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Natural Plant Extracts and Microbial Antagonists to Control Fungal Pathogens and Improve the Productivity of Zucchini (*Cucurbita pepo* L.) In Vitro and in Greenhouse

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Abstract: Background: Natural plant extracts and microbial antagonists have the potential for use in increasing the fungal resistance and productivity of horticulture plants. Methods: The purpose of this study was to evaluate the ability of both natural plant extracts and microbial antagonists as a biotical control of some fungal pathogens, i.e., *Fusarium* ssp., *Exserohilum* ssp. and *Nigrospora* ssp., along with improving the growth and productivity performance of zucchini under greenhouse conditions. *Eucalyptus camaldulensis* leaf extract (LE), *Citrus sinensis* LE, *Ficus benghalensis* fruit extract (FE), and two microbial antagonists *Pseudomonas fluorescens* (accession no. MW647093) and *Trichoderma viride* (accession no. MW647090) were tested under in vitro and in vivo conditions. Through morphological characteristics and the internal transcribed spacer (ITS) region, *Fusarium solani* (accession no. MW947256), *F. oxysporum* (accession no. MW947254), *Exserohilum rostratum* (accession no. MW947255), and *Nigrospora lacticolonia* (accession no. MW947253) were identified. HPLC analysis was used for the identification of phenolic compounds (PCs) and flavonoid compounds (FCs) in the extracts. Results: The highest inhibition percentage of fungal growth (IPFG) against *F. oxysporum* was obtained with *P. fluorescens*, *T. viride*, and *E. camaldulensis* LE (4000 mg/L); *F. solani* with *P. fluorescens*, *T. viride*, and *C. sinensis* LE (4000 mg/L); *Exserohilum rostratum* with *P. fluorescens*, *Ficus benghalensis* FE (4000 mg/L) and *E. camaldulensis* LE (4000 mg/L), and *N. lacticolonia* with *P. fluorescens*. Using HPLC analysis, the abundant PCs in *E. camaldulensis* LE were pyrogallol, and caffeic acid, those in *C. sinensis* LE were syringic acid and ferulic acid, and those in *F. benghalensis* FE were gallic acid and syringic acid. In addition, the abundant FCs in *E. camaldulensis* LE were kaempferol, and naringin, those in *C. sinensis* LE were hesperidin and quercetin, and those in *F. benghalensis* FE were kaempferol and quercetin. Under greenhouse experiments, *T. viride* and *E. camaldulensis* LE (4000 mg/L) followed by *P. fluorescens* + *T. viride* treatments gave the best results of zucchini plants in terms of leaf area, fruits number per plant, yield per plant, and total yield (marketable and non-marketable). Conclusions: Plant extracts and bioagents can be used to control some zucchini fungal pathogens and increase the productivity performance of zucchini plants.

Keywords: *Eucalyptus camaldulensis*; *Citrus sinensis*; *Ficus benghalensis* crown rot; Fusarium wilt; ITS; natural plant extracts; *Pseudomonas fluorescens*; *Trichoderma viride*; marketable yield; zucchini plants

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1. Introduction

Zucchini (*Cucurbita pepo* L.) is a popular, seasonal vegetable crop that is cultivated in large areas of Egypt [1]. Zucchini is considered a low-calorie vegetable with health-promoting properties [2,3]. The fruits contain biologically active compounds including lutein, β-carotene, and folic acid, as well as vitamins and minerals [2,4,5]. Zucchini fruits have gained significant importance not only on the fresh food market but also as a raw material for various kinds of vegetable-based processed food items, especially in Mediterranean and European countries [6,7]. Zucchini is normally grown in soil extensively during the summer season and intensively under greenhouse conditions during the fall and winter seasons for national and international markets [8].

Zucchini plants grown in the field and under greenhouse conditions are usually infected by pathogens specific for Cucurbitaceae [9–11]. The most important among these pathogens are *Exserohilum rostratum* and *Nigrospora lacticolonia* [12–15], and fungal pathogens are considered as some of the most serious pathogens causing a significant reduction in date palm growth, production, and development [16–18]. Fusarium solani and *F. oxysporum* surviving in the soil environment as saprotrophic mycelium and chlamydospores are known to be pathogens of zucchini and other vegetables, causing plant decay due to the colonization of their underground organs [12,19]. The most important pathogens seem to be *F. solani* causing crown rot, *F. oxysporum* responsible for plant wilting, *E. rostratum* causing leaf spot and *N. lacticolonia* causing brown spot [13,15,20]. In recent years, researchers on biological control of fungal plant pathogens have placed much interest in increasing crop production by avoiding several problems linked to developing practices compatible with sustainable agriculture and chemical control. Microbial antagonists are being used to control fungal growth. Some of the most common antagonists—including *Pseudomonas fluorescens* and *Trichoderma viride*—have been utilized as the main mechanism against some fungal zucchini diseases [21]. *Pseudomonas fluorescens* is a harmless bacterial species that is found to protect the roots of plants from plant disease [22–24]. *Trichoderma* is used as a biological control agent against a wide range of commercially important plant pathogens [25–27]. They are known to produce a number of antibiotics, such as trichodermol A, harzianolide, and trichodermin [28–31].

Natural plant extracts modulate plant growth and are involved in plant defense responses, including limiting pathogen development [32]. They are used as antimicrobial agents against a broad spectrum of plant pathogenic fungi such as *Alternaria solani*, *Aspergillus fumigatus*, *A. niger*, *Trichoderma longibrachiatum*, *A. flavus*, *A. fumigatus*, *Fusarium solani*, *F. oxysporum*, *Bipolaris orzyae*, *Botrytis cinerea*, *Curvularia lunata*, *F. verticilliodies*, and *F. graminearum* [33–39]. Moreover, these can be used against plant bacterial pathogens such as *Pectobacterium carotovorum*, *Pectobacterium atrosepticum*, *Dickeya solani*, and *Agrobacterium tumefaciens* [40]. The action of natural compounds such as terpenoids, phenolics, and alkaloids are not specific, and their effects on pathogens are versatile [41]. However, some studies found that flavonoids were not associated with antifungal activity [42], while other works reported that the inhibition of fungal growth was mainly due to flavonoids [34,43]. Natural bioactive compounds used in plant protection kill pathogens (fungicidal effect) or limit their development (fungistatic effect), as well as induce plant defense reactions as elicitors [41].

This study was aimed at investigating the effects of natural plant extracts and microbial antagonists on the growth and total yield of zucchini plants under greenhouse conditions, to evaluate the diversity of the fungal isolates colonizing zucchini, isolation and by identification through morphological characteristics and the internal transcribed spacer (ITS) region. In addition, we planned to evaluate the plant extracts (*Eucalyptus camaldulensis* leaves, *Citrus sinensis* leaves, and *Ficus benghalensis* fruits) and microbial antagonists (*Pseudomonas fluorescens* and *Trichoderma viride*) to control selected Zucchini fungal pathogens in vitro.

2. Materials and Methods

2.1. Isolation of the Fungal Pathogens

Fungi were isolated from naturally infected zucchini plant variety AZIAD F1. (Figure 1). The fruit pieces were directly placed on a Potato Dextrose Agar (PDA) medium, into sterile Petri dishes. Plates were incubated at 25 °C for 7 days as described by Pitt and Hocking [44] and Abass et al. [17]. A standard tissue isolation technique was used to obtain fungal pathogen cultures as described by Naik et al. [45]. The fungus was identified in the laboratory using culturing, microscopic, and molecular methods.

Figure 1. Symptoms of fungal diseases in naturally infected zucchini plants.

2.2. Morphological Identification of Fungal Isolates

The hyphae and conidia were examined in one-week-old colonies grown on PDA plates. The morphological identification was performed according to Matsushima [46]. Macroscopic characteristics included color, growth rate, and colony features, while microscopic characteristics including conidial size and shape were observed using a microscope (Reichert-jung, Dexter, MI, USA). Morphological characterizations were completed as described in previous works [15,47–50].

2.3. Isolation and Molecular Characterization of Genomic DNA Using ITS and Sequence Analysis

DNA extraction was performed from four tested fungal isolates grown on (PDA) medium. Fresh mycelia samples were collected from one-week-old cultures. The extraction of total genomic DNA of each fungal isolates was carried out according to previous recommendations [51–53].

Amplification of ITS regions of the rDNA was conducted using the universal primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′). The PCR amplification reactions were performed in a total volume of 25 µL, containing 3 µL of template DNA, 12.5 µL of PCR Green Master Mix (Thermo Scientific™, Gloucester, UK), 0.5 µL each of the universal forward primer (ITS1), reverse primer (ITS4) and 8.5 µL of molecular-grade water. The cycle initial denaturation step at 98 °C of 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 20 s; and a final extension for 10 min at 72 °C. Amplified products were separated on a 1.5% agarose gel in 0.5X TBE buffer (Tris-borate-EDTA), pre-stained with ethidium bromide (1 µg/mL) at 65 V for 15 min, GeneRuler 1 kb DNA ladder was used as a marker, and visualized under a UV transilluminator over ultraviolet light. The ITS region was sent for sequencing (Macrogen, Scientific Services Company, Seoul, Korea) [51–55], and thereafter identified by comparison with all available sequences in the National Centre for Biotechnology Information (NCBI) using the Basic Alignment Sequence Tool (BLAST).

2.4. Evaluation of Bioagents and Plant Extracts against Zucchini Fungal Pathogens In Vitro and In Vivo

2.4.1. Efficacy of Biological Control In Vitro

Two biological controls, namely, *Pseudomonas fluorescens* (accession number MW647093) and *Trichoderma viride* (accession number MW647090), were identified through DNA extraction as described in previous studies [43]. These biological controls were evaluated for their efficacy against some fungal isolated from zucchini plants using a dual-culture

technique [35,43,53]. PDA medium (15 mL) was poured into 90 mm diameter Petri dishes and allowed to solidify for 15 min. Then, a 5 mm disc from each of the fungal pathogens was taken from growing margins of a one-week-old culture and placed at one end of the Petri dish that continued the PDA medium. The fungus was centered between two *P. fluorescens* lines in Petri dishes and incubated for one week at 28 °C. In the case of the fungal antagonist, the isolated *T. viride* strain (5 mm disc) was inoculated on the opposite side of the same Petri dish. The activity of the antagonistic organisms was recorded by measuring the colony diameter in each treatment and comparing it to the control value [43,53].

2.4.2. Preparation of Plant Extracts and Their HPLC Analysis

Eucalyptus camaldulensis leaves, *Citrus sinensis* leaves, and *Ficus benghalensis* fruits were collected from Alexandria, Egypt, during June 2019. The samples were air-dried under laboratory conditions and ground using a small laboratory Wiley mill. Approximately 50 g of each of the *E. camaldulensis* leaves, *C. sinensis* leaves, and *F. benghalensis* fruits was extracted with distilled water by the soaking method for 24 h under room temperature [35,56].

The phenolic compounds from the water extracts of each of the *E. camaldulensis* leaves, *C. sinensis* leaves, and *F. benghalensis* fruits were identified by HPLC (Agilent 1100) was composed of two LC pump, a UV/Vis detector, and C18 column (125 mm × 4.60 mm, 5 µm particle size). Chromatograms were obtained and analyzed using the Agilent ChemStation. Phenolic acids were separated by employing a gradient mobile phase of two solvents—Solvent A (Methanol) and Solvent B [Acetic acid in water (1:25)]. The gradient program was started with 100% B and was held at this concentration for the first 3 min. This was followed by 50% eluent A for the next 5 min after which the concentration of A was increased to 80% for the next 2 min and then reduced to 50% again for the following 5 min detection wavelength at 250 nm. Therefore, the order of phenolic compounds was according to authenticate standard compounds by using this mobile phase.

The identification of flavonoid compounds from those extracts was performed by HPLC (Agilent 1100), composed of two LC pumps, a UV/Vis detector, and C18 column (250 × 4.6 mm, 5 µm). The mobile phase was acetonitrile (A) and 0.2% (*v*/*v*) aqueous formic acid (B) with an isocratic elution (70:30) program. The detection wavelength was set at 360 nm.

2.4.3. Bioactivity In Vitro

The plant extracts were prepared at the concentrations 4000, 2000, 1000 and 500 mg/L by dissolving the extract in dimethyl sulfoxide (DMSO 0.01%) and were tested against the growth of the four fungal isolates. Fresh growth from each fungal isolate was harvested from 7-day-old culture grown on PDA media. The agar dilution method was through the integration of different coveted concentrations of the extracts into an agar medium, followed by a single 5 mm culture disk of the isolate taken from actively growing cultures and placed in the middle of the Petri dishes [57]. The Petri dishes were incubated at 28 °C for one week, the controls were contained only PDA medium and fungal discs in the middle. The inhibition percentage of fungal growth (IPFG)% of the tested fungi was calculated with the following formula [35,55,58]:

Inhibition percentage of fungal growth (IPFG)% = [(Growth in control − Growth in treatment)/Growth in control] × 100.

Growth values in the control and in the treatment are the average diameters (mm) of fungal colonies.

2.5. Experiments under Greenhouse Conditions

Two successive experiments were carried out during 2019 and 2020. The first experiment began in late September and ended in mid-December. The second experiment began in late December and ended in early February under greenhouse conditions at Abis Experimental Farm Station (31°13′ N latitude, 29°59′ E longitude), Faculty of Agriculture, Alexandria University, Egypt. During the period of Zucchini (*Cucurbita pepo* L.) growth,

the average temperature (°C) condition under the greenhouse experimental is shown in Figure 2.

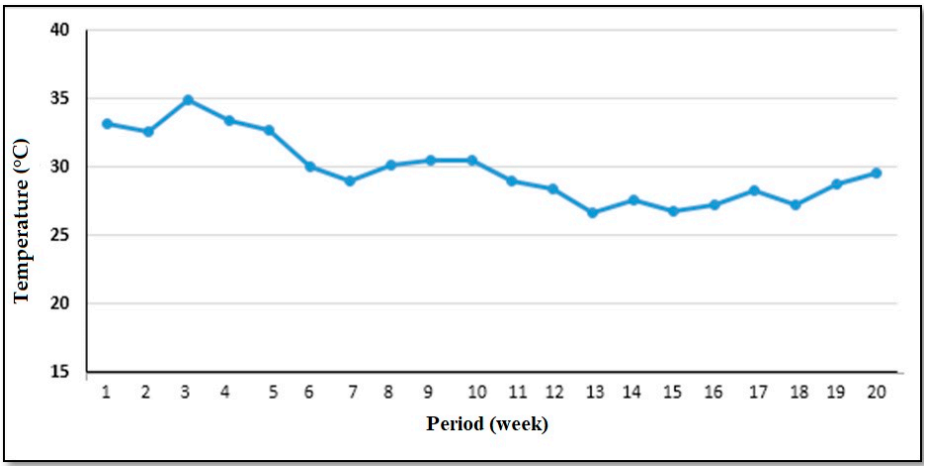

Figure 2. Average temperature (°C) under greenhouse conditions during the 2019 and 2020 experimental periods for zucchini growth.

AZIAD F1 cultivar was used in this study, and this cultivar was imported from the Sakata Tackey company Japan vegetable seed. It is a desirable variety in the Egyptian market and bears low temperatures and high production [59].

Seeds for Zucchini variety AZIAD F1 were sown in late September and also in late December, in the first and second experiments, respectively, in clay soil. The row spacing was 30 cm between the plants and 1 m between the lines in a row. The experimental layout was a randomized complete block design (RCBD). Each treatment had three replicates, and each replicate had seven plots, where each plot had 35 plants. Nitrogen, phosphorus, and potassium fertilizers were fertigated at rates of 60, 70 and 100 kg N, P_2O_5 and K_2O fed^{-1}, respectively. Ammonium sulfate $(NH_4)_2SO_4$, (20.5% N), phosphoric acid (58%), and potassium sulfate (48% K_2O) were the sources of N, P_2O5, and K_2O, respectively. The drip irrigation system consisted of laterals GR of 16 mm in diameter with drippers at 0.3 m distance. The drippers had a discharge rate of 4 L h^{-1}. Water irrigation was applied through the drip irrigation system.

Seven treatments (Table 1) were used in the present work. A drench of 50 mL suspension from each treatment was prepared. Inoculation with *T. viride* was mixed thoroughly with the soil, then watered and left to insure establishment and distribution of the inoculum in soil. Inoculation with *P. fluorescens* was sprayed on the plant. Inoculation with *T. viride* + *P. fluorescens* and the application of plant extracts (*E. camaldulensis* LE, *C. sinensis* LE, and *F. benghalensis* FE) were sprayed on the plant, respectively. In the case of plant extracts, the concentration used was selected based on the in vitro results to investigate its ability to reduce the incidence of fungal diseases in zucchini plants under greenhouse conditions [34,43].

All treatments were added to plants four times during the entire growing season of zucchini plants. The first addition was after two weeks from the sowing date then additions were added weekly.

After 45 days from sowing, the leaf number per plant and the leaf area were measured using the following equation:

Leaf area = −5.25 + 0.67(MLTD) + 1.48(ML) + 0.74(TD); where (ML) midrib length and (ML) distance between tertiary lobes according to Fargo et al. [60].

Harvesting was initiated after 50 days of the sowing date and lasted for 6 weeks. Fruits were harvested twice a week. Fruits number per plant, yield per plant (kg), and total yield (marketable and non-marketable kilograms per square meter) were recorded after each harvesting accumulatively.

Table 1. Treatments used in the greenhouse experiment.

Treatment	Concentration
Control	Without any biological agents
Eucalyptus camaldulensis LE	4000 mg/L
Citrus sinensis LE	4000 mg/L
Ficus benghalensis FE	4000 mg/L
Trichoderma viride (accession number MW647090)	10^6 spore/mL
Pseudomonas fluorescens (accession number MW647093)	10^8 CFU/mL
T. viride + *P. fluorescens*	10^6 spore/mL + 10^8 CFU/mL

2.6. Statistical Analysis

The reduction in linear growth of *F. oxysporum*, *F. solani*, *E. rostratum*, and *N. lacticolonia* as affected by the different concentrations of *E. camaldulensis* leaves, *C. sinensis* leaves, *F. benghalensis* fruits extracts, and the biocontrol agents *P. fluorescens* and *T. viride* was analyzed using analysis of variance in a completely randomized design using SAS (Statistical Analysis System), and compared with the values for of the control. Data obtained from the field experiment were statistically analyzed using one-way ANOVA. The means among the treatments were compared using minimum significant difference measured by LSD test at alpha 0.05 [61,62].

3. Results

3.1. Isolation of the Fungal Pathogen

Four pure cultures of fungal isolates were obtained from infected zucchini plants cultivar as shown in Figure 3. Based on the morphological and molecular identification, *Fusarium solani*, *F. oxysporum*, *Exserohilum rostratum*, and *Nigrospora lacticolonia* were identified.

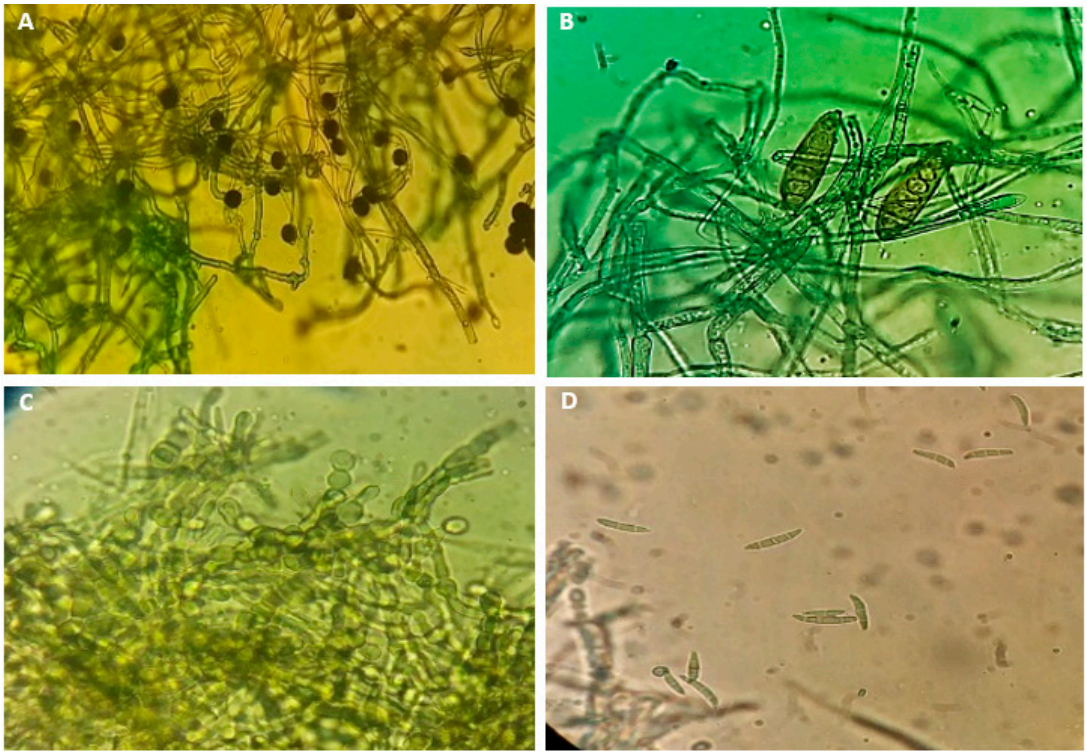

Figure 3. Morphological characteristics of the conidia, *N. lacticolonia* (**A**), *E. rostratum* (**B**), chlamydospores and conidia of *F. oxysporum* ((**C**,**D**), respectively) and conidia of *F. solani* at 40× magnification.

3.2. Morphological Characteristics of Fungal Isolates

Based on morphological characteristics (Figure 3), all the isolates can be divided into three different morphotypes. The colony color of *F. solani* on PDA plates was white-creamy to white-greyish, the pigmentation was colorless and white-creamy with dark brown zonation; microscopic characteristics included conidiogenous cell was long and branched monophialides and the macroconidia septation was 3–7. In the case of *F. oxysporum*, the colony color and pigmentation were pale to dark peach; microscopic characteristics, macroconidia morphology was straight and relatively slender, the apical cell morphology was tapered and curved, the basal morphology was foot-shaped, the macroconidia septation was the three most common. The characteristics of *E. rostratum* colonies on PDA plates were deep-brown, circular with abundant aerial mycelia that appeared woolly or cottony. The conidiophores were slightly curved, erected, and septate. The basal cell in a conidiophore was swollen. *N. lacticolonia* in the color of the culture was initially orange, with dark brown patches in the reverse, *N. lacticolonia* in the smaller ellipsoidal conidia (Figure 3).

3.3. Molecular Identification Based on rDNA-ITS Sequences

The amplified DNA fragments were purified and both strands were sequenced through the ITS region. The sequence data alongside BLAST search proved the identity to be *F. solani* (accession no. MW947256), *F. oxysporum* (accession no. MW947254), *E. rostratum* (accession no. MW947255), and *N. lacticolonia* (accession no. MW947253).

3.4. Evaluation of Bioagents and Plant Extracts against Zucchini Fungal Pathogens In Vitro

The data presented in Table 2 show the highly significant effects of the tested biocontrol agents *T. viride* (Accession no. MW647090) and *P. fluorescens* (accession no. MW647093) against the four isolates of *F. oxysporum*, *F. solani*, *E. rostratum*, and *N. lacticolonia*.

Table 2. Antifungal activity of *P. fluorescens* and *T. viride* bioagents and *E. camaldulensis* LE, *C. sinensis* LE, and *F. benghalensis* FE on *F. oxysporum*, *F. solani*, *N. lacticolonia*, and *E. rostratum* isolates under in vitro condition.

Treatment	Conc.	*F. oxysporum*	*F. solani*	*E. rostratum*	*N. lacticolonia*
Control		0.00 H	0.00 G	0.00 I	0.00 K
E. camaldulensis LE	4000 mg/L	77.80 C	78.53 ± 1.27 BC	78.53 ± 1.27 C	74.83 ± 1.32 C
	2000 mg/L	74.83 ± 1.32 D	76.33 ± 1.27 CD	75.93 ± 1.72 D	72.56 ± 1.27 C
	1000 mg/L	68.90 ± 2.20 E	73.66 ± 0.63 DE	73.66± 0.63 E	69.26 ± 0.63 D
	500 mg/L	66.70 F	71.10 E	71.10 ± 1.10 G	66.70 ± 1.10 EF
C. sinensis LE	4000 mg/L	72.93 ± 0.63 D	71.10 E	77.80 C	68.90 ± 2.20 DE
	2000 mg/L	70.00 ± 1.10 E	71.50 ± 4.50 E	74.06 ± 1.32 E	62.93 ± 1.27 G
	1000 mg/L	66.70 ± 1.10 F	67.43 ± 0.63 F	70.36 ± 1.27 G	60.36 ± 0.63 HI
	500 mg/L	65.20 ± 0.69 F	65.56 ± 1.15 F	68.53 ± 0.63 H	59.63 ± 4.16 I
Ficus benghalensis FE	4000 mg/L	68.90 ± 2.20 E	80.00 ± 2.20 B	81.46 ± 1.27 B	66.33 ± 0.63 F
	2000 mg/L	65.96 ± 0.63 F	74.83 ± 1.32 D	77.43 ± 2.28 CD	62.56 ± 0.63 GH
	1000 mg/L	62.56± 1.68 G	71.46 ± 2.28 E	72.93± 0.63 EF	59.26 ± 1.27 I
	500 mg/L	61.10 ± 1.10 G	71.46 ± 2.28 E	71.46 ± 0.63 FG	55.60 J
P. fluorescens	10^8 CFU/mL	94.80 ± 0.69 A *	92.20 ± 1.10 A	96.70 A	97.80 A
T. viride	10^6 spore/mL	80.03 ± 1.10 B	81.10 ± 1.10 B	68.16 ± 0.63 H	78.90 ± 1.10B
LSD 0.05		1.967	2.892	1.829	2.466

*: Means with the same letter/s within the same column are not significantly different according to LSD at 0.05 level of probability.

Moreover, the visual observation of the activity of plant extracts (*Eucalyptus camaldulensis* LE, *Citrus sinensis* LE, and *Ficus benghalensis* FE showed promising antifungal activity against the four isolates. Any increase in extract concentration led to an increase in the mycelial inhibition percentage of fungi. *P. fluorescens*, *T. viride* and *E. camaldulensis* LE (4000 mg/L) showed the highest activity against *Fusarium oxysporum* growth with inhibition percentages of fungal growth (IPFG) of 94.8%, 80.03% and 77.8%, respectively. *P. fluorescens*, *T. viride*, and *C. sinensis* LE (4000 mg/L) were observed as the highest IPFG against *Fusarium solani* with values of 92.2%, 81.10%, and 80%, respectively, followed by *E. camaldulensis* LE (4000 mg/L) with 78.53%. The highest IPFGs against *E. rostratum* were observed as *P. fluorescens*, *F. benghalensis* FE (4000 mg/L), and *E. camaldulensis* LE (4000 mg/L) with percentages of 96.7%, 81.46% and 78.53%, respectively. The highest inhibition percentage of fungal growth (IPFG) by *P. fluorescens* was 97.8% against *N. lacticolonia*, followed by antagonistic properties of *T. viride* (78.9%) and *E. camaldulensis* LE (4000 mg/L) with 74.83%.

T. viride inhibited the mycelial growth of all fungal isolates but could not overgrow the pathogen until 3 to 4 days. Furthermore, conidia production decreased compared to the control plates. *T. viride* hyphae coiled around hyphae of fungal isolates causing vacuolization and disintegration of isolates hyphae indicating strong antagonistic activity of the *T. viride* isolate, followed by the activity found against the fungal isolates when the *E. camaldulensis* LE, *C. sinensis* LE, and *F. benghalensis* FE were applied at a concentration of 4000 mg/L. Figure 4 shows the visual observations of the antifungal activity of *P. fluorescens*, *T. viride* and plant extract for example (*C. sinensis* LE) against *F. solani*, *F. oxysporum*, *E. rostratum*, and *N. lacticolonia*.

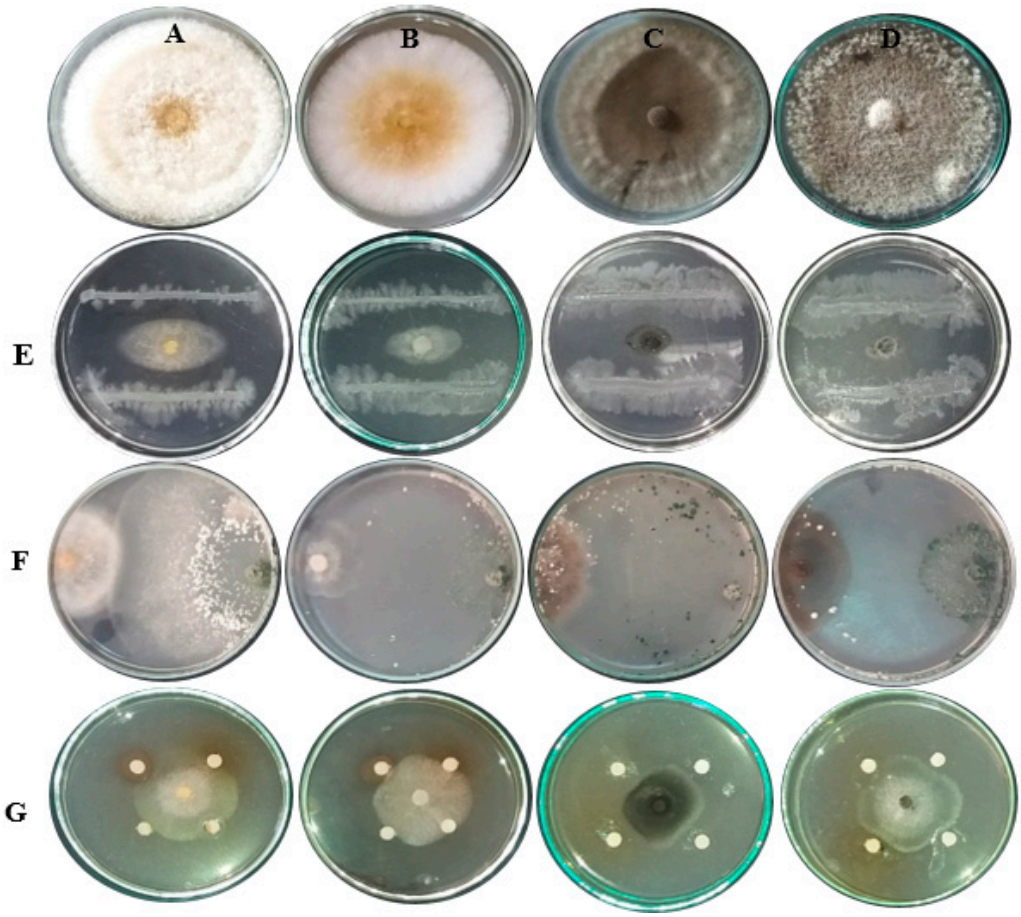

Figure 4. Visual observations of the antifungal activity of *P. fluorescens* (**E**), *T. viride* (**F**) and *C. sinensis* LE (**G**), against *F. solani* (**A**), *F. oxysporum* (**B**), *E. rostratum* (**C**) and *N. lacticolonia* (**D**).

3.5. HPLC Analysis of Extracts

The phenolic compounds (PCs) and flavonoid compounds (FCs) identified in the studied extracts are presented in Table 3 and Figure 5. The most abundant PCs in *E. camaldulensis* LE were pyrogallol (13.63 µg/mL), caffeic acid (7.41 µg/mL), and *p*-coumaric acid (6.33 µg/mL), and the abundant FCs were kaempferol (15.03 µg/mL), naringin (14.16 µg/mL), 7-OH flavone (12.09 µg/mL), and quercetin (11.14 µg/mL). *C. sinensis* LE showed the presence of abundant PCs of syringic acid (8.42 µg/mL) and ferulic acid 7.56 µg/mL), while the abundant FCs were hesperidin (14.19 µg/mL), quercetin (9.52 µg/mL) and kaempferol (6.14 µg/mL). In *F. benghalensis* FE, the abundant PCs were gallic acid (10.42 µg/mL), *p*-coumaric acid (5.14 µg/mL), and syringic acid (5.22 µg/mL), while the abundant FCs were kaempferol (12.06 µg/mL), quercetin (8.14 µg/mL), and hesperidin (6.44 µg/mL).

Table 3. Phenolic and flavonoid compounds identified in *E. camaldulensis* LE, *C. sinensis* LE, and *F. benghalensis* FE.

RT (min) *	Compound	Phenolic Compounds (µg/mL)		
		***E. camaldulensis* LE**	***C. sinensis* LE**	***F. benghalensis* FE**
5.0	Syringic acid	5.12	8.42	5.22
6.2	*p*-Coumaric acid	6.33	5.17	5.14
8.0	Caffeic acid	7.41	5.23	4.69
8.8	Pyrogallol	13.63	ND	ND
10.0	Gallic acid	4.98	2.33	10.42
10.5	Protocatechuic acid	ND	ND	3.08
11.1	Ferulic acid	5.17	7.56	ND
		Flavonoid Compounds (µg/mL)		
3.0	Rutin	5.19	ND	4.12
4.0	7-OH flavone	12.09	ND	ND
5.3	Naringin	14.16	5.33	3.66
7.0	Quercetin	11.14	9.52	8.14
8.0	Kaempferol	15.03	6.14	12.06
10.0	Hesperidin	0.69	14.19	6.44
12.01	Catechin	ND	4.06	ND

* RT: Retention time (min). ND: Not detected.

3.6. Characterization of Zucchini Vegetative Growth

The vegetative growth parameters, e.g., number of leaves per plant and leaf area (cm^2), were significant, depending on the treatments with natural plant extract and bio-agent antagonists in the two experiments in (Figure 6a,b and Figure 7a,b). Regardless of the biological antagonists, there were significantly more leaves of the plants treated by inoculation T. viride, and E. camaldulensis LE (400 mg/L), followed by *T. viride* + *P. fluorescens* in the two experiments. However, *T. viride* and *E. camaldulensis* LE gave the highest leaf area (cm^2), in the two growing experiments. Plants in the control treatment showed the smallest leaf size with the lowest number of leaves per plant compared with the plants treated with plant extracts and microbial agents.

3.7. Total Yield

Regarding the influences of the microbial antagonist factors on the number of fruits per plant, this varied from 27.8 to 28.6 in the case of *E. camaldulensis* LE to 25.7–26.4 in the case of *T. viride*, *P. fluorescens* + *T. viride* (25.1–25.9/plant), and *P. fluorescens* (24.3–25.2/plant) treatments. Moreover, it showed statistically positive results in both growing experiments (Table 4). Similarly, yield per plant was 1.950–1.847 kg and 1.830–1.787 kg for the *T. viride* and *E. camaldulensis* LE treatments, respectively, in both experiments.

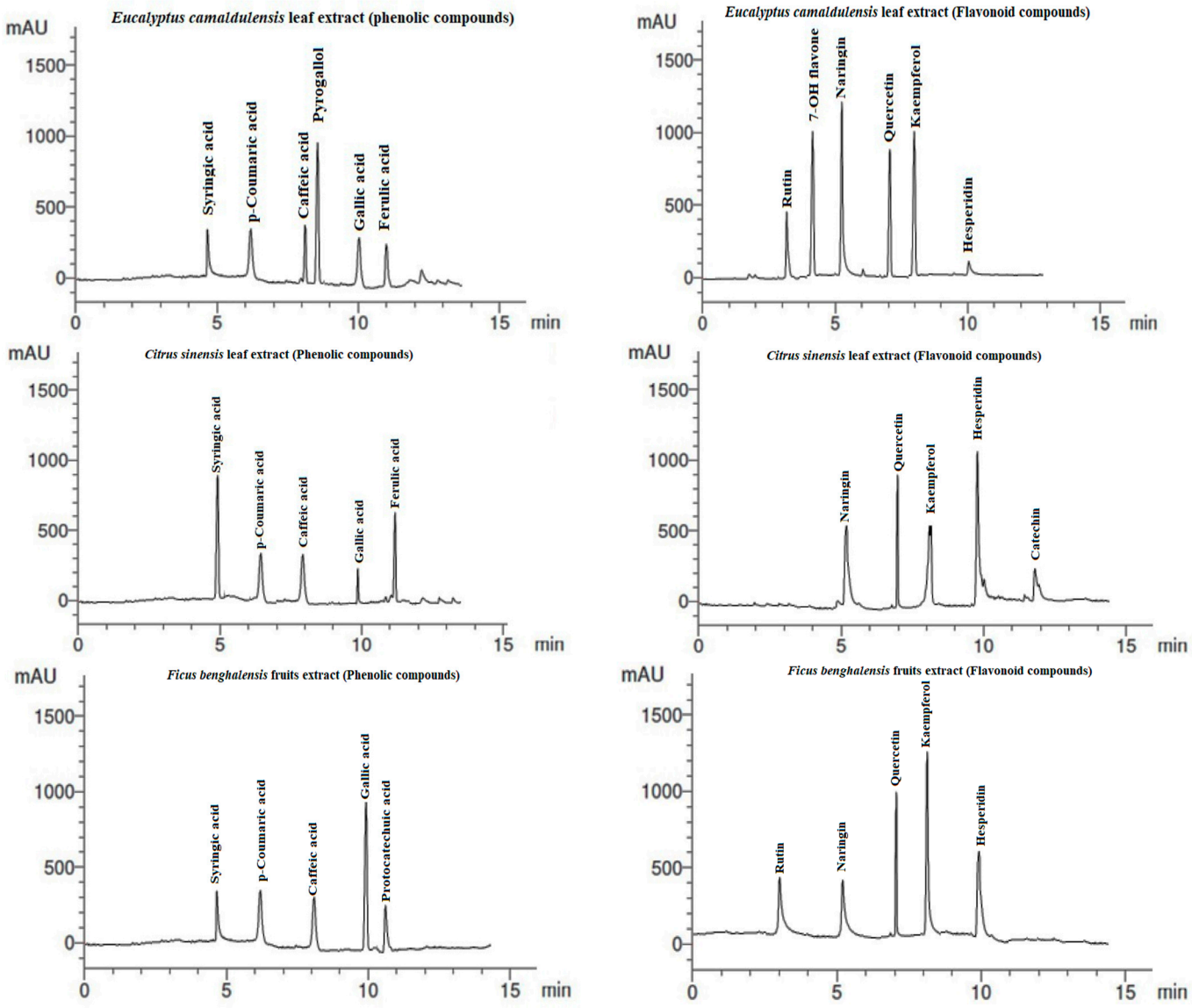

Figure 5. HPLC of phenolic and flavonoid compounds identified in water extracts from *E. camaldulensis* leaves, *C. sinensis* leaves, and *F. benghalensis* fruits.

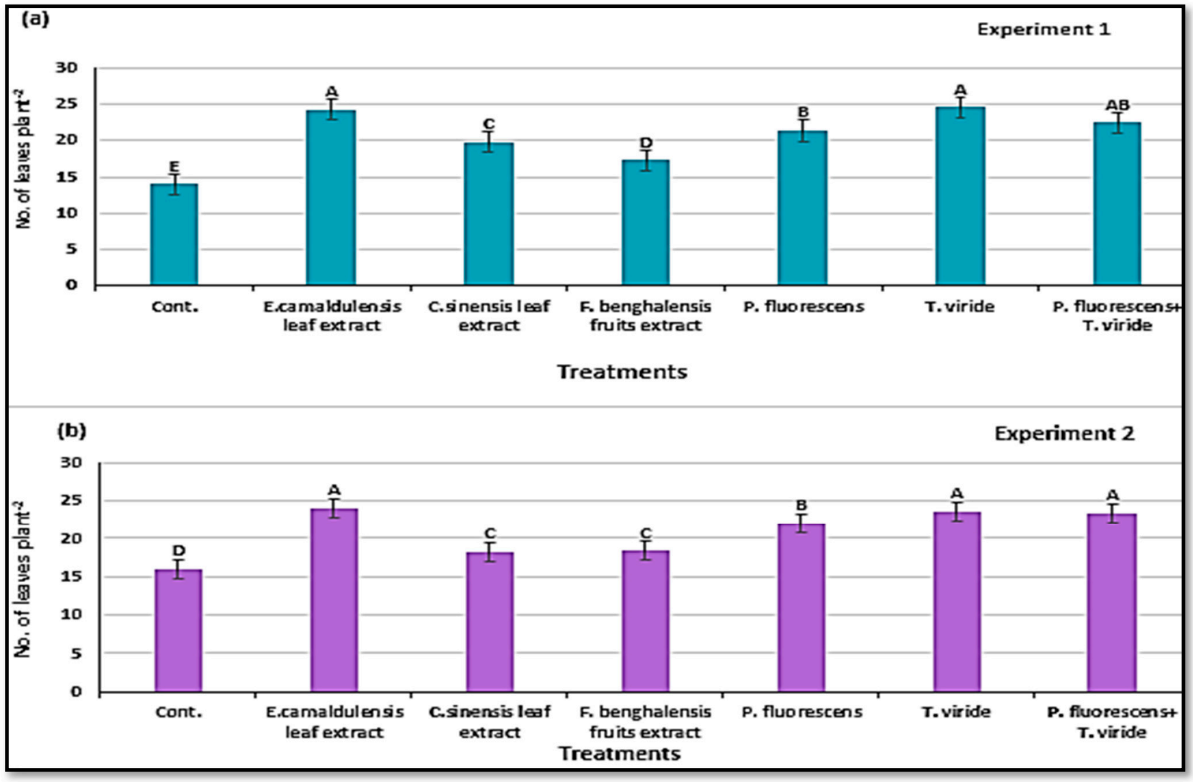

Figure 6. Number of leaves per plant (means ± S.E) of zucchini as affected by the natural plant extracts and microbial antagonists. Letters in figure indicate the means ± S.E of treatments with the same letter/s were not significantly different according to LSD at 0.05 level of probability. (**a**) Experiment 1; (**b**) experiment 2.

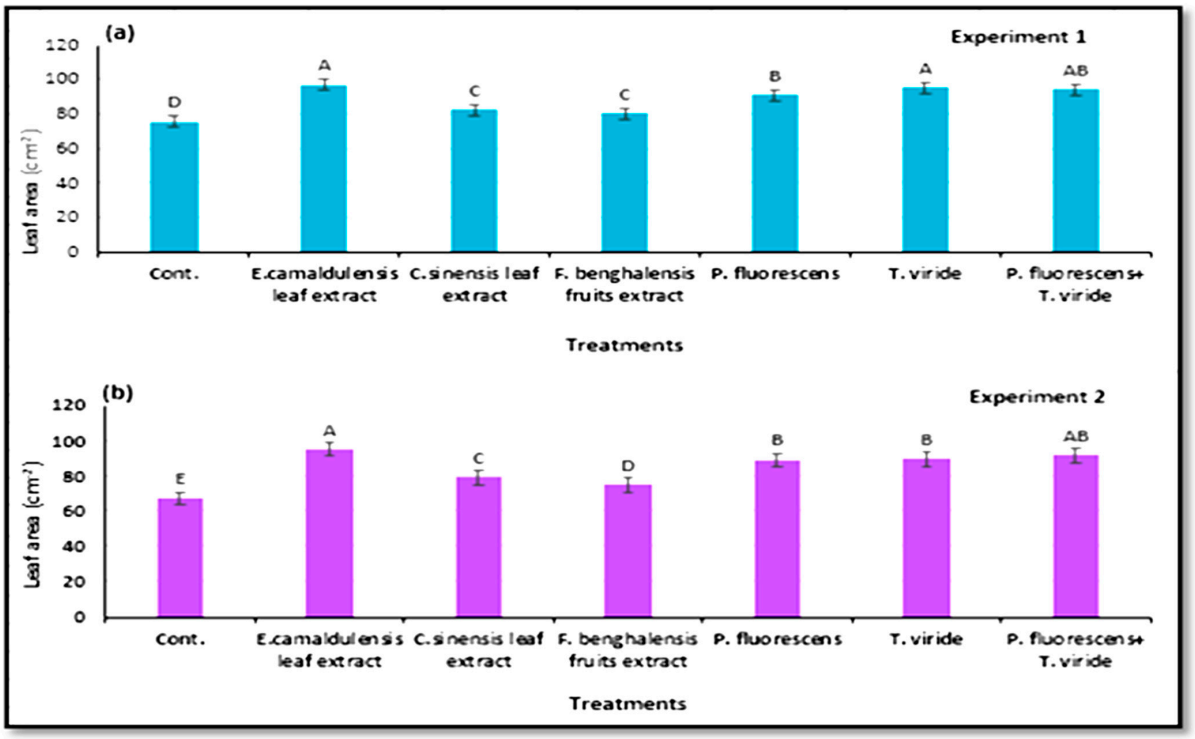

Figure 7. Leaf area (means ± S.E) of zucchini as affected by the natural plant extracts and microbial antagonists. Letters in Figure indicate the means ± S.E of treatments with the same letter/s were not significantly different according to LSD at 0.05 level of probability. (**a**) Experiment 1; (**b**) experiment 2.

Table 4. Number of fruit per plant and total yield per plant (kg) of zucchini as affected by natural plant extracts and microbial antagonists.

Treatment	Experiment 1		Experiment 1	
	No. of Fruits Per Plant	**Yield Per Plant (Kg)**	**No. of Fruits Per Plant**	**Yield Per Plant (Kg)**
Control	16.5 ± 1.23 D *	0.964 ± 0.24 E	17.3 ± 1.87 E	0.978 ± 0.26 E
E. camaldulensis LE	27.8 ± 2.76 A	1.830 ± 0.22 A	28.6 ± 2.43 A	1.787 ± 0.26 A
C. sinensis LE	20.3 ± 1.17 C	1.323 ± 0.22 C	21.2 ± 1.16 C	1.293 ± 0.21 C
F. benghalensis FL	19.9 ± 1.23 C	1.184 ± 0.24 D	19.3 ± 1.76 D	1.197 ± 0.12 D
P. fluorescens	24.3 ± 2.21 B	1.590 ± 0.53 B	25.2 ± 2.21 B	1.476 ± 0.23 B
T. viride	25.7 ± 2.23 B	1.950 ± 0.52 A	26.4 ± 2.24 AB	1.847 ± 0.36 A
P. fluorescens ± *T. viride*	25.1 ± 2.45 B	1.723 ± 0.42 AB	25.9 ± 2.12 B	1.656 ± 0.23 B

*: Means with the same letter/s within the same column are not significantly different according to LSD at 0.05 level of probability.

Data in Table 5 and Figure 8a,b show that the total yield per square meter and marketable yield were affected by the natural plant extracts and microbial antagonists studied. *T. viride* and *E. camaldulensis* LE showed the highest marketable yield (5.83 and 5.52 kg m^{-2}) and (5.84 and 5.50 kg m^{-2}) in the first and second experiment, respectively, without significant differences between them, followed by the treatment *P. fluorescens* + *T. viride*. The control treatment reduced the marketable yield between 41–39.9% and 41.1–41.3% with respect to *T. viride* and *E. camaldulensis* LE in the first and second experiment, respectively.

Non-marketable yield was also significantly affected by the natural plant extracts and microbial antagonists. Microbial and *E. camaldulensis* LE resulted in the lowest non-marketable yield of zucchini fruits. Non-marketable yield represented small percentages of 5.75%, 10.15%, 11.13% and 4.09% as affected by the treatments *P. fluorescens* +*T. viride*, *T. viride*, *P. fluorescens*, and *E. camaldulensis* LE, respectively, in the first experiment, while it was 5.61%, 10.23 %, 10.16% and 7.55%, respectively, in the second experiment with respect to total fruit yield (Table 5).

Table 5. Marketable yield and non-marketable yield (kg.m^{-2}) of zucchini as affected by natural plant extracts and microbial antagonists.

Treatments	Experiment 2		Experiment 1	
	Non-Marketable Yield (Kg·m^{-2})	**Marketable Yield (Kg·m^{-2})**	**Non-Marketable Yield (Kg·m^{-2})**	**Marketable Yield (Kg·m^{-2})**
Control	0.85 ± 0.05 A	2.42 ± 0.03 D	0.77 ± 0.01 A	2.44 ± 0.02 D *
E. camaldulensis LE	0.45 ± 0.01 D	5.53 ± 0.12 A	0.25 ± 0.01 D	5.84 ± 0.05 A
C. sinensis LE	0.62 ± 0.01 C	3.70 ± 0.21 C	0.50 ± 0.01 C	3.90 ± 0.11 B
F. benghalensis FE	0.78 ± 0.02 B	3.21 ± 0.31 C	0.64 ± 0.05 B	3.30 ± 0.21 C
P. fluorescens	0.50 ± 0.02 D	4.41 ± 0.11 B	0.59 ± 0.03 B	4.73 ± 0.31 AB
T. viride	0.63 ± 0.02 C	5.55 ± 0.01 A	0.66 ± 0.02 B	5.83 ± 0.14 A
P. fluorescens + *T. viride*	0.31 ± 0.01 E	5.47 ± 0.01 A	0.32 ± 0.01 D	5.42 ± 0.15 A

*: Means with the same letter/s within the same column are not significantly different according to LSD at 0.05 level of probability.

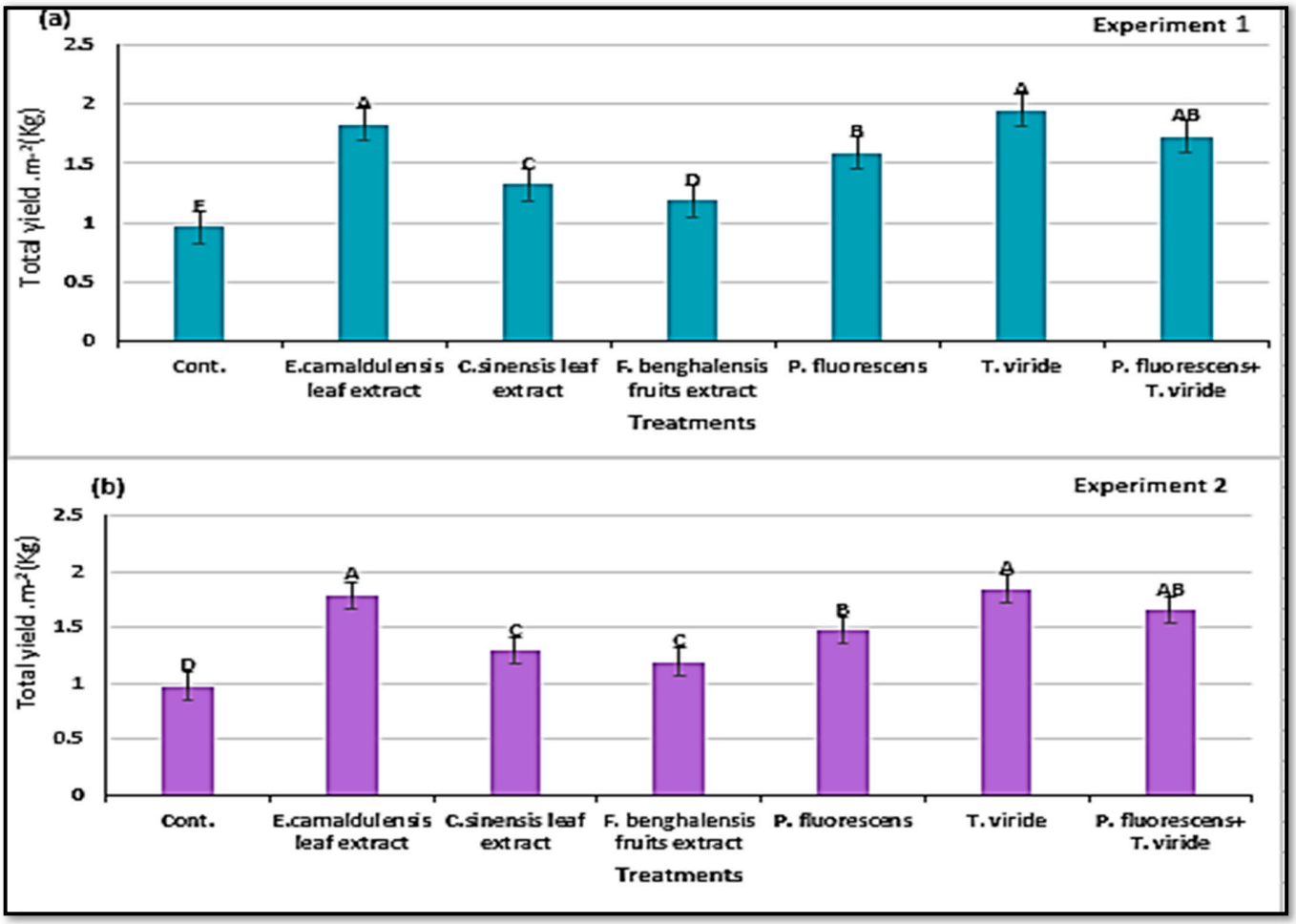

Figure 8. Total yield per square meter (means ± S.E) of zucchini as affected by the natural plant extracts and microbial antagonists. Letters in figure indicate the means ± S.E of treatments with the same letter/s were not significantly different according to LSD at 0.05 level of probability. (**a**) Experiment 1; (**b**) experiment 2.

4. Discussion

The uses of plant extracts and microbial bioagents as eco-friendly treatments for the management of plant diseases have recorded high significance in recent years [43,63–68]. The literature supports the implementation of biostimulant and biocontrol tools due to nature, antimicrobial activity, easy biodegradability, non-phytotoxicity, and resistance in the host. In agroecological practices, there are clear demonstrations of their potential to reduce chemical inputs, save energy and provide farmers with new opportunities for sustainable fertilization and disease control due to their being readily available [43,69,70].

The present work was mainly conducted to study the effect of natural plant extracts and microbial antagonists to control some zucchini fungal pathogens for this purpose. The tested hypothesis assumed that natural plant extracts and microbial antagonists would result in an increase in the growth and yield parameters of zucchini plants. The results indicated that the growth parameters were positively and significantly influenced by a very promising magnitude. With regard to some measurements such as leaves number per plant and leaf area in both experiments of cultivation, they have improved positively by the application of plant extracts and microbial bioagents. The results also showed that all the microbial bioagent treatments resulted in a significant increase in the growth criteria of zucchini plants, and significant superiority was found in the integration treatment on all biological control agents for *T. viride* in increasing the number of leaves, leaf area (cm^2), and marketable yield (kg).

The in vitro treatments using *P. fluorescens* showed high efficiency in reducing the pathogen growth, where the biocontrol effect was better due to its rapid growth and sporulation, resulting in higher competition success for space and available resources compared to other species [71–73]. However, few studies have conducted in vivo experiments on fungi disease control by *P. fluorescens*, *T. viride*, *P. fluorescens* with *T. viride*, and some plant extracts [74,75].

According to this study, biological control agents could be the best alternative and showed significant results against soil-borne pathogens on zucchini plants. Previously, agro-industrial waste mixed with biological control agents (*Lactobacillus plantarum*, *L. casei*, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, *Saccharomyces* sp., *Streptococcus lactis*, and *Streptomyces* sp.) was shown as a promising strategy against verticillium wilt of olive [76–81]. In addition, potential biocontrol agents against *F. oxysporum* f.sp. cubense (Foc) races STR4 and TR4 on banana plants was achieved [82].

However, *Trichoderma* produce phytohormones, vitamins, and solubilizing minerals in addition to their role in direct inhibition of pathogen growth, ultimately, zucchini plants can be easily grown up in field conditions [83,84]. Many strains of *P. fluorescens* are known to enhance plant growth promotion and reduce the severity of various diseases. The efficacy of the bacterial antagonists in controlling fungal diseases was often better alone, and sometimes in combination with fungicides [85]. The highest reduction in disease severity under greenhouse conditions was detected in tomato seedlings plants treated with formulated *Bacillus amyloliquefaciens* (74.4%), *P. aeruginosa* (66.7%), *P. fluorescens* (40%) and *B. subtilis* (53.3%) [68]. The antagonistic effect of *Trichoderma* towards *F. oxysporum* f.sp. vanillae, *P. meadii*, and *Colletotrichum vanillae* in vanilla, where the coculture of the phytopathogens and *Trichodema* clearly showed dominance of the *Trichoderma* species [86].

The biological activities of *E. camaldulensis* LE, *C. sinensis* LE, and *F. benghalensis* FE against the isolated fungi could be rested to the presence of PCs and FCs. According to the HPLC chromatograms the abundant PCs compound identified in *E. camaldulensis* LE was pyrogallol, caffeic acid and *p*-coumaric acid, which belongs to the class of cinnamate and is widely distributed in nature [87]. Flavonoids protect plants from different biotic and abiotic stresses and act as unique UV-filter, functioning as signal molecules, allelopathic compounds, phytoalexins, detoxifying agents, and antimicrobial defensive compounds. Moreover, FCs have roles against frost hardiness, drought resistance, and may play a functional role in plant heat acclimation, freezing tolerance, and photo protection [88–91]. Resistance mechanisms refer to traits that inhibit or limit attack, while tolerance strategies do not limit attack but reduce or offset consequences on the plant fitness by adjusting its physiology to buffer the effects of herbivory or diseases [92]. In earlier, several research workers have demonstrated that some plants extract possess antifungal activity against several plant diseases [33,34,38,93]. Similar to the present findings, *E. camaldulensis* LE has potential as an antifungal agent, where it is able to act as a moderate antifungal agent against household molds, and wood rot fungi [94], and phytopathogenic fungi [95]. Recently, leaf extracts of several aromatic plants were found to have a strong inhibitory effect against fungi in vitro and in vivo [96,97].

The application of extracts even in small amounts and after a single administration in certain stages of crop development proved that PCs alter the growth, nutrition, or resistance of organisms or organs, leading to an improvement in crop quality and quantity with an increase in production [35,98–100]. Previously, benzoic acid, quinol, salicylic acid, myricetin, and rutin were identified as abundant polyphenolic compounds found in *E. camaldulensis* bark extract with promising antifungal activity against the growth of *F. culmorum*, *Rhizoctonia solani*, and *Botrytis cinerea* [101]. n-Hexane oily extracts from *E. camaldulensis* aerial parts when applied showed promising antifungal activity against *F. culmorum*, *R. solani*, and *P. chrysogenum* [93]. In the present study, pyrogallol was found in high concentration in leaf extract but it was not found in bark extract of *E. camaldulensis* [101].

The polyphenolic composition of flavonoids and phenolic acids in the soluble fractions of the methanolic extracts of *E. camaldulensis* showed the presence of gallic, protocatechuic,

vanillic, and ellagic acids, and protocatechic aldehyde was identified, along with eriodictyol, quercetin, naringenin, vanillin, naringin, quercitrin, luteolin, and kaempferol [102]. Extract from the leaves of *E. camaldulensis* showed four major components HHDP-glucopyranose, chlorogenic acid, and phloroglucinol derivatives [103].

E. camaldulensis LE showed good antifungal activity against *Penicillium funiculosum*, *Penicillium ochrochloron*, *Aspergillus niger*, *A. flavus*, *Rhizoctonia solani*, and *F. oxisporum*, and these activity could be related to ellagic acid, quercetin 3-O-rhamnoside, quercetin 3-O-b-D-glucuronide, caffeic acid, chlorogenic acid, ferulic acid, and *p*-coumaric acid [104]. *Eucalyptus* LE in water, methanol, and *n*-hexane proved more effective than stem and bark for controlling the growth of *F. solani* [105]. Early blight of tomato caused by *Alternaria solani* was inhibited by 49.31% when *E. camaldulensis* extract was applied [106]. Aqueous LEs from *E. camaldulensis* exhibited highly pronounced antifungal potential against *Alternaria alternata*, *Drechslera hawaiiensis*, and *D. tetramera* [107].

C. sinensis LE showed moderate activity against the isolated and identified fungi *F. oxysporum*, *F. solani*, *N. lacticolonia* and *E. rostratum* isolates compared to other bio-assayed extracts. *C. sinensis* LE showed an inhibition percentage of 26.12% against the growth of *F. oxysporum* at 10% concertation [108]. Leaves are rich in flavonoids, flavonols, polymerized phenols and hydrolyzable tannins content [109]. PCs present in *Citrus* indicates their role as an antimicrobial agent since they are extensively used in preventing the diseases caused by bacteria or fungi compared to bactericides or fungicides [109–112]. Moreover, other FCs such as hesperetin, naringenin, hesperidin, cyanidin 3-glucoside, limocitrin, quercetagetin, quercitrin, and kaempferol derivatives were isolated from different parts of *C. sinensis* [113–115].

The bioactivity of *F. benghalensis* FE could be related to the presence of PCs and FCs [116–118]; several PCs and FCs were also identified in different parts from *F. benghalensis*, such as gallic acid, rhein, anthraquinone, gallocatechin, theaflavin-3,3′-digallate, and flavone in leaves [119]. Chlorogenic acid, caffeic acid, naringenin, quercetin, kaempferol, malondialdehyde, and morin from root extract and cyanidin 3-glucoside, chlorogenic acid, caffeic acid and quercetin from FE were identified [120]. Fruit extracts had significant antibacterial activity but no antifungal activity [117].

Ellagic acid, gallic acid, rutin, myricetin, and naringenin were the major compounds identified in methanolic extract of *Musa paradisiaca* L. peels, with good antifungal activity against the growth of *F. culmorum*, and *Rhizoctonia solani* [121]. PCs and FCs from plant extracts are considered to be bacteriostatic and fungistatic [33], where they cause the swelling of hyphal tips, leaking of plasma, cell wall distortion, plasma seeping around hyphae abnormal branching or fusion of hyphae, and consequent wrinkling of the hypha surface [122].

Phenolic and flavonoid molecules are of interest in industry sectors such as in nutraceutical product formulation as therapeutic agents for diabetes and cancer [123–125], in food as additives and preservatives [124,126], in cosmetics as UV-protection and antioxidant agents [127,128], and in the textile industry and packaging as antimicrobial agents [129,130]. However, most prior studies were carried out at the in vitro scale, and there is a lack of information about their in vivo action resulting from their bioavailability and absorption [124,131].

p-Hydroxybenzoic, *p*-coumaric, and protocatechuic acids as commercial compounds with an encapsulation technique such as atomization/coagulation were used for protection [128], and the commercial compound catechin is used in encapsulation techniques as an inclusion complex to increase solubility and resistance to heat, light, and oxygen [132]. Phenolic extract from *Punica granatum* L. was used commercially for spray drying to improve storage stability (4 °C per 90 days) and for the protection of bioactivities [133]. Catechin from Grape juice was used in spray drying to improve thermal resistance [134]. In addition, quercetin was used as a commercial compound using the superparamagnetic iron oxide nanoparticles technique to increase bioavailability [135].

Finally, according to the in vitro results, future evaluation using naturally or artificially infected plants should be carried out using those bioagents and plant extracts, especially *Eucalyptus camaldulensis* leaf extract.

5. Conclusions

Plant extracts and microbial antagonists for the control of fungal pathogens such as *Fusarium oxysporum*, *Fusarium solani*, *Exserohilum rostratum* and *Nigrospora lacticolonia* are associated with beneficial effects in zucchini plants. Improved productivity of zucchini plants was obtained in terms of leaf area, fruit number per plant, yield per plant (kg), and total yield (marketable and non-marketable kilograms per square meter). The results for the three studied plant extracts and bioagents against zucchini fungal pathogens in vivo suggest that *Trichoderma viride* and *Eucalyptus camaldulensis* leaf extract have the superior effect against zucchini fungal pathogens, followed by *Pseudomonas fluorescens* with *Trichoderma viride*. In the future, we suggest evaluating plant extracts and bioagents on naturally infected plants; this could possibly reduce the amount of agricultural chemicals used in the control, which may leak into other ecosystems, and thus help to reduce their environmental burdens on agriculture.

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