PeLCD through virus-vector control, and (C) strategies for improvement of PeLCD resistance in chili.

1.5 Fungi as biological control agents

Apart from their ability to improve nutrient uptake and nitrogen use in plants, fungi also have biocontrol capabilities. They can aid in the fight against pests like nematodes and microbial pathogens (bacteria, fungi, and viruses) that infect various parts of the plant such as roots, foliage, and fruits. They offer protection against diseases with the help of processes like mycoparasitism, competition for resources with pathogens, antibiosis, conferring ISR to the host plant, and mycovirus-mediated cross-protection **(Singh and Giri, 2017)**. Among biocontrol agents, fungi from the *Trichoderma* genus are very well-established as potent agents **(De Palma *et al.*, 2019)**.

1.6 *Trichoderma* spp. a promising bioagent against PLCV

1.6.1 History of the Taxonomy of *Trichoderma* spp.

The term *Trichoderma* was first introduced into mycology in 1794 by Persoon **(Bissett, 1991a)**, but its classification remained obscure and contradictory until 1969 when **Rifai** made the first attempt to produce a workable classification system of the kind. The division of the genus *Trichoderma* into species has been the subject of many studies and much controversy. In the living kingdom, the limits of the "species" are based on the possibility of interbreeding between individuals. However, the anamorphic fungi of the genus *Trichoderma*, as such, have no known sexual reproduction, and this character cannot, therefore, be used for their systematics. This is based on the cultural aspects and the morphology of the sporogenous apparatus, as well as on molecular biology techniques **(Harman and Kubicek, 2014)**. If we briefly list the most important dates that have marked the systematics of *Trichoderma*, we quickly realize that their taxonomic positioning was not easy.

In 1794, **Persoon** described the first *Trichoderma* sp and established four species.

In 1821 **Fries** classified *Trichoderma* spp. among the Gasteromycetes.

In 1860, controversies on this systematic began, and **Tulasne** contradicted **Fries** since he did not find teleomorphic (sexual) forms in this genus.

In 1871, faced with the growing number of species encountered, **Harz** insisted on the importance of morphological characters under optical microscopy (especially the phialides).

In 1916, **Waksman** described what he found to be six new strains of *Trichoderma* using macroscopic criteria, different from those advocated by **Harz**.

In 1926, **Abbot** identified four species of *Trichoderma* according to criteria different from the previous ones. Until 1939, Abbot's reasoning remains valid, but alongside completely independent identifications.

In 1939, **Bisby** tried to bring order to these systems by proposing a single species: *Trichoderma viride*. For 24 years, any fungal species with green spores was considered to be *Trichoderma* spp.

In 1963, the work of **Gutter and Monbasher** ended the previous system by demonstrating the variability of *Trichoderma* species according to environmental conditions.

In 1969, aware of all this controversy, **Rifai** proposed a classification that could be used with the concept of "aggregate species", based on microscopic characters. “An aggregated species is an entity composed of groups of very similar species that are difficult to separate”. Nine aggregated species are created (*T. aureoviridae* Rifai, *T. hamatum* Bain, *T. harzianum* Rifai, *T. koningii* Oudemans, *T. longibrachiatum* Rifai, *T. piluliferum* Webster and Rifai, *T. polysporum* Rifai, *T pseudokoningii* Rifai, and *T. viridae* Gray) while tolerating some variability within each aggregate species **(Rifai, 1969)**. Compared to many previous classification systems, this system seems to be the easiest to use by the scientific community, especially since it was recently improved by **Bissett (1991b, 1984)**.

In 1991, Bissett proposed the concept of "section" to deal with the growing number of new species of *Trichoderma*, unrelated to the aggregated species. Based on the morphology of conidiophores and phialides, it groups aggregated species into five sections (Figure 6) (*Trichoderma, Pachybasium, Hypocreanum, Longibrachiatum, and Saturnisporum*) **(Leuchtmann *et al.*, 1996)**. Bissett's taxonomic system is supported by molecular biology approaches (PCR) to respond to the positioning of newly identified *Trichoderma* species (whose teleomorph forms are often unidentified) and remains the most reliable currently.

Over 200 species of the genus Hypocrea have been identified, but are rarely cultivable, and therefore little described in modern terms **(Harman and Kubicek, 2014)**. Under certain unknown conditions, Hypocrea sp. (teleomorphs) transform definitively into *Trichoderma* sp. (anamorphs). It is then thought that evolution led to the disappearance of the sexual mode for the establishment of a genus with exclusively asexual reproduction **(Harman and Kubicek, 2014)**. Molecular biology now reveals to us that genetically different species of *Trichoderma* present spectacular morphological similarities and their characteristics overlap, which explains the long controversy known by this genus before **(Sugiyama, 1987)**. Modern mushroom taxonomy has abolished the phylum Deuteromycotina, to which the genus *Trichoderma* belonged. The current taxonomic position of *Trichoderma* is as follows **(Bissett *et al.*, 2015)**: Phylum: Amastigomycota and/or Eumycetes; Division: Ascomycota; Subdivision: Pezizomycotina; Class: Sordariomycetes; Subclass: Hypocreomycetidae; Order: Hypocreales; Family: Hypocraceae; Genus: Trichoderma

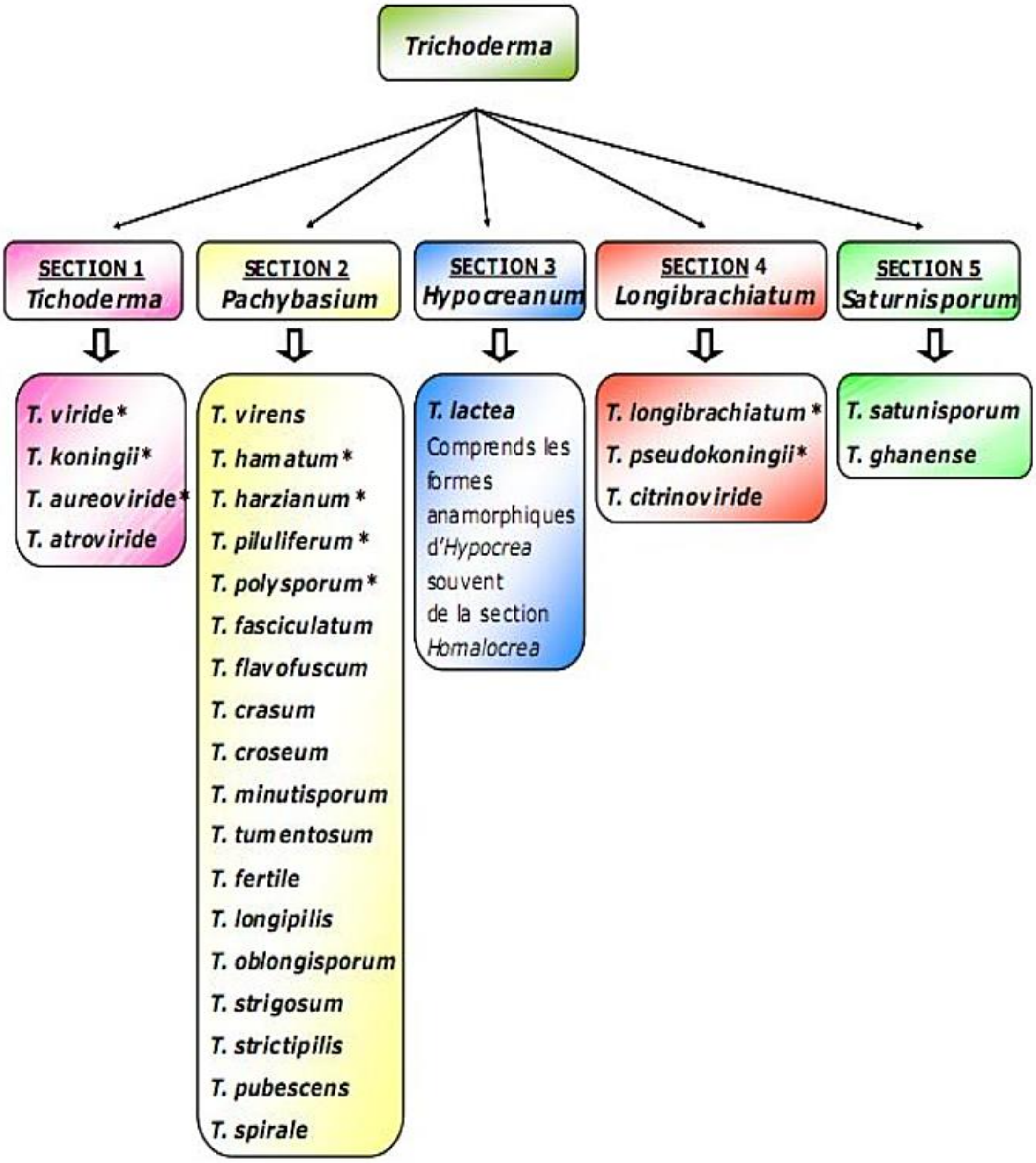


Figure 6: Representation of the 5 systemic sections of Trichoderma and some of the species belonging to it according to Bisset (1991a and b).
The sign * indicates the aggregated species of Rifai (1969).

1.6.2 Ecology

Trichoderma is the most widespread genus in the soil and is found in a wide variety of habitats. This may be due to the competitive nature of *Trichoderma* species and the possibility of producing various metabolites **(Gams and Bissett, 2002)**. According to **Kubicek *et al*. (2003)**, the abundance of *Trichoderma* spp. in ecosystems is due to their ability to produce various bioactive substances and enzymes. They are, therefore, a link in the biological chains. *Trichoderma* is commonly found in soil, wood, and plant debris **(Dubos, 1985)**. *Trichoderma* species are favored by the presence of a large number of roots which they easily colonize **(Gams and Bissett, 2002)**. The dynamics of Trichoderma populations are linked to environmental conditions, a strong desiccation of the soil for a long period leads to a decline in populations **(Papavizas, 1985)**. However, if the soil is hydrated, the propagule rate returns to its original value.

1.6.3 Morphology

Since the revision of the genus *Trichoderma* by **Rifai, (1969)**, morphological characteristics have been used to characterize and distinguish *Trichoderma* species **(Bissett *et al.*, 2015)**. In addition, **Samuels *et al.* (2015**) provided detailed observations on the morphological characters of defined species of *Trichoderma.*

1.6.3.1 Macroscopic characterization

Certain colony characteristics such as growth rate, pigmentation, pustule formation, and odor may be characteristic criteria of a species. However, colony appearance does not provide sufficient information for characterization due to the difficulty in establishing an accurate description **(Harman and Kubicek, 2014)**. The genus *Trichoderma* is characterized by a rapid and intensive growth rate with abundant sporulation **(Harman and Kubicek, 2014)**. According to **(Samuels *et al.*, 2015)**, the majority of *Trichoderma* cultures grow rapidly at temperatures between 25 °C and 30 °C and generally, no growth is observed at 35 °C. However, some species grow well at 35 °C. This served as an important distinguishing criterion between morphologically similar species. For example, *T. harzianum* can be distinguished from morphologically similar species, such as *T. aggressivum* and *T. atroviride* by growing them at 35 °C. After 96 hours of incubation at 35 °C, the diameter of the colonies of *T. aggressivum* and *T. atroviride* does not exceed 5 mm, while that of *T. harzianum* shows good growth and sporulation **(Samuels, 1996)**.

Trichoderma is easily recognizable in culture thanks to the generally green color of its spores **(Dubos, 1985)**. *Trichoderma* conidia give rise to an initially white mycelium. Two days later, a green color is visible on the aerial parts of the mycelium, corresponding to conidiogenesis **(Chabasse *et al.*, 2002)**. The conidia typically form within a week in compact or slightly flaky tufts of greenish, whitish, and sometimes yellowish color. The colonies are stained according to the pigmentation of the phialides. Characteristics of mycelium development and pigmentation can be better observed in a rich medium such as PDA **(Samuels *et al.*, 2015)**.

Some species of *Trichoderma* shed a yellow-colored pigment in the PDA medium, and others, such as *T. viride*, produce a characteristic odor similar to a "coconut" odor **(Gams and Bissett, 2002; Sharma and Singh, 2014)**.

1.6.3.2 Microscopic characterization

Trichoderma species usually form vegetative hyphae that are septate, hyaline, and smooth-walled **(Gams and Bissett, 2002)**. The conidiophores (Figure 7) are strongly branched. Side branches produced from main branches may or may not be paired and sometimes may be branched. Normally, the branch form at a 90° angle to the main branch. Paired branches have a pyramidal structure. Conidiophores have a conical or pyramidal shape. Very branched, they carry phialides in the form of flanges or skittles. In turn, phialides carry the spores (phialospores or conidia). Flask-like structures called phialides are found at the tips of the conidiophores. Phialospores, also known as conidia, are produced at the end of phialides where they accumulate to form a conidial head **(Gams and Bissett, 2002)**. In some species, the main branches are terminated by sterile or fertile elongations **(Samuels *et al.*, 2002)**.

The conidia are unicellular and either ellipsoidal (3-5×2-4 µm, L/l = 1.3) or globular (L/l < 1.3). They are usually green, or sometimes colorless, grayish, or brownish. Their surfaces are generally smooths, but rough-surfaced conidia are found in a few species such as *T. viride* **(Gams and Bissett, 2002; Samuels *et al.*, 2002)**.

Chlamydospores play a role in survival. They are large, thick-walled vegetative cells with condensed cytoplasm **(Lin and Heitman, 2005)**. These unicellular globulars to subglobular chlamydospores are formed inside or at the ends of the hyphae. They are colorless, pale yellow or greenish **(Gams and Bissett, 2002; Samuels *et al.*, 2002)**. These generally unicellular chlamydospores can be multicellular in some species such as *Trichoderma stromaticum* **(Samuels *et al.*, 2002; Sharma and Singh, 2014)**.

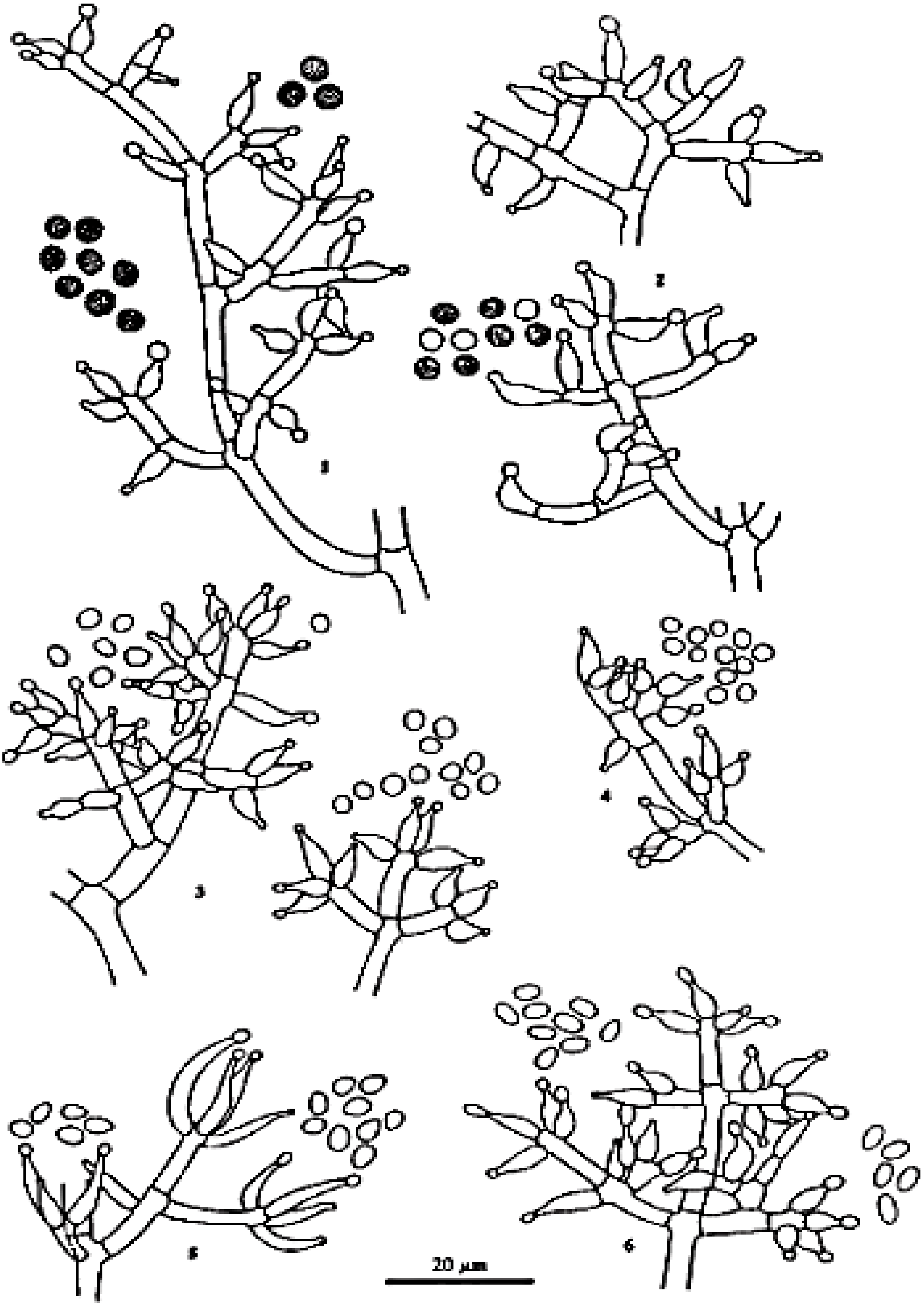


Figure 7: Morphological appearance of conidia, conidiophores, and phialides of Trichoderma spp.: 1. T.Viride; 2. T. Atroviride; 3. T. Harzianum; 4.T.Inhamatum; 5. T. aureovirid; 6. T. koningii (Gams and Bissett, 2002).

1.6.3.3 Molecular characterization

The morphological identification of fungi of the genus *Trichoderma* is very complex and represents a break in the studies concerning these microorganisms. Based on reproductive structures, this identification is difficult and sometimes even impossible to achieve **(Johnson *et al.*, 2002)**. it is due to the complexity of the proposed identification keys, based on the morphological criteria that are sometimes difficult to identify **(Ko, 1998; Wu *et al.*, 2003)**. Molecular methods based on DNA sequence polymorphism and PCR amplification have proved very useful in the characterization of isolates of this genus, both in identification studies and in the development of phylogenetic classifications **(Kullnig-Gradinger *et al.*, 2002)**.

Genes encoding ribosomal RNAs (rDNA) in *Trichoderma* are the regions most used in phylogenetic and taxonomic studies **(Lieckfeldt *et al.*, 1999; Samuels *et al.*, 2002)**. These molecules present in all organisms are involved in the process of protein synthesis. The sequences are organized into tandem repeat units of the genes encoding 18S rRNA, 5.8S rRNA, and 28S rRNA. Each ribosomal gene is separated by intergenic spaces. The spaces between 18S rDNA and 5.8S rDNA and between 5.8S rDNA and 28S rDNA are internal spaces of transcribed ribosomal regions (ITS1 and ITS4 respectively) that are then, excised during rRNA maturation. The outer space of the ribosomal transcribed regions (ETS) and the intergenic space (IGS) separate the large and the small ribosomal subunit (Figure 8). The existence of these contiguous and conserved regions differs during evolution and allows the comparison of organisms at the level of species, family, order, class, and even kingdom **(Kageyama *et al.*, 1997)**. Indeed, the coding regions are highly conserved, which makes it possible to compare relatively distant organisms. On the other hand, the much less conserved internal regions make it possible to compare close organisms.

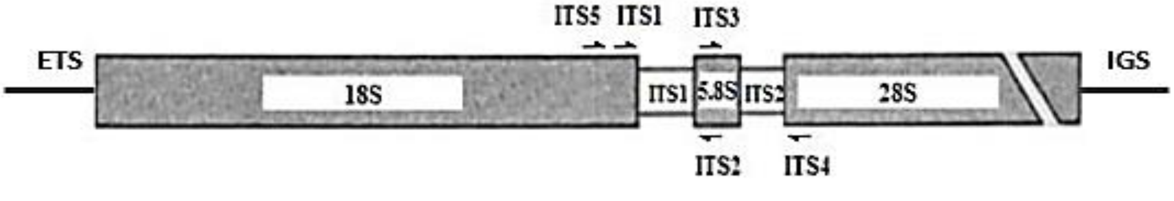


Figure 8: Schematic representation of rDNA showing the 18S, 5.8S and 28S genes with the ITS1 and ITS4 regions (White et al., 1990).

The nucleotide sequences of the ITS regions are excessively used since **(White *et al.*, 1990)** developed universal primers allowing their amplification by PCR (polymerase chain reaction). Indeed, several authors have been interested in the sequencing of these regions, especially in the characterization of fungi of the genus *Trichoderma* **(Druzhinina *et al.*, 2011; Kullnig-Gradinger *et al.*, 2002; Singh *et al.*, 2014)**.

1.6.4 *Trichoderma*: biological control agent and plant growth promoter

According to the definition of **Cook and Baker (1983)**, biological control consists in reducing the density of a pathogenic agent and/or its activity (the infectious potential) by implementing one or more organisms other than a man. The use of a biological control method does not seek to prevent the total eradication of the invasive species but, its objective is to reduce its numbers sufficiently and sustainably to take them below a threshold of harmfulness, ecologically and /or economically acceptable. The main objective is therefore to restore a sustainable balance between the biological control agent and the pathogen **(Suty, 2010)**.

The use of *Trichoderma* in biological control to protect cultivated plants dates back to the 1930s. Their antagonistic properties have been known for a long time since the first publication mentions them in 1887. However, the in-depth study of the phenomenon of antagonism and its application as means of combating plant parasites only began between the two world wars. The models studied were mainly interested in soil parasites but already, but in 1952 Wood reported the effectiveness of *Trichoderma viride* in controlling Botrytis cinerea on lettuce.

1.6.4.1 Importance of *Trichoderma* spp. in agriculture

Trichoderma spp. are predominant over wide geographic regions in all climatic zones and can be isolated from nearly every soil, decaying wood, compost or other organic matter **(Hoyos-Carvajal *et al.*, 2009)**. *Trichoderma* spp. are remarkable for their rapid growth and capability of utilising diverse substrates. Biopreparations based on *Trichoderma* spp. as an effective biocontrol agent for plant diseases caused by soil-borne fungi have been reported. Its uses keep growing in importance as the demand for more environmentally friendly management strategies for plant disease increases **(Divya Rani and Sudini, 2013; Akrami and Yousefi, 2015)**. It has been estimated that 90% of all antagonistic fungi used in plant protection belong to this genus (**Benitez *et al*. 2004**). Some of the species are of economic importance because they produce enzymes and antibiotics. According to **(Hoyos-Carvajal *et al*., 2009)**, *Trichoderma* spp. can form intimate associations with plant roots, providing an endemic level of biological control or stimulating plant growth by producing soluble forms of mineral nutrients and growth-promoting metabolites. For these facts, this genus is of great interest for sustainable agriculture.

1.6.4.2 Biocontrol capacity of *Trichoderma* spp.

Trichoderma spp. is remarkable for its rapid growth and the capability of utilizing diverse substrates. Its uses keep growing in importance as the demand for more environmentally friendly management strategies for plant disease increases **(Akrami and Yousefi, 2015; Divya Rani and Sudini, 2013)**. It has been estimated that 90% of all antagonistic fungi used in plant protection belong to this genus. *Trichoderma* spp promotes plant growth, increases nutrient availability, improves crop production, activates an efficient reactive oxygen species (ROS) detoxification system, produces antimicrobial compounds against pathogens, and increases sensitivity to respond successfully to later pathogen invasion termed as induced systemic resistance (ISR) **(Shoresh *et al.*, 2010; Shoresh and Harman, 2008; Vinale *et al.*, 2008)**. Trichoderma species are soil-borne filamentous fungi known for their utility in many plants health benefit applications **(Kumar *et al.*, 2021)**. Its strains deploy a complex mechanism in pathogen control that includes colonizing the soil and root of the host, inhabiting a physical space, and evading the multiplication of the phytopathogens while concomitantly producing cell wall-degrading enzymes and antimicrobial metabolites to kill the pathogens, inducing plant defense mechanisms, promoting plant development and improving plant tolerance to biotic and abiotic stressors **(Zin and Badaluddin, 2020)**.

The induction of specific resistance mechanisms in the host plant in response to colonization by *Trichoderma* is an obvious principle in the protective capacity of this plant pathogen control agent **(Shoresh *et al.*, 2010)**. This resistance can be either localized or systemic (Figure 9). This then implies indirect protection resulting at the cellular level by anatomical, metabolic, and physiological reactions stimulating the expression of a variety of compounds involved in the defense of the plant such as proteins linked to pathogenicity (PR: pathogenesis-related), phytoalexins, phenylalanine ammonia-lyase (PAL) and phenolic compounds **(Harman *et al.*, 2004; Shoresh *et al.*, 2010)**.

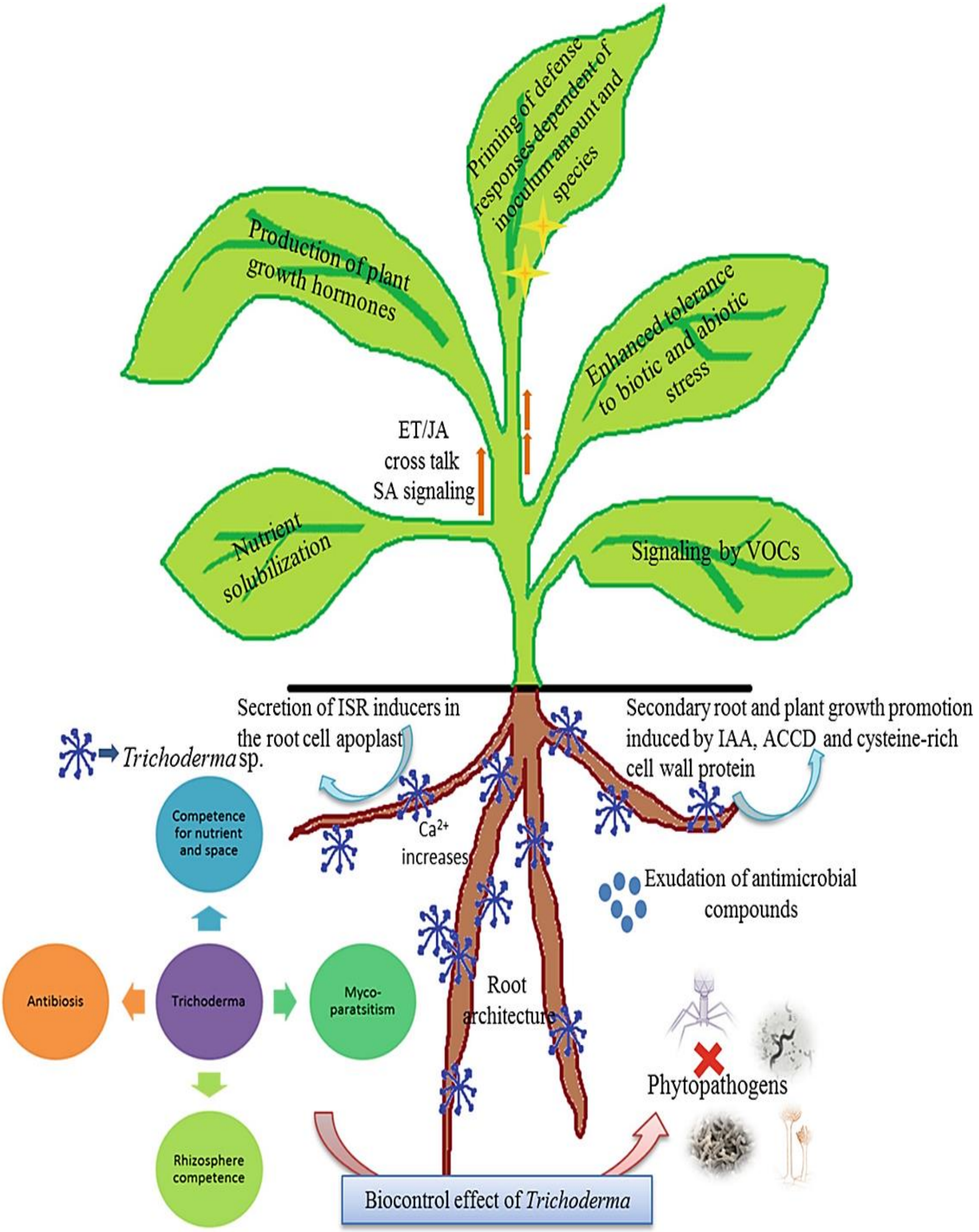


Figure 9: Plant defense, priming, and systemic resistance as mechanisms for Trichoderma spp. biocontrol (Gupta and Bar, 2020).

Chapter II
MATERIALS AND METHODS

2 CHAPTER II: MATERIALS AND METHODS

2.1 MATERIAL

2.1.1 Plant material

The *Capsicum annuum* seeds used in the framework of this study were the red variety (Safi variety) which are the most cultivated and contains 5 times more provitamins A than yellow variety (Big Sun). Before each test, seeds with no cracks or other visible deformations were surface sterilized with 1 % sodium hypochlorite solution for 5 minutes, rinsed thrice with sterilized distilled water and air dried.

2.1.2 Characteristics of substrates used in pot experiments

The experimental soil, (Rhodic kanduidlut; U.S. soil taxonomy) was collected from an agricultural farm near the University of Yaoundé I. The averaged chemical and elemental composition are [P: 1.54 ppm; K: 0.28 Cmol/kg; Ca: 3.97 Cmol/kg; CH_3COONa: 0.52 mM; Clay: 51.9%; Mg: 0.92 Cmol/kg; Al: 0.43 Cmol/kg; Na: 0.023 Cmol/kg; pH (water): 5.89; Mn: 0.02 Cmol/kg; N: 11.17]. The soil was air-dried, sieved (4 mm), and mixed along with river sand at 3:1 (W/W). The resulting mix was then autoclaved (121 °C, 1 h) twice.

2.1.3 Fungi species and origin

The *Trichoderma* isolates (Figure 10) were obtained from the core collection of the Antimicrobial and Biocontrol Agents Unit of the Laboratory for Phytobiochemistry and medicinal plant studies, Cameroon. They were selected based on their biological control against fungi **(Eke *et al.*, 2016b)**. Before experiments, inoculums were prepared by inoculating young colonies of each BCA in a conical flask seeded with Potato dextrose broth medium (PDB, Himedia) for seven days under constant stirring (150 rpm). Afterward, the resulting suspension was filtered and adjusted at desired titer (10^6 spores/mL) using the Mallassez counting chamber.

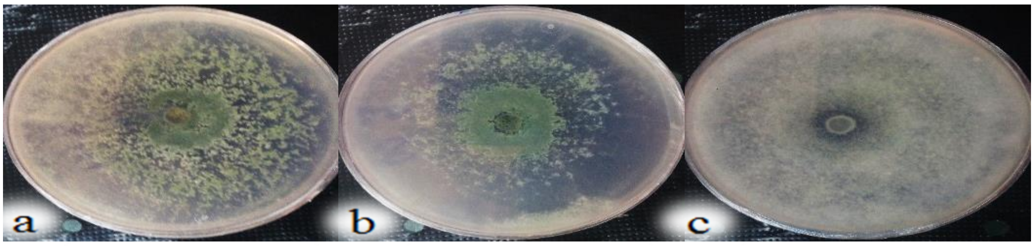


Figure 10: Trichoderma strains used in this study (a: Trichoderma I1, b: Trichoderma I2, c: Trichoderma I3) (Kepngop 2022, in this study).

2.1.4 Source of whiteflies and rearing

Whiteflies (*Bemisia* spp.) used as a vector in this study were collected on pepper seedlings showing PeLCD symptoms as described by **Kumar *et al.* (2006)** in commercial hot pepper (Chilli pepper) farm in Bangangte, West Region, Cameroon (Longitude 10°50’00” E; Latitude 5°15’00” N) and some field in Yaounde central region, Cameroon (E 11°29'53.4192"; N 3°51'50.7708"). In each field, 20 plants were randomly selected along two diagonal transects across the field and whiteflies were counted from the top five leaves of ten plants per transect. Whiteflies were aspirated alive directly in glass vials and those for identification were directly preserve in 95% ethanol in vials, before being stored in the freezer at -20°C.

Their identity was verified by Professor Kekeunou Sevilor of the Zoology Laboratory, Department of Animal Biology and Physiology, University of Yaoundé I, Cameroon. The collected insects were identified using the morphological protocols described by many authors **(Bellows *et al.*, 1994; Delvare and Aberlenc, 1989; Malumphy *et al.*, 2009; Martin, 1987; Martin and Mound, 2007).** The insects were reared in isolation on young pepper seedlings grown in net cages on a laboratory bench (Figure 11). Two weeks before the tests, viruliferous whiteflies were produced by feeding matured *Bemisia* spp on diseased (*PeLCD*) pepper seedlings maintained in an insect-proof net cage. During the suppression test, the viruliferous whiteflies that were created were utilised to inoculate experimental plants.

Figure 11: Cages used during rearing and infection of pepper plant (Kepngop, 2022 in this study).

2.2 METHODS

2.2.1 *Trichoderma* isolates identification

2.2.1.1 Microscopic identification of *Trichoderma*

The three isolated *Trichoderma* were identified at the genus level according to **Barnett and Hunter, 1972**. Thereafter, the identity was taken to the species level based on the microscopic observations following the taxonomic keys of **Rifai (1969),** and the online interactive key provided by **Samuels (1996)**. The Phialids' shape and size, spores and colony aspects and color on PDA were the main characteristics evaluated. The microscopic examination was carried out after mounting the cultures in lactophenol cotton blue. The isolated ones displaying the same cultural and microscopic features were clustered in morphotypes. For our work, among isolates, the three used for this work were taken to genomic identification to avoid duplicates.

2.2.1.2 Molecular identification of *Trichoderma* isolates

a) Genomic DNA extraction

The identity of the studied BCA was ascertained based on the 5.8s ribosomal DNA gene sequences. Before the PCR, the hyphal tips of each *Trichoderma* isolate were grown in an Eppendorf tube filled with Potato Dextrose Broth (PDB, Himedia, India) for 72 hours at 28±2 ^{o}C. Mycelial mats were spun down by centrifugation (13000 rpm, 5 min) and washed with 500 µL TE (TrisCl, EDTA) buffer. Later on, 300 µL of extraction buffer (200 mM Tris HCl, pH 8.5, 250 mM NaCl, 0.5% SDS, and 25 mM EDTA) was added to each pellet and crushed with liquid nitrogen. Subsequently, 150 µL of 3M Sodium acetate, pH 5.2 was added and tubes were placed at -20 ^{o}C for 30 minutes. The tubes were thereafter centrifuged (1000 rpm, 10 min). The supernatant was transferred to another sterile Eppendorf tube. An equal volume of isopropanol was added and kept for 10 minutes at room temperature. DNA was precipitated by centrifugation (12000 rpm, 10 min). After washing with 70% ethanol, the pellet was dried for 5 minutes, resuspended in 50 µL TE buffer, and preserved at -20 ^{o}C for further use.

b) Polymerase Chain Reaction

PCR products of the 5.8s ribosomal fragment (approximately 600 bp) were produced using the ITS 1 and ITS 4 primers, using a thermocycler (Applied Biosystems™ GeneAmp^{R} PCR system 9700), under the following conditions: first cycle of denaturation at 95°C for 2 min, were followed by 35 cycles of denaturation at 94°C for one min, primer annealing at 55°C for 1 minute, elongation at 72°C for 2 minutes and final extension at 72°C for 10 min and finale hold at 4°C. A total reaction mixture of 25 µL was made up of PCR buffer (0.2X), PCR water (14.95 µL), $MgCl_2$ (0.16 mM), dNTP (0.144 mM), ITS1 (forward primer, 0.01 µM), ITS4 (reverse primer 0.01 µM), Taq polymerase (0.5 mU/µL) and genomic DNA (100 ng). The PCR products were electrophoresed in 1% agarose gel stained in SYBR Gold (Invitrogen, Oregon, USA.) at 100 V for 30 min in gels buffered with 1×TAE buffer. DNA bands were visualized while using UVP GelStudio plus and only samples with intact bands were selected for sequencing.

Table 2: Primers characteristics (White et al., 1990)

Primers	Sequences (5'-3')	Length	Tm (ºC)	GC (%)
ITS-1	TCCGTTGGTGAACCAGCGG	19	53	58
ITS-4	TCCTCCGCTTATTGATATGC	20	50	45

ITS (Internal Transcribed Spacer region of fungal ribosomal DNA), **Tm** (melting temperature), **GC** (Percentage of nucleotide G and C)

c) Sequencing and phylogeny

PCR products were sent to Psomogen Inc. (Rockville, MD, USA) for purification and direct bi-directionally sequenced to obtain maximum length. DNA sequences data were assembled manually through the 5.0 BioEdit sequence analyser software (Ridom GmbH., Würzburg, Germany). The sequences were assembled into contigs using CLC Main Workbench 22 (QIAGEN, Aarhus, Denmark). Multiple alignments of edited sequences were performed using Clustal W in MEGA version 11 **(Kumar *et al.*, 2016)**, and the sequences were trimmed. Construction of a maximum-likelihood phylogenetic tree was performed using MEGA with 1000 bootstrap replicates the identities of each studied isolate were confirmed by the closest match method **(Sheoran *et al.*, 2015).** Sequences were blasted using GenBank's (NCBI) Blastn and selected reference sequences with 99% to 100% identity to our 5.8s ribosomal sequences were included in the phylogenetic tree for comparison with previously published haplotypes **(Márquez *et al.*, 2007).**

2.2.2 Evaluation of the capacity of *Trichoderma* isolates to produce phytohormone

2.2.2.1 Evaluation of the capacity of *Trichoderma* isolates to produce indole acetic acid

Principle

In the presence of Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution) indole acetic acid (IAA) reacts to form a pink color which presents a maximum absorbance at 530 nm.

Procedure

Indole acetic acid is a hormone involved in plant root development. The capability of a given BCA living endophytically to produce IAA is a prove of potential triggered host plant growth. A spectrophotometric method, described by **Goswami *et al.*, 2013** was utilized to detect and quantify IAA produced by the tested *Trichoderma*. Supernatant from 72 hours broth culture (PDB, Himedia India) supplemented with Tryptophan (200µg/mL) and NaCl (2% was mixed in a 1:1 ratio with salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). The development of the pink color indicated the production of IAA. The optical density readout was done at 530 nm (Tecan Infinite M200 microplate reader), and the concentration of IAA was estimated using a standard curve of pure IAA prepared at the range of 10-100µg/mL.

2.2.2.2 Evaluation of the capacity of *Trichoderma* isolates to produce Salicylic acid

Principle

In the presence of ferric chloride, salicylic acid is characterized by the formation of purple coloration. This purple complex presents a maximum of absorption at 527 nm and the amount of salicylic acid is proportional to the absorbance.

Procedure

The production of SA by *Trichoderma* was quantified according to **Meyer *et al.* (1992)**. *Trichoderma*'s conidial load of 10^6 cells/L was seeded in test tubes containing succinate medium and incubated under constant stirring (100 rpm) for 48 hours (28±2°C). Later on, the suspension was centrifuged (6000 g, 5 min) and acidified (pH 1.8) with HCl (1N). Salicylic acid was afterward extracted with chloroform and the solvent evaporated with a rotary evaporator (BUCHI 001) set at 40°C. The water-free extract was then re-suspended in water, and 5µL of ferric chloride solution ($FeCl_3$) was added. The appearance of purple coloration indicated SA production. The optical density (OD) of the purple ferric-salicylic acid complex developed was measured at 527 nm (Tecan Infinite M200 microplate reader). The concentration of SA released by each *Trichoderma* single or in consortia was estimated against a standard curve of pure SA plotted with a concentration range of 10-100mM.

2.2.3 Assay for rhizospheric efficiency

2.2.3.1 Endophytic power

- **Inoculum preparation, seed inoculation, and gnotobiotic growth.**

To make sure that the studied BCA's can colonize the inner parts of pepper seedlings, inoculums of each *Trichoderma* were prepared and made to 10^6 conidia/mL using a sterile saline solution **(Singh *et al.*, 2016)**. Their respective double and triple combinations of *Trichoderma* (v/v) were also prepared by mixing up the equal volume of each counterpart. Peppers seeds were surface-disinfected by soaking in 1.5% sodium hypochlorite for 5 min, followed by two runs of rinses with sterilized distilled water. The sterile seeds were dipped in conidia suspensions for two hours and subsequently placed in Petri plates provided with sterile moistened tissue paper **(Eke *et al.*, 2016a).** The control seeds were dipped in sterile distilled water. Both the controls and inoculated plates were incubated at ambient conditions for two weeks.

- ***Trichoderma* recovery and enumeration**

Young Pepper seedlings (1g) from each treatment were surface disinfected in 1.5 % Sodium Hypochlorite (5 min) and 70% Ethanol (1 min), followed by three times rinsing with SDW (sterilized distilled water). The sterilized seedlings were afterward ground in 10 mL sterile Phosphate Buffered Saline (PBS; pH 7.2) and allowed to settle for 15 minutes. The supernatants were separated from the residues by centrifugation at 5000 rpm for 5min. This supernatant was further diluted and seeded onto chloramphenicol amended (100 mg/L) PDA (Himedia. India) at 28^{o}C. *Trichoderma* colonies were counted in the Petri dishes and expressed in terms of colony-forming unit (CFU) per gram of fresh weight **(Sheoran *et al.*, 2015)**. In addition, the hyphal tips of each emerging *Trichoderma* were seeded onto a fresh PDA medium, and their cultural and microscopic characteristics were compared to the initial inoculums **(Rifai, 1969; Samuels, 1996).** The experiment was performed in triplicate and repeated twice.

- **Assessment of *in vitro* cohabitation of the Biological Control Agent**

Principle

It consists to grow many strains of biological control agents in the same petri dish to evaluate if there can be antagonism between them.

Procedure

It is established that consortia of BCA's rather than single are likely to perform well in open field conditions over single BCA. To ascertain the ability of tested *Trichoderma* to share the same niche, PDA media was prepared and poured in different petri dishes and then, 5-day old mycelia plug of each *Trichoderma* isolate, was seeded at opposite positions on the PDA medium as T1 with T2, T1 with T8, T2 with T8 and T1T2T8 and incubated after. The cohabitation was positive in case no inhibition zone was formed at the contact zones of the antagonists.

2.2.3.2 Evaluation of the effect of *Trichoderma* on seed germination

The BCA and their consortia were grown onto PDA medium for one week and the spore was harvested in sterilized saline solution (0.85%) and adjusted at 10^6 spores/mL as described by **Singh *et al.*, 2016**. To evaluate the impact of *Trichoderma* on pepper seed germination, surface disinfected seeds were treated with Carboxymethyl Cellulose (CMC, 1.5%) as an adhesive and coated with *Trichoderma* conidia suspension (10^6 conidia/mL). Seed treated with CMC only served as control**.** The seeds were placed in Petri plates provided with two layers of tissue paper plotted with sterile distilled water. Five Plates of 25 seeds each were prepared for each treatment. Seeds were considered to have sprouted when the radicle was 3 folds the size of the seeds. The number of germinated seed were monitored daily till total constant values and the following germination parameters were calculated as described at (1) (2) (3).

The germination means time (GMT)

$$\mathbf{GMT = \sum D \times n / \sum n} \qquad \mathbf{(1)}$$

Where; GMT; germination mean-time; D: days of seeds germination evaluation (3, 6, 9, 12, and 15 days) and n; the number of seeds germinated at each day D.

The Germination Index **(Eke *et al.*, 2019a)**

$$\mathbf{GI = \sum N / \sum D} \qquad \mathbf{(2)}$$

Where; GI; is the germination index; D days of seeds germination evaluation (3, 6, 9, 12, and 15 days) and N is the number of seeds germinated on the days D.

Germination Percentage (GP)

GP = (SG/N)*100 **(3)**

Where: GP: Germination Percentage; SG: Seeds Germinated and N: Total number of seeds.

After 15 days of plant germination, the radicle length was measured. The IAA and SA contents of the seedlings were estimated as described previously by **Goswami *et al.*, 2013)** and **Meyer *et al.*, 1992)** respectively.

2.2.4 *In planta* Peppers Leaf Curl Virus (*PeLCV*) suppression experiment

2.2.4.1 Whitefly: rearing, genotype and vector efficiency

a) Whitefly rearing

The insects collected in Bangangte in a field with a high disease incidence were reared on young pepper seedlings grown in net cages on a laboratory bench at ambient conditions for the multiplication of viruliferous whiteflies. The thus generated viruliferous whiteflies were used to inoculate experimental plants during the suppression test.

b) Whitefly genotyping

DNA extraction

The extraction was carried out in the molecular laboratory at the International Institute of Tropical Agriculture (IITA) in Dar es Salaam, Tanzania as described by **Misaka *et al.* (2020)** with slight modification. The insects were added to 3µL of lysis buffer in a 1.5 mL Eppendorf tube then macerated and another 20µ of lysis buffer was added. The lysis buffer contained 10 mM Tris-HCl (pH 8.0, 50 mM KCL, 2.5 mM MgCl, 0.45% Tween-20, 0.01% Gelatine, and 60 µg/mL Proteinase). The mixture was then vortex shaken and spun down and immediately incubated on ice for 15 min. This was followed by incubation at 55°C in a water bath for 30 min. and the lysate was stored at -20°C for downstream use. For PCR use, the lysate was diluted while using sterile DPEC-treated water in a ratio of 1:9.

Mitochondrial Cytochrome Oxidase I (MtCOI) amplification and sequencing

PCR products of the mtCOI fragment (approximately 850 bp) were produced using the forward primer 2195-Bt-F (5’-TGRTTTTTTGGTCATCCRGAAGT-3’) and C012-Bt-sh2-R (5’-TTTACTGCACTTTCTGCC-3’) **(Mugerwa *et al.*, 2018)** using a thermocycler (Applied Biosystems™ GeneAmp^R PCR system 9700), under the following conditions: first cycle of denaturation at 95°C for 5 min, were followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 54°C for 30 s, 72°C for 45 s and final extension at 72°C for 10 min. A total reaction mixture of 25 µL was made up of 1X QuickLoad Master Mix (New England Biolabs, UK), 1 mM MgCl, 0.24µM of each primer, 2 µL DNA, and sterile distilled water.

The PCR products were electrophoresed in 1% agarose gel stained in SYBR Gold (Invitrogen, Oregon, USA.) at 100 V for 30 min in gels buffered with 1×TAE buffer. DNA bands were visualized while using UVP GelStudio plus and only samples with intact bands were selected for sequencing. PCR products were sent to Psomogen Inc. (Rockville, MD, USA) for purification and direct sequencing. DNA sequences were manually edited while using Ridom Trace Edit v1.1.0 software (Ridom GmbH., Würzburg, Germany). The sequences were assembled into contigs using CLC Main Workbench 22 (QIAGEN, Aarhus, Denmark). Multiple alignments of edited sequences were performed using Clustal W in MEGA version 11 **(Kumar *et al.*, 2016)**, and the sequences were trimmed. Construction of a maximum-likelihood phylogenetic tree was performed using MEGA with 1000 bootstrap replicates. Sequences were blasted using GenBank’s (NCBI) Blastn and selected reference sequences with 99% to 100% identity to our mtCOI sequences were included in the phylogenetic tree for comparison with previously published haplotypes.

c) Whitefly, begomovirus vector efficiency

For begomovirus detection, PCR reactions were carried out using the begomovirus detection primer pairs (Rojas-F: 5’-TGTGAAGGCCCATGTAAGGTCCAGTC-3’, Rojas-R: 5’-ACAGGGTTAGAGGCATGAGTACATGCC-3’), amplifying the partial coat protein gene of approximatively 1200 bp nucleotides. PCR reactions were performed in a volume of 20 µL containing 1 µL total nucleic acids, 1 µL of each primer (10 µM), 1 µL dNTPs (10 mM mix), 2 µL MgCl (25 mM), 4 µL 5 × reaction buffer, 0.5 U/µL Go-Taq DNA polymerase (Promega, Madison, WI, USA), and nuclease-free water. Amplification was carried out under the following cycling conditions: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; followed by an incubation period at 72°C for 5 min. The PCR products were electrophoresed in 1% agarose gel stained in SYBR Gold (Invitrogen, Oregon, USA.) at 100 V for 60 min in gels buffered with 1×TAE buffer. DNA bands were visualized while using UVP GelStudio plus

2.2.4.2 Soil drenching and growth condition

The BCA inoculums (single and combination) were grown on PDA medium for one week and the spore was harvested in sterilized saline solution (0.85%) and adjusted at 10^6 spores/mL by counting in hemacytometer as described by **Singh *et al.*, 2016**. Two weeks old seedlings, pregerminated in axenic conditions were sown in 5 Liters pots filled with 3 plants either inoculated (test pots) or uninoculated (control). The pots (tests and controls) were arranged in insect-proof cages following a randomised block design with 7 replications. Each replication comprised 10 pots with a spacing of 60× 45 cm as described by **Thakur *et al.*, 2019.**

2.2.4.3 Begomovirus inoculation and experimental layout

For infection, viruliferous whitefly colonies propagated on symptomatic pepper plants in insect-proof cages were utilized as virus sources **(Senanayake *et al.*, 2012; Thakur *et al.*, 2019).** Diseased chili pepper plants hosting around a hundred viruliferous whiteflies were removed from the cage and shaken into the cage containing *Trichoderma*-treated and untreated seedlings to maximize infection and allow to stand at equal distance of all the treatment for homogeneity purposes during 48 hours **(Thakur *et al.*, 2019).**

The trial was a 5×8×1 factorial, arranged following a completely randomized block design (CRBD) with 10 replications per treatment. The treatments were codified as follows: Control (treatment without *Trichoderma* and viral infection), *Bemisia* (treatment with only *Bemisia sp*), *Bemisia* + T1 (treatment with *Bemisia sp* and *T. polysporum* T1), *Bemisia* + T2 (treatment with *Bemisia sp* and *T. atroviridae* T2), *Bemisia* + T8 (treatment with *Bemisia sp* and *T. harzianum* T8). And consortium treatments: *Bemisia* + T1T2, *Bemisia* + T1T8, *Bemisia* + T2T8 and *Bemisia* + T1T2T8. The pots were watered regularly and grown for 60 days.

After 60 days of emergence (DAE), shoot and root were removed for each treatment, shoot and root length (cm) were taken using a caliper and after, dry weights (g) were recorded upon drying at 60°C until constant mass. The disease incidence (DI) was determined (4) for each treatment as the total number of plants displaying **PeLCVD** symptoms over the total number per treatment as follows **Camara *et al.*, 2013**.

$$\mathbf{DI\ (\%) = (SP/N) * 100} \qquad \mathbf{(4)}$$

Where SP = represents the symptomatic plant, N = total number of plants in each group

In order to assess symptom severity, 5 classes (0, 1, 2, 3, and 4) disease rating scale was adopted **(Lapidot *et al.*, 1997)** with

0 = no visible symptoms; 1 = very slight leaf curl; 2 = substantial curl with or without yellowing; 3 = substantial curl with substantial yellowing; 4 = Substantial curl + yellowing + Stunting or

death of the plant. The Disease Severity (DS) was thereafter calculated using the formula (5) adapted from **(Filion *et al.*, 2003):**

$$\mathbf{DS\ (\%) = (\sum (IDi)/ND) * 100} \qquad \mathbf{(5)}$$

Where; I = the number of affected plants having a similar degree of infection, Di = degree of infection on a given plant, N = the total number of plants examined, and D = highest degree of infection (on the 1–4 scale).

2.2.4.4 Evaluation of the effect *Trichoderma* spp. Treatments on the biomarkers accumulation

a) Evaluation of total phenolic content

- **Preparation of phenolic extract**

The phenolic extracts were prepared as described by **Tchameni *et al.* (2011).** Briefly, 5g of dried pepper plant from a representative sample was ground and extracted with 50 mL of 70% methanol at room temperature for 1 hour, then filtered with Whatman n°1 filter paper, and the methanol thoroughly evaporated at 40 °C using a rotary evaporator (BUCHI 001). The aqueous phase obtained was adjusted to 70 ml with sterile distilled water, and de-pigmented by adding 50 mL of 40% ammonium sulfate [$(NH_4)_2SO_4$], 1.5 mL of 80% phosphoric acid (H_3PO_4) and 50 mL of petroleum ether. The ether phase was discarded and the aqueous phase of the sample was extracted four times with 50 mL ethyl acetate. The subsequent aqueous phase was then discarded and the organic phase was dried by adding 5 g of magnesium sulfate ($MgSO_4$) and filtering after 5 min using Whatman n°1 filter paper. The clear organic phase obtained was dried at 40 °C under a vacuum using a rotary evaporator to afford the extract that was subsequently used for the experiments.

- **Determination of the total phenolic content**

Principle

In basic medium, Folin-ciocalteu reagent is reduced in presence of phenolic compound resulting in the formation of a blue molybdenum-tungsten complex that is measured spectrophotometrically at 760 nm and the intensity increases linearly with the concentration of phenolics in the reaction medium.

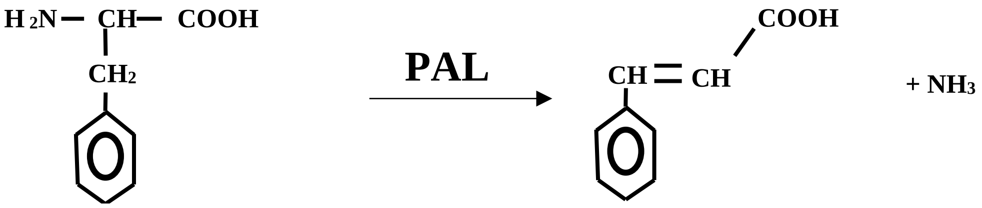
Procedure

The main secondary metabolite used by the plant for defense against biotic attack is phenolic compounds. The total phenolic content was determined using the Folin-ciocalteu assay protocol described by **Siddhuraju *et al.* (2002).** Hence, 50 µL of the above-prepared extract at 0.5 g/mL in water was mixed with 3 mL of distilled water, 250 µL of Folin-ciocalteu's reagent, and 750 µL of 70% Sodium Carbonate and the mixture vortexed thoroughly and further incubated for 8 min at room temperature. Then, 950 µL of distilled water was added to the reaction mixture and allowed to incubate for 2 h at room temperature. Gallic acid was used as standard in the same experimental conditions. The absorbance was recorded at 760 nm against a blank using a Tecan Infinite M200 microplate reader. The experiments were performed in triplicate and the results were expressed as Gallic acid equivalents (mg of GAE/g sample) through a regression ($r^2 = 0.99$) curve plotted from pure Gallic acid.

b) Assay of Phenylalanine Ammonia Lyase (PAL) specific activity

Principle

In basic medium, phenylalanine is converted into trans-cinnamic acid in the presence of phenylalanine ammonia lyase enzyme. This trans-cinnamic acid shows maximum absorbance at 290°C



Phénylalanine

Acide trans-cinnamique

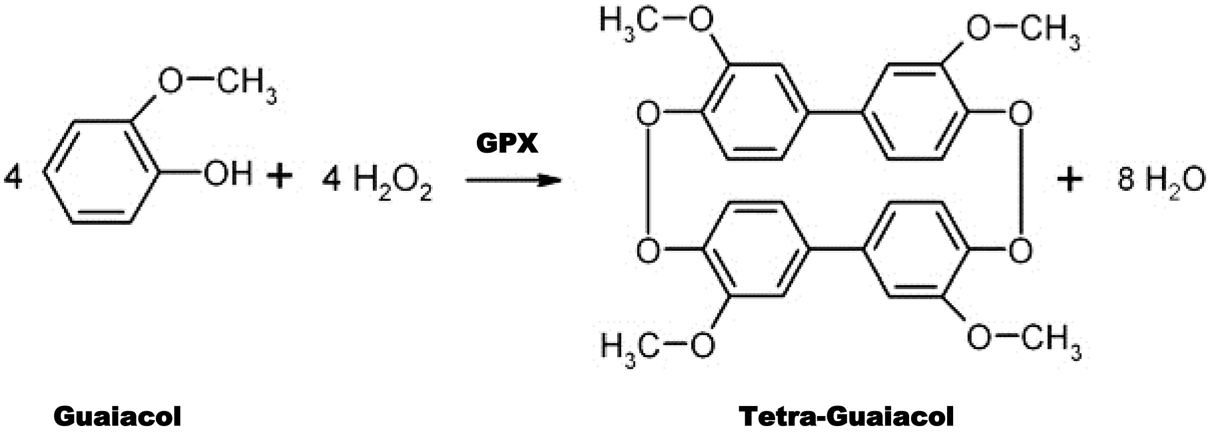
Procedure

The increase in specific activity of the phenylpropanoid pathway is often indicative of a strengthened innate immune system. The PAL extraction was done by the protocol described by **Yingsanga *et al.* (2008).** Fresh root samples (0.5 g) were crushed with 20 mL of 50 mM sodium phosphate buffer (pH 8.8). The extracts were filtered and the filtrates were centrifuged at 13,000g at 4°C for 30 min. PAL activity was measured in the supernatant following the method of **Whetten and Sederoff (1992)**. An assay mixture, containing 100 µL of enzyme extract, 500 µL of 50mM sodium phosphate buffer (pH 8.8), and 600µL of 1 mM Phenylalanine was allowed to stand for 1 h at room temperature followed by the addition of 2 mL of 2 N Hydrochloric acids as reaction stopper. The absorbance of the released trans-cinnamic acid was measured at 290 nm using a microplate reader (Tecan Infinite M200) and the result was expressed in terms of OD290/min/mg protein.

c) Assay of Guaiacol peroxidase (GPX) specific activity

Principle

In the presence of hydrogen peroxide and guaiacol, guaiacol peroxidase enzyme oxidise guaiacol into tetra guaiacol which shows a maximum absorbance at 470°C.



Procedure

Peroxidase is an enzyme involved in numerous physiological processes in response to biotic and abiotic stresses. It leads to the biosynthesis of defense-related secondary metabolites contributing to stress mitigation. The enzyme activity was assayed by measuring the increase in absorbance at 470 nm (Tecan Infinite M200) as a result of guaiacol oxidation to tetra guaiacol by the pro-oxidant (H_2O_2) **(Zheng and van Huystee, 1992).** The reaction mixture contained 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 transform per mg of enzyme extract per min (mmol/mg/min).

d) Assay of superoxide dismutase (SOD) specific activity

Principle

In basic medium and presence of EDTA, riboflavin and methionine, the capacity of superoxide dismutase to inhibit the reduction of Nitroblue tetrazolium is assessed and the amount of reduction is evaluated by measuring the absorbance at 560 nm.

Procedure

In higher plants, the SOD enzyme is an antioxidant enzyme that protects cellular components from being oxidized by reactive oxygen species generated by stressors. An increase in the specific activity of this enzyme is indicative of a stressed host. SOD activity was assayed as described by **Beauchamp and Fridovich (1971)** based on the measurement of the inhibition of the photochemical reduction of Nitroblue tetrazolium NBT. The 3 mL assay mixture was made up of 13 mM L-methionine, 63 mM NBT, 0.05M sodium carbonate 13 mM riboflavin, 0.1 mM EDTA, and 0.5 mL enzyme sample. The mixture was kept under two fluorescent lamps (15W) for 15 min at 25°C, followed by dark for 15 min. The absorbance was then recorded at 560 nm (Tecan Infinite M200 microplate reader). One unit of the SOD activity was defined as the amount of enzyme required to inhibit the reduction of NBT by 50%. The result was expressed in SOD unit/mg fresh weight.

2.2.5 Effect of *Trichoderma* consortium (T2-T8) on pepper yield and fruit quality

2.2.5.1 Field experiment

a) Experimental farm and experiment 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 above sea level within the 5th agroecological zone of Cameroon. The so-called site is characterized by a bimodal rainfall (1500 mm) pattern and the Sandy-clay-loam complex, characterized by Organic matter (3.62%), C/N ratio (20.5), cation exchange capacity (29 meq/100 g), and pH of 5.2 **(Fokom *et al.*, 2019).** Weeds were removed and taken off to the farm. The soil was plowed using the conventional manual hoe-plowing tillage system (15–20 cm depth). Peppers plant was pre-germinated in sterilized soil with formulated biopesticide of *Trichoderma* and was sown in trays after 45 days and grown in axenic conditions in the nursery before transplanting in the field at a spacing of 60 cm between rows and 40 cm between plants. The overall treatment was set in a randomized complete block design with three replications per treatment. The spacing between plots in each replication was 1m. Each block constituted control (C) and *Trichoderma*-treated seedlings (T2-T8). During the production, some vegetative parameters were recorded (shoot length by using a caliper, number of fruits per plant by harvest and counting, fruit weight by weighting, leaf surface by calculating with parameter recorded in leaf, Nitrogen and Phosphorus content photosynthetic pigments and yield of production by using the **formula 4**) and fruit was harvest for fruit quality evaluation.

$$\mathbf{Yield}\ \left(\frac{\mathbf{kg}}{\mathbf{ha}}\right) = \frac{\boldsymbol{weight/parcel}\ (\boldsymbol{g})}{\boldsymbol{area/plot}\ (\mathbf{m^2})}\ \boldsymbol{x}\ \frac{\mathbf{10000\ m^2}}{\mathbf{1}\ \boldsymbol{ha}}\ \boldsymbol{x}\ \frac{\mathbf{1\ kg}}{\mathbf{1000}\ \boldsymbol{g}} \qquad \mathbf{(4)}$$

b) Estimation of nitrogen and phosphorus content

Principle

In an acid medium (H_2SO_4), the presence of phosphorus is revealed by the addition of an ammonium molybdate solution which produces a yellow crystalline precipitation of ammonium phosphor-molybdate. This Phosphor-molybdate complex reacts with amino-naphtol sulphonic acid to form a blue molybdenum complex showing a maximum absorbance at 710 nm.

$$Na_2HPO_4 + 12\ (NH_4)_2MO_4 + H_2SO_4 \longrightarrow (NH_4)_3PO_4MOO_3H_2O + H_2SO_4$$

(NH4)3PO4MOO3H2O + (NH4)2SO4 + Na2SO4 + H2O $\longrightarrow$ **Blue complex**

Procedure

Young leaves were sampled, oven-dried (80°C) until constant mass, and ground to powder. Subsamples of dried powder were subjected to acid digestion **(Kjeldahl, 1883)** and the total P and N were estimated. The total P was determined as described by **Watanabe and Olsen (1965)**. Briefly, an aliquot 3mL was transferred to a 15 mL volumetric flask and 4 mL of yellow solution (2.5 M H_2So_4: 4%; (NH4) $6Mo_7O_{24}$ $4H_2O$: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 was done 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 ($R^2 = 0.976$).

Principle

In a basic medium, the presence of nitrogen is revealed by the formation of a complex with the nitroprusside showing a maximum absorbance at 625 nm.

Procedure

The N content was estimated as described by **Hevia and Cioccia (1988)**. Thus, 3 mL of pre-digested pepper leaves was mixed up with phenol nitroprusside and alkaline hypochlorite, then incubated (20°C, 15 min). OD was measured at 625 nm (Tecan Infinite M200) 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).

c) Photosynthetic pigment measurement.

Principle

Pigments absorb naturally, so it consists to evaluate the spectrophotometric absorbance of acetone plant extract at various wavelengths (470, 646, 663nm) and then, these absorbances measurements are applied to a standard equation.

Procedure

Photosynthetic pigments were extracted by maceration of leaflets from each treatment in 10 mL acetone (80%) for 48h at 4°C. The homogenates were centrifuged (5000g; 5min) and ODs (Tecan Infinite M200) were measured at 470, 646, and 663nm, respectively. Chlorophylls content were calculated according to the formula described by **Lichtenthaler and Wellburn, (1983)** as follows:

Chla (µg/ml) = 12.21 A_{663} – 2.81 A_{646}
Chlb (µg/mL) = 20.13 A_{646} – 5.03 A_{663}
229*Carot (µg/mL) = 1000 A_{470} – 3.27 Chla – 104 Chlb.

2.2.5.2 Fruit quality evaluation

After 120 days, the fruit was harvested and prepared for postharvest quality and shelf-life studies, fruits harvesting was carried out at the mature stage when 50% of the plants attained the fruits maturity stage. Fruits with bruises, a sign of infection, or those different from the group were discarded from the samples. Uniform, unblemished pepper fruits having similar size and color were then selected and hand washed with tap water to remove soil particles and reduce microbial population on the surface. Then, the fruits were surface dried with a soft cloth and subdivided and stored in a freeze at 4°C, at room temperature in three replications during 0, 5, 10, 15 and 20 days. Some parameters of the quality as Total soluble solids, Moisture content, pH, capsaicinoids, carotenoids, ascorbic acids, physiological weight loss, and phenols contents were evaluated:

a) Total soluble solids (TSS).

Principle

It consists to put a drop of diluted fruit juice on a prism of the refractometer and read the measurement

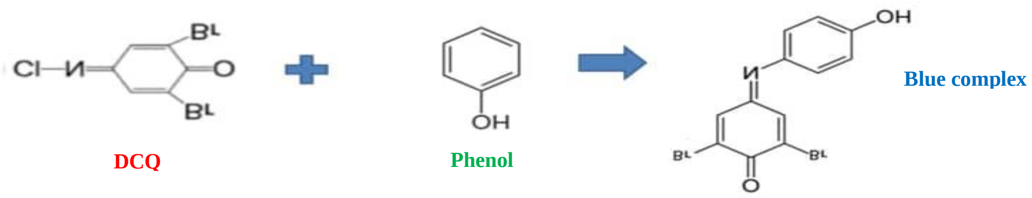
Procedure

The total soluble solids Juice of pepper fruits was extracted from a 10 g bulk sample of 3–4 fruits in a blender as described by **Antoniali *et al.* (2007)**. The homogenized sample was filtered using a funnel with filter paper in a beaker. The filtrate was taken for TSS determination. The TSS was determined by hand refractometer with a range of 0 to 32 °Brix (Bx), and a resolution of 0.2 °Bx by placing 1 to 2 drops of clear juice on the prism. Between samples, the prism of the refractometer was cleaned with tissue paper soaked in methanol, washed with distilled water, and dried before use. The refractometer was standardized against distilled water (0 °Bx TSS).

b) Colorimetric quantification of capsaicinoids

Principle

In presence of 2.6-Dichloroquinone-4-chloroimide (DCQ) and ammonia, a blue complex is formed with capsaicinoids showing a maximum absorbance at 600 nm. The value of the absorbance is proportional to capsaicinoids content.



Procedure

Extraction of capsaicinoids

Five-gram of pepper fruit was ground with a home blender for 3 minutes and then a five-fold volume of acetone was added to the extract at 50°C for 1 hour in triplicate. Centrifuged supernatant was taken for colorimetric analysis.

Quantification of capsaicinoids

Chromogenic substances by origin are water-soluble components with high polarity extracted with acetone and they affect the chromogenic reactions of capsaicinoids. As described by **Ryu *et al.* (2017)** to remove chromogenic substances, it is important to first dry the extract. One mL of acetone extract from chili pepper was transferred into a glass test tube and completely dried. Five milliliters of n-hexane were added and the mixture was allowed to remain at room temperature for 10 minutes to dissolve the extract. Then, the 4-mL n-hexane layer was carefully taken to a new tube without any solid materials tagging along. To 4 mL of n-hexane solution,10 mL of 0.05N NaOH was added and the mixture was intensely vortexed. The supernatant was removed. One milliliter from the NaOH layer left was taken and then mixed with 50 mL of 1N HCl, 50 mL of 0.1% DCQ, and 50 mL of 2.5% ammonia solution in order. The mixture was reacted at room temperature for 10 minutes and the absorbance was measured at 600 nm. For the standard, 5 mg capsaicin was dissolved in 10 mL of methanol and 50 mL, 100 mL, 200 mL, and 400 mL of the solution were transferred into glass tubes with the methanol removed by rota-evaporation. Each standard was dissolved in 4 mL of hexane and then extracted with the NaOH solution for color development as mentioned earlier.

c) Moisture content

Principle

It consists to evaluate all water present in fruit by drying until constant weight and comparing its final weight with the first weight without drying.

Procedure

This parameter was determined using a 10 g sample from each treatment (cool and ambient storage) that was first weighed then, cut into pieces and dried in a forced air circulation oven at 70 °C to constant weight. After this, a difference was evaluated between the two weights as described by **Antoniali *et al.* (2007)** and the results were expressed in percentages.

d) Physiological weight loss

Principle

It consists to evaluate the weight variation of fruit in the interval of each five days until 20 days.

Procedure

Physiological weight loss (PWL) was determined following the method described by **Samira *et al.* (2013)**; Stored fruits (ambient and cool storage) from each treatment were weighed at the start of the experiment and 5 days intervals until 20 days. The differential weight loss was calculated for each interval and converted into percentages by dividing the change by the initial weight recorded on each sampling interval.

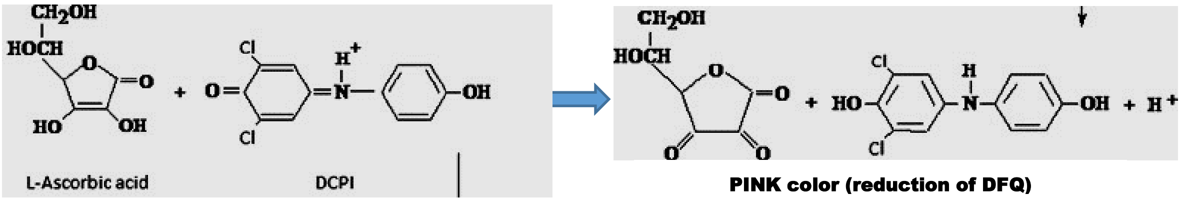
e) PH of juice

Ten grams of pepper was mixed with 90 ml of distilled water and homogenized in a blender as described by **Antoniali *et al.* (2007)**; The homogenized sample was filtered using a funnel with filter paper in a beaker and the pH value of the filtrate was measured by a pH meter.

f) Ascorbic acid content

Principle

In acidic medium, ascorbic acid forms a pink complex in presence of 2.6-dichlorophenolindophenol (DFQ) which persists for more than five seconds. The amount of ascorbic acid is obtained by calculating using a titration equation.



Procedure

The amount of ascorbic acid was determined by titrating 3 g of blended pulp homogenized with 50 ml of oxalic acid at a concentration of 12%. The titration was done using the 2.6 dichlorophenolindophenol method **(Antoniali *et al.*, 2007)**; Then ascorbic acid content (%) was calculated from the titration value, dye factor, dilution, and volume of the sample, and the result was expressed in mg of ascorbic acid per 100 g of pulp.

g) Carotenoids content

Principle

It consists to evaluate the absorbance of fruit juice at 3 wavelengths 470, 646, and 663nm and after the resulting absorbance measurement are then applied to the standard equation.

Procedure

Carotenoids were extracted by maceration of fresh pepper fruit from each treatment in 10 mL acetone (80%) for 48h at 4°C. The homogenate obtained was centrifuged (5000g; 5min) and ODs (Tecan Infinite M200) were measured at 470, 646, and 663nm, respectively. The carotenoid content was assessed according to the formula described by **Lichtenthaler and Wellburn (1983)** as follows:

$229 * \text{Carot}\ (\mu g/mL) = 1000\ A_{470} - 3.27\ (12.21\ A_{663} - 2.81\ A_{646}) - 104\ (20.13\ A_{646} - 5.03\ A_{663})$

h) Phenols contents

Principle

In basic medium, Folin ciocalteu reagent is reduced in presence of phenolic compound resulting in the production of a blue molybdenum-tungsten complex that is measured spectrophotometrically at 760 nm and the intensity increases linearly with the concentration of phenolics in the reaction medium.

Procedure

The total phenolic content was determined using the Folin-ciocalteu methods described by **Siddhuraju *et al.* (2002)**. Thus, 50 µL of prepared extract at 0.5 g/mL in distilled was thoroughly mixed with 250 µL of Folin-ciocalteu's reagent and 750 µL 70% Sodium Carbonate, respectively. The mixture was incubated for 8 min at room temperature and 950 µL of distilled water was added and allowed to incubate 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 were expressed as Gallic acid equivalents (mg of GAE/g sample) through a regression curve plotted from pure Gallic acid ($r^2 = 0.99$).

2.3 Data analysis

The raw data collected from the overall experiments were analysed by ANOVA, with the Tukey's multiple comparisons-based tests or the paired t-test at a 5 % level of significance through GraphPad PRISM (5.0 version) statistical software. All graphs were drawn with GraphPad PRISM and Origin Pro software 2024 was used to evaluate the correlation and relation between treatment and storage conditions through principal component analysis.

Chapter III

RESULTS AND DISCUSSION

3 CHAPTER III: RESULTS AND DISCUSSION

3.1 Results

3.1.1 Characterisation and functional analysis of endophytic *Trichoderma* spp as a biological control agent

3.1.1.1 Cultural and genomic identification of *Trichoderma* isolates

The cultural characteristics of *Trichoderma* spp. were evaluated visually and under a light microscope. After two days of incubation on PDA, all of the isolates had hyaline to white hyphae that became light to dark green depending on the isolate. The whole colony became greenish as a result of conidia production, which occurred from the center of the culture outward. The overall pattern of the colonies ranged from uniform lawn to undulated circular green rings. *Trichoderma* spp. was identified by the recorded characteristics **(Rifai, 1969).**

The electrophoretic profile of the amplification products (Figure 12) showed the effective amplification of the internal transcribed spacer (ITS) regions of our *Trichoderma* mycelium with a presence of a bands around 600bp for the sample and no amplification in negative control. This result was confirmed by the amplification of the positive control which were a Trichoderma already molecularly characterised. The isolated *Trichoderma* strains were identified as *T. polysporum* T1, *T. atroviridae* T2, and *T. harzianum* T8 by sequencing the ITS genes (Figure 12) and a BLAST search in the NCBI database (Figure 13).

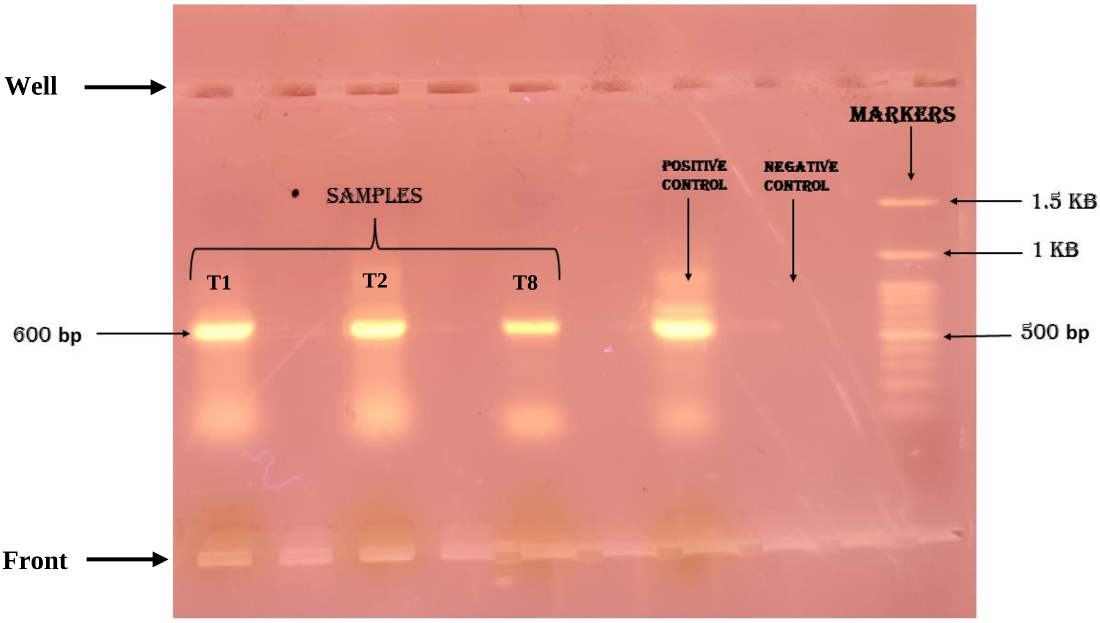


Figure 12: Electrophoretic profile of the amplification products of ITS regions* (600bp) *of isolated Trichoderma.

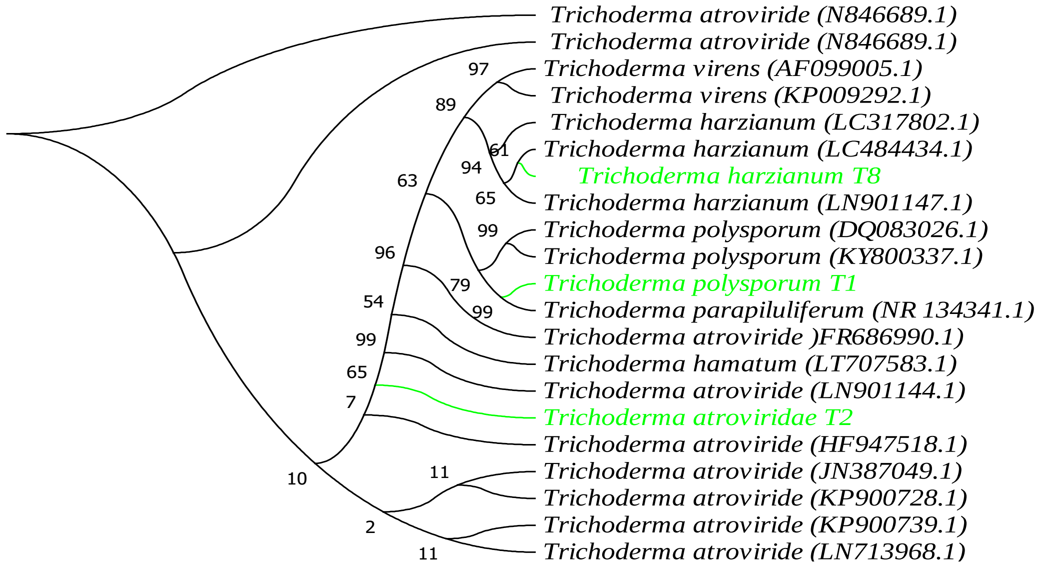


Figure 13: Phylogeny of Trichoderma isolates. Molecular tools coupled with taxonomy analysis using ITS regions of rDNA and BLAST search for the three isolates showed 100% homology with T. atroviride (HF947518), T. polysporum (NR134341), and T. harzianum (LC484434).

3.1.1.2 *Trichoderma* spp. hormonal (IAA and SA) profiling

The *Trichoderma* species can synthesize both IAA and SA as revealed by the figures 14 below. In general, the binary application of the BCA's elicited more IAA (Figure 14a) and SA (Figure 14b) than the single application and the consortium of *T. atroviridae* and *T. harzianum* (T2T8) was the best producer, inducing more IAA (100 g/mL) biosynthesis; while the triple combination of *T. atroviridae*, *T. polysporum*, and *T. harzianum* (T1T2T8) released more SA than any other treatment, culminating at 18 µg/mL. The amount of IAA was released more than SA but the best production of SA was recorded by the consortium of T1T2T8 around 18mM.

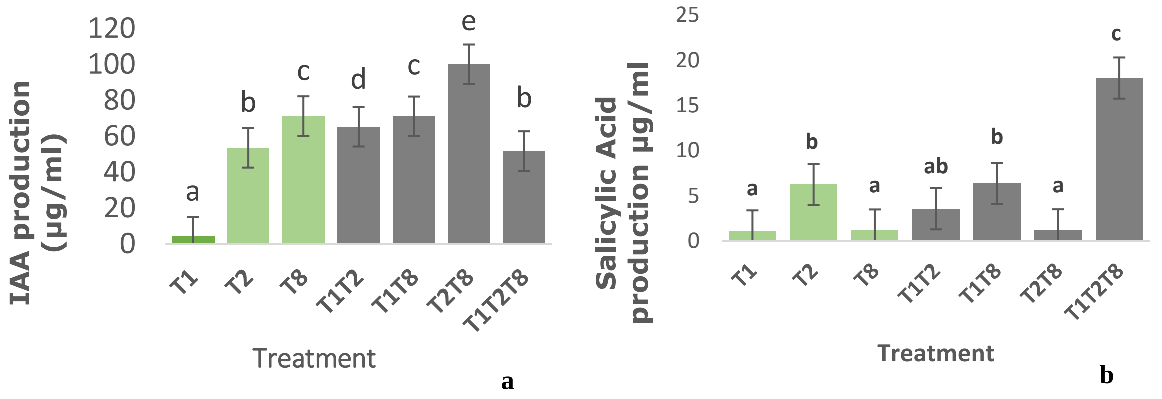


Figure 14: Quantitative estimation of Indole Acetic Acid (a) and Salicylic Acid (b) produced by T. polysporum T1, T. atroviridae T2 and T. harzianum T8 and combination of T1, T2 and T8. Bars bearing the same letter a, b, c, d are not significantly different at P≤0.05.

3.1.1.3 Tissue colonization and cohabitation abilities of *Trichoderma*

The results show that BCAs and their consortia could invade and survive endophytically in chili pepper plants. *T. atroviridae* T2 was shown to be the most invasive (50 CFU/g fresh weight) when tested alone, followed by *T. harzianum* T8 (32 CFU/g fresh weight). *T. polysporum* T1 in combination with either *T. atroviridae* T2 or *T. harzianum* T8 resulted in decreased tissue colonization with 9 and 20.5 CFU/fresh weight respectively. In the first hand, this indicates an inhibited action of *T. polysporum* T1 on the other bioagents in terms of colonization also confirmed by the value of the combination of the 3 bioagents. On the other hand, a synergistic impact was seen when the greatest colonizers were combined. (*T. atroviridae* T2 or *T. harzianum* T8), culminating at as high as 122.5 CFU per unit of fresh matter (Figure 15a). Inhibition zones were only found at the contact zones of *T. Polysporum* T1 and *T. atroviridae* T2 hyphae elsewhere. The ability to share the same ecological niche and host was demonstrated by all other tested BCA (Figure 15b).

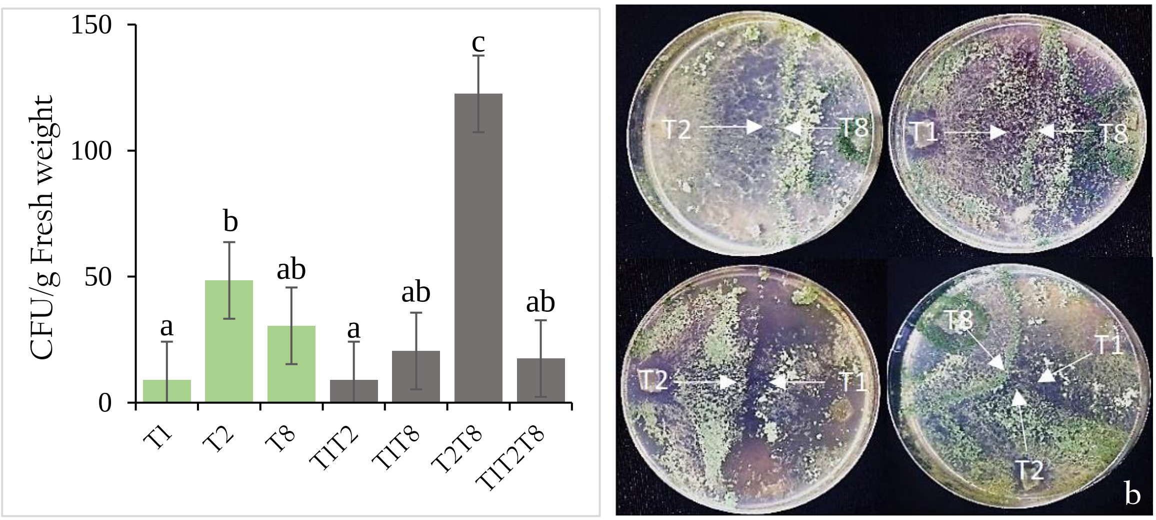


Figure 15: Endophytic efficiency of Trichoderma spp. in chili pepper plant grown in gnotobiotic condition (a) and cohabitation ability on PDA medium (b). T1: T. polysporum T1, T2: T. atroviridae T2, T8: T. harzianum T8 and combination of T1, T2 and T8. Bars bearing the same letter are not significantly different at P≤0.05.

3.1.1.4 Seed biopriming with *Trichoderma* spp

- **Effect of *Trichoderma* inoculation on the seeds germination**

In general, except for the combined application of T2 and T8, the final number of sprouted seeds per treatment and the total germination index (Table 3) were greater in all *Trichoderma*-inoculated treatments compared to the corresponding controls. Similarly, no statistically significant difference in mean sprouting time was seen in either *Trichoderma*-inoculated (single and consortia) or water controls, demonstrating that there are no negative effects of the bioagents on the seeds germination at the tested dosages. However, *Trichoderma* stimulated significantly the radicle length of germinated seed of pepper in comparison of the control without any treatment (Figure 16).

Table 3: Effects of Trichoderma on seeds germination.

Treatment	**Germination parameters**		
	GMT (Days)	GI	GP (%)
Control	8.6 ± 0.21^{a}	0.95 ± 0.07^{a}	88.9^{a}
T1	8.7 ± 0.33^{a}	1.01 ± 0.02^{a}	95.6^{a}
T2	8.0 ± 0.69^{a}	1.16 ± 0.12^{a}	100^{a}
T8	8.3 ± 0.42^{a}	1.12 ± 0.07^{a}	100^{a}
T1T2	8.0 ± 0.57^{a}	1.05 ± 0.07^{a}	93.3^{a}
T1T8	8.1 ± 0.45^{a}	1.07 ± 0.07^{a}	93.3^{a}
T2T8	9.0 ± 0.29^{a}	0.81 ± 0.04^{a}	80.0^{a}
T1T2T8	8.3 ± 0.83^{a}	1.12 ± 0.14^{a}	100^{a}

GMT: germination meantime, **GI**: germination index, **GP**: germination percentage. **T1**: *T. polysporum* T1, **T2**: *T. atriviridae* T2, **T8:** *T. hazianum* T8 and combination of *Trichoderma*. Columns bearing the same letter are not significantly different at $P \leq 0.05$.

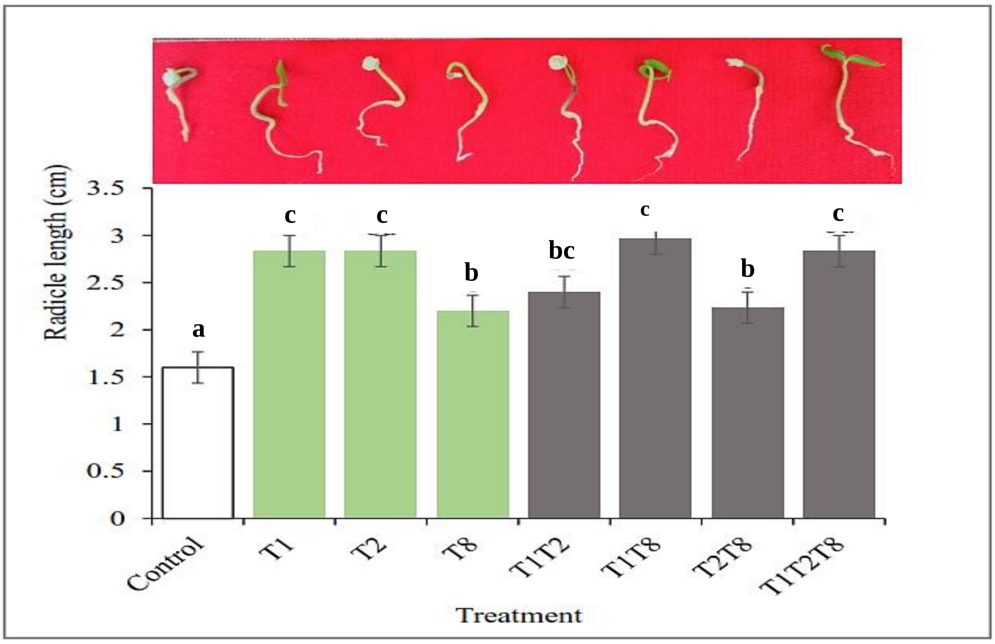


Figure 16: Effect of chili pepper seeds biopriming with Trichoderma spp. on the seed germination and radicle length. Control (without any treatment), T1 T. polysporum T1, T2 T. atroviridae T2, T8 T. harzianum T8, T1T2, T1T8, T2T8, and T1T2T8. bars bearing the same letter are not significantly different at P≤0.05.

IAA contents in peppers seedlings

The Indole Acetic Acid (IAA) hormone was present in all plants. However, only *T. harzianum* T8 increased IAA content in a single treatment compared to the control (non-primed plant) ($P \leq 0.05$). In contrast to the control treatment, the combined application of T2T8 and T1T2T8 revealed a significant increase in IAA ($P \leq 0.05$) (Figure 17a). Overall, these two binary treatments produced the highest IAA concentration, reaching up to 21g/mL.

SA contents in peppers seedling

The SA content in germinated chili peppers plants was recorded (Figure 17b). It is noteworthy that, in contrast to IAA, *T. harzianum* T8 was less sensitive compared to the other bioagents evaluated alone. *T. atroviridae* T2 was the most effective treatment (11.89 mM). Meanwhile, as compared to single applications, combined treatments performed poorly. However, the binary application of *T. polysporum* T1 and *T. harzianum* T2 was the most effective treatment (7.12 mM).

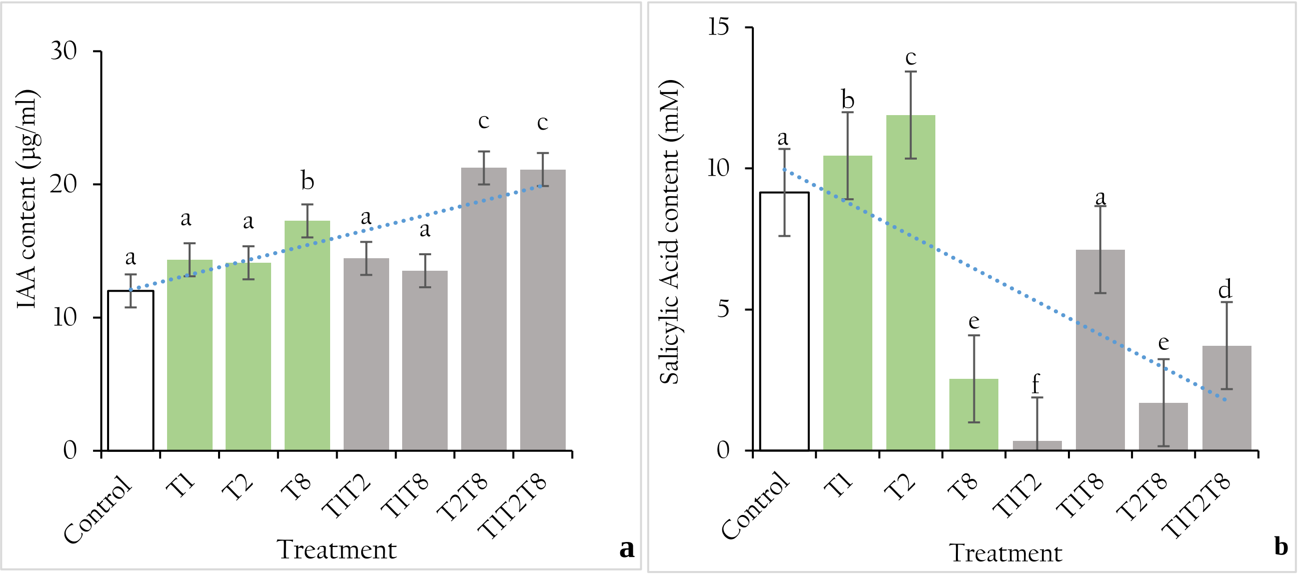


Figure 17: Quantitative estimation of Indole Acetic Acid (a) and Salicylic Acid (b) contents in Trichoderma-inoculated or not pepper seedlings. Control (without any treatment), T1 T. polysporum T1, T2 T. atroviridae T2, T8 T. harzianum T8, and combination T1T2, T1T8, T1T2T8. Bars bearing the same letter are not significantly different at P≤0.05.

3.1.2 Assessment of the biocontrol efficacy of *Trichoderma* spp. against pepper leaf curl disease (*PeLCD)*

3.1.2.1 Genotype and begomovirus identification

The presence of virus in the vector was confirmed by the presence of amplification observed at 1.2 Kb and confirmed by the positive control which was the already sequence begomovirus. Insects without virus used for the treatment of control plants showed no amplification as confirmed by the negative control (Figure 18). After the sequencing of the vector DNA, the sequences obtained from pepper and other vegetable plant were grouped into one phylogenetical group *Bemisia tabaci* Mediterranean (MED) which was the predominant haplotype (Figure 19). In contrast cassava plant was colonized by various mitotype of *Bemisia tabaci*.

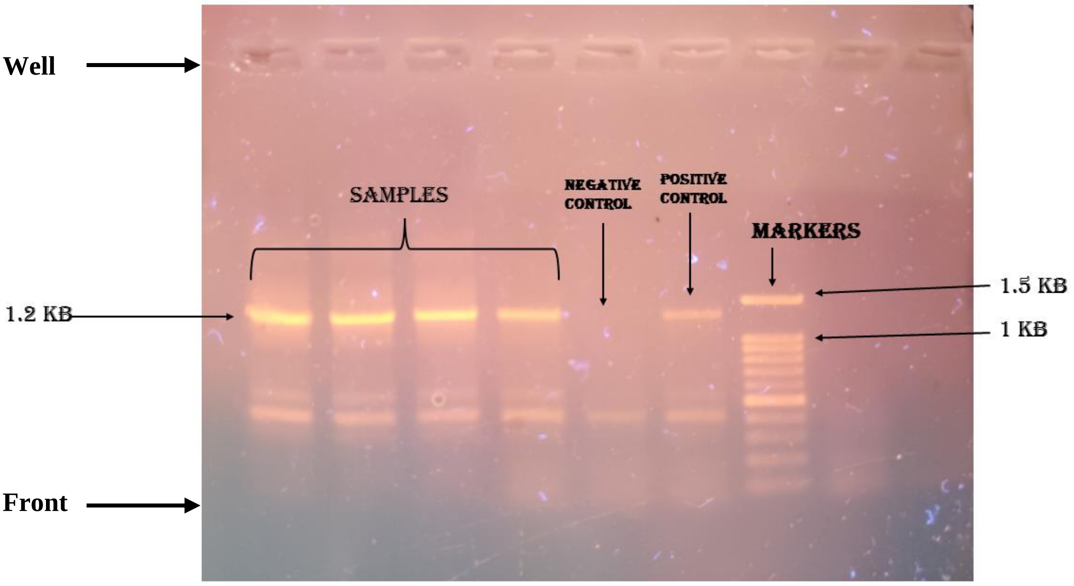


Figure 18: Electrophoretic profile of the amplification products of the partial coat proteins gene of the Begomovirus* (1200 bp) *extracted in B. tabaci whiteflies collected on pepper plant

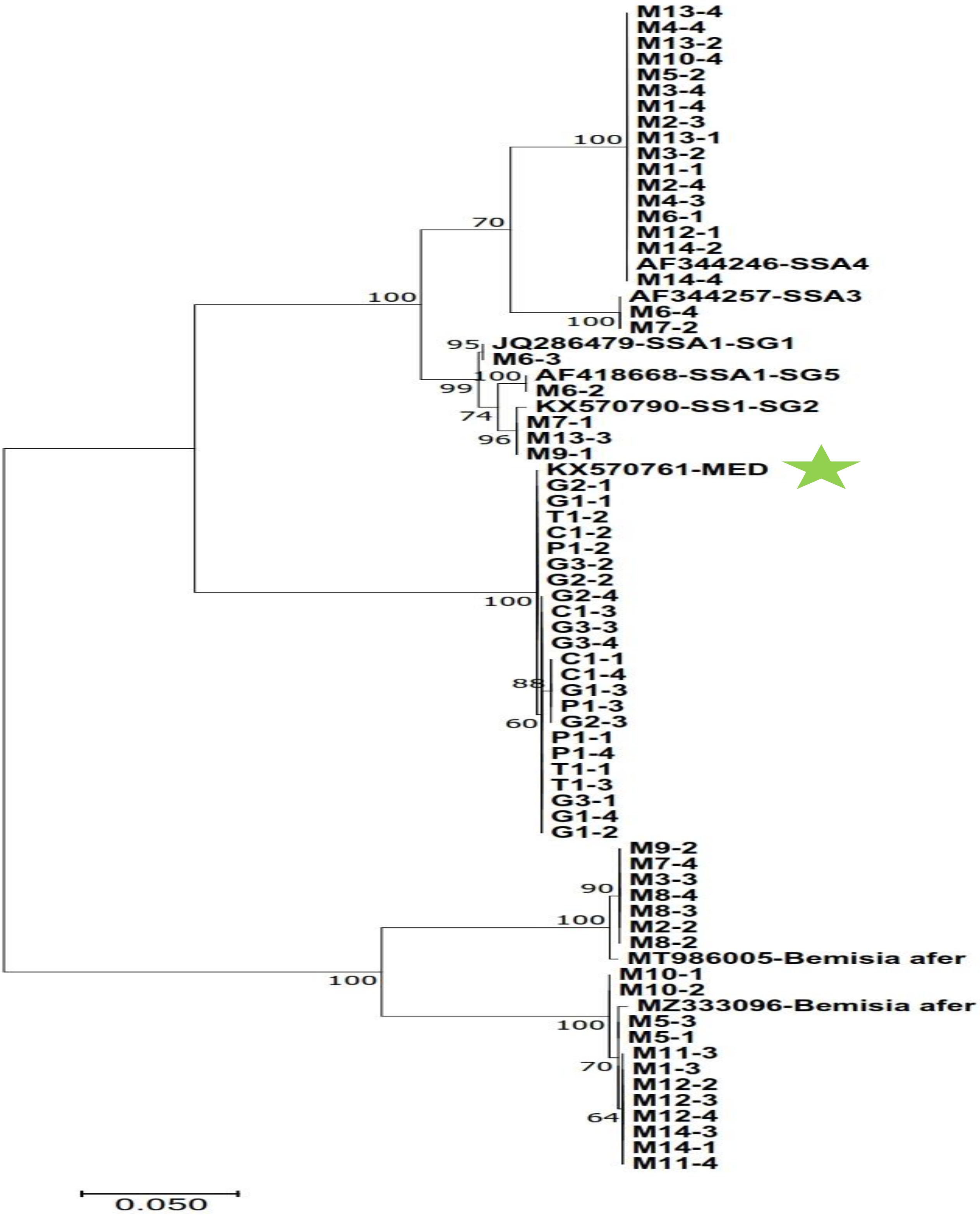


Figure 19: Phylogenetic relationships of the B. tabaci whiteflies collected on P (pepper). First Number represents the field and second number of the whiteflies picked in each field vials.

3.1.2.2 In planta effect of chili pepper priming by *Trichoderma* on PeLCD

Typical pepper leaf curl virus Disease (PeLCVD) signs such as poor growth, plant stunting, leaf yellowing, and curling were observed in plants emerging from control (non-inoculated) pots, confirming effective Begomovirus transmission in the test plants (Figure 20). *Trichoderma* priming could not completely suppress the disease in the plantlets examined (PeLCD incidence = 100%). In contrast, *Trichoderma* inoculation drastically reduced the severity of PeLCVD. The decrease in PeLCVD severity varied from 34 to 50 percent, with the treatments T8, T1, T1T8, and T2T8 achieving the greatest reduction of PeLCVD severity (50 %) (Table 4).

Table 4: Effect of chili pepper biopriming by Trichoderma on PeLCVD reduction

Treatment	***PeLCVD* severity (%)**	**Protection rate (%)**	***PeLCVD* incidence (%)**	**Protection rate (%)**
Control	-	-	0	-
Bemisia	100^{a}	0.0	100	0
Bemisia + T1	50.0^{b}	50.0	100	0
Bemisia +T2	58.3^{b}	41.7	100	0
Bemisia + T8	50.0^{b}	50.0	100	0
Bemisia + (T1T2)	58.3^{b}	41.7	100	0
Bemisia + (T1T8)	50.0^{b}	50.0	100	0
Bemisia + (T2T8)	50.0^{b}	50.0	100	0
Bemisia + (T1T2T8)	66.6^{b}	33.4	100	0

Control (plant without treatment, positive control), ***Bemisia*** (negative control: plant infected only by *PeLCV* transmit by whiteflies *Bemisia tabaci* MED), ***Bemisia* + T1** (plant treated with *Trichoderma polysporum T1* and infected by *PeLCV)*, **Bemisia + T2** (plant treat with *Trichoderma atroviridae* T2 and infected by *PeLCV*), ***Bemisia* + T8** (plant treat with *Trichoderma harzianum* T8 and infected by *PeLCV*), ***Bemisia* + T1T2** (plant treat with *Trichoderma polysporum* T1 and *atroviridae* T2 and infected by *PeLCV*), ***Bemisia* + T1T8** (plant treat with *Trichoderma polysporum* T1 and *harzianum* T8 and infected by *PeLCV*), ***Bemisia* + T2T8** (plant treat with *Trichoderma atroviridae* T2 and *harzianum* T8 and infected by *PeLCV*), ***Bemisia* + T1T2T8** (plant treated with *Trichoderma polysporum* T1, *atroviridae* T2 and *harzianum* T8 and infected by *PeLCV*). The values with a different letter in the same column are significantly different at 5% level by using the Duncan test.

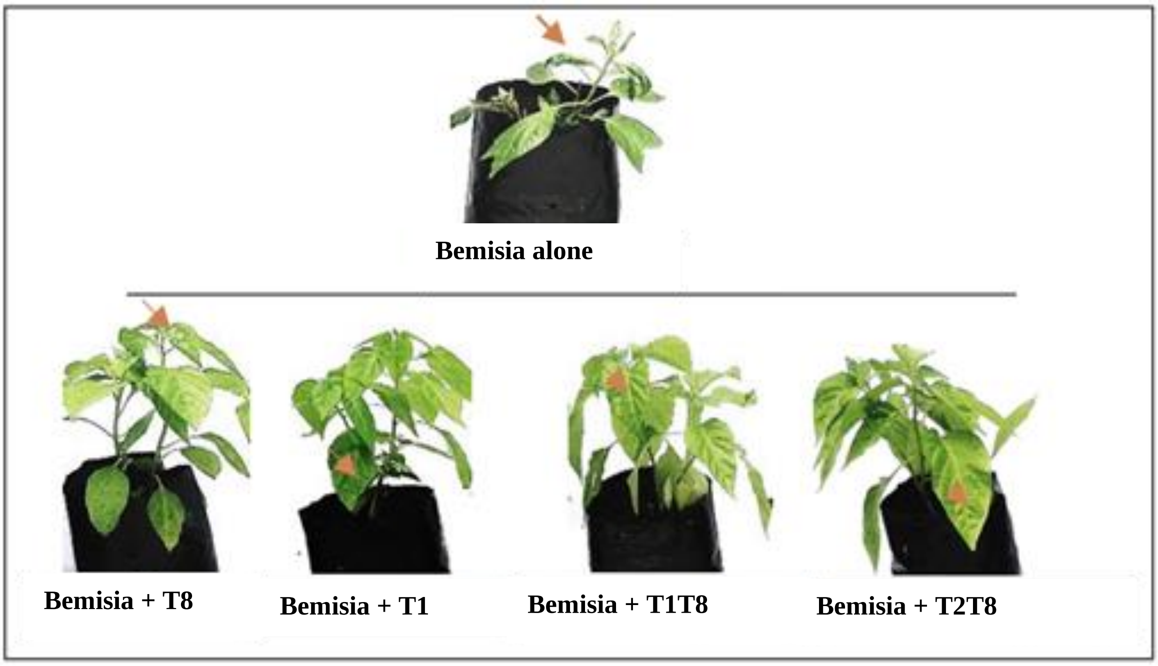


Figure 20: Effect of Trichoderma spp. priming or not on pepper growth and PeLCVD suppression. Bemisia alone (negative control, plant infected only by PeLCV transmitted by whiteflies Bemisia tabaci MED), Bemisia + T1 (plant treated with Trichoderma polysporum T1 and infected by PeLCV), Bemisia + T8 (plant treated with Trichoderma harzianum T8 and infected by PeLCV), Bemisia + T1T8 (plant treat by Trichoderma polysporum T1 and harzianum T8 and infected by PeLCV), Bemisia + T2T8 (plant treat by Trichoderma atroviridae T2 and harzianum T8 and infected by PeLCV.

3.1.2.3 Effect of *Trichoderma* biopriming on chili pepper growth under Begomovirus pressure

When compared to Begomovirus-infected plants alone, *Trichoderma* inoculation significantly improved all growth parameters examined ($P \leq 0.05$). There was no significant difference between all inoculated plants by *Trichoderma* alone or in consortia. However, the combinations T2T8 (17.66 cm) and T1T2T8 (31.33 cm) produced the best shoot and root expansions. *T. polysporum* T1 infected plants produced much more dry matter than the other treatments (Table 5). These results were confirmed by the observation of plant growth of different treatment where we can see a stunting aspect of the plant only infected by virus in comparison of plant treated with *Trichoderma* (Figure 21).

Table 5: Effect of pepper priming with Trichoderma spp. on some growth parameter of pepper plant under PeLCV pressure

Treatment	**Shoot length (Cm)**	**Root length (Cm)**	**[1]SDM (g)**	**[2]RDM (g)**	**Root/ shoot**
Control	13.0 ± 1^{b}	18.67 ± 2.2^{a}	$0,62 \pm 0.001^{a}$	0.13 ± 0.001^{a}	1.44
Bemisia	8 ± 1^{a}	10.33 ± 1.53^{b}	0.71 ± 0.001^{a}	0.12 ± 0.001^{a}	1.29
Bemisia + T1	16.0 ± 1.73^{b}	28.67 ± 1.16^{c}	3.99 ± 0.001^{b}	3.42 ± 0.001^{b}	1.79
Bemisia +T2	15.8 ± 1.89^{b}	29.67 ± 2.52^{c}	3.58 ± 0.001^{c}	1.83 ± 0.041^{c}	1.87
Bemisia + T8	17.66 ± 0.58^{b}	17.83 ± 2.75^{a}	2.57 ± 0.001^{d}	1.28 ± 0.001^{d}	1.01
Bemisia + (T1T2)	17.33 ± 0.76^{b}	21.33 ± 2.52^{d}	2.79 ± 0.001^{e}	2.20 ± 0.001^{e}	1.23
Bemisia + (T1T8)	14.17 ± 2.02^{b}	28.67 ± 8.14^{c}	2.77 ± 0.001^{e}	2.89 ± 0.001^{f}	2.02
Bemisia + (T2T8)	17.66 ± 2.08^{b}	20.17 ± 2.75^{d}	3.60 ± 0.001^{c}	1.71 ± 0.001^{c}	1.14
Bemisia+ (T1T2T8)	14.83 ± 1.04^{b}	31.33 ± 5.77^{c}	3.40 ± 0.001^{c}	1.76 ± 0.001^{c}	2.11

[1] **Shoot Dry Matter** [2] **Root Dry Matter. Control** (plant without treatment, positive control), ***Bemisia*** (negative control: plant infected only by *PeLCV* transmitted by whiteflies *Bemisia tabaci* MED), ***Bemisia* + T1** (plant treated with *Trichoderma polysporum T1* and infected by *PeLCV)*, ***Bemisia* + T2** (plant treated with *Trichoderma atroviridae* T2 and infected by *PeLCV*), ***Bemisia* + T8** (plant treated with *Trichoderma harzianum* T8 and infected by *PeLCV*), ***Bemisia* + T1T2** (plant treated with *Trichoderma polysporum* T1 and *atroviridae* T2 and infected by *PeLCV*), ***Bemisia* + T1T8** (plant treated with *Trichoderma polysporum* T1 and *harzianum* T8 and infected by *PeLCV*), ***Bemisia* + T2T8** (plant treated with *Trichoderma atroviridae* T2 and *harzianum* T8 and infected by *PeLCV*), ***Bemisia* + T1T2T8** (plant treated with *Trichoderma polysporum* T1, *atroviridae* T2 and *harzianum* T8 and infected by *PeLCV*). The values with a different letter in the same column are significantly different at 5% level by using the Duncan test.

Figure 21: Pepper plant bioprimed by Trichoderma growing in net cage in presence of bemisia sp vector of Pepper Leaf Curl Virus. a : Bemisia + T1T2T3, b : Bemisia + T2, c : Bemisia, d : Bemisia + T8, e : Bemisia + T2, f : Bemisia, g : Bemisia + T1T2, h : Bemisia + T1T8, and i : Bemisia + T2T8.

3.1.2.4 Effect of *Trichoderma* spp. treatment on the biomarkers accumulation

Effect of *Trichoderma* spp. treatment on the total phenolic content

In general, the soluble phenol content is upgraded significantly (P≤0.05) in all treatments as compared to non-primed plants (50 to 183%) (Figure 22a). Furthermore, the consortium treatments stimulated more phenol production, with increases ranging from 83% to 183%. The binary applications T1T8 showed the greatest increase (17.29g/mL/g dry weight) (P≤ 0.05). These findings show that the tested *Trichoderma* modulates the abundance of phenol content in several plant biological processes, including defense, in either a single or in consortia.

Effect of *Trichoderma* spp. treatment on the specific activity of phenylalanine ammonia-lyase (PAL)

Pepper plants bioprimed with *Trichoderma*, either alone or in consortia, efficiently regulated the PAL-specific activities as compared to their non-inoculated counterparts. Except for treatments T1T8 and T1T2T8, which were not significant (P≤0.05) all *Trichoderma*-inoculated pots showed a reduction in PAL activity (Figure 22b). The pathogen-infected and untreated plantlets (*Bermisia* alone) had the highest activity (5.671 OD/min/mg protein). The consequences of *Trichoderma* invasion on the phenylpropanoids biosynthesis pathway are well depicted.

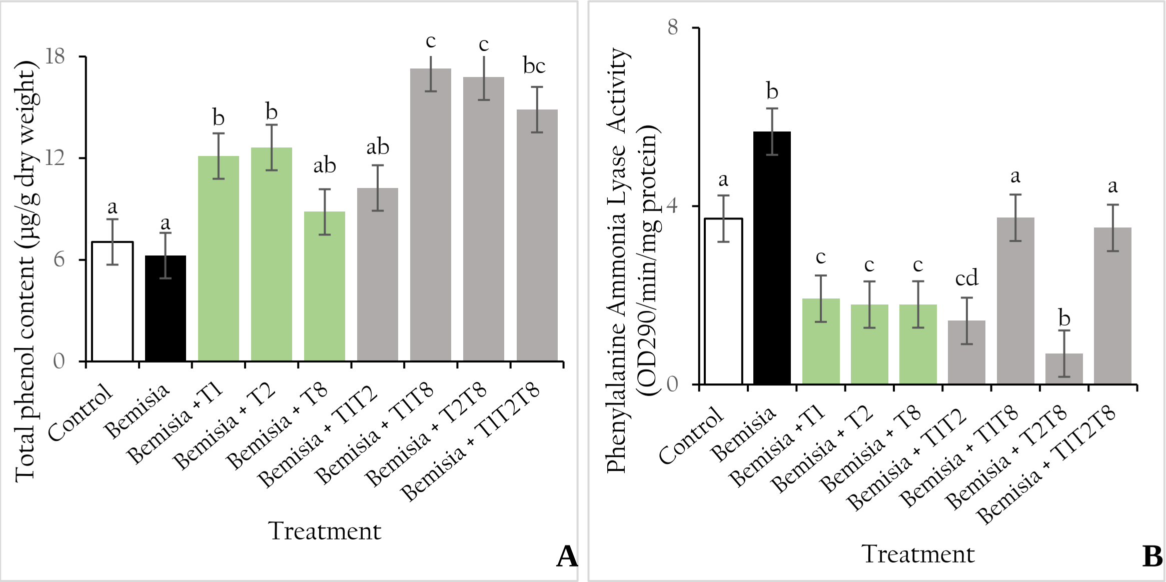


Figure 22: Effect of biopriming pepper plant by Trichoderma on the total phenol content (A) and activity of PAL enzyme (B) under PeLCV pressure. Control (plant without treatment, positive control), Bemisia (negative control plant infected only by PeLCV transmitted by whiteflies Bemisia tabaci MED), Bemisia + T1 (plant treated with Trichoderma polysporum T1 and infected by PeLCV), Bemisia + T2 (plant treated with Trichoderma atroviridae T2 and infected by PeLCV), Bemisia + T8 (plant treated with Trichoderma harzianum T8 and infected by PeLCV), Bemisia + T1T2, Bemisia + T1T8, Bemisia + T2T8, Bemisia + T1T2T8. The values with different letters in bars are significantly different at the 5% level

Effect of *Trichoderma* spp. treatment on the specific activity of superoxide dismutase (SOD).

The variations in SOD activity in *Trichoderma* primed versus unprimed pepper plants (control without *Trichoderma* spp.) are shown (Figure 23A). It is worth noting that only treatments T2, T8, and T1T8 showed a significant change when compared to the control (no treatment) (P≤0.05). In comparison to the *Bemisia* treatment, there was no significant change with *Trichoderma* primed plants in single or consortia (P≤0.05) but T8 (0.243 mmol/min/g of proteins) as a single and T1T8 (0.228 mmol/min/g of proteins) as consortia showed the highest SOD activity. Overall, our findings show that *Trichoderma* activates several molecular pathways for plant defense and other purposes during host root colonization.

- **Effect of *Trichoderma* spp. treatment on the specific activity of guaiacol peroxidase (GPx)**

The guaiacol peroxidase activity (GPx) in pepper plant shoot tissues was tested in uninfected and *Trichoderma* inoculated plants (Figure 23B). When comparing *Bemisia* infected and inoculated plants to non-primed plants, a significant change in guaiacol specific activity was found. Plants grown in *Trichoderma* infected pots in single or in combination treatments exhibited a significant (P≤0.05) increase in GPx activity compared to un-inoculated plants. There was no significant difference (P≤0.05) between inoculated plants with *Trichoderma* alone or in consortia and *Bemisia* treatments; however, T1T2T8 showed a significant difference (P<0.05) with the best GPx activity.

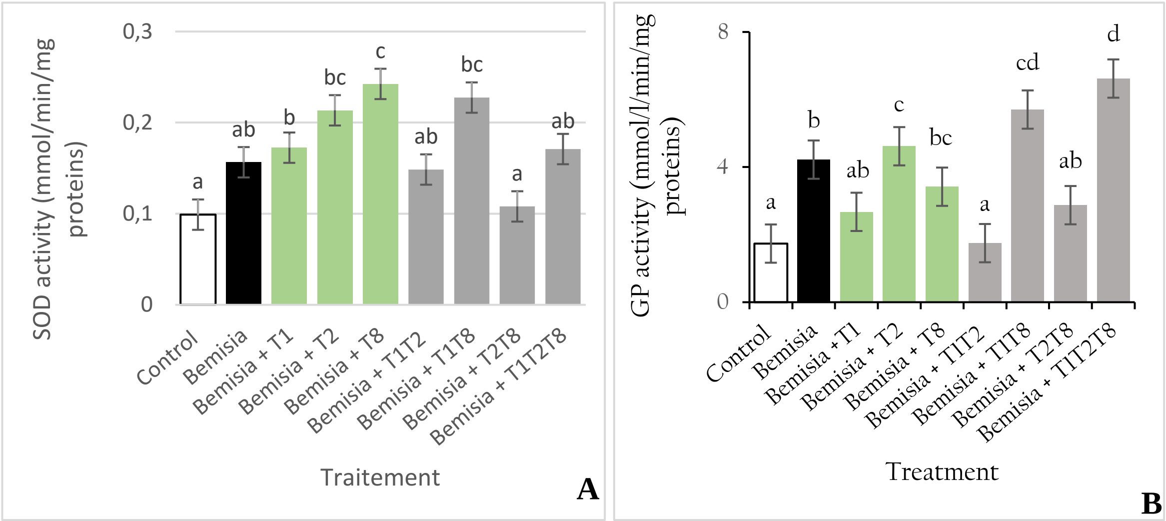


Figure 23: Effect of biopriming pepper plant by Trichoderma spp. on the activity of SOD enzyme (A) and GP enzyme (B) under PeLCVD pressure. Control (plant without treatment, positive control), Bemisia (negative control plant infected only by PeLCV transmitted by whiteflies Bemisia tabaci MED), Bemisia + T1 (plant treated with Trichoderma polysporum T1 and infected by PeLCV), Bemisia + T2 (plant treated with Trichoderma atroviridae T2 and infected by PeLCV), Bemisia + T8 (plant treated with Trichoderma harzianum T8 and infected by PeLCV), Bemisia + T1T2, Bemisia + T1T8, Bemisia + T2T8, Bemisia + T1T2T8. The values with a different letter in bars are significantly different at 5% level by using turkey test.

3.1.3 Effect of *Trichoderma* spp. consortium (T2-T8) on pepper growth and fruit quality.

3.1.3.1 Effect of *Trichoderma* spp. consortium (T2-T8) on growth and yield of chili pepper.

Nearly all growth parameters evaluated as leaf area (LA), fruit weight (FW), fruit diameter (FD), and production yield (PY) were significantly enhanced upon seedlings drenching with the *Trichoderma* consortium as referred to uninoculated controls (P≤0.05). Concretely, 23,5%, 54%, and 23,5% increases were recorded in FW, FD, and PY respectively, compared to uninoculated counterparts. Except for LA where no significant difference was recorded (Table 6, Figure 24 and Figure 25).

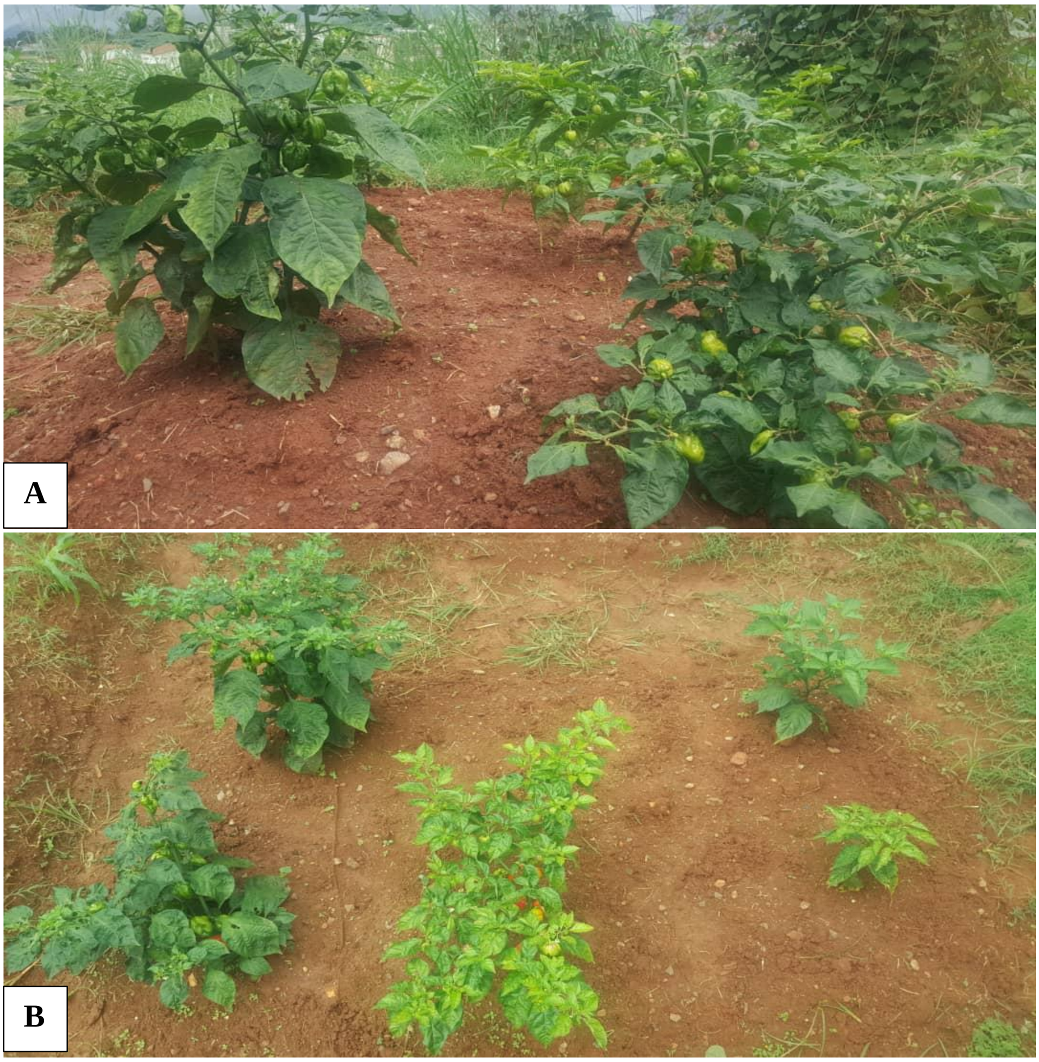


Figure 24: Pepper plant grown in open field. (A, plant treated (T2-T8) and B, control) (Kepngop, 2022 in this study).

Table 6: Effect of Trichoderma consortium (T2-T8) on the peppers plant productivity

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

Column bearing the same letter are not significantly different at $P \leq 0.05$; * or ** stand for level of significance.

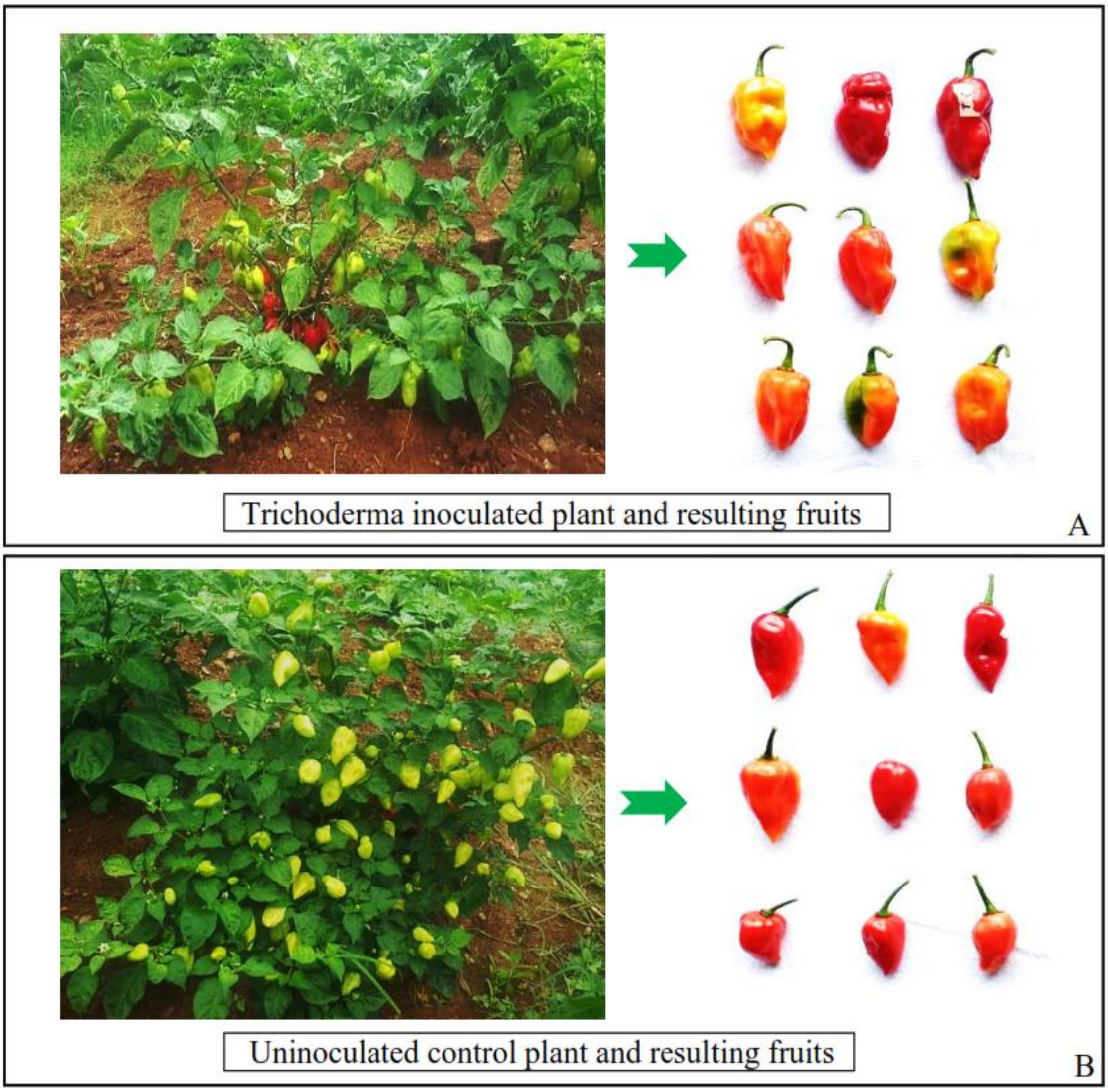


Figure 25: Differential fruits bearing capacity and ripen chili pepper sizes as affected (A) or not (B) by the combination T2T8 Trichoderma (Kepngop, 2022 in this study).

3.1.3.2 Effect of *Trichoderma* spp. fertilization on Phosphorus and nitrogen uptake.

The phosphorus and nitrogen contents of chili plant tissues were significantly enhanced upon biofertilizer application. Notably, 0.048 mg/g fresh matter total phosphorus was recorded in control plants meanwhile 0.1 mg/g/fresh matter was found in *Trichoderma*-treated plantlets, accounting for 108% more P compared to control plants (Figure 26). Likewise, 1.162 mg/g fresh weight was obtained in *Trichoderma*-primed pepper plants, 220% folds higher than un-treated counterparts which culminated at 0.363 mg/g dry weight. This indicates the ability of the so said formulation to boost essential nutrient assimilation.

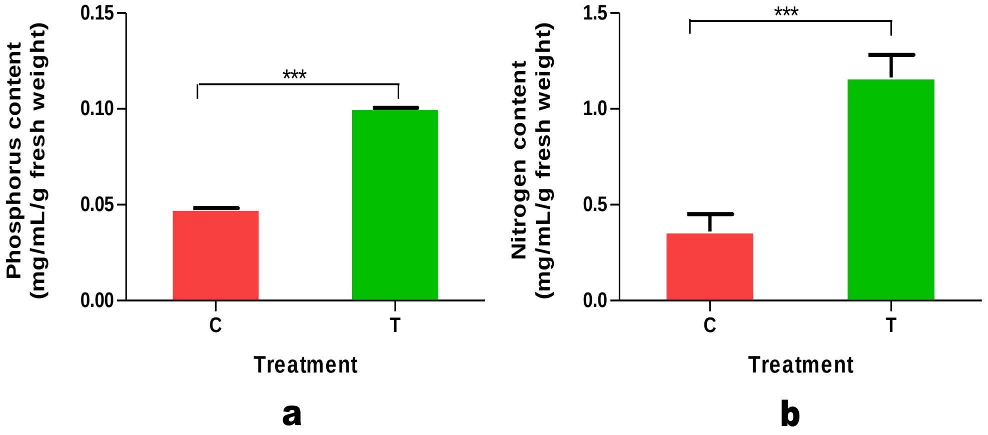


Figure 26: Effect of Trichoderma spp. fertilization on chili pepper Phosphorus (a) and Nitrogen (b) absorption during plant growth. C: un-inoculated controls T: Trichoderma-treated plants. *** level of significance at P≤0.05.

3.1.3.3 Effect of *Trichoderma* spp. consortium (T2-T8) on IAA and photosynthetic pigment biosynthesis.

The production of IAA was significantly increased in *Trichoderma*-inoculated plants compared to un-inoculated counterparts (P≤0.05). For instance, 42.47µg/g of fresh weight IAA was produced upon *Trichoderma* bio-priming, accounting for 76.8% more compared to 24.01µg/g fresh matter recorded for untreated controls. For photosynthetic pigments, 122%, 11%, 113% and 48% more Chla, Chl_b, Chl $_{(a+b)}$, and carotenoids respectively were recovered from pepper plants upon bio-fertilization, as referred to as uninoculated counterparts. Likewise, the ratio Chl_a/Chl_b was 99.9% higher in *Trichoderma*-inoculated plants relative to un-primed plants (Table 7).

Table 7: Effect of Trichoderma consortium (T2-T8) on the IAA hormone and photosynthetics pigments content of chili pepper plant

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	**	***	**	***	***	*

Colum bearing the same letter are not different significantly at $P \leq 0.05$ and *, **, *** show the level of significance.at $P \leq 0.05$.

3.1.3.4 Effect of *Trichoderma* spp. consortium (T2-T8) on fruit quality and conservation

- **Effect of the biofertilization on physiological weight loss (PWL) of pepper fruit**

The storage environment has affected significantly ($P \leq 0.05$) the variation of the percent weight loss of the pepper varieties (figure 27). During storage at ambient conditions, plants treated with *Trichoderma* (T) were found to have the highest percentage of weight loss of 19.36%, 38.85%, 60.96%, and 85.41%, respectively after 5, 10, 15, and 20 days of storage. However, in the cool storage, *Trichoderma* treated plant showed the lowest percentage of weight loss (0.28%, 1.89%, 3.38%, and 4.67% respectively) on the same date. The difference in weight loss of fruits under the two storage environments tended to narrow down.

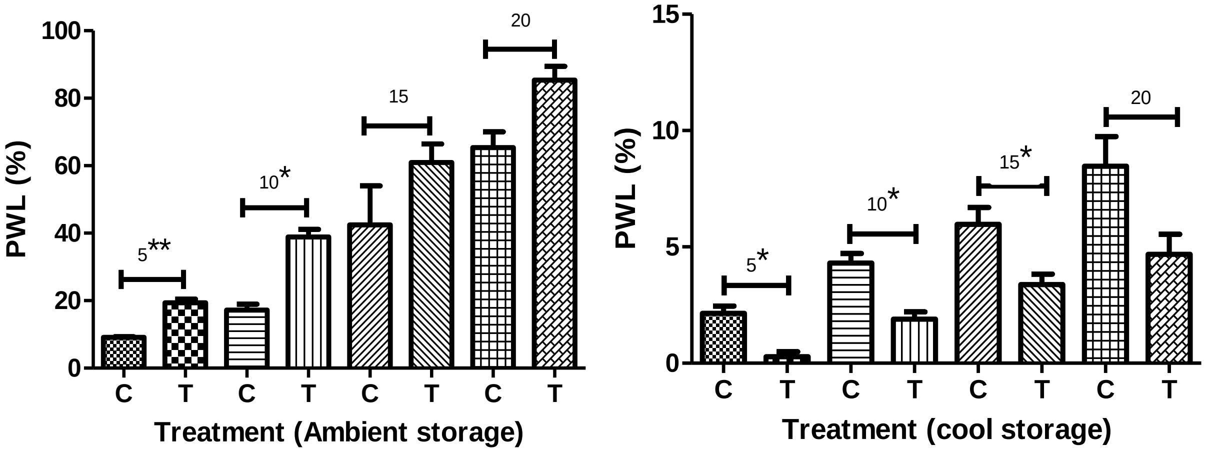


Figure 27: Effect of Trichoderma biofertilization of chili pepper plant on the Physiological Weight Loss (PWL) of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance at $P \leq 0.05$ between treatments

Effect of the biofertilization on the Moisture content of pepper fruit

The moisture content of fruits under two treatment conditions showed no significant variation (P≤0.05) during the storage periods except in ambient storage on days 15 and 20 (figure 28). During the storage fruits treated with *Trichoderma* retained more moisture compared to the control but not significantly. At ambient conditions, *Trichoderma* treated plant showed a fruit with more moisture content than the control (P≤0.05) only after 15 and 20 days. Cool storage showed no significant difference between all treatments during the 20 days of storage.

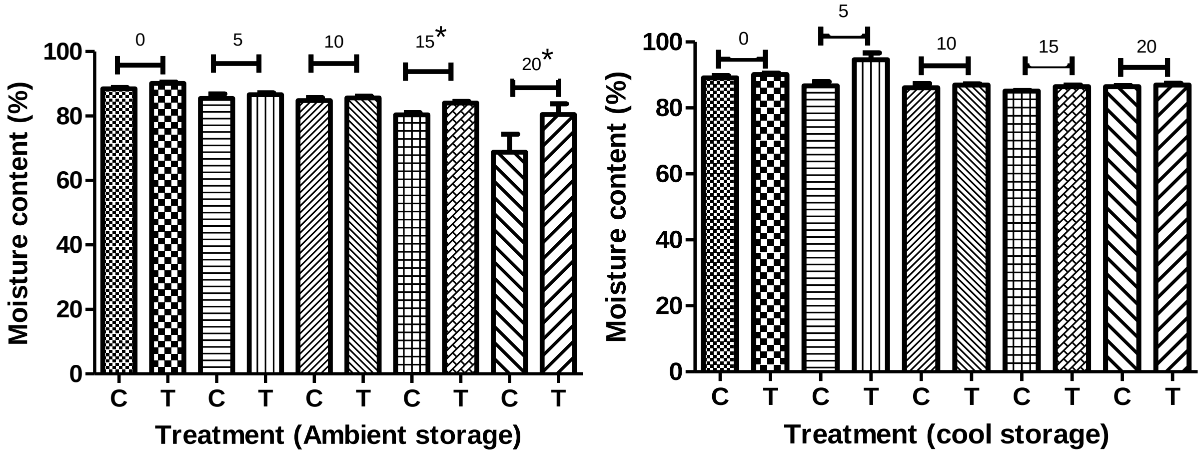


Figure 28: Effect of Trichoderma biofertilization of chili pepper on the moisture content of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance at P≤0.05 between treatments.

Effect of the biofertilization on Total Solid Soluble (TSS) of pepper fruit

The data in Figure 29 displays the TSS contents of peppers fruits treated with *Trichoderma* and subjected to two storage conditions. There was a significant (P≤0.05) difference in the TSS contents of the stored fruits. In all storage environments, *Trichoderma* treatment showed lower TSS (3°Bx) than control fruit treatment with higher TSS (5.5°Bx) at the beginning of the storage and then control showed a reduction of TSS over time and *Trichoderma* an increasing over time. Thus, *Trichoderma* treatment of plant allow plant to increase or limit the degradation over the time. At ambient temperature, except after 10 and 20 days of storage, no significant (P≤0.05) difference was recorded between *Trichoderma* and the control plant. However, in cool storage, except after 5 and 20 days no significant difference (P≤0.05) was recorded, a significant difference was recorded between treatments.

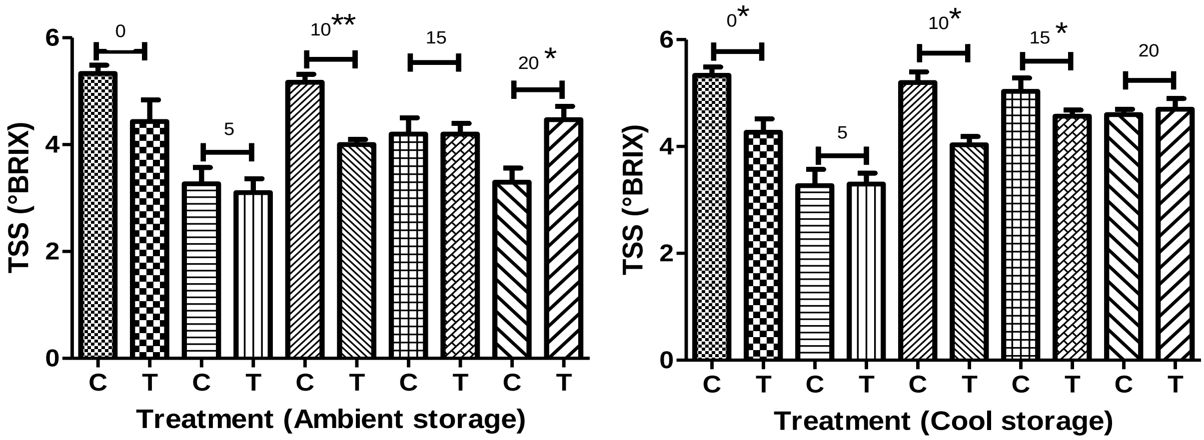


Figure 29: Effect of Trichoderma biofertilization of chili pepper fruits on Total Solid Soluble (TSS) content of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance.at P≤0.05 between treatments.

Effect of the biofertilization on Ascorbic acid content of pepper fruit

A significant difference (P≤0.05) in the ascorbic acid content of pepper fruits was observed between *Trichoderma* fruit (293.225mg) and control (213.264 mg) in 100 g of FW (Figure 30). The ascorbic acid content of all hot pepper harvested and stored in cool conditions showed a decrease of ascorbic acid content after 5 days and an increment until day 20 for both treatments. However, in ambient conditions the control has showed an increase of ascorbic acid on day 5 and then steadily declined until day 20. For *Trichoderma*-treated fruit, a gradual increase was recorded during all the storage days. Overall, in cool conditions like showed by plant treated by *Trichoderma* spp. control fruits have showed a gradual increase of ascorbic acid content over time of conservation but *Trichoderma* spp. treat fruit have showed the same increase over the time like in ambient storage.

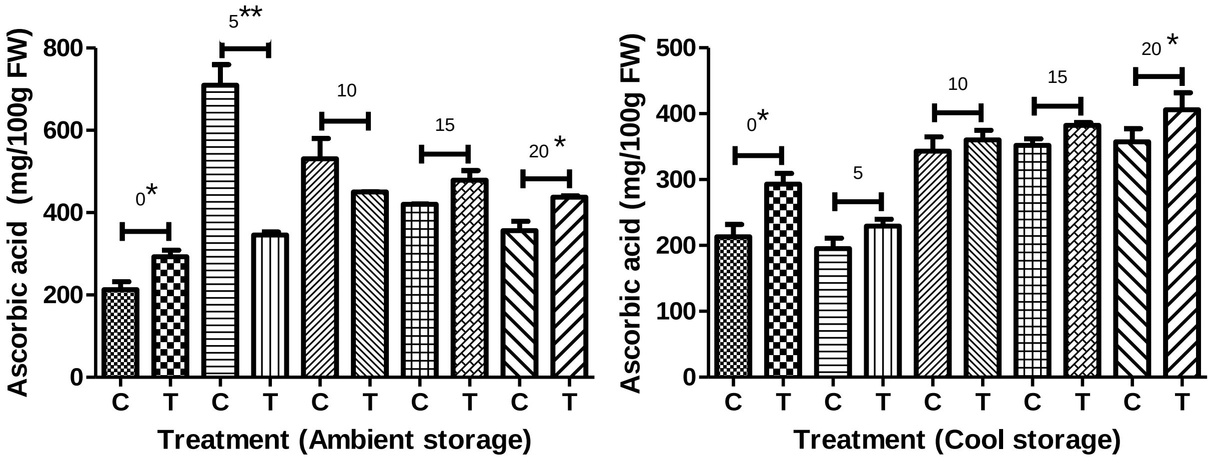


Figure 30: Effect of Trichoderma biofertilization of chili pepper on Ascorbic acid content of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance.at P≤0.05 between treatments.

Effect of the biofertilization on the pH values of pepper fruit

A significant difference ($P \leq 0.05$) in the pH content of pepper fruit was observed between treatments considered in this study throughout the storage period except for some few days (Figure 31). In general, an increase in the pH value during storage was confirmed in both storage for the control and fruit harvested from plants treated with *Trichoderma*. In ambient conditions, the increase was gradual for the two treatments with a high PH value for *Trichoderma*-treated fruit which were significative during 5, 10 and 20. But in cool condition, the values of PH were more conserved over the days for each treatment with high values for Trichoderma-treated fruit which were significative during days 5, 10, and 15.

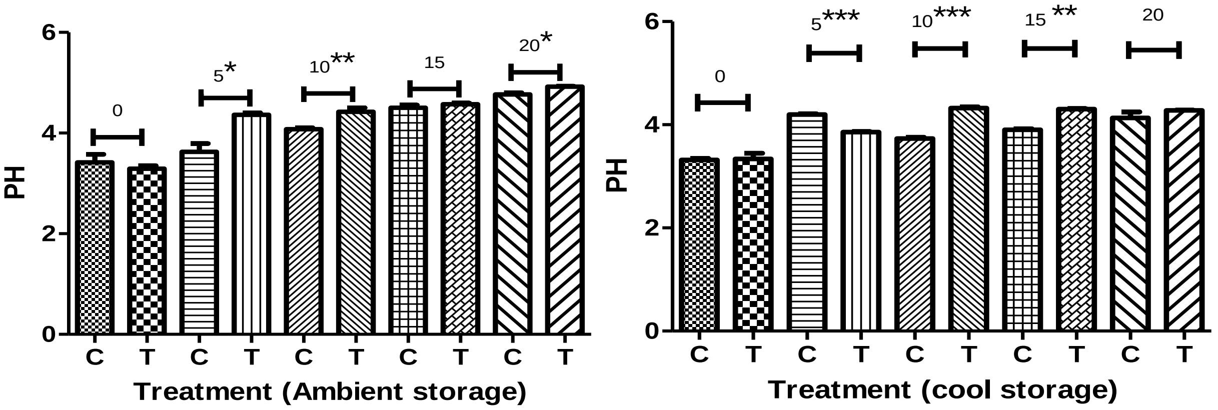


Figure 31: Effect of chili pepper biofertilization on the PH values of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance.at $P<0.05$ between treatments.

Effect of the biofertilization on capsaicin content of pepper fruits

The data in Figure 32 displays the pungency (capsaicin content) of hot pepper harvested at the ripening stage and subjected to two storage conditions. The capsaicin content of hot pepper was significantly different ($P \leq 0.05$) at harvest and during storage. At harvest, high capsaicin content was recorded in fruit harvested from plants treated with *Trichoderma* (1.01 mg/g FW) compared to control fruit (0.7 mg/g FW). Fruits of all pepper treatments recorded significantly more pungency value during storage compared to at harvest. Overall, capsaicin content varied from 1.01 to 3.29 mg/g FW for fruit harvested from plants treated with *Trichoderma* and from 0.7 to 1.77 mg/g FW in the control plant. High capsaicin content was also observed in peppers subjected to ambient storage conditions than in those stored in the cool environment.

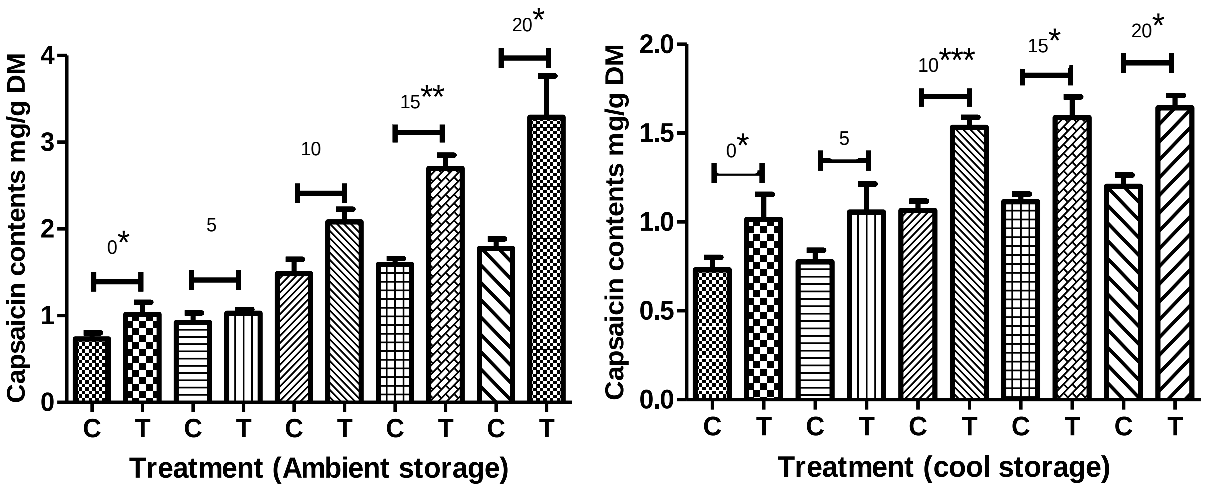


Figure 32: Effect of Trichoderma biofertilization of chili pepper on the Capsaicin contents of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance.at P<0.05 between treatments.

Effect of the biofertilization on total Phenolic content of pepper fruit

The quantification of total phenol content in hot pepper showed a significant difference (P≤0.05) between the two treatments during storage. At harvest, fruit harvested from plants treated with *Trichoderma* showed a high (323.5 mg /g DW) and a significant difference compared to fruit harvested from the control plant (195.5 mg/g DW) (Figure 33). In both treatments, fruit harvest from plants treated with *Trichoderma* showed no significant variation of phenol content during the harvest. However, fruit harvested from the control plant showed a steady increase during the first 10 days of storage, afterward, no significant variation was recorded. Overall, fruit harvested from plants treated with *Trichoderma* spp. showed higher total phenolic content than fruit harvested from control plants during storage in both storage conditions.

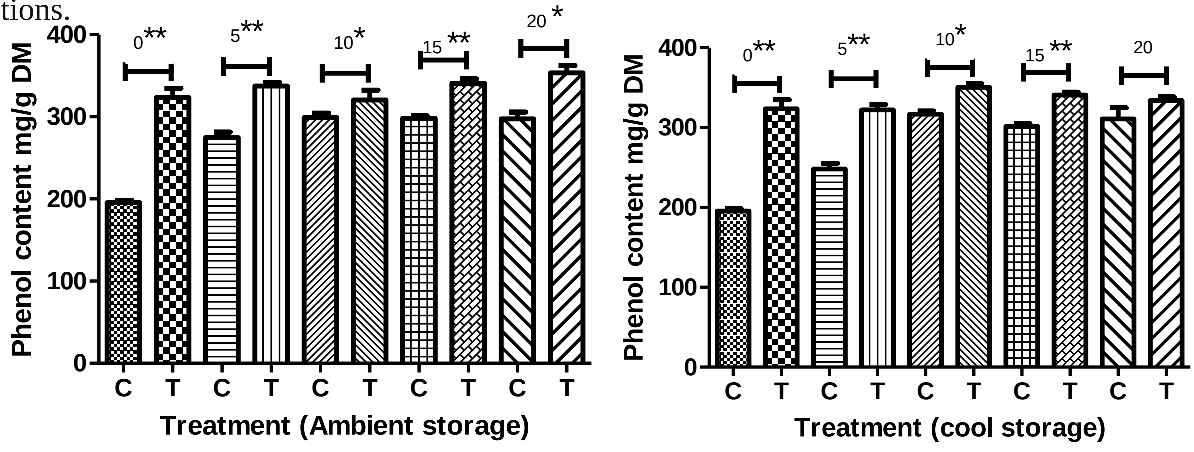


Figure 33: Effect of Trichoderma fertilization of chili pepper on total phenols contents of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance.at P<0.05 between treatments.

Effect of the biofertilization on carotenoids content of pepper fruit

Figure 34 represents the effect of culture treatment and storage environment on carotenoid content in hot peppers fruit. Fruits harvested from plant treated with Trichoderma and store in ambient and cool storage have showed higher content of carotenoids during all the time of storage (P≤0.05). In ambient storage, *Trichoderma*-treated fruits showed an increase of the carotenoids content over the time compare to control fruits where the increase were light. But in Cool storage, excepted the days 15 and 20 where the increase in each treatment was significant, the others days the amount in each treatment (*Trichoderma* and control) were constant. The higher value (4.25 µg/g FW) was recorded in ambient conditions with fruit harvested from plants treated with *Trichoderma* and the lowest value (1.84 µg/g FW) was recorded in cool conditions with fruit harvested from control plant. Fruit harvested from pepper plants treated with *Trichoderma* showed the best carotenoid content at the harvest stage and during all the storage period.

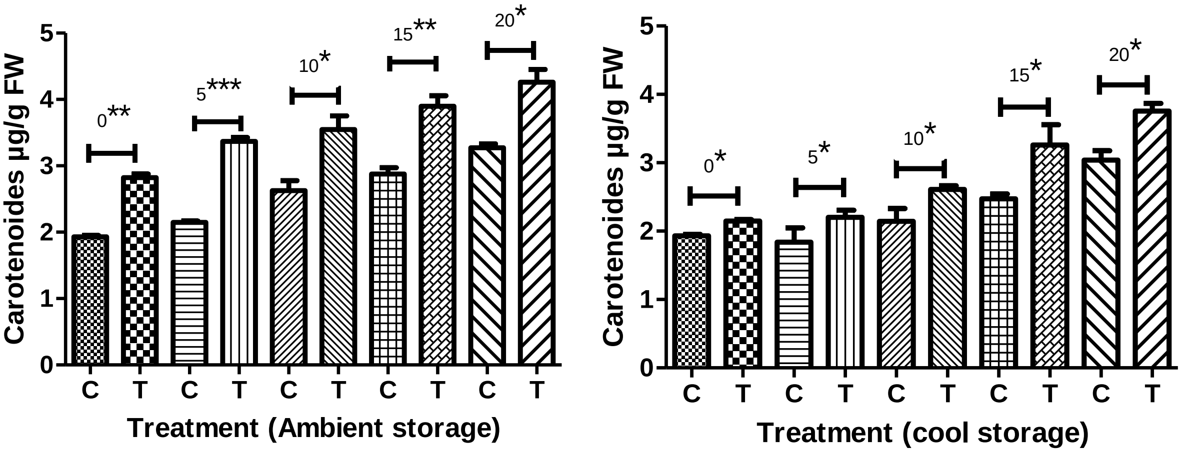


Figure 34: Effect of Trichoderma biofertilization of chili pepper on carotenoids contents of peppers fruits after harvest and storage during 20 days. C: un-inoculated controls T: Trichoderma-treated plants. 0 to 20 represents days of storage and *, **, *** show the level of significance.at P≤0.05 between treatments.

Correlation and Principal Component Analysis

The correlation coefficients between phytochemical content is summarized in figure 35A. Significant (P≤0.05) positive correlations were found between many parameters. The highest correlation between PWL, pH (R^2= 0.77), Capsaicinoids (R^2=0.86), and Carotenoids (R^2= 0.72) was obtained and also between PWL, ascorbic acid (0.37) and Phenol (R^2= 0.32). A highly significant correlation (R^2= 0.86) was found between PWL and capsaicinoids, indicating that the burns sensation of pepper leads to fruit physiology. It's also important to note that capsaicinoids, phenol, and carotenoids were significantly and positively correlated showing group variation of different biochemical compounds affected the physiology of the plant. However, only TSS and moisture content were not significantly correlated showing that the shift of these parameters is not link to other during the conservation.

The PCA (Figure 35B) illustrates the relationship between the parameters evaluated in this study, and how they are influenced during storage and their environment. The PCA clearly showed a cluster of the four biochemical compounds analysed together, as evidenced by their correlation coefficients. The second cluster contained TSS, and the third cluster grouped the moisture content, according to the significant correlations found between the respective groups of parameters.

Based on this plot, it is possible to confirm that after storage, TA 20 (fruit harvested on *Trichoderma* treated plant and stored at ambient temperature during 20 days) at the left had the highest overall capsaicinoids, pH, PWL, carotenoids, ascorbic acids, and phenols whereas CC 0, CC 5, CC 10, CA 5, TA 0 and CA 0 located in the opposite position, showed the lowest compounds contents but highest TSS content.

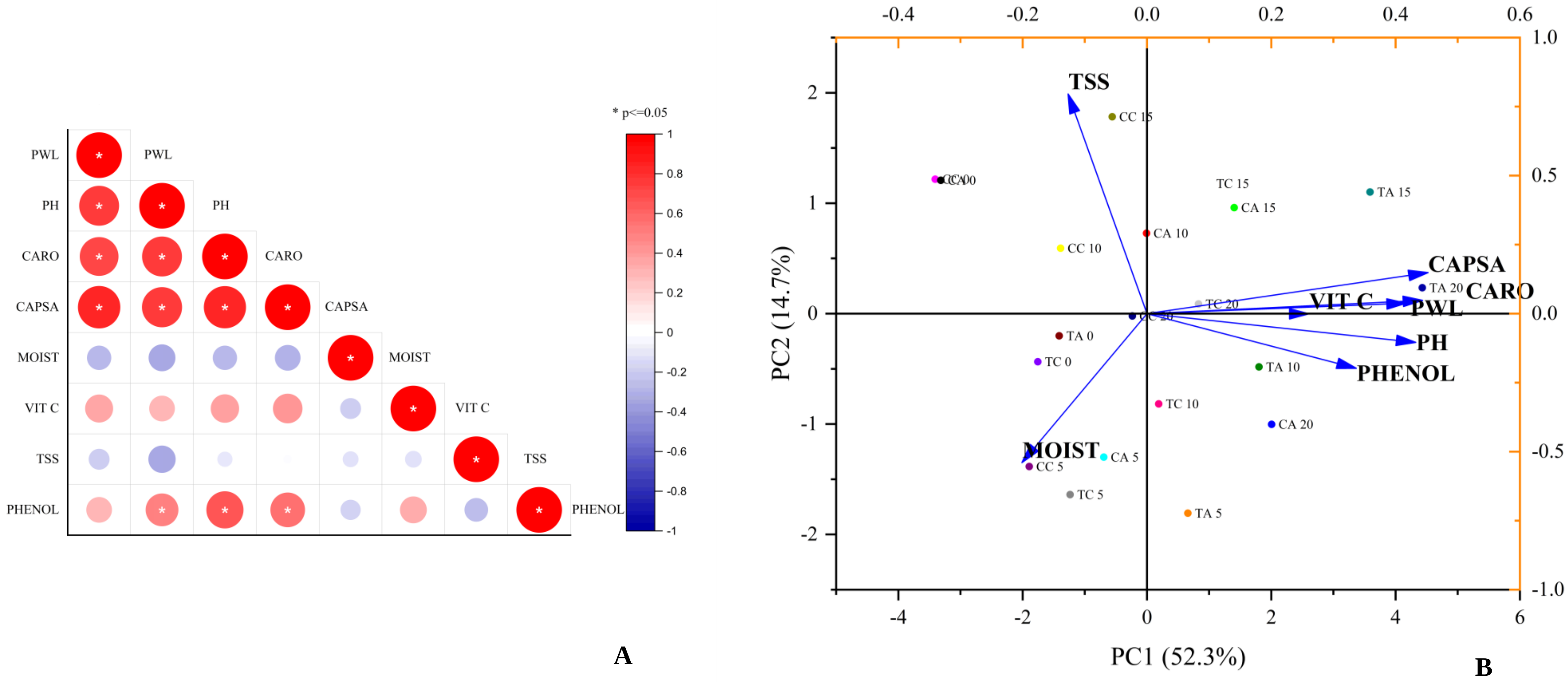


Figure 35: Principal component analysis of phytochemical content of fruits treated and not with bio fertilisation after 20 days of storage in cold (4°C) and ambient conditions

TA = **fruit treated with *Trichoderma* at Ambient condition,** CA = **Control fruit at ambient condition,** TC = **fruit treated with *Trichoderma* at cool condition,** CC = **Control fruit at Cool condition, 0; 5; 10; 15; 20 represents days of storage.** Phenols= **Total phenolics,** TSS = **Total soluble solids,** PWL = **percentage weight loss,** Caro = **Carotenoids content,** capsa= **capsaicinoids content, moist= moisture content,** Vit C= **Ascorbic acid content.**

3.2 Discussion

Biological control is a good alternative for eco-friendly and healthy products but it is important to select a biocontrol agent with some criteria. *Trichoderma* showed an ability to produce Salicylic Acid (SA) and auxin indole3-acetic acid (IAA) hormone *in vitro* and it confirms the work of **Egamberdieva *et al.* (2017)** who showed that phytohormones produced by microbes are an important tool for increasing the abiotic and biotic stress tolerance of plants, providing potential practical applications under changing or extreme environmental conditions. Auxin or indole3-acetic acid (IAA) are important phytohormones and were shown to promote several growths and developmental events, such as cell division, elongation, and differentiation **(Asgher *et al.*, 2015).** SA is also an important phytohormone and it has been extensively studied because it influences various plant developmental processes as well as its role in resistance to abiotic and biotic stresses **(Vlot *et al.*, 2009).** In the context of biotic stress SA is a crucial player in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) as well as effector-triggered immunity (ETI) **(Jones and Dangl, 2006)**. All *Trichoderma* treatments stimulated seeds germination but no significant difference was recorded with the control treatment however, the radicle length of germinated seeds was significantly different from the control showing the capability of *Trichoderma* to stimulate rapid growth of radicle for efficient and prompt nutrition of plants which is recorded most of the time by agronomic growth parameters. *Trichoderma* used here, single or in consortia has shown its capacity to colonize chili plants but the cohabitation of different *Trichoderma* was not efficient for all combinations. This could be due to the competition for food and the effect of the presence of many microorganisms which can be synergistic or antagonistic. However *in vitro* and *in vivo*, the consortia made up of T2 and T8 have shown the best ability for inner part plant colonization. After seeds germination, the content of phytohormones was evaluated and a significant difference between *Trichoderma* treatment and control for the IAA content was recorded and a more significant correlation with the endophytic colonization was obtained showing the implication of *Trichoderma* on plant growth; but, a low quantity of Salicylic acid was recorded which is normal because IAA and SA production are antagonists as shown by **Denancé *et al.* (2013)** and confirmed by a negative and significant correlation between SA and IAA. In addition, SA and IAA didn’t trigger the same pathways in plant development.

The severity and difficulties in controlling viral infections are undoubtedly attributable to their physiopathology. Viruses, in contrast to phytophagous diseases, impair the host's innate immune system and subtilize the cell machinery to establish themselves in plant tissues **(Li *et***

***al.*, 2014).** The reduction of *pepper leaf curl disease* using *Trichoderma* inoculants is revealed in this work for the first time. In the framework of this study, typical symptoms such as leaves curling and yellowing, whole plant stunting, and eventually total death was recorded in chili plantlets infected by begomovirus in the nursery, indicating a successful transfer of the virus from the viruliferous vector (*Bemisia tabaci* MED) to young pepper seedlings. Meanwhile, all *Trichoderma*-primed seedlings (single and consortium treatments) showed a substantial reduction in *PeLCD* severity, reaching up to 50%. Similar outputs were obtained by **Muvea *et al.*, 2018** in an onion infected with the iris yellow spot virus (IYSV) and treated with the beneficial fungus *Hypocrea lixii.* The Elicitation of the plant immune system is typically the primary and most likely mechanism for protecting the host from viral invasion. In this study*, in vitro* and *in planta* results revealed that *Trichoderma,* both alone and in combination, may boost the production of salicylic acid, a hormone known to trigger systemic acquired resistance (SAR) in a variety of host plants against diseases **(Dempsey and Klessig, 2017).** Moreover, all *Trichoderma* primed (single or consortium) and infected (virus) seedlings synthesised substantially more phenolic compounds compared to the non-primed virus-free control and non-pre-primed chili pepper seedlings. This result indicates a three-way interaction between viral assault, phenol accumulation, and disease suppression in resistant cultivars as observed by **Siddique *et al.* (2014)** who discovered a significantly higher phenolic content in resistant cotton genotypes than in susceptible ones following *cotton leaf curl Burewala virus* ingress. The decrease in phenolic compounds in virus-infected plants only (*Bemisia tabaci* MED), could be the result of viral-driven host defense-related gene repression as mentioned by **Markakis *et al.*, 2010.** This observation was confirmed by agronomic parameter evaluated because all the *Trichoderma*-treated plants significantly improved shoot length (up to 50%), root length (up to 67%), and shoot and root dry matter (up to 82.2% and 96.5% respectively). This might be ascribed to disease suppression as asserted by **Eke *et al.* (2016)**, or simply due to the augmented synthesis of IAA as observed in this study. This plant growth hormone is largely reported to control various growth processes such as meristematic cell maintenance and formation and cell enlargement and division, leading to vigorous plants **(Su *et al.*, 2011a; Zhang *et al.*, 2013).** In general, the boost between consortium partners was most illustrated with the consortium T2-T8 because, for all parameters evaluated against disease suppression, the consortium among *T. atroviridae* T2 and *T. harzianum* T8 was present among the best performances. These findings are consistent with those of **Eke *et al.*, 2016a,** which stated that a single BCA fighting with a pathogen is more likely to fail than a consortium application since the former are considered to reinforce each other, resulting in a better outcome.

Several studies have also shown that PAL activation and subsequent increase in phenolic content in plants is a general response related to disease resistance, suggesting that the greater the resistance profile, the lower the activation of the defense mechanism. This might explain the contradictory activity of PAL in comparison to the total content of phenolic compounds in the plants examined. In this work, two destressing enzymes were tested to see if there were any changes in their specific activity when inoculated or not with BCAs under viral pressure. Some pathogens may benefit from an increase in reactive oxidative substrate (ROS) levels generated in host cells during the hypersensitive reaction triggered during the early stages of pathogen ingress as a facilitating factor to invade the host **(Díaz-Vivancos *et al*., 2008)**. Superoxide dismutase (SOD) is one of the most significant radical scavengers catalyzing the dismutation of the superoxide radical into hydrogen peroxide and oxygen **(Hameed and Iqbal, 2014)**. The obtained results for peroxidase showed a non-significant difference between *Trichoderma*-treated plants and *Bemisia tabaci* MED treatments alone irrespective of the improved protection. This indifference in destressing enzyme activity was previously observed in resistant cotton **(Siddique *et al.*, 2014).** One of the most fundamental differences between susceptible and resistant plant defense mechanisms against a pathogen is the speed of early localized ROS accumulation at the site of the pathogen (including viruses) infection. The accumulated ROS is an efficient way of removing the invader before any plant oxidative stress or disease signs develop **(Hernández *et al.*, 2016)**. This might have occurred in this work, given the significantly higher activity of destressing enzymes in virus-free and unprimed plants compared to *Trichoderma-treated* plants that showed more resistance to the *PeLCD*. These observations could be confirmed by the finding of **De Palma *et al.* (2019)** and **Mayo *et al.* (2016)** who reported the capacity of *Trichoderma* to fine-tune plant gene expression for efficient and strategic use of the plant defense machinery.

Despite tremendous beneficial effects associated with *Trichoderma* colonization to plant fitness, their efficacy in climatically fluctuating environments and various soil conditions has been hampering their full integration into agricultural systems for more sustainability **(Alfiky and Weisskopf, 2021; Rivera-Méndez *et al.*, 2020)**. Herein, a fungal inoculant formulated from propagules units of two *Trichoderma* sp. namely; *T. atriviridae* T2 and *T. harzianum* T8 bearing each outstanding attribute was utilized. Subsequently, *Trichoderma* fertilization led to significantly triggered N and P accumulation in peppers plant tissues despite the difficulties related to the extraction of this essential nutrient from the soil chemical complexes **(Fernández *et al.*, 2007)**. While ***Li et al.* (2015)** described the capacity of *Trichoderma* to improve P absorption by tomatoes in P-deficient hydroponic culture, through solubilisation and chelation, many other attempts demonstrated the ability of *Trichoderma* inoculants to change the soil pH, and produce phosphatases to solubilize soil insoluble phosphorus, rendering them bio-available to plants **(Eke *et al.*, 2019b)**. Besides, **Fiorentino *et al.* (2018)** showed that *Trichoderma*-based inoculants were capable of improving N uptake in N-deficient land, favoring its absorption in native soil conditions, or inciting efficient use of this essential nutrient in N-enriched soils. Ultimately, N and P are building blocks of important macromolecules such as chlorophyll, proteins, DNA, and many others. Their subsequent deficiency leads to repressing photosynthetic efficiency, plant development, and thus limited productivity **(Veronica *et al.*, 2017)**. Substantial accumulation of photosynthetic pigments, culminating at 122% for $Chl_{a,}$ 11% for Chl_b, 113% for Total chlorophyll ($Chl_{(a+b)}$), 99% for the ratio of $Chl_a/Chl_{b,}$ and 48% for carotenoids were recorded in response to *Trichoderma* bio fertilization. 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, rubisco accumulation and numerous components involved in the light and dark reactions (Calvin cycle**)** **(Harman *et al.*, 2021)**. Our results indicated that the biosynthesis of IAA got increased by 76% in response to *Trichoderma* application. The so-said hormone is indeed well-known to control various plant growth processes such as meristematic cell maintenance and formation and cell enlargement and division, leading to vigorous plants **(Su *et al.*, 2011b; Zhang *et al.*, 2013)**. Therefore, the Treatment of pepper plant by *Trichoderma* induced the increase of azote and phosphorus uptake, which impact the IAA and photosynthetic pigment production leads by a better development of the plant than control plant. It was clearly shown that a better plant leads to a good fruit quality. A higher percentage of weight loss in pepper stored at ambient conditions was recorded compared to those stored in a cool environment. This can be related to the RH and temperature surrounding the product. The cool environment had more air humidity as well as cooler than the ambient storage conditions, reducing excessive moisture loss from the product. The quality of most fruits and vegetables is affected by water loss during storage, which depends on the temperature and RH of the storage conditions **(Perez *et al.*, 2004). Hardenburg, 1986** mentioned that storage under low temperatures is the most efficient method to maintain the quality of fruits and vegetables due to its effects on reducing respiration rate, ethylene production, ripening, senescence, and root development. High temperature increases the vapor pressure difference between the fruit and the surrounding, which is the driving potential for faster moisture transfer from the fruit to the surrounding air **(Salunkhe *et al.*, 1991).** Accordingly, the higher physiological weight loss shown at ambient conditions can be associated with increased cell wall degradation leading to exposure of cell water for easy evaporation combined with higher membrane permeability due to faster metabolism and ripening rate at high-temperature storage **(Dumville and Fry, 2000).** Therefore, *Trichoderma* could increase fruit metabolism by increasing fruit enzyme activities and water content. This observation could explain the higher percentage of water loss in ambient storage of fruit treated with *Trichoderma* and the beneficial effect of the cool storage on the inhibition of these enzymes activities which lead to the reduction of the percentage of water loss. However, the none significant difference recorded between control plants and *Trichoderma*-treated plants could be due to the use of the same varieties of peppers as ascribed by **Maalekuu *et al.* 2006** who noted that each genotype characterised by some factor such as their cuticular wax content, the difference in cell membrane degradative enzymes and their effects on membrane integrity and membrane lipid composition which are responsible of the difference in moisture loss rate among different genotypes.

The presence of TSS in all fruit at different storage environments indicated that even at reduced temperatures, starch was being converted into sugars **(Getinet *et al.*, 2008; Antoniali *et al.*, 2007)**. Generally, the rate at which the TSS levels increased was faster at ambient temperature than in the cooling chamber. An increase in TSS content could potentially be attributed to moisture loss by the fruits and conversion of organic acids to sugars while the decrease over the storage period may be explained by the respiratory use of the solids by the fruit cells **(Atta-Aly and Bercht, 1995).** This study showed that a plant treated by *Trichoderma* can enhance ascorbic acid content in pepper fruit and this fruit can manage ascorbic acid breakdown during conservation because the differences in nutritional composition are determined by the cultivar, the growing conditions and fruit maturity and that further changes can occur during postharvest handling and storage **(Bosland *et al.*, 2012)**. In one hand, the slow variation of ascorbic acid content in cool conditions between both treatments could be due to the low temperature in the former storage (lower by 10.0 °C) than the ambient condition; which might have retarded aging through the reduction of respiration rate. On the other hand, a high temperature which is known to increase enzymatic catalysis that leads to the biochemical breakdown of compounds in fruit and vegetables could be responsible for the reduction of ascorbic acid content of control pepper stored at ambient conditions **(Yoshida *et al.*, 1984)**. This observation could explain the ability of fruits treated by *Trichoderma* to retard aging through the reduction of respiration rate and therefore the breakdown of ascorbic acid during storage.

The increase of the pH values of pepper over the time of storage was in agreement with the previous findings by **González Estrada *et al.* (2018)**. At the harvest, there was no significant difference between the two treatments at both conditions this could be explained by the finding of **Antoniali *et al.* 2007** who reported that there was no significant difference in pH values of pepper during ripening. According to **Mizrach *et al.* 1997** carbohydrate and acid metabolism are closely connected during the postharvest ripening period which would thus raise the pH of the product. Capsaicin which is an alkaloid compound responsible for the pungency of hot peppers was more important in the fruit store in ambient conditions than those store in cool environment. Therefore, this increasing concentration of capsaicinoids under warmer temperatures than at lower temperatures might be due to faster ripening as well as loss in moisture content of the fruits **(Sathiyamurthy *et al.*, 2002)**. It is assumed that peroxidases catalyse capsaicinoid oxidation and play a central role in its metabolism in pepper **(Sung *et al.*, 2005)**. Peroxidase activity is lower in fruits that have low moisture content **(Bernal *et al.*, 1995)**. Lower peroxidase and capsaicin oxidase activity mean that the oxidation or breakdown of capsaicin will be slower when fruits have low moisture. Thus, both higher rates of synthesis and lower rates of degradation could also contribute to the greater concentration of capsaicin in fruits with low moisture content **(Sung *et al.*, 2005)**.

Phenols are secondary metabolites that are an important tool for the antioxidant efficacy of some fruit. This phenolic content in fruit is influenced by the activity of PPO and the loss of water. The combined action of the two is responsible for the loss of the phenolic compounds in fruit due to its degradation during storage. This can explain why in the fruit harvested from control plant we recorded a variation of the amount of phenolic compound in the both storage environment while in fruit harvested in plant treated by *Trichoderma*, from the harvest time until the end of the storage the amount of phenol was constant in the both storage environment showing the ability of Trichoderma to increase the antioxidant ability of pepper fruit. Therefore, **Khan *et al.* 2017** reported an increasing of the antioxidant compounds amount such as ascorbic acid and carotenoids in ripe fruits due to the application of 100% *Trichoderma* bio-compost in field conditions. The content of carotenoids in peppers was stated to be significantly affected by environmental and agricultural factors **(Nour *et al.*, 2013)**.

The Principal Component Analysis illustrates the relationship between the parameters evaluated in this study, and how they are influenced during storage and their environment. The PCA clearly shows (Pink color) a cluster of the four biochemical compounds analysed together, as evidenced by their correlation coefficients. In general, the parameters PWL, PH, capsaicinoids, carotenoids, ascorbic acid and phenol were positively correlated showing the link which exist between all these parameters. As demonstrated previously, to maintain all these antioxidant coupound (capsaicinoids, carotenoids, ascorbic acid and phenol) fruit should show a lower PWL to reduce activity of metabolism and PH is well known in the implication of metabolism and enzyme activity. This correlation could also show that the treatment of pepper plant with *Trichoderma* spp. has a big impact on the amount of antioxidant compound produce and maintained over the time of the storage.

Based on this plot, the treatment TA 20, TA 15, and TA 10 are in the same alignment like the parameters capsaicinoids, pH, PWL, carotenoids, ascorbic acids, and phenols showing that at these times these treatments were the best and there were containing the majority of compound whereas CC 0, CC 5, CC 10, CC 15 and CA 0 located in the opposite position of these parameters showed the lowest compounds contents. In general, PCA revealed that *Trichoderma* treatment caused a shift in the phytochemical content of pepper fruits the pattern of shifting varied with the environment of storage while ambient conditions showed the best phytochemical content during the 20 days of storage. PCA results confirm the correlation analysis and demonstrate the usefulness of this multivariate analysis in the classification of fruits and vegetables based on their AA and individual antioxidant compounds.

CONCLUSION RECOMMENDATIONS AND PERSPECTIVES

4 CONCLUSION, RECOMMENDATION AND PERSPECTIVES

4.1 Conclusion

This work aimed to evaluate the effects of some *Trichoderma* against Pepper Leaf curl virus Disease and chili fruit quality. Accordingly, the following conclusions have been drawn.

- Characterization of *Trichoderma Polysporum* T1, *Trichoderma Atroviridae* T2, and *Trichoderma Hazianum* T8. These *Trichoderma* shows the capacity to colonize the inner part of the plant, produce and release plant hormones. They are not toxic for peppers seeds, and some consortia stimulate radicle growth and regulate hormonal production by plants according to the plant's physiological status. Based on this work it is better to use endophytic biological control agents than others because they can interfere directly with all plant development,
- Work demonstrated *Trichoderma*'s capacity to lower *PeLCVD* severity up to 50% which was effective by the modulation of some biochemist and agronomics parameters Overall, the consortium T2T8 performs best. there is no alternative against plant virus disease, so *Trichoderma* can be a serious alternative,
- In the end, a consortium of *Trichoderma* T2T8 shows the capability to enhance plant productivity by upgrading photosynthetic pigment production, plant nutrition (100%) and IAA production in peppers during open-field production. This result confirms the increase in fruit quality through the treatment of plants by *Trichoderma*. This application increases the content of all micronutrients evaluated which is important for human health. *Trichoderma* also enhances the maintenance of fruit quality during conservation for up to 20 days. It is strongly advocated to use a consortium of biological control than a single one to have a better result also the use of *Trichoderma* is a good alternative because it can help not only for plant defense but also for fruit quality.

This work showed that endophytic *Trichoderma* especially the consortium made by *Trichoderma atroviridae* T2 and *harzianum* T8 is a good biological control agent which reduced pepper leaf curl disease by 50% and globally enhanced chili fruit quality even after conservation during 20 days.

4.2 Recommendations

Biological control agents can help to reduce utilization of chemical pesticides and therefore preserve well-being. Viral disease is a huge problem in agriculture and it is not possible to fight against it when a plant is infected. Based on this work, *Trichoderma* can be an alternative all this purpose. Before growing a pepper plant, it is important to pre-germinate its seed in soil supplemented with endophytic Trichoderma to help the plant fight against viral disease and also enhance fruit quality before and after conservation.

4.3 Perspectives

The obtained results clearly show the need to carry out further investigations. Hence, we intend to

- To evaluate the effect of *Trichoderma* on the Post-transcriptional gene silencing system of plant.
- To do a RNAi transformation of Trichoderma to optimize its capacity
- To do an epidemiology to identify different type of virus affecting plant in all agroecology zone of Cameroon.

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RESEARCH ARTICLE

Biological elicitor potential of endospheric *Trichoderma* and derived consortia against *pepper* (*Capsicum annuum L.*) *leaf curl virus*

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ABSTRACT

Pepper leaf curl virus (*PeLCV*), transmitted by whitefly is a cosmopolitan plant viral disease hampering a wide range of crops. Chemical elicitors have been used to curb the trend regardless of their setbacks on environment and human kind. Herein, we report the suppressive effects of *Trichoderma Polysporum* T1, *Atroviridae* T2 and *Harzianum* T8 and their consortia against *PeLCV*. They endophytically colonised pepper tissue with significant shift in auxin leading to accumulated biological yield. Likewise, *Trichoderma*-induced innate host immunity priming and stress tolerance may be mediated by the triggered phenylpropanoid biosynthesis (183% more phenolic content). The capability of *Trichoderma* bioagents to release salicylic acid under *in vitro* conditions appear to have prominent role in orchestrating *PeLCV* suppression (up to 50%). Interestingly, no difference was recorded in the specific activity of the destressing enzymes indicating a likely early ROS accumulation at infection sites leading to restricted virus spread. Overall, *Trichoderma* could help to counteract viral diseases.

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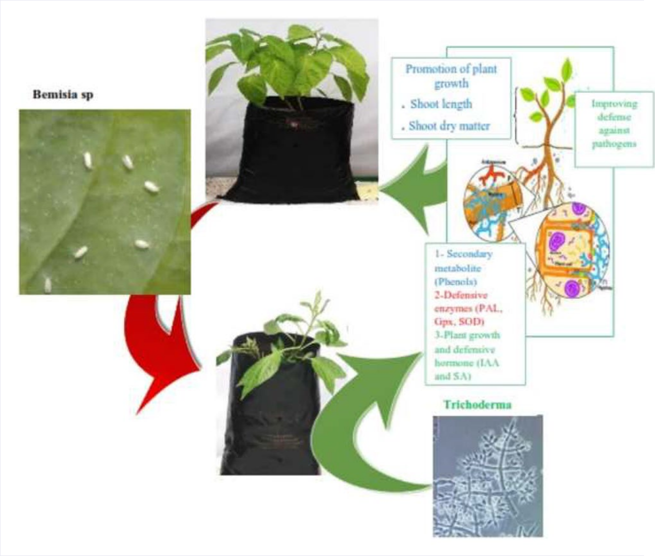
HIGHLIGHTS

- T. harzianum T8, Atroviridae T2 and Polysporum T1 have the capability to reduced PeLCV in peppers plants
- These Trichoderma showed a best capacity in consortia than single against PeLCV
- These Trichoderma produce some phytohormones and help plant for growing and plant defence against biotic attack.

GRAPHICAL ABSTRACT



1. Introduction

Vegetable crops are crucial to the food, health, and livelihood of the world's rural populations, including those in Cameroon. Vegetables are essential income crops for traders and smallholder farmers in under developed countries owing to their flavour, colour, and taste, as well as their high vitamin content (Moriones, Praveen, and Chakraborty 2017). Attempts at domestication of vegetables for food and commercial purposes were envisioned centuries ago in order to sustain the world's ever-growing population, particularly in Africa, where food security has always been a pressing issue. Despite substantial improvements in crop improvement in recent years, tremendous efforts are still required to achieve a stable balance between requirements and supplies.

The global vegetable production trend reveals a steady decline, which has been attributed to a set of constraints topped by diseases, among which viral infestations are of paramount importance globally (Tolin and Fayad 2016). Germiniviruses, which cause Pepper Leaves Curling (PeLCV) and are transmitted by whiteflies (*B. tabaci*), are among the most economically significant plant viral diseases (Gangwar and Gangwar 2018; Harrison and Robinson 1999). The former harms both weeds and main commodities including but not limited to common beans (*Phaseolus vulgaris* L.), cassava (*Manihot esculenta*), cotton (*Gossypium* spp.), squash (*Cucurbita* spp.), Okra (*Abelmoschus esculentus*), tomato (*Solanum lycopersicum*) and chilli peppers (*Capsicum annuum* L.), causing up to a billions USD annual loss worldwide (Gangwar and Gangwar 2018; Stansly and Naranjo 2010). A new recent survey conducted in Western and Central Africa has thrown further insights on the prevalence and destructive character of begomovirus towards major indigenous staples (Leke et al. 2011; Thakur et al. 2019). In Cameroon, for instance, an outbreak of a highly deadly viral infection was reported in several commercial chilli pepper farms (Achiangia, Ngimoh, and Annih 2013) Given their virulence, broad host range, and dissemination, such diseases require special attention since they have the propensity to become epidemic, having a negative effect on local and global food security.

However, neither local researchers nor the government has addressed this issue, regardless of the fact that a rapid diagnosis of an outbreaks and the adoption of effective control measures would be essential to anticipate on the losses. In endemic regions, a variety of chemically diverse synthetic pesticides, such as organophosphate, organochlorine, and others, are now used to target whiteflies as the primary vector (Oliveira et al. 2013). Nonetheless, the intrinsic carcinogenic, mutagenic and teratogenic hazards of such synthetic pesticides, as well as the emergence of resistant whiteflies (Naveen et al. 2017; Shadmany, Omar, and Muhamad 2015), have motivated the quest for a more environmentally friendly alternative (Faoro and Gozzo 2015).

As a result, small RNAs, recognised as key regulators of host plant resistance gene expression to dwarf viral-induced plant diseases, hold a lot of promise (Djami-Tchatchou and Dubery 2015). However, in addition to their transiency, fine monitoring of miRNA activities is necessary prior to implementation in modern farming to avoid "off-type" outcomes (Faoro and Gozzo 2015; Walters, Ratsep, and Havis 2013). Similarly, attempts are being made to harness endophytes, which share similar functions and work in tandem with their hosts to improve crop fitness under adverse conditions (Eke, Fokou, et al. 2016; Niu et al. 2017). Endophytes, or plant-inhabiting microorganisms, are believed to detect physiological changes in stressed plants and adjust plant gene expression accordingly (De Palma et al. 2019; Liu et al. 2017). Mayo et al. (2016) revealed a suppression of circumstantial irrelevant genes in favour of defense-related counterparts in common bean (*P. vulgaris* L) primed with *Trichoderma velutinum* under Rhizoctonia solani assault.

Many further studies have demonstrated *Trichoderma* species' ability to systemically induce resistance in a variety of hosts against numerous phytophagous fungi (Eke, Fokou, et al. 2016) bacteria (Salas-Marina et al. 2015), nematodes (Al-Hazmi and Tariq Javeed 2016), fungi (Mousumi Das, Haridas, and Sabu 2019) and viruses (El-Sharkawy et al. 2014) Meanwhile, the diversity of the earth's ecosystems, as well as the notion of coevolution, emphasises the significance of exploring *Trichoderma* from geographically diverse regions. Since their metabolomics and subsequent efficiency may vary accordingly. Consortium of biological control agents (BCA) are presently strongly advocated over single BCA since they are likely to be more effective and transposable in open field (Eke, Chatue, et al. 2016; Wehner et al. 2010). In this study, we tested endophytic *Trichoderma* and their consortia for the growth and suppression of *PeLCV* in Pepper plants in the laboratory.

2. Materials and methods

2.1. Source of whiteflies and rearing

Whiteflies (*Bemisia* spp.) used as vector in this study were collected on pepper seedlings with characteristic *PeLCV* symptoms (Kumar et al. 2006) in commercial hot pepper (Chilli pepper) farm in Bangangte, West Region, Cameroon (Longitude 10°50'00" E; Latitude 5°15'00" N). Their identity was ascertained by Professor Kekeunou Sevilor at the Laboratory of Zoology, Department of Animal's Biology and Physiology of the University of Yaoundé I, Cameroon. The collected insects were identified using the protocols described by many authors (Bellows et al. 1994; Delvare and Aberlenc 1989; Martin 1987; Malumphy et al. 2009; Martin and Mound 2007)

The insects were reared in isolation on young pepper seedlings grown in net cages on a laboratory bench in net cages. Two weeks before the tests, viruliferous whiteflies were produced by feeding matured *Bemisia* spp on diseased (*PeLCV*) pepper seedlings maintained in an insect proof net cage. During the suppression test, the viruliferous whiteflies that were created were utilised to inoculate experimental plants.

2.2. *Trichoderma* isolates and identity confirmation

2.2.1. Source of *Trichoderma* spp

The *Trichoderma* (BCA) isolates were obtained from the Antimicrobial and Biocontrol Agents Unit of Cameroon's Laboratory for Phytobiochemistry and Medicinal Plant Studies. They were chosen for their biological control of fungal pathogens (Eke, Fokou, et al. 2016). Inoculums were prepared prior to tests by inoculating young colonies of each BCA in a conical flask seeded with Potato dextrose broth medium (PDB, Himedia) for 7 days with constant stirring (150 rpm). After that, the suspension was filtered and adjusted to the desired titre (10^6 spores/mL) using a Mallassez haemocytometer.

2.2.2. *Trichoderma identity*

2.2.2.1. *Genomic DNA extraction.* The 5.8 s ribosomal DNA gene sequences were used to determine the identity of the investigated BCA. Prior to the PCR, the hyphal tips of each *Trichoderma* isolate were cultured for 72 hours at 28 °C in an Eppendorf tube filled with Potato Dextrose Broth (PDB, Himedia, India). Mycelial mats were centrifuged (13,000 rpm, 5 minutes) and rinsed with 500 L TE (TrisCl, EDTA) buffer. Afterward, each pellet received 300 L of extraction buffer (200 mM Tris HCl, pH 8.5, 250 mM NaCl, 0.5% SDS, and 25 mM EDTA) and was crushed using liquid nitrogen. Following that, 150 L of 3 M Sodium acetate, pH 5.2 was added to the tubes, which were then stored at −20 °C for 30 minutes and centrifuged. The supernatant was then transferred to a new clean Eppendorf tube. An equal volume of isopropanol was added and the mixture was left at room temperature for 10 minutes. Centrifugation was used to precipitate DNA (12,000 rpm, 10 min). After washing with 70% ethanol, the pellet was dried for 5 minutes before being re-suspended in 50 L TE buffer and stored at −20 °C for future use.

2.2.3. *5.8 s ribosomal DNA gene amplification*

For the PCR, reaction mixtures (25 L) containing PCR buffer (0.2X), PCR water (14.95 L), MgCl2 (0.16 mM), dNTP (0.144 mM), ITS1 (forward primer, 0.01 M), ITS4 (reverse primer, 0.01 M), Taq polymerase (0.5 mU/L), and genomic DNA (100 ng) were prepared for each isolate. The thermal cycler programme was as follows: 95 °C for 2 minutes, 95 °C for 1 minute (35 cycles). Primer annealing for 1 minute at 55 °C, elongation for 2 minutes at 72 °C, final extension for 10 minutes at 72 °C, and final hold at 4 °C. Agarose gel electrophoresis was used to visualise the PCR results. The gel was operated at 75 V for 30 minutes before being examined with a UV trans-illuminator at 254 nm wave length. The gel pictures were created using Gel Doc XR + Imaging system (Bio-Rad Laboratories Inc., Germany).

2.2.4. *Sequencing and phylogeny*

To achieve maximal length, PCR products were bi-directionally sequenced. Using the 5.0 BioEdit sequence analyser programme. The sequencing data were end trimmed and contigs assembled. then, the assembled sequences were used for blast searches in the NCBI nucleotide database(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blast/BlastSearch&LI 160 NK LOC=blasthome). The identities of each separate study were confirmed using the closest match approach (Sheoran et al. 2015). The total sequences were then aligned in Fasta text format with Clustal W, which is built with Mega program (Version 7.0). To generate the phylogenic tree, the neighbour-joining method with Kimura parameter distances was used. Percentage of similarities was used to group species (Márquez, Bills, and Zabalgogeazcoa 2007).

2.3. Assay for phytohormones production

2.3.1. Indole acetic acid production

Indole acetic acid (IAA) is a hormone that aids in the growth of plant roots. The capacity of a specific BCA living endophytically to produce IAA is a promise of fostered host plant development, A spectrophotometric method, described by Goswami et al. (2013) was utilised to detect and quantify IAA production by the tested BCa's. Supernatant from 72 hours broth culture (PDB, Himedia India) supplemented with Tryptophan (200 µg/mL) and NaCl (2% was mixed in 1:1 ratio with salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). Development of pink colour indicated the production of IAA. The optical density readout was done at 530 nm (Tecan Infinite M200 microplate reader) and the concentration of IAA was estimated using a standard curve of pure IAA prepared at the range of 10–100 µg/ml.

2.3.2. Salicylic acid production

Salicylic acid is a plant hormone that controls many aspects of plant growth and development, as well as resistance to abiotic and biotic stressors. The evaluation of the production of SA by *Trichoderma* spp. was carried-out according to Meyer, Azelvandre, and Georges (1992). Trichoderma's conidial load of 10^6 cells/L was seeded in test tubes containing succinate medium and incubated under constant stirring (100 rpm) for 72 hours (28 ± 2 °C). Later on, the suspension was centrifuged (6000 g, 5 min) and acidified (pH 1.8) with HCl (1 N). Salicylic acid was then extracted with Chloroform and the solvent evaporated with rotary evaporator (BUCHI 001) set at 40 °C. The water-free extract was then re-suspended in water and 5 µL of ferric chloride solution ($FeCl_3$) was added. The appearance of purple colouration indicated SA production. The OD of the purple ferric-salicylic acid complex developed was measured at 527 nm (Tecan Infinite M200 microplate reader). The concentration of SA released by each BCA was estimated against a standard curve of pure SA plotted with concentration range of 10–100 mM.

2.4. Assay for rhizospheric efficiency

2.4.1. Endophytic power

2.4.1.1. Inoculum preparation, seed inoculation and gnotobiotic growth. To ensure that the investigated BCAs could colonise the interior parts of pepper seedlings, inoculums of each *Trichoderma* were produced and diluted to 10^6 conidia/mL in sterile saline solution (Singh et al. 2016). Their respective double and triple combinations (v/v) were likewise made by combining equal parts of each counterpart. Surface-disinfected pepper seeds were surface-disinfected by soaking them in 1.5% sodium hypochlorite for 5 minutes, followed by two rinses with sterile distilled water (SDW). The sterile seeds were immersed in conidia solutions for two hours before being put onto Petri plates lined with sterile wet tissue paper (Eke, Fokou, et al. 2016). The control seeds were dipped in sterile distilled water. Both controls and inoculated plates were incubated (25 ± 2 °C) in growth chamber with 12/12 photoperiod for two weeks.

2.4.1.2. Trichoderma recovery and enumeration. Young Pepper seedlings (1 g) from each treatment were surface disinfected in 2% Sodium Hypochlorite (5 min) and 70% Ethanol (1 min), followed by three times rinsing with SDW. The sterilized seedlings were afterwards grounded in 10 mL sterile Phosphate Buffered Saline (PBS; pH 7.2), and allow to settle for 15 minutes. The supernatants were separated from the residues and further diluted and seeded onto chloramphenicol amended (100 mg/L) PDA (Himedia. India) at 28 °C. *Trichoderma* colonies were counted and expressed in terms of colony forming unit (CFU) per gram of fresh weight (Sheoran et al. 2015). At mean time, hyphal tips of each emerging *Trichoderma* were seeded onto fresh PDA medium and their cultural and microscopic characteristics were compared to initial original inoculums (Rifai 1969; Samuels 1996). The experiment was performed in triplicate and repeated twice (Table 1).

2.4.2. Assessment of in vitro cohabitation of the BCA

It has been proven that consortia of BCAs, rather than a single BCA, are more likely to function effectively in open field settings. To

Table 1. Estimation of *Trichoderma* growth ability on PDA medium supplemented with NaCl at differing concentration.

	Growth Inhibition (%)		
Conc (mmol)	T1	T2	T8
0	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
250	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
500	30 ± 0.8a	38.2 ± 0.8b	36.5 ± 1.7b
750	56,5 ± 1.7a	53.5 ± 0.8b	61.8 ± 4.2c
1000	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a

^{Conc} concentration. ^{T1}*T. polysporum* T1, ^{T2}*T. atroviridae T2*, ^{T8}*T. harzianum* T8. Line bearing the same letter are not significantly different at $p < 0.05$.

determine if the tested *Trichoderma* isolates might share the same niche, 5 days old mycelia plugs of each *Trichoderma* isolate were planted at opposite locations on PDA media and cultured. Cohabitation was positive if no inhibition zone formed at the antagonists contact zones.

2.4.3. Salinity stress tolerance

Soil salinity might have a significant impact on the development and efficiency of BCAs (Mohamed and Haggag 2006). To test the salt tolerance of the studied BCAs, mycelial discs were plated on PDA media containing varying concentrations of NaCl (0, 250, 500, 750, and 1000 mmol) and cultured (28 °C, 7 days). Three replicates per plate were prepared, and the whole test was repeated twice. The colony diameter was measured daily, and the development of the fungi under stressed and unstressed circumstances was compared.

2.4.4. Assessment of seed germination and early seedling growth

The BCAs and their consortia were cultured for one week on PDA medium, and the spores were collected in sterile saline solution (0.85%) and adjusted to 10^6 spores/mL, as described by Singh et al. (2016). To test the impact of *Trichoderma* on Pepper seed germination, surface disinfected seeds were coated with *Trichoderma* conidia suspension (10^6 conidia/mL) and coated with Carboxymethyl Cellulose (CMC, 1.5%) as an adhesive. CMC-treated seed was solely used as a control (Singh et al. 2016). The seeds were placed in Petri dishes that were lined with two layers of tissue paper and sterile distilled water. For each treatment, five plates of 25 seeds were prepared. When the radicle was three times the size of the seed, the seed was considered to have sprouted. The number of germinated seeds was counted on a daily basis until final count, and the following germination parameters were computed (1) (2) (3)

The germination means time (GMT) (Benech Arnold et al. 1991)

$$\mathrm{GMT} = \sum \mathrm{D} \times \mathrm{n} / \sum \mathrm{n} \tag{1}$$

Where; GMT; germination mean time; D: days of seeds germination evaluation (3, 6, 9, 12 and 15 days) and n; number of seed germinated at each day D.

The Germination Index (Eke et al. 2019)

$$\mathrm{GI} = \sum \mathrm{N} / \sum \mathrm{D} \tag{2}$$

Where; GI; being germination index; D days of seeds germination evaluation (3, 6, 9, 12 and 15 days) and N; number of seed germinated at the days D.

Germination Percentage (GP)

$$GP = \left(SG / N\right) * 100 \tag{3}$$

Where: GP: Germination Percentage; SG: Seeds Germinated and N: Total number of seeds.

After 15 days, the radicle length was measured. The IAA and SA contents of the seedlings were estimated as described by Goswami et al. (2013) and Meyer, Azelvandre, and Georges (1992) respectively.

2.5. In planta PeLCV suppression experiment

2.5.1. Potting soil preparation and sowing

The experimental soil, (Rhodic kanduidlut; U.S. soil taxonomy) was collected in agricultural farm near the University of Yaoundé I. The averaged chemical and elemental composition are [P: 1.54 ppm; K: 0.28 Cmol/kg; Ca: 3.97 Cmol/kg; CHCOONa: 0.52 mM; Clay: 51.9%; Mg: 0.92 Cmol/kg; Al: 0.43 Cmol/kg; Na: 0.023 Cmol/kg; pH (water): 5.89; Mn: 0.02 Cmol/kg; N: 11.17]. The soil was air-dried, sieved (4-mm) and mixed along with river sand at 3:1 (W/W). The resulting mix was then autoclaved (121 °C, 1 h) twice.

2.5.2. Soil drenching and growth condition

The above-mentioned BCA inoculums (single and combination) were used to inoculate the soil (10^6 Conidia/g soil) (Eke, Fokou, et al. 2016). 2 weeks old seedlings pregerminated under axenic conditions were planted in 5 Litre pots filled with inoculated (test pots) or uninoculated (control pots) soil (control). The pots (tests and controls) were set up in insect proof cages according to Thakur et al. (2019).

2.5.3. Begomovirus inoculation and experimental layout

Viruliferous whitefly colonies grown on symptomatic pepper plants in insect-proof cages were used as viral sources for infection (Senanayake, Varma, and Mandal 2012; Thakur et al. 2019). Diseased chilli pepper plants with hundreds of viruliferous whiteflies were taken from the cage and shaken into the cage with *Trichoderma*-treated and untreated seedlings to promote infection and enable plants to stand at similar distances from all treatments for uniformity reasons for 48 hours (Thakur et al. 2019).

The trial was a 5 × 8 × 1 factorial arranged following a completely randomized block design (CRBD) with 10 replications per treatment. The treatments were codified as follows: Control (treatment without *Trichoderma* and viral infection), *Bemisia* (treatment with only *Bemisia* spp), *Bemisia* + T1 (treatment with *Bemisia* spp and *T. polysporum* T1), *Bemisia* + T2 (treatment with *Bemisia* spp and *T. atroviridae* T2), *Bemisia* + T8 (treatment with *Bemisia* spp and *T. harzianum* T8). And consortium treatments: *Bemisia* + T1T2, *Bemisia* + T1T8, *Bemisia* + T2T8 and *Bemisia* + T1T2T8. The pots were watered regularly and grown for 60 days.

2.5.4. Recording of growth and PeLCV parameters

60 DAE, shoot and root dry weight (g) were recorded upon drying at 60 °C until constant mass (using balance), shoot and root length (cm), shoot and root height were also measured. Later, the disease incidence (DI) was calculated (4) for each treatment as the total number of plants showing *PeLCV* symptoms divided by the total number of plants per treatment, as shown below (4) Camara et al. (2013).

$$\mathrm{DI}\ (\%) = (\mathrm{SP}/\mathrm{N}) * 100 \tag{4}$$

Where SP = represent symptomatic plant, N = total number of plants in each group

For disease severity, a 5 classes (0, 1, 2, 3 and 4) disease rating scale was adopted (Lapidot et al. 1997) with

0 = no visible symptoms; 1 = very slight leaf curl; 2 = substantial curl with or without yellowing; 3 = substantial curl with substantial yellowing; 4 = Substantial curl + yellowing + Stunting or death of the plant. The Disease Severity (DS) was thereafter calculated using the formula (5) adapted from Filion, St-Arnaud, and Jabaji-Hare (2003):

$$\mathrm{DS}\ (\%) = (\Sigma\ (\mathrm{IDi})/\mathrm{ND}) * 100 \tag{5}$$

Where; I = number of affected plants having similar degree of infection, Di = degree of infection on a given plant, N = total number of plants examined, and D = highest degree of infection (on the 1–4 scale).

2.5.5. Changes in some biochemical markers of resistance

2.5.5.1. Preparation of phenolic extract.

The phenolic extracts were prepared as describe by Tchameni et al. (2011). Briefly, 5 g (powder) of pepper plant previously dry for 1 h at room temperature and then, filtrated through Whatman n°1 filter paper, the methanol was thoroughly evaporated at 40 °C using a rotary evaporator (BUCHI 001). After adjustment of aqueous phase to 70 mL with distilled water, the mixture was subsequently depigmented using successive washing with 50 mL of 40% ammonium sulphate [$(NH4)_2SO_4$], 1.5 mL of 80% phosphoric acid (H_3PO4), and 50 mL of petroleum ether. Finally, the aqueous phase was repeatedly extracted with 4 × 50 mL ethyl acetate. Five grams of anhydrous magnesium sulphate ($MgSO_4$) were added to the pooled organic fractions and filtered after 5 min using Whatman n°1 filter paper. Subsequent evaporation under vacuum at 40 °C using a rotary evaporator resulted in the extract that was further used for the experiments.

2.5.5.2. Determination of the total phenolic content. The main secondary metabolite used by plant for defence against biotic attack are phenolic compounds. The total phenolic content was determined using the Folin-ciocalteu assay protocol as described by Siddhuraju, Mohan, and Becker (2002). 50 µL of the above-prepared extract at 0.5 g/mL in water were mixed with 3 mL of distilled water, 250 µL of Folin-ciocalteu's reagent, and 750 µL of 70% Sodium Carbonate and the mixture vortexed thoroughly and further incubated for 8 min at room temperature. Then, 950 µL of distilled water were added to the reaction mixture and allowed to incubate for 2 h at room temperature. Gallic acid was used as standard in the same experimental conditions. The absorbance was recorded at 760 nm against a blank using Tecan Infinite M200 microplate reader. The experiments were performed in triplicate and the results expressed as Gallic acid equivalents (mg of GAE/g sample) through a regression ($r^2 = 0.99$) curve plotted from pure Gallic acid.

2.5.5.3. Assay of phenylalanine ammonia lyase specific activity. The increase in specific activity of the phenyl-propanoid pathway is often indicative of strengthened innate immune system. The PAL extraction was done by the protocol described by Yingsanga et al. (2008). Fresh root samples (0.5 g) were crushed in with 20 mL of 50 mM sodium phosphate buffer (pH 8.8). The extracts were filtered and the filtrates were centrifuged at 13,000 g at 4 °C for 30 min. PAL activity was measured in the supernatant following the method of Whetten and Sederoff (1992). An assay mixture, containing 100 µL of enzyme extract, 500 µL of 50 mM sodium phosphate buffer (pH 8.8), and 600 µL of 1 mM Phenylalanine was allowed to stand for 1 h at room temperature followed by addition of 2 mL of 2 N Hydrochloric acids as reaction stopper. The absorbance of the released trans-cinnamic acid was measured at 290 nm using microplate reader (Tecan Infinite M200) and result was expressed in terms of OD290/min/mg protein.

2.5.5.4. Assay of gaiacol peroxidase specific activity. Peroxidase enzymes are engaged in a number of physiological processes in response to biotic and abiotic stressors. It promotes the biosynthesis of defence-related secondary metabolites that aid in withstanding stresses. The enzyme activity was determined by measuring the rise in absorbance at 470 nm (Tecan Infinite M200) caused by the pro-oxidant (H_2O_2) oxidation of guaiacol to tetraguacol (Zheng and van Huystee 1992). The reaction mixture comprised 0.1 mL of guaiacol at 1% (v/v), 10 mM sodium phosphate at PH 6.0, 0.1 mL of 0.3% (v/v) H_2O_2, and 0.3 mL enzyme extract. The enzyme activity was measured in mmol of substrate transformed per mg of enzyme extract per minute (mmol/mg/min).

2.5.5.5. Assay of superoxide dismutase specific activity. SOD enzyme is an antioxidant enzyme found in higher plants that protects cellular components from oxidation by reactive oxygen species generated by the stress. An increase in this enzyme's specific activity indicates a stressed host. SOD activity was assessed by examining the reduction of the photochemical reduction of Nitroblue tetrazolium NBT, as reported by Beauchamp and Fridovich (1971). 13 mM L-methionine, 63 mM NBT, 0.05 M sodium carbonate, 13 mM riboflavin, 0.1 mM EDTA (Himedia product), and 0.5 mL enzyme sample were mixed in a 3 mL assay mixture. The mixture was exposed to two fluorescent lamps (15 W) for 15 minutes at 25 °C, followed by 15 minutes in the dark. The absorbance was then measured at 560 nm (Tecan Infinite M200 microplate reader). One unit of SOD activity was defined as the quantity of enzyme necessary to prevent NBT decrease by 50%. The result was reported as SOD unit/mg fresh weight.

2.6. Statistical analyses

The obtained numerical data from the overall experiments were normalized and submitted to analysis of variance (ANOVA) with the SIGMAPLOT 11.0 statistical software. The Duncan's paired based comparison test was used to determine the differences between mean values. The differences in means were validated when the mean errors fell below the significance level of 5%

3. Results

3.1. Cultural and genomic identification of *Trichoderma* isolate

The cultural characteristics of *Trichoderma* spp. were evaluated visually and under a light microscope. After two days of incubation on PDA, all of the isolates had hyaline to white hyphae that became light to dark green depending on the isolate. The whole colony became greenish as a result of conidia production, which occurred from the centre of the culture outward. The overall pattern of the colonies ranged from uniform lawn to undulated circular green rings. *Trichoderma* spp. was identified by the recorded characteristics (Rifai 1969).

The isolated *Trichoderma* strains were identified as *T. polysporum* T1, *T. atroviridae* T2, and *T. harzianum* T8 by sequencing of the ITS genes and a BLAST search in the NCBI database (Figure 1).

3.2. *Trichoderma* hormonal (IAA and SA) profiling

The hormonal profiles of the studied *Trichoderma* species (Figure 2a and b) revealed that they had the competence to synthesise and release IAA and SA in their environment. The intensity of production varying

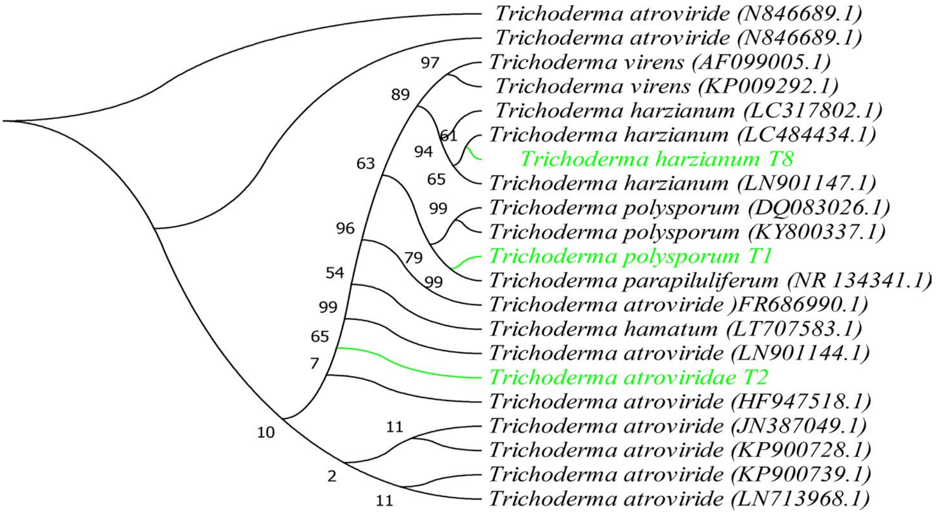


Figure 1. Phylogeny of *Trichoderma* isolates. Molecular tools coupled with taxonomy analysis using ITS regions of rDNA and BLAST search for the three isolates showed 100% homology with *T. atroviride* (HF947518), *T. polysporum* (NR134341) and *T. harzianum* (LC484434).

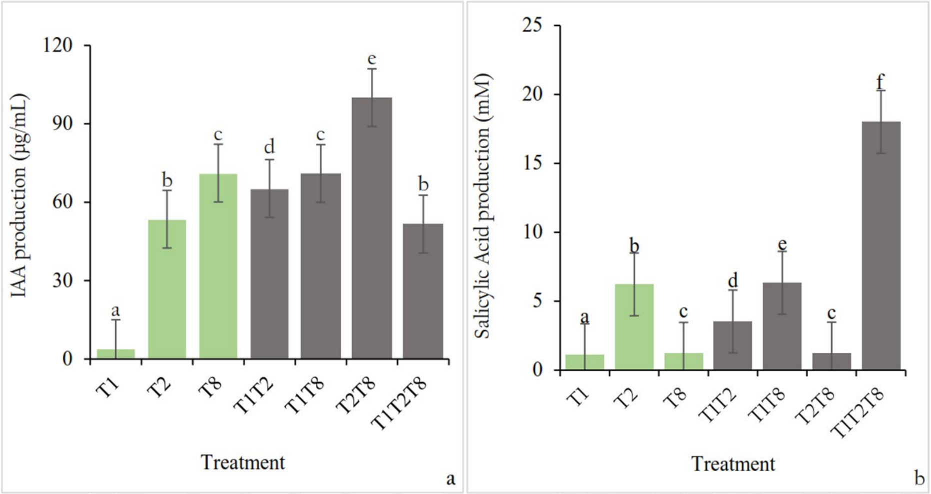


Figure 2. Quantitative estimation of Indole Acetic Acid (a) and Salicylic Acid (b) produced by *T. polysporum* T1, *T. atroviridae* T2 and *T. harzianum* T8 and combination of T1, T2 and T8. Column bearing the same letter are not significantly different at $p = 0.05$.

from one BCA to another. In general, the binary application of the BCA's elicited more IAA (Figure 2a) and SA (Figure 2b), with the consortium of *T. atroviridae* and *T. harzianum* (T2T8) inducing more IAA (100 g/mL) biosynthesis, while the triple combination of *T. atroviridae*, *T. polysporum*, and *T. harzianum* (T1T2T8) releasing more than any other treatment, culminating at 18 µg/mL.

3.3. Tissue colonization and cohabitation abilities of Trichoderma

The results show that BCAs and their consortia could invade and survive endophytically in chilli pepper plants ($p < 0.05$). *T. atroviridae* T2 was shown to be the most invasive (50 CFU/g fresh weight) when tested alone, followed by *T. harzianum* T8 (32 CFU/g fresh weight). *T. polysporum* T1 in combination with either *T. atroviridae* T2 or *T. harzianum* T8 resulted in decreased tissue colonisation, indicating an inhibited action of *T. polysporum* T1 on the other bioagents in terms of colonisation. On the other hand, a synergistic impact was seen when the greatest colonisers were combined. (*T. atroviridae* T2 or *T. harzianum* T8), culminating at as high as 122.5 CFU per unit fresh matter (Figure 3a). Inhibition zones were only found at the contact zones of *T. Polysporum* T1 and *T. atroviridae* T2 hyphae elsewhere. The ability to share the same ecological niche and host was demonstrated by all other tested BCA (Figure 3b).

3.4. Saline stress tolerance

To investigate Trichoderma's sensitivity to salt (NaCl) stress, all colonies were cultured on PDA medium supplemented with differing quantities of NaCl (0, 250, 500, 750, 1000 mmol.). At 250 mmol NaCl, the *Trichoderma* strains tested grew. However, the development of all the BCA's was completely suppressed at higher doses (NaCl = 1000 mmol.). Despite this, *Trichoderma* has demonstrated the capacity to tolerate salt concentrations of up to 750 mmol. *T. atroviridae* T2 being the best BCA.

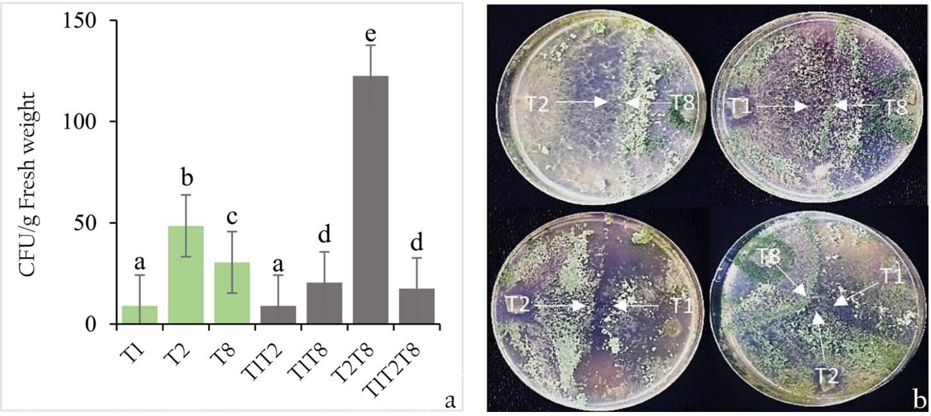


Figure 3. Endophic efficiency of *Trichoderma* spp in chilli pepper plant grown in gnotobiotic condition (a) and cohabitation ability on PDA medium (b). T1: *T. polysporum* T1, T2: *T. atroviridae* T2, T8: *T. harzianum* T8 and combination of T1, T2 and T8. Column bearing the same letter are not significantly different at $p < 0.05$.

3.5. Seed biopriming with Trichoderma spp

3.5.1. Importance of Trichoderma inoculation on seeds germination

In general, with the exception of the combined application of T2 and T8, the final number of sprouted seeds per treatment and the total germination index (Table 2) were greater in all *Trichoderma*-inoculated treatments compared to the corresponding controls. Similarly, no statistically significant difference in mean sprouting time was seen in either *Trichoderma*-inoculated (single and consortia) or water controls, demonstrating the non–lethal effects of the bioagents on the seeds at the tested dosages.

The radicle growth was significantly ($p < 0.05$) affected by *Trichoderma* inoculation compared to the control (Figure 4), the best result was recorded with the consortium T1T8 (2.97 cm).

3.5.2. IAA contents in peppers seedlings

The findings suggest that IAA hormone was present in all primed plants. However, only *T. harzianum* T8 increased IAA content in a single

Table 2. Effects of *Trichoderma* towards seeds germination.

	Germination parameters		
Treatment	GMT (Days)	GI	GP (%)
Control	8.6 ± 0.21	0.95 ± 0.07	88.9
T1	8.7 ± 0.33	1.01 ± 0.02	95.6
T2	8.0 ± 0.69	1.16 ± 0.12	100
T8	8.3 ± 0.42	1.12 ± 0.07	100
T1T2	8.0 ± 0.57	1.05 ± 0.07	93.3
T1T8	8.1 ± 0.45	1.07 ± 0.07	93.3
T2T8	9.0 ± 0.29	0.81 ± 0.04	80.0
T1T2T8	8.3 ± 0.83	1.12 ± 0.14	100

^{GMT} germination mean time, ^{GI} germination index, ^{GP} germination percentage. ^{T1}*T. polysporum* T1, ^{T2}*T. atriviridae* T2, ^{T8}*T. hazianum* T8 and combination of *Trichoderma*.

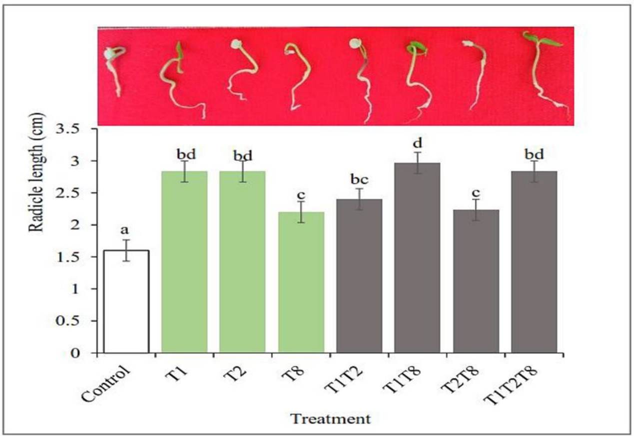


Figure 4. Effect of chilli pepper seeds biopriming with *Trichoderma* spp. on the seeds germination and radicle length. Control (without any treatment), T1 *T. polysporum* T1, T2 *T. atroviridae* T2, T8 *T. harzianum* T8, T1T2, T1T8, T2T8 and T1T2T8.

treatment compared to the control (non-primed plant) ($p < 0.05$). In contrast to the control treatment, the binary application of T2T8 and T1T2T8 revealed a substantial increase in IAA ($p < 0.05$) (Figure 5a). Overall, these two binary treatments produced the highest IAA concentration, reaching up to 21 g/ml. This finding displayed the capability of consortia to improve IAA synthesis over control or single inoculation.

3.5.3. SA contents in peppers seedling

The SA content in germinated chilli peppers plants was recorded (Figure 5b). It is noteworthy that, in contrast to IAA, *T. harzianum* T8 was less sensitive compared to the other bioagents evaluated alone. *T. atroviridae* T2 was the most effective treatment (11.89 mM). Meanwhile, as compared to single applications, combined treatments performed poorly. The binary application of *T. polysporum* T1 and *T. harzianum* T2 was the most effective treatment (7.12 mM).

3.6. In planta PeLCV suppression experiment

3.6.1. Effect of chili pepper priming on PeLCV

Typical *pepper leaf curl virus* (*PeLCV*) signs such as poor growth, plant stunting, leaf yellowing, and curling were clearly observed in plants emerging from control (non-inoculated) pots, confirming effective Begomovirus transmission in the test plants (Figure 6). *Trichoderma* priming could not completely suppress the disease in the plantlets examined (*PeLCV* incidence = 100%). In contrast, *Trichoderma* inoculation dramatically reduced

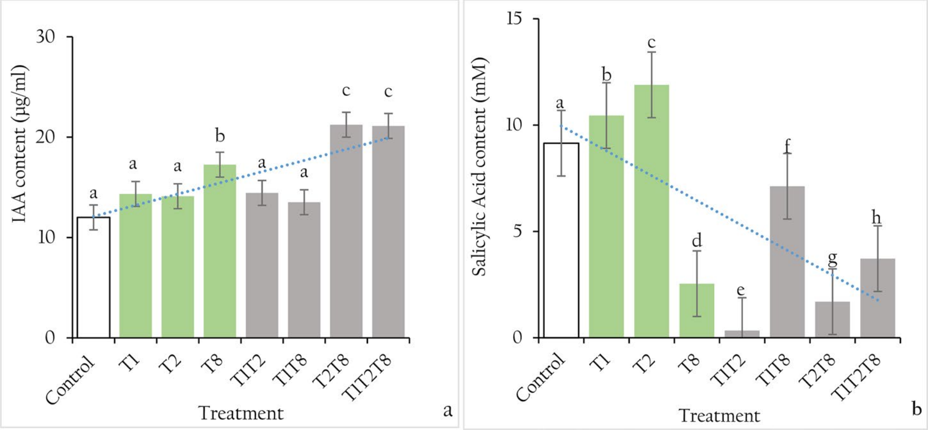


Figure 5. Quantitative estimation of Indole Acetic Acid (a) and Salicylic Acid (b) content in *Trichoderma*-inoculated or not pepper seedling. Control (without any treatment), T1 *T. polysporum* T1, T2 *T. atroviridae* T2, T8 *T. harzianum* T8, and combination T1T2, T1T8, T2T8 and T1T2T8.

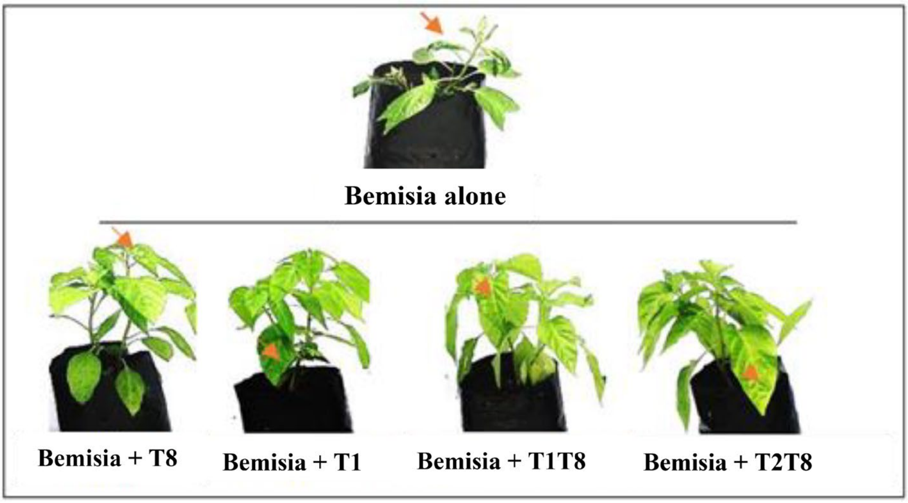


Figure 6. Effect of *Trichoderma* priming or not on pepper growth and *PeLCV* suppression. *Bemisia* alone (negative control plant infected only by *PeLCV* transmit by whiteflies *Bemisia* sp), *Bemisia* + T1 (plant infected by *PeLCV* and treat by *Trichoderma Polysporum T1*), *Bemisia* + T8 (plant infected by *PeLCV* and treat by *Trichoderma harzianum* T8), *Bemisia* + (T1T8) (plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1 and *Harzianum* T8), *Bemisia* + (T2T8) (plant infected by *PeLCV* and treat by *Trichoderma Atroviridae* T2 and *Harzianum* T8).

the severity of *PeLCV*. The decrease of *PeLCV* severity varied from 34 to 50 percent, with the treatments T8, T1, T1T8, and T2T8 achieving the greatest reduction of *PeLCV* severity (50%) (Table 3).

3.6.2. Effect of Trichoderma biopriming on chili pepper growth under Begomovirus pressure

When compared to Begomovirus infected plants alone, *Trichoderma* inoculation significantly improved all growth parameters examined

Table 3. Effect of chili pepper biopriming by *Trichoderma* on *PeLCV* suppression.

Treatment	*PeLCV* severity (%)	Protection rate (%)	*PeLCV* incidence (%)	Protection rate (%)
Control	0^c	–	0	–
Bemisia	100^a	0.0	100	0
Bemisia + T1	50.0^b	50.0	100	0
Bemisia +T2	58.3^b	41.7	100	0
Bemisia + T8	50.0^b	50.0	100	0
Bemisia + (T1T2)	58.3^b	41.7	100	0
Bemisia + (T1T8)	50.0^b	50.0	100	0
Bemisia + (T2T8)	50.0^b	50.0	100	0
Bemisia + (T1T2T8)	66.6^b	33.4	100	0

$^{\text{Control}}$ plant without treatment, positive control, $^{\text{Bemisia}}$ negative control plant infected only by *PeLCV* transmit by whiteflies *Bemisia* sp, $^{\text{Bemisia+ T1}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum T1*, $^{\text{Bemisia+ T2}}$ plant infected by *PeLCV* and treat by *Trichoderma atroviridae* T2, $^{\text{Bemisia+ T8}}$ plant infected by *PeLCV* and treat by *Trichoderma harzianum* T8, $^{\text{Bemisia+ T1T2}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1 and *Atroviridae* T2, $^{\text{Bemisia+ T1T8}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1 and *Harzianum* T8, $^{\text{Bemisia+ T2T8}}$ plant infected by *PeLCV* and treat by *Trichoderma Atroviridae* T2 and *Harzianum* T8, $^{\text{Bemisia+ T1T2T8}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1, *Atroviridae* T2 and *Harzianum* T8. The values with different letter in a same column are significantly different at 5% level by using Duncan test.

($p < 0.05$). There was no significant difference between all inoculated plants by *Trichoderma* alone or in consortia. However, the combinations T2T8 (17.66 cm) and T1T2T8 (31.33 cm) produced the best shoot and root expansions. *T. polysporum* T1 infected plants produced much more dry matter than the other treatments (Table 4).

3.6.3. Change in selected biochemical markers of resistance

3.6.3.1. Change in total Trichoderma harzianum and Trichoderma viride phenolic content. In general, the soluble phenol content rose significantly ($p < 0.05$) in all treatments as compared to non-primed plants. The mean increase above the control varied from 50% to 183%. (Figure 7a). Furthermore, the consortium treatments stimulated greater phenol production, with increases ranging from 83% to 183%. The binary applications T1T8 showed the greatest increase (17.29 g/mL/g dry weight) ($p < 0.05$). Overall, these findings show that the tested *Trichoderma* modulates the abundance of phenol content in several plant biological processes, including defence, in either a single or multiple application.

3.6.3.2. Change in specific activity of phenylalanine ammonia lyase. The findings indicated that pepper plants bioprimed with *Trichoderma*, either alone or in consortia, efficiently regulated the PAL specific activities as compared to their non-inoculated counterparts. Except for treatments T1T8 and T1T2T8, which were not significant ($p < 0.05$) all *Trichoderma*-inoculated pots showed a reduction in PAL activity (Figure 7b). The pathogen-infected

Table 4. Effect of pepper priming with *Trichoderma* on the growth of pepper under *PeLCV* pressure.

Treatment	Shoot length (Cm)	Root length (Cm)	[1]SDM (g)	[2]RDM (g)	Root/shoot
Control	13.0 ± 1^{b}	18.67 ± 2.2^{ac}	$0{,}62 \pm 0.001^{b}$	0.13 ± 0.001^{a}	1.44
Bemisia	8 ± 1^{a}	10.33 ± 1.53^{a}	0.71 ± 0.001^{a}	0.12 ± 0.001^{a}	1.29
Bemisia + T1	16.0 ± 1.73^{b}	28.67 ± 1.16^{bcd}	3.99 ± 0.001^{c}	3.42 ± 0.001^{b}	1.79
Bemisia +T2	15.8 ± 1.89^{b}	29.67 ± 2.52^{bd}	3.58 ± 0.001^{d}	1.83 ± 0.041^{c}	1.87
Bemisia + T8	17.66 ± 0.58^{b}	17.83 ± 2.75^{ac}	2.57 ± 0.001^{e}	1.28 ± 0.001^{d}	1.01
Bemisia + (T1T2)	17.33 ± 0.76^{b}	21.33 ± 2.52^{abcd}	2.79 ± 0.001^{f}	2.20 ± 0.001^{e}	1.23
Bemisia + (T1T8)	14.17 ± 2.02^{b}	28.67 ± 8.14^{bcd}	2.77 ± 0.001^{g}	2.89 ± 0.001^{f}	2.02
Bemisia + (T2T8)	17.66 ± 2.08^{b}	20.17 ± 2.75^{abcd}	3.60 ± 0.001^{h}	1.71 ± 0.001^{g}	1.14
Bemisia + (T1T2T8)	14.83 ± 1.04^{b}	31.33 ± 5.77^{d}	3.40 ± 0.001^{i}	1.76 ± 0.001^{h}	2.11

[1] Shoot dry matter

[2] Root dry matter.

$^{\text{Control}}$ plant without treatment, positive control, $^{\text{Bemisia}}$ negative control plant infected only by *PeLCV* transmit by whiteflies *Bemisia* sp, $^{\text{Bemisia+ T1}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum T1*, $^{\text{Bemisia+ T2}}$ plant infected by *PeLCV* and treat by *Trichoderma atroviridae* T2, $^{\text{Bemisia+ T8}}$ plant infected by *PeLCV* and treat by *Trichoderma harzianum* T8, $^{\text{Bemisia+ T1T2}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1 and *Atroviridae* T2, $^{\text{Bemisia+ T1T8}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1 and *Harzianum* T8, $^{\text{Bemisia+ T2T8}}$ plant infected by *PeLCV* and treat by *Trichoderma Atroviridae* T2 and *Harzianum* T8, $^{\text{Bemisia+ T1T2T8}}$ plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1, *Atroviridae* T2 and *Harzianum* T8. The values with different letter in a same column are significantly different at 5% level by using Duncan test.

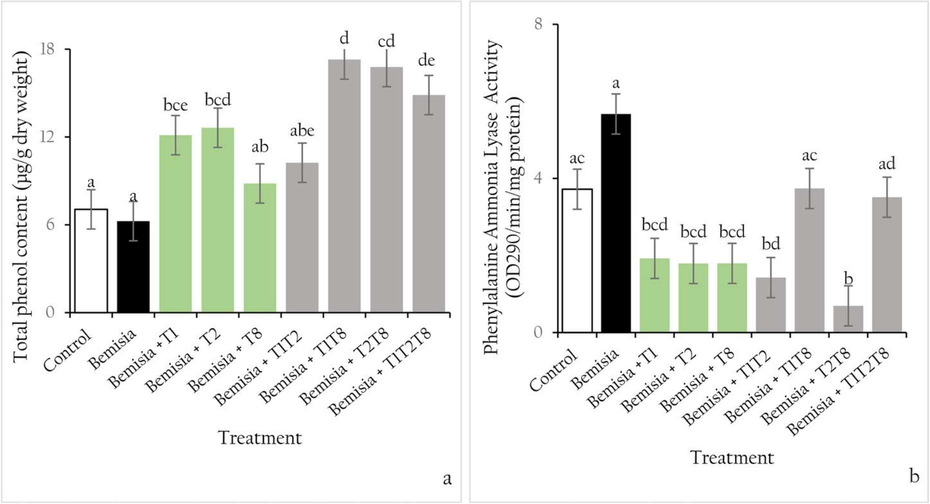


Figure 7. Effect of biopriming pepper plant by *Trichoderma* on the total phenol content (a) and activity of PAL enzyme (b) under *PeLCV* pressure. Control (plant without treatment, positive control), *Bemisia* (negative control plant infected only by *PeLCV* transmit by white-flies *Bemisia* sp), *Bemisia* + T1 (plant infected by *PeLCV* and treat by *Trichoderma Polysporum T1*), *Bemisia* + T2 (plant infected by *PeLCV* and treat by *Trichoderma atroviridae* T2), *Bemisia* + T8 (plant infected by *PeLCV* and treat by *Trichoderma harzianum* T8), *Bemisia* + T1T2, *Bemisia* + T1T8, *Bemisia* + T2T8, *Bemisia* + T1T2T8. The values with different letter in a same column are significantly different at 5% level by using Duncan test.

and untreated plantlets (*Bermisia* alone) had the highest activity (5.671 OD/min/mg protein). The consequences of *Trichoderma* invasion on the phenylpropanoids biosynthesis pathway are well depicted.

3.6.3.3. Change in specific activity of gaiacol peroxidase. The gaiacol peroxidase activity (GPx) in pepper plant shoot tissues was tested in un-infected and *Trichoderma* inoculated plants (Figure 8b). When comparing *Bemisia* infected and inoculated plants to non-primed plants, a significant change in gaiacol specific activity was found. Plants grown in *Trichoderma* infected pots in single or multiple treatments exhibited a significant ($p < 0.05$) increase in GPx activity compared to un-inoculated plants. This study used just T2 treatment as a single BCA's t and T1T2T8 as a consortium. There was no significant difference (P0.05) between inoculated plants with *Trichoderma* alone or in consortia and *Bemisia* treatments; however, T1T2T8 showed a significant difference ($p < 0.05$). The best GPx activity was found with a T1T2T8 consortium.

3.6.3.4. Change in specific activity of super oxide dismutase. The variations in SOD activity in *Trichoderma* primed versus unprimed pepper plants are demonstrated (Figure 8a). It is worth noting that only treatments T2, T8, and T1T8 showed a significant change when compared to the control

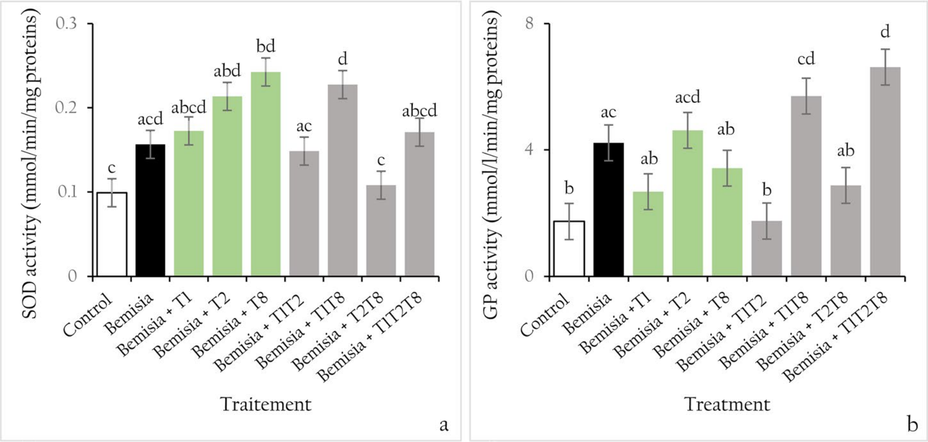


Figure 8. Effect of biopriming pepper plant by *Trichoderma* on the activity of SOD enzyme (a) and GP enzyme (b) under *PeLCV* pressure. Control (plant without treatment, positive control), *Bemisia* (negative control plant infected only by *PeLCV* transmit by whiteflies *Bemisia* sp), *Bemisia* + T1 (plant infected by *PeLCV* and treat by *Trichoderma Polysporum* T1), *Bemisia* + T2 (plant infected by *PeLCV* and treat by *Trichoderma atroviridae* T2), *Bemisia* + T8 (plant infected by *PeLCV* and treat by *Trichoderma harzianum* T8), *Bemisia* + T1T2, *Bemisia* + T1T8, *Bemisia* + T2T8, *Bemisia* + T1T2T8. The values with different letter in a same column are significantly different at 5% level by using Duncan test.

(no treatment) ($p = 0.05$). In comparison to the *Bemisia* treatment, there was no significant change with *Trichoderma* primed plants in single or consortia ($p = 0.05$). T8 (0.243 mmol/min/g of proteins) as a single and T1T8 (0.228 mmol/min/g of proteins) as a binary showed the highest SOD activity. Overall, our findings show that *Trichoderma* activates several molecular pathways for plant defence and other purposes during host root colonisation.

4. Discussion

The severity and difficulties in controlling viral infections are undoubtedly attributable to their physiopathology. Viruses, in contrast to phytophagous diseases, impair the host's innate immune system and subtilize the cell machinery in order to establish themselves in plant tissues (Li et al. 2014). In fact, the plant's functionality, production, and survival are all jeopardised. Aside from the drawbacks associated with vector (mainly insect) elimination, the transiency and chimeric outputs attributed to genetic engineering, such as the use of small RNAs, clearly underlines the quest of a sustainable control approach for viral infections in agriculture. The suppression of *pepper leaf curl virus* using *Trichoderma* inoculants is revealed in this work for the first time. In the framework of this study, typical symptoms such as leaves curling and yellowing, whole plant stunting, and eventually total death were recorded in chilli plantlets infested by begomovirus in nursery, indicating a successful transfer of the virus from viruliferous vector (*Bemisia* spp) to young pepper seedlings. Meanwhile, all *Trichoderma*-primed seedlings (single and consortium treatments) showed a substantial reduction in *PeLCV* severity, reaching up to 50%. Similar outputs were obtained by Muvea et al. (2018) in onion infected with the iris yellow spot virus (IYSV) vectored by *Thrips tabaci* and challenged with the beneficial fungus *Hypocrea lixii*. The authors pointed out the vector repellence and/or the antibiosis as probable mode of action underlying the disease suppression. It has been proven that promising biocontrol agents, such as *Trichoderma* species, have the ability to synthesis hundreds of biologically active chemicals, including a wide spectrum of anti-bacterial Trichodermin (Leylaie and Zafari 2018), antifungal Trichokonin (Shi et al. 2012) and antiviral Gliotoxin (Mukherjee, Horwitz, and Kenerley 2012). However, elicitation of the plant immune system is typically the primary and most likely mechanism for protecting the host from viral invasion. In this sense, our data indicate that host priming with *Trichoderma* inoculants alters chilli pepper metabolic signalling. For example, ours in vitro and in planta results revealed that the studied *Trichoderma,* both alone and in combination, may boost salicylic acid synthesis in young pepper plants. The so-called inductor being consistently proven to trigger systemic acquired resistance (SAR) in a variety of host plants against diseases. (Dempsey and Klessig 2017). Such resistance necessitates increased expression of defensive genes, which leads to increased production of defensive chemicals, the most important of which are phenolic compounds (Pieterse et al. 2014). All *Trichoderma* primed (single or consortium) and infected (virus) seedlings synthesised substantially more phenolic compounds in comparison to the non-primed virus-free control and non-pre-primed chilli pepper seedlings. Siddique et al. (2014) discovered a significantly higher phenolic content in resistant cotton genotypes than in susceptible ones following *cotton leaf curl Burewala virus* ingress. Herein, compared to the virus-free non-primed controls and virus-infested non-primed chilli pepper seedlings, all *Trichoderma*-primed (single or in consortium) and infested (virus) seedlings synthetized significantly more phenolic compounds indicating a three-way interaction between viral assault, phenol accumulation, and disease suppression in resistant cultivars. Also, among the inducible compounds, the synthesis of insects repellent volatile terpene like β-acoradiene, β-cubebene, β-cedrene, β-bisabolene, β-himachalene, γ-himachalene, limonene and citral are reported to increase upon *Trichoderma* inoculation (Du et al. 2016; Lee et al. 2016). The decrease in phenolic compounds in virus-infested plants only (*Bermisia* spp), could be the result of viral-driven host defence-related genes repression as mentioned by Markakis et al. (2010). It is noteworthy that all the *Trichoderma*-primed plantlets inoculation singly or in consortia significantly improved shoot length (up to 50%), root length (up to 67%) and shoot and root dry matter (up to 82.2% and 96.5% respectively). This might be ascribed to disease suppression as asserted by Eke, Chatue, et al. (2016), or simply due to the augmented synthesis of IAA as observed in this study. This so-called plant growth hormone, is largely reported to control various growth processes such as meristematic cells maintenance and formation and cell enlargement and division, leading to vigorous plants (Su, Liu, and Zhang 2011; Zhang et al. 2013). Overall, the consortium among *T. atroviridae* T2 and *T. harzianum* T8 was the best treatment. These findings are consistent with those of Eke, Chatue, et al. 2016, which stated that single BCA's fighting with a pathogen is likely to fail than a consortium application since the former are considered to reinforce each other, resulting in a better outcome. This phenomenon is still being unravelled since their success is influenced on by the mechanism of action of the partners. Eke, Chatue, et al. (2016) provided a preliminary insight, suggesting that the combination of bioagents with diverse modes of action is likely to be synergistic since they are thought to each implicate in a separate function, diverting the competition. In general, the boost between consortium partners was illustrated in this study with 152.6% and 301.6% more IAA production with the consortium T2-T8 than with the solo application of T8 and T2, respectively.

Several studies have also shown that PAL activation and subsequent increase in phenolic content in plants is a general response related with disease resistance, suggesting that the greater the resistance profile, the lower the activation of the defence mechanism. This might explain the contradictory activity of PAL in comparison to the total content of phenolic compounds in the plants examined. In this work, two destressing enzymes were tested to see if there were any changes in their specific activity when inoculated or not with BCA's under viral pressure. Some pathogens may benefit from an increase in reactive oxidative substrate (ROS) levels generated in host cells during the hypersensitive reaction triggered during the early stages of pathogen ingress as a facilitating factor to invade the host (Díaz-Vivancos et al. 2008). Superoxide dismutase (SOD) is one of the most significant radical scavengers catalysing the dismutation of the superoxide radical into hydrogen peroxide and oxygen (Hameed and Iqbal 2014). The obtained results for peroxidase showed non-significant difference between *Trichoderma*-treated plants and *Bemisia* spp treatments alone irrespective of the improved protection. This indifference in destressing enzyme activity was previously observed in resistant cotton (Siddique et al. 2014). Actually, one of the most fundamental differences between susceptible and resistant plant defence mechanisms against a pathogen is the speed of early localised ROS accumulation at the site of pathogen (including viruses) infection. The accumulated ROS are an efficient way of removing the invader before any plant oxidative stress or disease signs develop (Hernández et al. 2016). This might have occurred in this work, given the significantly higher activity of destressing enzymes in virus-free and unprimed plants compared to *Trichoderma* treated plants that showed more resistance to the *PeLCV*. These observations could be confirmed by the finding of De Palma et al. (2019) and Mayo et al. (2016) reporting the capacity of *Trichoderma* to fine-tuned plant gene expression for an efficient and strategic use of the plant defence machinery.

The current study demonstrated *Trichoderma*'s capacity to lower *PeLCV* severity by increasing systemic acquired resistances via SA synthesis, which was effective via phenolic compound production. *Trichoderma* substantially increased plant growth by producing auxin. Likewise, *PeLCV* disease decreased by producing destressing enzyme (SOD, GPx). Overall, the consortium T2T8 performed best, confirming the hypothesis that a single bioagent competing with a pathogen is likely to fail than a consortium application.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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

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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:

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

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

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

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).

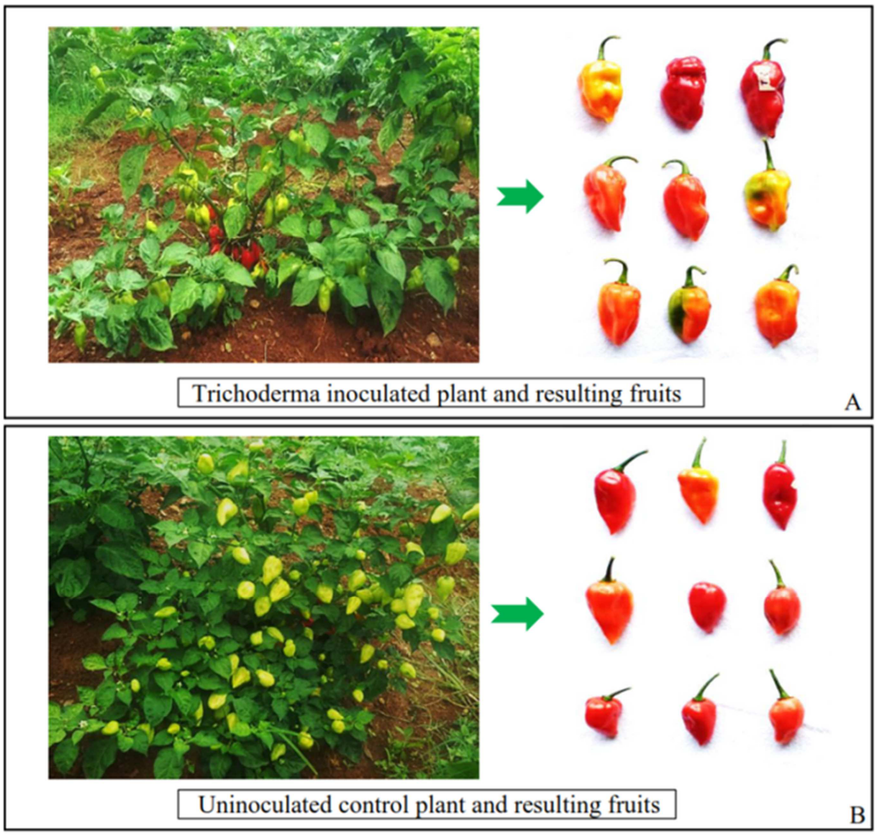

Figure 2. *Differential fruits bearing capacity and ripen chili pepper fruits size as affected (A) or not (B) by the tested Trichoderma-based bio-fertilizer.*

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 ± 10,2^a	8.66 ± 1.15^a	3753.5 ± 51^a
Biofertilizer (T)	71.25 ± 6.50^a	926.9 ± 6,7^b	13.33 ± 0.58^b	4634.5 ± 33.5^b
Significance ($P \leq 0.05$)	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.

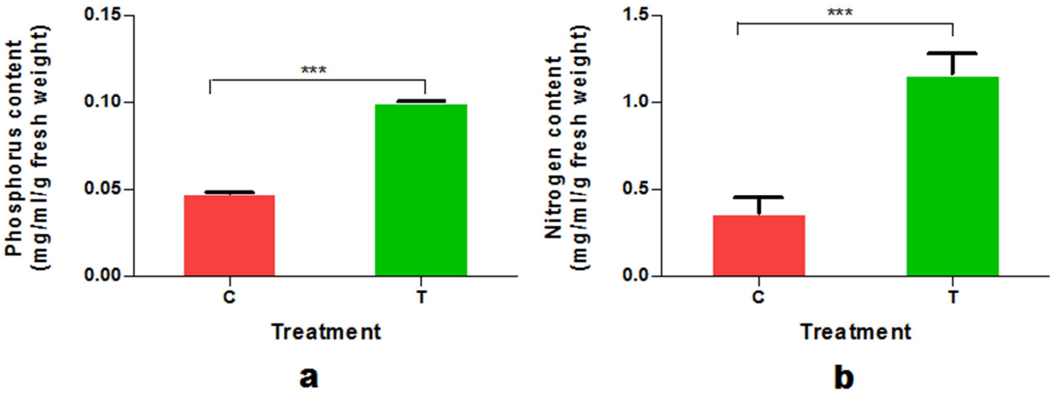

Figure 3. *Effect of Trichoderma fertilization on chili pepper Phosphorus (a) and Nitrogen (b) absorption. C: un-inoculated controls T: Trichoderma-treated plants.*

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 ($P \leq 0.05$). 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.

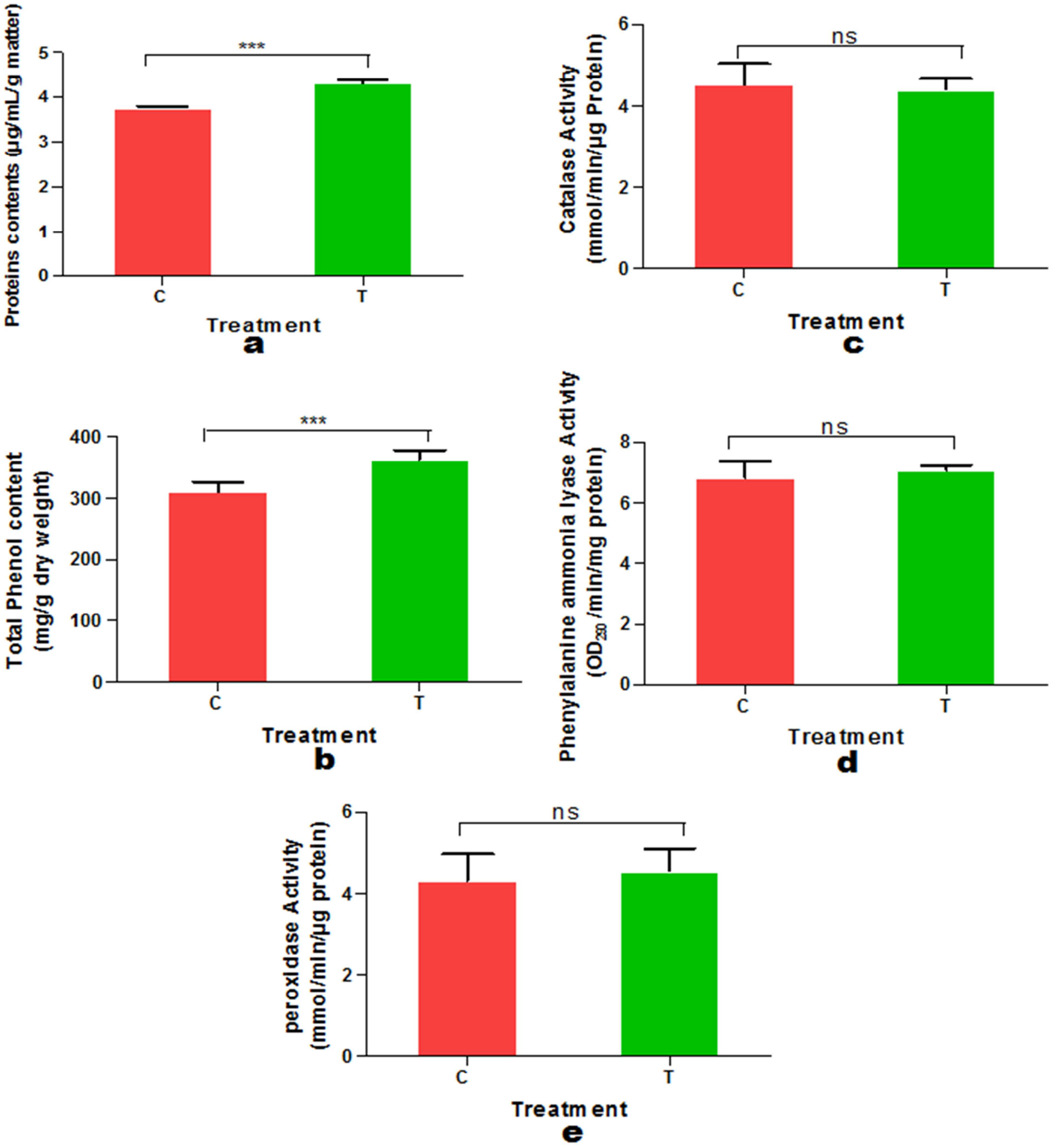

Figure 4. *Effect of bio-fertilization on Proteins (a), phenol content (b) and specific activities of CAT (c), PAL (d) and POX (e). C stands for control (un-primed) and T for Trichoderma-inoculated plants.*

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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insects

Article

Genetic Diversity of Whiteflies Colonizing Crops and Their Associated Endosymbionts in Three Agroecological Zones of Cameroon

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Simple Summary: *Bemisia tabaci* is as a major pest of vegetable crops in Cameroon, and several species have developed resistance against insecticides. Here, we investigated the frequency of infection by endosymbiont and the genetic diversity of whiteflies in Cameroon. Mitochondrial cytochrome oxidase I (mtCOI) markers and Kompetitive Allele Specific PCR (KASP) were used for the characterization. Overall, an analysis of the mtCOI sequences showed six mitotypes of *Bemisia tabaci*, and two distinct clades of *Bemisia afer* and *Trialeurodes vaporariorum*. *Bemisia tabaci* mitotypes identified included: Mediterranean (MED) on tomato, pepper, okra, and melon; and sub-Saharan Africa (SSA) groups and sub-groups (SG)–SSA1-SG1, SSA1-SG2, SSA1-SG5, SSA3, and SSA4 on cassava. The six mitotypes of cassava *B. tabaci* were split into three SNP haplogroups including sub-Saharan Africa–West Africa (SSA-WA), sub-Saharan East and Central Africa (SSA-ECA), and SSA4 by KASP genotyping. The endosymbionts identified infecting the whiteflies were *Arsenophonus*, *Rickettsia*, and *Wolbachia*.

Abstract: *Bemisia tabaci* (Gennadius) is as a major pest of vegetable crops in Cameroon. These sap-sucking insects are the main vector of many viruses infecting plants, and several cryptic species have developed resistance against insecticides. Nevertheless, there is very little information about whitefly species on vegetable crops and the endosymbionts that infect them in Cameroon. Here, we investigated the genetic diversity of whiteflies and their frequency of infection by endosymbionts in Cameroon. Ninety-two whitefly samples were collected and characterized using mitochondrial cytochrome oxidase I (mtCOI) markers and Kompetitive Allele Specific PCR (KASP). The analysis of mtCOI sequences of whiteflies indicated the presence of six cryptic species (mitotypes) of *Bemisia tabaci*, and two distinct clades of *Bemisia afer* and *Trialeurodes vaporariorum*. *Bemisia tabaci* mitotypes identified included: MED on tomato, pepper, okra, and melon; and SSA1-SG1, SSA1-SG2, SSA1-SG5, SSA3, and SSA4 on cassava. The MED mitotype predominated in all regions on the solanaceous crops, suggesting that MED is probably the main phytovirus vector in Cameroonian vegetable cropping systems. The more diverse cassava-colonizing *B. tabaci* were split into three haplogroups (SNP-based grouping) including SSA-WA, SSA4, and SSA-ECA using KASP genotyping. This is the first time that SSA-ECA has been reported in Cameroon. This haplogroup is predominant in regions currently affected by the severe cassava mosaic virus disease (CMD) and cassava brown streak virus disease (CBSD) pandemics. Three endosymbionts including *Arsenophonus*, *Rickettsia*, and *Wolbachia* were present in female whiteflies tested in this study with varying frequency. *Arsenophonus*, which has been shown to influence the adaptability of whiteflies, was more frequent in the MED mitotype (75%). *Cardinium* and *Hamiltonella* were absent in all whitefly samples. These findings add to the knowledge on the diversity of whiteflies and their associated endosymbionts, which, when combined, influence virus epidemics and responses to whitefly control measures, especially insecticides.

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1. Introduction

The whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) is a significant pest that damages many plants worldwide such as ornamentals, vegetables, legumes, and cotton [1]. Whitefly species of this complex are extremely polyphagous insects, and they are found in greenhouses and crop fields in temperate and tropical regions [2,3]. In sub-tropical and tropical countries, *B. tabaci* is one of the principal pests, especially on the main food and cash crops such as cassava, cotton, sweet potato, tobacco, and tomato [4]. These whiteflies have a severe impact on the economic activity and food security of many farmers and populations, since agriculture is one of the main economic activities [4]. *Bemisia tabaci* whiteflies have the ability to carry viruses with semi-persistent and persistent mechanisms and can vector viruses belonging to at least five genera (*Begomovirus*, *Crinivirus*, *Ipomovirus*, *Carlavirus*, and *Torradovirus*) [5]. Their sap-sucking feeding also causes physical damage to plants, although the most damage caused is by the vectoring of over 300 plant viruses [1]. Economic losses due to *B. tabaci* were estimated at USD 10 billion from 1980 to 2000 [6] in the US, and over USD 1 billion annually on cassava in Africa [7]. *Bemisia tabaci* has been described as a cryptic species complex with more than 39 morphologically indistinguishable species [3,8,9]. Most of these species are localized in geographic regions **[10]** except a few, among which two occur worldwide and are invasive: the “Mediterranean” or “MED” species (previously referred as ‘Biotype Q’) and the “Middle East–Asia Minor” species (“MEAM1”—previously referred to as ‘Biotype B’) [2]. Among these whitefly species, there are many that colonize Solanaceae [11,12] and these have shown the ability to rapidly develop resistance to chemical insecticides [4,13]. In Africa, despite the harmful impact on potential natural enemies and on the environment, chemical pesticides are widely used to control *B. tabaci* populations. Whiteflies are infected with facultative endosymbiont bacteria that have been implicated in their pest status, as they have been shown to affect tolerance to insecticides [14], virus transmission efficiency [15,16], and high-temperature tolerance [17]. As with many other arthropods, endosymbiotic bacteria are also widespread in *B. tabaci* [18]. Endosymbionts are present in many parts of insects, such as the whitefly body cavity, hemolymph, or intracellularly in special cells called bacteriocytes [19]. Members of the *Bemisia* species complex carry a primary endosymbiotic bacterium called *Candidatus Portiera aleyrodidarum* [20,21], which is fixed in populations and confined to bacteriocyte cells, with a main role in the regulation of amino acid-deficient diets in all whiteflies [20]. This bacterium is essential for host survival and development and has a long co-evolutionary history with all members of the subfamily Aleyrodidae [20,22,23]. In addition to the primary endosymbiont, many different secondary endosymbionts may be present, such as *Rickettsia* [24], *Wolbachia* [25,26], *Hamiltonella* and *Arsenophonus* [20,21], *Cardinium* [27], and *Fritschea* [28]. These have been reported from *Bemisia* populations around the world. Several of these secondary endosymbionts interfere with host physiology, ecology, and reproduction [29–31], and they may have effects on rapid evolutionary shifts [32], thermotolerance [17], resistance to insecticides [14], host fitness [33], defense against pathogens [34], and virus transmission ability [15,35–37].

Several *Bemisia* mitotype populations around the world have been surveyed for infection with endosymbionts and showed a clear variation in the infection frequency within the *Bemisia* genetic groups [18,38–40]. For example, in populations tested from China, *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Hamiltonella*, and *Cardinium* were detected in MEAM1 and MED populations [41]. *Arsenophonus*, *Cardinium*, *Rickettsia*, and *Wolbachia* were detected in native whiteflies of Africa [39], China [38], and India [42], but not *Hamiltonella* and *Fritschea*. A study by Gorsane et al. [43] hypothesized that the presence of *Cardinium* in MEAM1 and *Cardinium*, *Fritschea*, and *Wolbachia* in MED may explain the differences in infestation status possibly due to plant host variability, site to site variations, and the influence of chemical insecticides. Also, *Hamiltonella* in MED and *Rickettsia* in MEAM1 populations are also reported to increase the acquisition, retention, and transmission of the Tomato yellow leaf curl virus [44,45]. However, recent work on cassava whiteflies indicated that the coinfection of *Bemisia tabaci* colonies of sub-Saharan Africa 1 sub-group 3 (SSA1-SG3) by two secondary endosymbiotic bacteria *Arsenophonus* and *Rickettsia* reduced their ability to transmit East African cassava mosaic virus—Uganda (EACMV-UG) and these whiteflies also showed lower adult emergence, slower development, and lower virus retention abilities than those free of bacteria [46]. Skaljac et al. [47] showed that *Hamiltonella* and *Arsenophonus* were strictly localized to the bacteriocytes during all developmental stages in *T. vaporariorum.* However, they are less likely to be able to manipulate their host's reproduction since this requires invading reproductive organs outside the bacteriocyte. This observation suggests that *Hamiltonella* and *Arsenophonus* in *T. vaporariorum* are involved in a functional advantage rather than its reproduction [47].

For effective whitefly management, therefore, it is important to make the correct species identification, including the identification of intracellular bacterial communities. Many aspects of this species complex remain unknown, such as the degree of genetic isolation between some species, their geographical distribution, as well as the within-species genetic diversity and endosymbiont species being carried by whiteflies. Knowing which species of whitefly is present in Cameroon and its associated endosymbionts is crucial for the management of this pest. Because these cryptic species of whitefly are morphologically indistinguishable [3], we used molecular tools (mitochondrial cytochrome oxidase I sequences—mtCOI and Kompetitive Allele Specific PCR–KASP) for identification. In the study reported here, the endosymbiont colonization frequency and results from whitefly mtCOI sequencing and KASP were combined to provide an overall assessment of the genetic diversity of *B. tabaci* in Cameroon.

2. Materials and Methods

2.1. Whitefly Sampling and Population Study

2.1.1. Field Survey

Field surveys were conducted in four regions of Cameroon by using standardized diagnostic protocols described by many authors [48–50]. This involved collecting data and sampling (counting whiteflies and collecting specimens for molecular identification) from cassava, tomatoes, pepper, and okra young plants. The number and distribution of collection sites varied, according to the number of fields that were found in each location and the relative abundance of *B. tabaci* in those fields. A total of 24 fields were randomly chosen and surveyed from the four regions. Whitefly samples were collected from fifteen fields of cassava (*Manihot esculenta* Crantz), four fields of tomato (4) (*Solanum lycopersicum* L.), three fields of okra (*Abelmoschus esculentus* L.), one field of melon (*Cucumis melo* L.) and one field of pepper (*Capsicum annuum* L.) across twelve locations in three (monomodal rainforest, bimodal rainforest, and highland) agroecological zones across Cameroon (Table 1). The locations included Ngoa Ekelle, Minkoameyos, Awae, Bangangte, Manko'o, Mantem, Nyang, Ngohsi, Djibeeng, Balngong, Bandoumou, and Melen. *Bemisia tabaci*-colonizing tomatoes were collected from two locations and four fields (Manko'o in the highland agroecological zone and Awae in the bimodal rainforest agroecological zone). *Bemisia tabaci* on cassava were sampled from 10 locations and 15 fields (Ngoa Ekelle, Minkoameyos, Awae, Balngong, and Bandoumou, all located in the bimodal rainforest agroecological zone, Bangangte in the highland agroecological zone, and Mantem, Nyang, Ngohsi, and Djibeeng in the monomodal rainforest). On okra, *B. tabaci* were collected from two locations and three fields (Minkoameyos and Awae located in the bimodal rainforest agroecological zone). On melon, *B. tabaci* were collected from one location and one field (Ngoa Ekelle in the bimodal rainforest agroecological zone). On pepper, *B. tabaci* were collected from one location and one field (Melen in the bimodal rainforest agroecological zone).

Table 1. The range of climate characteristics (annual rainfall, relative humidity, and temperature) of the three agroecological zones covered in the study.

Region	Average Mean Temperature (°C)	Annual Relative Humidity (%)	Average Rainfall (mm)	Agroecological Zone
Southwest	22.6–33.6	66.1–86.2	2000–3700	Monomodal rainforest
Littoral	22.6–33.6	66.1–86.2	2000–3700	Monomodal rainforest (humid forest)
West	22.6–33.1	76.4–84.5	1800–2100	Western highland
Center	17.3–31.6	60–90	1500–2000	Bimodal rainforest (humid forest)

2.1.2. Data Recording and Storage

In each field, 20 plants were randomly selected along two diagonal transects across the field and whiteflies were counted from the top five leaves of vegetable and for cassava. Approximately 40 *B. tabaci* adult whiteflies were collected from each field and all whiteflies collected from a single field were considered to be a single sample. Whiteflies were aspirated alive and immediately preserved in 95% ethanol in vials, before being stored in the freezer at −20 °C.

2.2. Genetic Diversity of Sampled Whiteflies

2.2.1. DNA Extraction

DNA extraction was carried out in the molecular laboratory at the International Institute of Tropical Agriculture (IITA) in Dar es Salaam, Tanzania. The insects (single female whiteflies) were added to 3 µL of lysis buffer in a 1.5 mL Eppendorf tube, then macerated and another 20 µL of lysis buffer was added. The lysis buffer contained 10 mM Tris-HCl (pH 8.0, 50 mM KCL, 2.5 mM MgCl, 0.45% Tween–20, 0.01% Gelatine, and 60 µg/mL Proteinase). The mixture was then vortex shaken and spun down and immediately incubated on ice for 15 min. This was followed by incubation at 55 °C in a water bath for 30 min. The lysate was then stored at −20 °C for downstream use. For PCR use, the lysate was diluted while using sterile diethylpyrocarbonate (DPEC) treated water in a ratio of 1:9.

2.2.2. MtCOI Amplification and Sequencing

PCR products of the mtCOI fragment were produced using the forward primer 2195-Bt-F (5′-TGRTTTTTTGGTCATCCRGAAGT-3′) and C012-Bt-sh2-R (5′-TTTACTGCACTTTCTGCC-3′) [51] to target *Bemisia* whiteflies (~850 bp), and universal primers LCO-1490-F (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198-R (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) to target non-*Bemisia* whiteflies (~710 bp) using a thermocycler (Applied Biosystems™ GeneAmp^R PCR system 9700, Foster City, CA, USA), under the following conditions: first cycle of denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 40 s, and annealing at 54 °C for 30 s, 72 °C for 45 s, and the final extension at 72 °C for 10 min. A total reaction mixture of 25 µL was made up of 1X QuickLoad Master Mix (New England Biolabs, Hitchin, UK), 1 mM MgCl, 0.24 µM of each primer, 2 µL DNA, and nuclease-free water.

The PCR products were electrophoresed in a 1% agarose gel stained in GelRed (Biotium, Hayward, CA, USA) at 100 V for 30 min in gels buffered with a 1 × TAE buffer. DNA bands were visualized under ultraviolet light (UVP GelStudio PLUS, Analytik Jena, Upland, CA, USA) and only samples with intact bands were selected for sequencing. PCR products were sent to Psomagen Inc. (Rockville, MD, USA) for purification and direct sequencing. DNA sequences were manually edited using Ridom Trace Edit v1.1.0 software (Ridom GmbH., Würzburg, Germany). The sequences were assembled into contigs using CLC Main Workbench 22 (QIAGEN, Aarhus, Denmark). A multiple alignment of the edited sequences was performed using Clustal W in MEGA version 7 [52] and the sequences were trimmed. The construction of a maximum-likelihood phylogenetic tree was performed using MEGA with 1000 bootstrap replicates. Sequences were blasted using GenBank's

(NCBI) Blastn and selected reference sequences with 99% to 100% identity to our mtCOI sequences were included in the phylogenetic tree for comparison with previously published haplotypes. The outgroup *Bemisia afer* was included in the phylogenetic tree for *Bemisia tabaci*, while the outgroups *Bemisia afer*, *B. tabaci*, and *Aleurodicus dispersus* were included for *Trialeurodes vaporariorum*.

2.2.3. Kompetitive Allele-Specific PCR (KASP)

Kompetitive Allele-Specific PCR (KASP) was used to further distinguish the major genotypes of cassava-colonizing *B. tabaci* [53]. The KASP reaction mixture (10 µL) contained 5 µL 2X KASP master mix, 0.14 µL KASP primer assay mix, and 5 µL DNA template (1 µL of PCR product/DNA extract + 4 µL of sterile water). KASP genotyping was performed in a Strategene MX 3000P real-time PCR unit (Agilent Technologies, Santa Clara, CA, USA). The following cycling conditions were used: Stage 1: 30 °C for 60 s (pre-read); Stage 2: 94 °C for 15 min hot-start Taq activation (1 cycle); Stage 3: 94 °C for 20 s, 61 °C (61 °C decreasing 0.6 °C per cycle to achieve a final annealing/extension temperature of 55 °C) for 60 s (10 cycles); Stage 4: 94 °C for 20 s, 55 °C for 60 s (29 cycles); Stage 5: 94 °C for 20 s, 57 °C for 60 s (3 cycles); and Stage 6: 37 °C for 60 s (1 cycle, cooling) followed by an end-point fluorescent read. These conditions were used for three primers (BTS99-319, BTS22-762, and BTS55-473), while Stage 3: 94 °C for 20 s, 68 °C (68 °C decreasing 0.6 °C per cycle to achieve a final annealing/extension temperature of 62 °C) was used for the primer BTS613. The quality of genotyping cluster plots was visually assessed, and only samples in distinct clusters were considered for manual SNP calling, using the MxPro -Mx3000P software incorporated in the Strategene MX 3000P unit (Agilent Technologies, Santa Clara, CA, USA) and KlusterCaller (LGC Genomics, Teddington, UK). The KASP protocol for *B. tabaci* is described in detail in Wosula et al. [53].

2.3. Infectivity of Endosymbionts in Whiteflies

Screening for the Presence of Endosymbionts by PCR

The DNA extracts from 75 whitefly specimens that were identified through mtCOI sequencing were used for bacterial endosymbiont diagnoses. The PCR was performed using a total volume of 25 µL containing 2 µL template DNA, 12.5 µL OneTaq Quick-Load 2X Master Mix (New England Biolabs, Hitchin, UK) with Standard Buffer, 0.6 µL of primer (0.25 mM) (Table 2), 1 µL MgCl2 (25 mM) solution, and 8.9 µL of sterile water. A total of 35 cycles of amplification were carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA), and conditions were the same for all sets of primers except for the annealing temperature (Table 2): denaturation at 95 °C for 3 min and 94 °C for 30 s, annealing temperature as showed in Table 2 for 45 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min and held at 10 °C.

Table 2. Primer sequences and annealing temperatures used for PCR amplification of endosymbionts.

Target Gene	Primer Name	Sequence (5′→3′)	Reference	Amplicon Length	Annealing Temperature
Portiera 16S rDNA	28F 1098R	TGCAAGTCGAGCGGCATCAT AAAGTTCCCGCCTTATGCGT	[25]	1050 bp	58 °C
Arsenophonus 23S rDNA	Ars23S-1 Ars23S-2	CGTTTGATGAATTCATAGTCAAA GGTCCTCCAGTTAGTGTTACCCAAC	[30]	750 bp	58 °C
Rickettsia 16S rDNA	Rb-F Rb-R	GCTCAGAACGAACGCTATC GAAGGAAAGCATCTCTGC	[24]	960 bp	58 °C
Wolbachia *wsp* gene	81F 471R	TGGTCCAATAAGTGATGAAGAAA- CAAAAATTAAACGCTACTCCA	[39]	600 bp	53 °C

Table 2. *Cont.*

Target Gene	Primer Name	Sequence (5′→3′)	Reference	Amplicon Length	Annealing Temperature
Cardinium 16S rDNA	Card-F Card-R	TAGACACACACGAAAGTTCATGT GCATGCAATCTACTTTACACTGG	[39]	650 bp	57 °C
Hamiltonella 16S rDNA	Hb-F Hb-R	TGAGTAAAGTCTGGGAATCTGG AGTTCAAGACCGCAACCTC	[18]	730 bp	58 °C

Amplified PCR products were separated using 1% agarose gel electrophoresis, stained with GelRed (Biotium, Hayward, CA, USA) with a 100 bp ladder (NEB, England, UK), and then visualized under ultraviolet light (UVP GelStudio PLUS, Analytik Jena, Upland, CA, USA). All PCRs included a negative control (sterile water) to spot any DNA contamination, and a positive control to prevent false negatives.

3. Results

3.1. Whitefly Abundance

Mean whitefly counts varied with survey site (Table 3). The mean whitefly count per plant across the country was 8.0 for cassava, 29.7 for tomato, 31.6 for okra, 60.0 for melon, and 18.0 for pepper. At the regional level, the mean whitefly count varied from 2.9 in the Littoral region to 12.5 in the southwest region for cassava. In addition, mean whitefly counts on vegetable crops such as tomato, pepper, melon, and okra were high and ranged from 18 to 60. The highest whitefly mean count of 60 was recorded for the center region on Melon, while the Littoral region had the lowest mean (2.9) except on cassava. Field-level data showed many fields with whitefly counts higher than 50 per plant located in the center, southwest, and west regions.

Table 3. Whitefly abundance on different crops in three different agroecological zones of Cameroon.

Region	Division	Agroecological Zone	Number of Fields	Crop (Number of Fields)	Whitefly Abundance (Mean)
Southwest	Dian Koupemanengouba	Monomodal rainforest	2	Cassava	12.5
Littoral	Moungo	Monomodal rainforest (humid forest)	3	Cassava	2.9
West	Nde	Western highland	5	Cassava (2) Tomato (3)	5.9 37.3
Center	Mefou et Afamba Mefou et Akono Mfoundi	Bimodal rainforest (humid forest)	14	Cassava (8) Tomato (1) Okra (3) Melon (1) Pepper (1)	10.7 22.0 31.6 60.0 18.0

3.2. mtCOI Mitotypes of Whiteflies Colonizing Five Crop Plants in Cameroon

In total, 92 whitefly samples were sequenced, out of which 75 produced quality mtCOI sequences. There was a high level of diversity among *B. tabaci* populations that were collected from the sampled crop plants. The sequences obtained from whiteflies collected from pepper, okra, and melon were grouped into one phylogenetic group (MED), but whiteflies from tomato were grouped into two phylogenetically distinct groups: *B. tabaci* MED and *Trialeurodes vaporariorum* (Westwood). The sequences from cassava were grouped into five mitotypes of *B. tabaci* (SSA1-SG1, SSA1-SG2, SSA1-SG5, SSA3, and SSA4) and two *Bemisia afer* (Priesner and Hosny) clades (Figures 1–3). These groups were identified based on the topology of the phylogenetic tree and the clustering of the sequences that were obtained from this study relative to the reference sequences retrieved from GenBank. The predominant *B. tabaci* mitotype MED had a total of 23 whiteflies, which accounted for 30.3% of all the whiteflies collected from the four host plants (tomato, okra, melon, and pepper) and they were distributed in the two agroecological zones where these samples were collected (bimodal rainforest and highland). The second most abundant *B. tabaci* mitotype was SSA4 with 17 whiteflies (22.4%), with all of them being found on cassava, and they were distributed in all three agroecological zones (monomodal rainforest, bimodal rainforest, and highland). The other mitotypes occurring on cassava were SSA1-SG1 (1.3%) (monomodal rainforest agroecological zone), SSA1-SG2 (4.0%) (monomodal rainforest and bimodal rainforest agroecological zone), SSA1-SG5 (1.3%) (monomodal rainforest agroecological zone), and SSA3 (2.6%) (monomodal rainforest agroecological zone). *Bemisia afer* had two distinct clades with 19 whiteflies, which accounted for 25.3% of the sequences. It was present on cassava in all three sampled regions (monomodal rainforest, bimodal rainforest, and highland agroecological zone). *Trialeurodes vaporariorum* (10 whiteflies accounting for 13.2% of the sequences) was present only on tomato in the highland agroecological zone. Sequences from this study were deposited in the NCBI database accessions PP580858–PP580933.

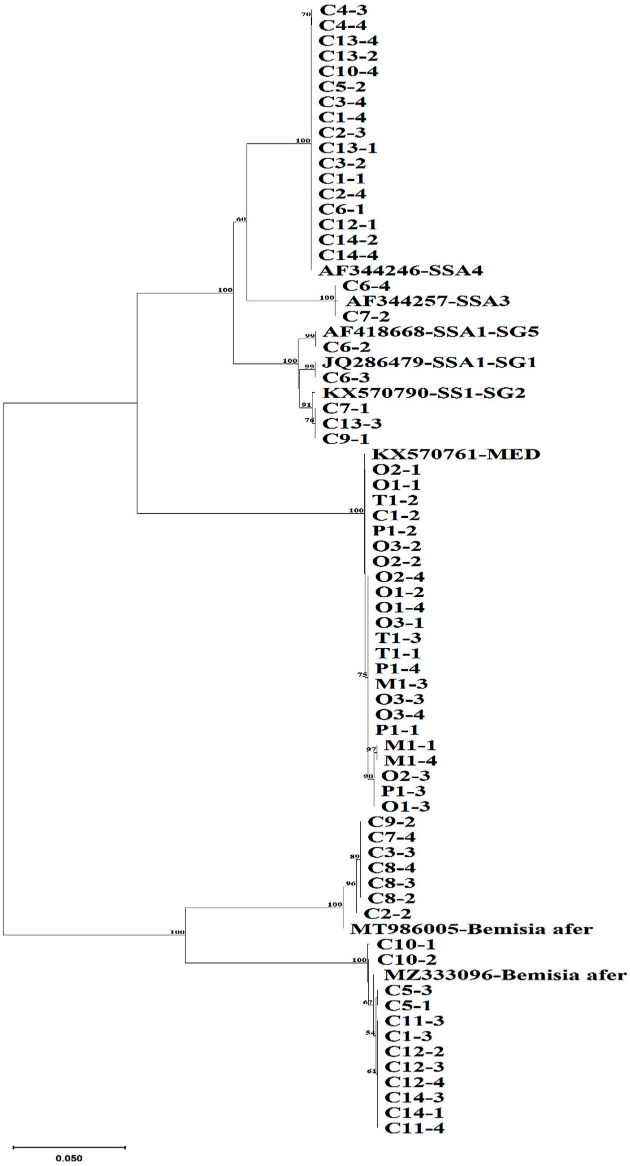

Figure 1. Phylogenetic relationships of the *Bemisia tabaci* whiteflies collected from crop plants in three agroecological zones of Cameroon. C (cassava plant), O (okra plant), T (tomato plant), P (pepper), and M (melon). The first number represents the field, and the second number is the sample number.

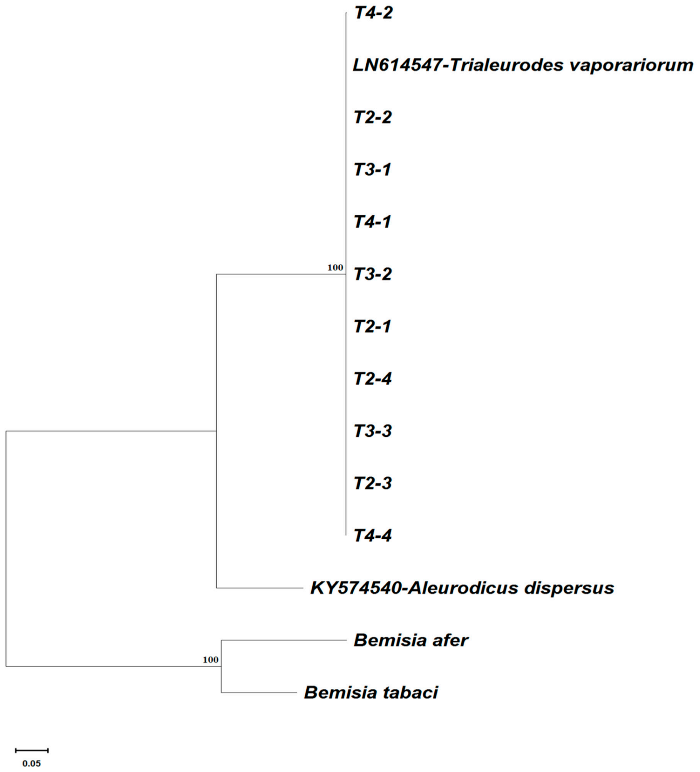

Figure 2. Phylogenetic relationships of the *Trialeurodes vaporariorum* whiteflies collected from tomato plants in three agroecological zones of Cameroon.

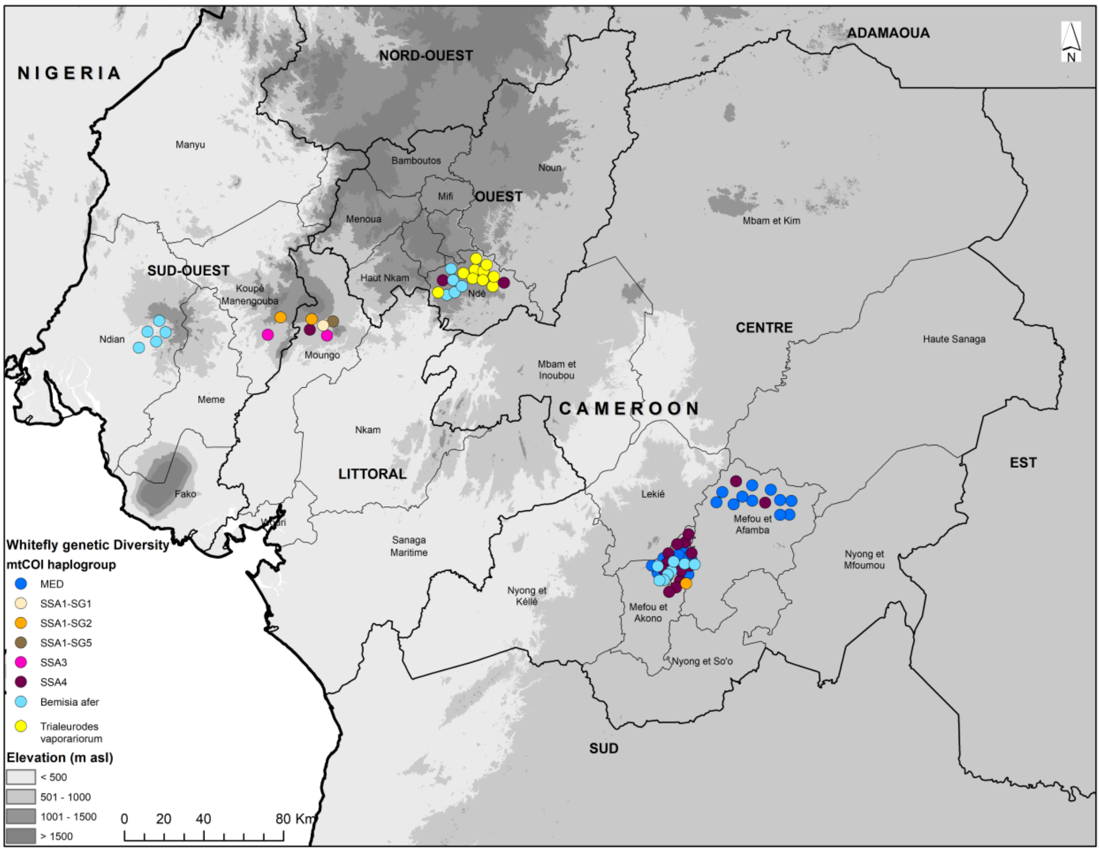

Figure 3. Genetic diversity according to mtCOI sequences of whiteflies collected on cassava, okra, tomato, pepper, and melon in Cameroon.

3.3. Kompetitive Allele Specific PCR Analysis (KASP)

The KASP genotyping results are based on SNP genotyping, and it has been designed to discriminate between the major genotypes of cassava *B. tabaci* whitefly. Twenty-four whiteflies that were identified as cassava *B. tabaci* based on mtCOI sequencing were further characterized using KASP genotyping. The SNP genotyping clusters for the selected four primers for representative samples are presented in Figures 4 and 5.

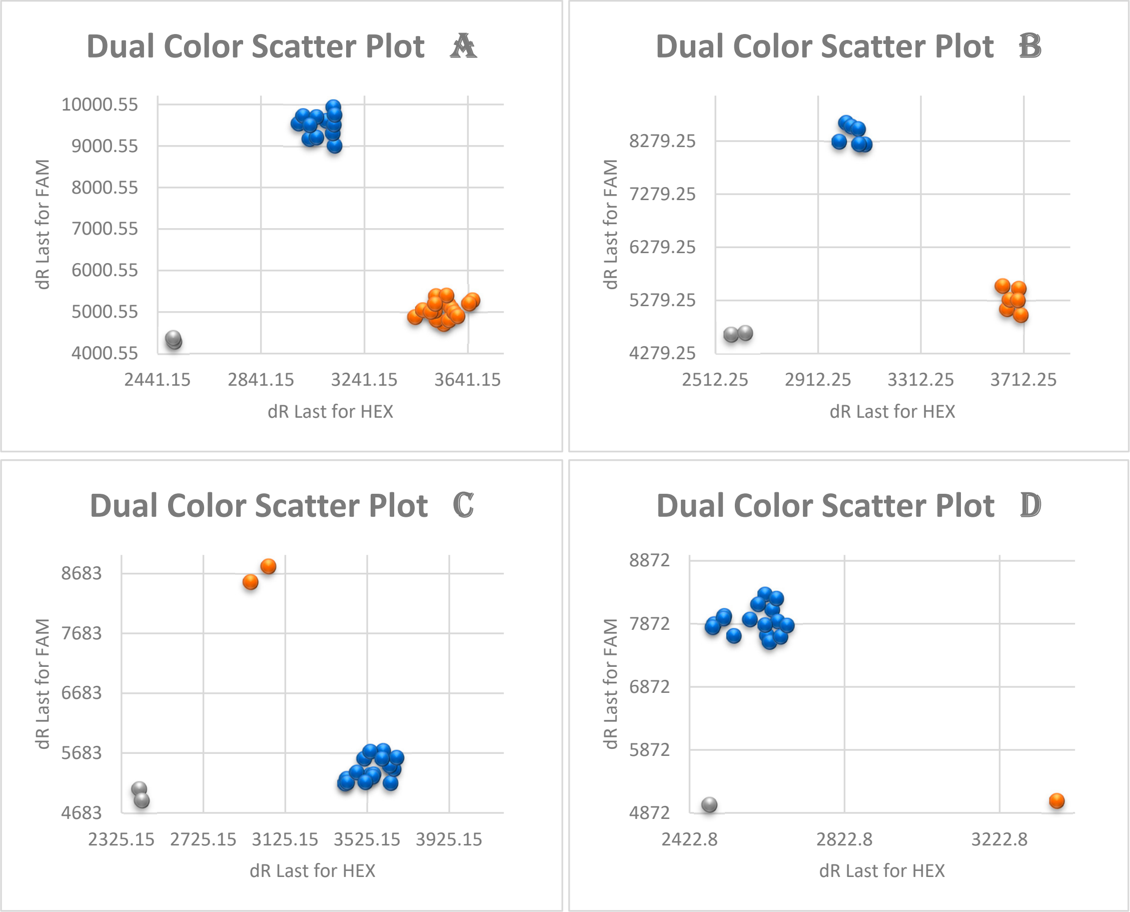

Figure 4. Cluster plots of four KASP SNP-based primers discriminating between major haplogroups of *Bemisia tabaci* whiteflies collected from cassava. Orange and blue represent the two distinct alleles, while grey dots represent negative controls. (**A**) = BTS99-319 (SSA-ECA and SSA-WA vs. SSA-ESA, SSA-CA, SSA2, and SSA4); (**B**) = BTS22-762 (SSA-ECA vs. SSA-WA); (**C**) = BTS613 (SSA-ESA and SSA-CA vs. SSA-ECA, SSA-WA, SSA2, and SSA4); and (**D**) = BTS55-473 (SSA2 vs. SSA4).

KASP genotyping split the 24 cassava whitefly samples into three haplogroups: SSA-ECA, SSA-WA, and SSA4. Haplogroup SSA4 with 15 samples out of the 24 (62.5%) was the most frequent, and included mtCOI mitotypes SSA4 (10), SSA3 (2), SSA1-SG2 (2), and SSA1-SG5 (1). The haplogroup SSA-ECA was the second most frequent with five samples (20.8%), all of which were designated as mitotype SSA4. The last haplogroup SSA-WA had four samples (16.7%) that were designated as mitotypes SSA4 (2), SSA1-SG2 (1), and SSA1-SG5 (1). This is the first study to report the designation of mitotype SSA4 samples into SNP haplogroup SSA-ECA (Table 4).

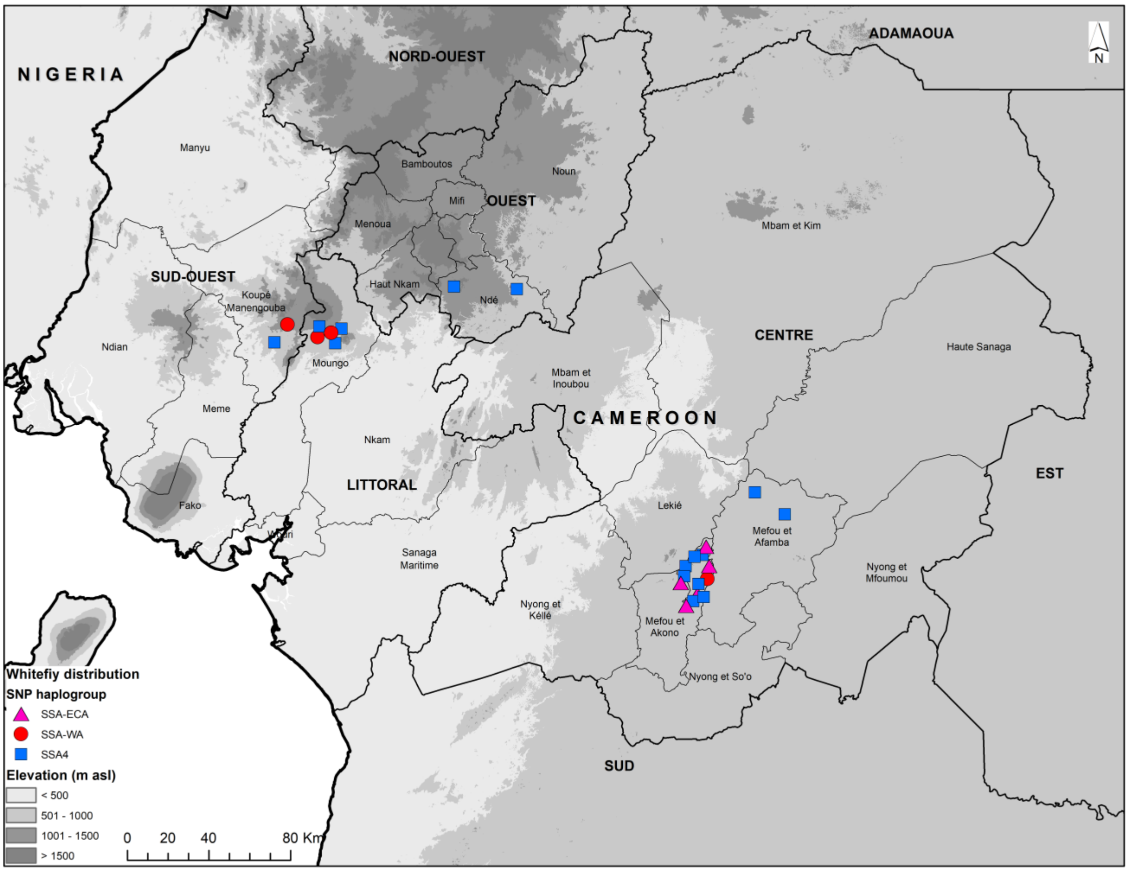

Figure 5. Cassava whiteflies (*Bemisia tabaci*) haplogroup distribution based on KASP SNP genotyping discrimination.

Table 4. The six SNP-based haplogroups and associated mtCOI sequences mitotypes of cassava whitefly *Bemisia tabaci*.

KASP Haplogroup	Mitotypes (mtCOI)	References
SSA-ECA	SSA1-SG1 SSA1-SG2, SSA1-SG1/SG2, SSA4	[54–57]; in this study
SSA-ESA	SSA1-SG3, SSA1-SG2	[54,56–58];
SSA-WA	SSAA1-SG1, SSA1-SG5, SSA2, SSA4	[56,57]; in this study
SSA-CA	SSA1-SG1, SSA1-SG2	[56]
SSA2	SSA2, SSA3, SSA4	[54,56,57]
SSA4	SSA4, SSA1-SG2, SSA1-SG5	[56,57]; in this study

The samples in the SSA4 group were from Ngoa Ekelle, Minkoameyos, Awae, Bangangte, Mantem, Nyang, Djibeeng, and Bandoumou located in the three agroecological zones. Samples in the SSA-ECA group were from Ngoa Ekelle, Balngong, and Bandoumou in the central part of Cameroon located in the bimodal rainforest agroecological zone, while those in SSA-WA were from Mantem and Nyang in the monomodal rainforest agroecological zone of western Cameroon, as well as from Ngoa ekelle and Awae in the bimodal rainforest agroecological zone in the central part of the country.

3.4. Frequency of Infection of Whiteflies by Three Endosymbionts

The primary endosymbiont *Portiera* was detected in 77% of the whiteflies identified by mtCOI and KASP (75). Therefore, only whiteflies with at least three specimens and bearing *Portiera* were used to evaluate the presence of secondary endosymbionts. The secondary endosymbionts were found in 78% (45 whiteflies infected out of 58 tested) of the insects, and their frequency varied significantly across the different whitefly populations. The SSA4 mitotype showed the highest percentage of no secondary endosymbiont with 50% of non-infection. However, none of the whitefly samples showed an infection by the endosymbionts *Hamiltonella* and *Cardinium* (Figure 6). The identified endosymbionts were more often in single infections in cassava-colonizing whiteflies than non-cassava whiteflies, except in *T. vaporariorium* where *Arsenophonus* singly infected 100% (7/7) of the specimens. In the MED mitotype, infection by the combination of *Arsenophonus* (A), *Wolbachia* (W), and *Rickettsia* (R) was the most represented (41%). Only A (18%) and W (6%) were identified in single infection and all other infections were in coinfection with *Arsenophonus* AR (23%) and AW (12%). Whiteflies collected on cassava and identified as *B. afer* had infections with all of the endosymbionts dominated by the single infections of A (38%) and W (23%). No triple infection was recorded but all possible double infections were detected as AR (8%), AW (23%), and RW (8%). The SSA4 mitotype was singly infected with *Wolbachia* (W) in 72% of the specimens followed by *Arsenophonus* (A) and *Rickettsia* (R) with 14% each. The SSA1-SG2 mitotype had single infections of W (33%) but 67% of this mitotype were not infected by the secondary endosymbiont (Figure 6). Considering only infected whiteflies, *Arsenophonus* (95.8%; 23/24) was the most frequently represented endosymbiont in non-cassava whiteflies (MED, *T. vaporariorium*). However, in cassava whiteflies (SSA4, SSA1-SG2, and *Bemisia afer*) *Wolbachia* was the most frequently represented (56.5%; 13/23).

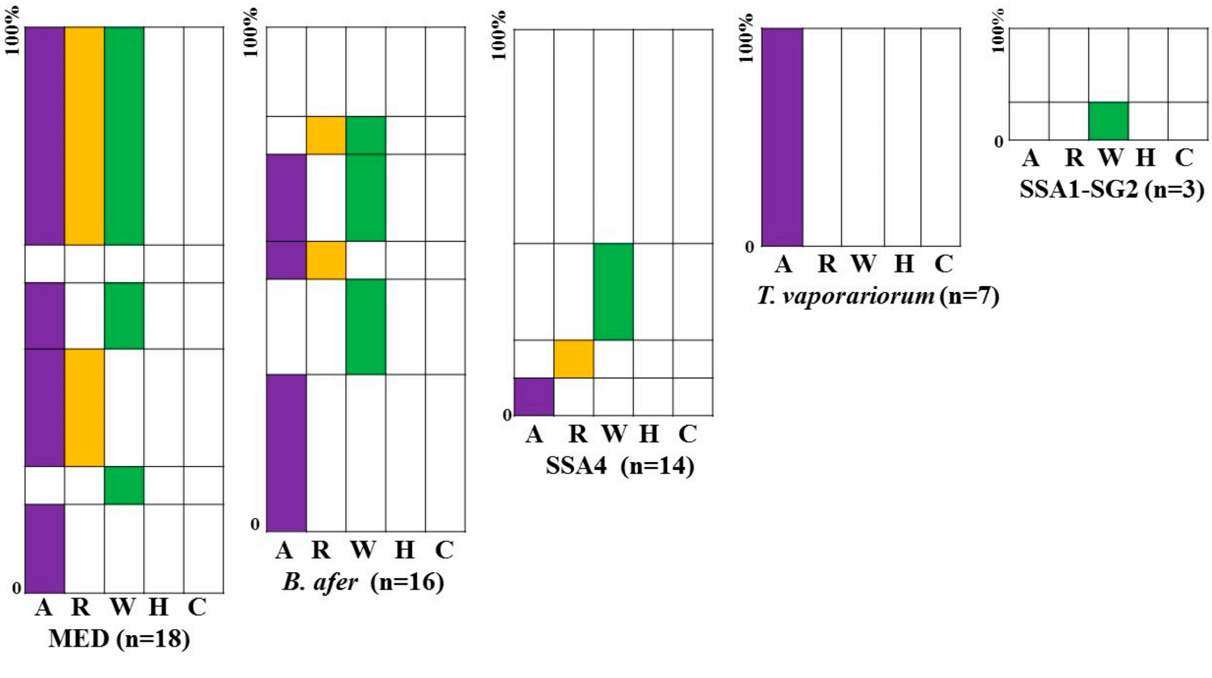

Figure 6. The frequency of infections of symbionts in different whitefly populations. The number of individuals tested and the genetic groups they belong to are indicated below each graph. The various colors represent the different secondary symbionts tested where A, *Arsenophonus*; R, *Rickettsia*; W, *Wolbachia*; H, *Hamiltonella*; and C, *Cardinium*. The combination of colors in the rows represents whiteflies that shared that particular complex of symbionts. The width of the row indicates the percentage of individuals that have that combination of symbionts. The numbers in brackets indicate the number of individuals tested in each of the genetic groups and sub-groups.

4. Discussion

The current study confirmed that whiteflies occur widely on vegetables and cassava throughout Cameroon, and their abundance depends on the host plant species and agroecological region. The number of whiteflies was higher on vegetables compared to cassava; this could be attributed to the fact that the mitotype MED found on vegetables is an invasive species known for rapid resistance development to insecticides, hence high populations [3]. The abundance of whiteflies in vegetables is linked with increased spread and the severity of virus diseases [59,60].

An analysis of the genetic diversity of the whiteflies collected showed that the three whitefly species commonly occurring on cassava and vegetables were *B. tabaci*, *B. afer*, and *T. vaporariorum*. Whilst *B. afer* and *B. tabaci* occurred widely throughout the agroecological zones sampled, *T. vaporariorum* was only reported from tomato in the highland zone. *T. vaporariorum* is commonly called the greenhouse whitefly and has a more temperate distribution than the two *Bemisia* species. In Africa, *T. vaporariorum* only occurs in high altitude and cooler regions [61].

Bemisia afer accounted for 25% of the *Bemisia* spp in samples from Cameroon and it was identified in all of the agroecological zones. Although *B. afer* is not currently considered to be a significant threat to cassava production in Africa, it has been shown to be an economically important viral vector in other crops, transmitting the sweet potato chlorotic stunt virus in sweet potatoes in Peru [62]. This highlights the importance of careful monitoring of this second cassava-colonizing *Bemisia* species in Africa.

The clustering of the cassava *B. tabaci* whitefly mitotypes SSA1-SG1, SSA1-SG2, SSA1-SG5, SSA3, and SSA4 into a distinct major clade separate from *B. tabaci* whiteflies that do not colonize cassava is consistent with what has been reported in other studies of *B. tabaci* from various cassava-growing countries in Africa [57,63]. The grouping of the MED mitotype is also consistent with what has been reported in previous studies [63]. We found that *B. tabaci* MED was predominant on okra, pepper, tomato, and melon in all of the sampled locations. MED is a globally important *B. tabaci* mitotype, which is thought to have originated from countries neighboring the Mediterranean basin, which include Algeria, Morocco, Egypt, and Sudan in Africa [3,64]. Consequently, there are numerous other reports of its prevalence on a wide range of crop and weed hosts [55,63,65–67]. *B. tabaci* MED has been reported to be extremely polyphagous and invasive [3], causing damage to both field and greenhouse crops [68]. It has also developed resistance to various insecticides under intensive production systems [69–71]. The mitotype *B. tabaci* MED was also the most abundant whitefly collected. As confirmed by the results on whitefly abundance, evidence elsewhere has suggested that begomovirus infection can increase *B tabaci* MED fecundity, which facilitates its spread [72]. The predominance of MED on major vegetable crops considered in this study confirms that this is the most important whitefly pest of vegetables in Cameroon.

In the studied cassava group of *B. tabaci*, the largest number of samples based on mtCOI sequencing were in the SSA4 mitotype, and these were widely distributed across the sampled locations. SSA4 has previously been reported on cassava in Cameroon accounting for 15% [73] and 37% [56] of the cassava whitefly samples. The findings from this study suggest SSA4 is increasing in dominance compared to other mitotypes. SSA1-SG1 and SSA1-SG5 were less frequent, as they were only detected at a single location each. In another study, a similar trend was reported for samples obtained from cassava in Cameroon, where 7% were identified as SSA1-SG1, while none were identified as SSA1-SG5 [56]. These results differ from other recent findings from East and Central Africa, which have shown SSA1-SG1 to be the predominant *B. tabaci* mitotype on cassava [7,54,57,74]. SSA2 was not encountered in the current study, although this may be a consequence of the absence of samples from northern parts of Cameroon. A similar result was reported elsewhere [73], where only SSA3 and SSA4 were recorded, although other research on cassava *B. tabaci* in Cameroon, which included samples from the north of the country, recorded SSA2 making up 44% of the total types found [56]. This trend of presence or absence of SSA2 depending on the duration of the surveys is not unique; it has been observed in East Africa where SSA2 was reported as being absent in samples collected from cassava [75,76], and then reported present with subsequent surveys [51,56,57,77]. In South Sudan, SSA2 was reported as the most predominant mitotype on cassava accounting for 75% of the samples that were collected from the cassava plants [55]. Similarly, the current study noted the presence of SSA1-SG5 and SSA1-SG2, which were not found in previous studies [56,57], although, importantly, the sample collection locations differed between the studies. An accurate identification of these species is critical for the effective management of whiteflies both as pests and as virus vectors. The development of the KASP diagnostic method, based on a large SNP dataset for *B. tabaci* in Africa, provided important means for distinguishing between the major genetic groupings of *B. tabaci* occurring on cassava in Africa [53]. This method allows for the identification of these haplogroups in laboratory procedures lasting a matter of hours and with no requirement for sequencing. This study builds on the importance of adopting KASP as a diagnostic method for cassava *B. tabaci* whiteflies as it reports for the first time mitotype SSA4 samples designated as SNP haplogroups SSA-ECA and SSA-WA (Table 4). An important outcome of SNPs' analysis and the application of KASP has been the recognition of an association between the haplogroup SSA-ECA and regions currently affected by severe CMD and CBSD pandemics [56].

The most common SNP haplogroup, however, was SSA4. For the first time, this haplogroup included samples that were designated as mitotypes SSA1-SG5 and SSA1-SG2. Previously, samples identified as SSA4 using KASP, based on SNPs' analysis, only had mitotypes SSA3 and SSA4 [53,56]. There were similar novel associations between SSA-WA and mitotype SSA4; previously, SSA-WA was reported to have mitotypes SSA1-SG5 (predominantly), SSA1-SG1, and SSA2 [56]. The present study also revealed that haplogroup SSA-ECA had all samples designated as mitotype SSA4. This is a first, as previously this SNP haplogroup only had samples of mitotypes SSA1-SG1, SSA1-SG2, and SSA1-SG1/SG2 [54–56]. Each of these sets of results provides further evidence of the weak association between identifications based on SNPs dispersed throughout the *B. tabaci* genome and identities derived from short, maternally-inherited mitochondrial COI sequences. Finally, this work shows, again, that there is not a good correlation between COI and KASP identities, which confirms the unreliability of using COI to identify *B. tabaci* genotypes.

The identification of *B. tabaci* haplogroup SSA-ECA raises a concern about the potential future spread of cassava viruses in Cameroon, as SSA-ECA is predominant in areas associated with severe epidemics of CMD and CBSD in East, Central, and Southern Africa [54,56]. It is important to note, however, that CBSD has not yet been identified in Cameroon and has only so far been reported from East and Southern Africa. The most westerly report of CBSD has been made from the eastern part of the Democratic Republic of Congo (DRC) [78].

Microbial endosymbionts represent an important component of the biology and ecology of invertebrates like *Bemisia*. Major facultative endosymbionts *Arsenophonus*, *Wolbachia*, *Hamiltonella*, *Cardinium*, and *Rickettsia* were evaluated for all whitefly genetic groupings and the infection frequencies were significantly correlated with the whitefly genotype. The current study showed that cassava whiteflies are mostly infected by single endosymbionts rather than multiple infections, as had been observed from previous studies elsewhere in Africa [39]. However, MED, which occurred on crop plants other than cassava, showed a higher level of endosymbiont coinfection. In this study MED had diverse secondary endosymbiont communities comprising *Arsenophonus*, *Rickettsia*, and *Wolbachia*. Similar studies have reported the presence of these endosymbionts in this mitotype [4,11,79–82]. MED individuals analyzed in this study were predominantly infected with *Arsenophonus*, which is comparable to findings from West Africa reporting a high prevalence of this endosymbiont in the ASL (=MED) mitotype [4]. By contrast, MED individuals collected from vegetables in Senegal were predominantly infected with *Hamiltonella* [11].

The cassava *B. tabaci* cryptic species in this study had diverse endosymbiont infections comprising *Arsenophonus*, *Rickettsia*, and *Wolbachia* as reported in other studies [39,79,83]. The work of Ghosh et al. [39] showed that cassava whiteflies are infected by *Arsenophonus*, *Rickettia, Wolbachia*, and *Cardinium* with the predominance of *Wolbachia* in Tanzania, Malawi, Uganda, and Nigeria. Tajebe et al. [79] reported *Arsenophonus*, *Rickettsia*, *Wolbachia*, *Hamiltonella*, and *Cardinium*, with *Arsenophonus* being the most prevalent in cassava *B. tabaci* whiteflies in Tanzania.

Trialeurodes vaporariorum was only infected with *Arsenophonus,* as noted elsewhere [84]. *Hamiltonella* and *Cardinium* were not detected in this study, although they have been reported in other studies on whiteflies collected from Africa [4,39,79,83]. Secondary endosymbionts in *B. tabaci* have been shown to have an influence on whitefly biology, survival, fecundity, heat tolerance, resistance/susceptibility to insecticides, and virus transmission [85]. Tajebe et al. [79] noted that the most striking feature of *B. tabaci* individuals sampled from the cassava virus pandemic that affected parts of Tanzania was the virtual absence of *Arsenophonus*. Furthermore, Ghosh et al. [46] reported that cassava *B. tabaci*, infected by *Arsenophonus* and *Rickettsia*, had decreased fitness and virus retention compared to whiteflies of the same type that were not infected by either endosymbiont. In our study, there was, overall, a much greater frequency of *Arsenophonus* in non-cassava whiteflies and *B. afer* compared to those *B. tabaci* (SSA4 and SSA1-SG2) individuals sampled from cassava. However, larger sample numbers of cassava *B. tabaci* would be required to draw stronger conclusions about possible differences in *Arsenophonus* frequency amongst the cassava-colonizing *B. tabaci* genotypes present in Cameroon. Since the absence of endosymbionts has been linked to greater fitness and virus retention capabilities, however, it will be important to monitor endosymbiont occurrence in future studies. For these reasons, knowledge about prevailing endosymbionts in surveyed whitefly populations is critical for influencing future research on the role of these bacteria in whiteflies and their effect on virus epidemics.

5. Conclusions

This work demonstrated the presence of six mitotypes of *Bemisia tabaci*, and two distinct clades of *Bemisia afer* and *Trialeurodes vaporariorum* on vegetables and cassava in Cameroon. *Bemisia tabaci* mitotypes identified included MED on vegetables and SSA1-SG1, SSA1-SG2, SSA1-SG5, SSA3, and SSA4 on cassava. The MED mitotype was widely distributed in all sampling regions and is almost certainly the main phytovirus vector in Cameroonian vegetable cropping systems. For the first time, we found the haplogroup SSA-ECA on cassava in Cameroon. This is a concerning new development, as this haplogroup is predominant in regions currently affected by the severe cassava mosaic virus disease (CMD) and cassava brown streak virus disease (CBSD) pandemics in Eastern and Central Africa. The whiteflies in this study were found to be infected with endosymbionts from three different genera (*Arsenophonus, Wolbachia*, and *Rickettsia*). None of the insects were infected by *Hamiltonella* and *Cardinium*. Moreover, the *Rickettsia* species, which are implicated in the resistance of insects to insecticides, entomopathogens, and natural enemies, was recorded in all whitefly species at varying levels. MED mitotype whiteflies were predominantly infected with *Arsenophonus*, which is implicated in the adaptability of whiteflies. These findings add to the knowledge of the diversity of whiteflies and associated endosymbionts, which, when combined, can influence virus epidemics and responses to whitefly control measures especially insecticides.

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