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Alternative Control of Tomato Wilt Using the Aqueous Extract of *Calotropis procera*

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Abstract: *Calotropis procera* (Aiton) Dryand (commonly known as the apple of sodom, calotrope, and giant milkweed) is an evergreen, perennial shrub of the Apocynaceae family, and is mainly found in arid and semi-arid regions. Previous studies have established the toxic effects of *Calotropis procera* (*C. procera*) (Aiton). Its extract is used as one of the vital alternatives to pesticides due to its effective impacts on several pathogens and to combat insect pests that cause severe damage to several crops, but so far, its effects on combating *Fusarium oxysporum* f. sp. *lycopersici* (FOL) have not yet been evaluated. Therefore, the current study focuses on evaluating the effects of the aqueous extract of this plant on FOL by studying physicochemical indicators. In Saudi Arabia and many other countries, such disease is considered as one of the critical factors affecting tomato growth and production. For the control of Fusarium wilt disease (Fwd), many strategies have been developed, but are limited in their use. Ten isolates of the pathogens were obtained from diseased tomato plants. These isolates were able to exhibit symptoms of tomato wilt, and they varied in their virulence. Isolate No. 5 caused the highest severity of the disease (73%). According to these results, we used this isolate in the following experiment, which was identified by ITS sequences as *F. oxysporum* f. sp. *lycopersici*. Following intensive screening studies, we selected aqueous plant extract of *C. procera* to study its effectiveness against Fwd of tomato plants and their antifungal activity or induce systemic resistance in plants. All concentrations of *C. procera* extracts suppressed growth of the pathogen. The highest reduction of mycelia growth was obtained by aqueous extract at 15%, it reduced the pathogen growth to 70.2%, relative to the control. In greenhouse conditions, the aqueous *C. procera* extract at 5, 10, and 15% significantly reduced Fwd of the tomato compared to the infected control (52.01, 69.86, and 83.6%, respectively). The use of aqueous *C. procera* extract at 5, 10, and 15% significantly enhanced the fresh and dry weight of tomato plants (g plant^{-1}) compared to inoculated plants (for fresh weight: 50, 66.6, and 86.6 and for dry weight: 60, 100, and 120%, respectively). Total phenols and flavonoids as well as antioxidant enzymes were increased in inoculated or non-inoculated tomato plants after being treated with aqueous extracts of *C. procera*.

Keywords: tomato wilt; antifungal activity; antifungal activity; systemic resistance; phenol content; flavonoid; peroxidase

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1. Introduction

Tomatoes (*Lycopersicum esculentum*, Mill) are one of the most profitable and popular vegetables grown worldwide, are ranked second after potatoes among vegetable plants, and the first among processed crops in terms of the areas planted and the production with vegetables, globally. However, the tomato plant faces major challenges in terms of its growth and productivity, the most important of which is its infection with many plant

pathogens, such as fungi, bacteria, viruses, nematodes, and biotic factors [1]. Root rots, damping-off, and wilt are among the important fungal diseases. *Fusarium oxysporum* is a well-known plant pathogen that causes significant harm to a variety of host plants [2]. Tomato wilt disease causes reduction in weight of tomato fruits and productivity [3,4].

Chemical control pollutes the environment and is harmful to human health, and the control effects decrease gradually due to fungicide resistance of pathogens. Bio-control has been identified as a potential and sustainable method due to less chance of environment pollution and low health risk compared with chemical controls. Several researchers have screened several plants for anti-fungal specifications, which are attributed to biologically active substances produced in the secondary metabolism of plants. *Calotropis* spp. for example, is known by the active ingredients in these products, albuminoids, colocynthisin phytosterol glycoside [5], *C. procera* leaf content alkaloids, glycosides, phenolic glycosides, and cardic glycoside [6]. In the same context, the leaf extract of *C. procera* has been shown to reduce the radial growth of *F. oxysporum,* which was isolated from cumin [7]. In addition, Baraka et al. [8] found that extracts of fruits such plant significantly reduced the infection of lupine by *R. solani*, *F. oxysporum*, and *F. solani*.

C. procera belongs to the Asclepiadaceae family, with a broad range of ecological amplitude. It is a salt-tolerant weed that thrives in drought-prone areas, such as lagoon borders and overgrazed pastures. It is commonly known as rubber tree, Sodom apple usher, calotrope, akando and arka. When cut or broken, all sections of the plant ooze white latex, which acts as a defense against fungus, insects, and viruses [9]. The whole plant contains a and b amyrin, giganteol, gigantin, teraxasterol, and b-sitosterol isogiganteol [10]. This plant has produced a vast number of secondary metabolites, including numerous flavonoids [11], Triterpenes [12], cardiac glycosides [13], and sterols [14]. This plant has attracted the attention of several authors for its multiple biological influences due to the fact that it contains a large number of biologically active compounds and is characterized by its toxic effects, its negative allelopathic potential, and its role in weed control [15,16]. In this regard, the active constituents of CLEs and their mode of action in disease control are still unknown due to the large number of potential molecular targets [15], the overlap of performance and interaction between them [17,18], and their interaction with a number of environmental factors [19]. Hussaien et al. [20] found that treated onion plant with *Citrullus colocynthis* increased phenolic content as well as antioxidant enzymes and possibly induction of resistance against onion blight disease. Many cellular components are protected by phenols and flavonoids, which play an important role in plant growth and development. Increased enzymatic activity can also lead to higher phenolic content and antioxidant enzyme activity [21]. POD activity helps in the regulation of hydrogen peroxide formation in the cell wall, which is required for the cross-linking of phenolic groups in response to external stressors [22].

The present work aimed to study the effectiveness of different concentrations of *C. procera* water extracts against the causal pathogen of tomato wilt *F. oxysporum* in vitro conditions and their effect on disease control of tomato wilt under a greenhouse experiment. The effect of the best concentration on seed germination and antioxidant enzymes (peroxidase and polyphenol oxidase), phenol, and flavonoid contents in tomato lives was also investigated. We further include the impact of the aqueous CLEs in order to identify the composition of extracts for the possibility to be used as alternative bio-pesticides to protect against *Fusarium oxysporum* f. sp. *lycopersici*.

2. Materials and Methods

2.1. Isolation of Fusarium Pathogen from Tomato Plant

Roots of tomato plants displaying wilt disease were collected from different locations in the city of Jeddah during the winter season. Infected plants were removed and washed thoroughly under running water to remove any soil particles that might have adhered to them. Then, diseased plants were cut into small pieces and dipped for 2 min in 70% ethyl alcohol to sterilize their surface and washed several times in sterile distilled water. The

pieces were dried by sterilized filter paper, then transferred individually into Petri dishes (9 cm) containing about 15 mL potato dextrose agar (PDA) supplemented by streptomycin antibiotic 400 mg/L^{-1} to avoid any contamination with bacteria. After, Petri plates were incubated for 7 days at 27 ± 2 °C. The plates were examined daily to observe the hyphal growth in the plates. Pure cultures of developing fungi were obtained by hyphal tip technique. The fungi pure culture was incubated on PDA for 7 days in slant agar at 27 ± 2 °C. The purified fungi were identified according to Nelson et al. [23] using fungal morphological and microscopical characteristics.

2.2. Pathogenicity Test

In our current experiment, 10 isolates were examined for their ability to cause wilting of tomato plants grown under greenhouse conditions using tomato hybrids (Super Strain-B) to evaluate the pathogenic capability of the tested fungal isolates. This study was conducted under greenhouse conditions using 30 cm sterilized pots, containing about 2 kg soil. Pots and soil were sterilized by autoclave at 121 °C. Fungal inoculum was prepared by growing each isolate fungus on sterilized grain sorghum medium (200 mL + 150 g + 4 g + 4 g + 50 g from water, grain sorghum, sucrose, and clean sand, respectively) and incubated for two weeks at 27 °C. The inoculum was mixed with the soil at 3% (*w*/*w*), and then irrigated for three times one week before transplanting to ensure even distribution and growth of each particular isolate.

Tomato seedlings (three 35-day-old seedlings) were planted in each infected pot, three replicates were used, and each one consisted of 5 pots (2 seedlings/pot). The experiment was repeated two times. After 90 days, the degree of disease was measured as was measured according to the color of brown vessels.

2.3. Determination of Disease Index

The disease index was estimated by measuring the internal discoloration (browning) area in the vascular bundle by measuring the longitudinal sector of the root using the scale described by Mousa et al. [1].

1 = 0–25% of vascular root bundles are brown, 2 = 26–50% of vascular root bundles are brown.

3 = 51–75% of vascular root bundles are brown, 4 = 76–100% of vascular root bundles are brown.

The percentage of internal discoloration was calculated by the formula:

$$\% \text{ of vascular browning} = (\text{Sum of vascular browning value}/4 \times \text{total number of plants}) \times 100 \quad (1)$$

2.4. Molecular Identification of F. oxysporum

The pathogenic fungal isolate, KAUK7, was purified by a single spore isolate and identified by its microscopic and microscopic properties [23]. To identify the ITS sequence, KAUK7 was sent to Solgent Company, Daejeon South Korea by DNA extraction and PCR was used to amplify the tRNA region covering ITS1 and ITS2 in two rounds [24]. The nucleotide sequence data of the ITS2 and ITS2 regions were subjected to pairwise alignment by the method of Lipman and Pearson [25] using the program "GENETYX-MAC" (Genetyx Corp., Osaka, Japan) and sequences were further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) available from: https://www.ncbi.nlm.nih.gov/ (accessed on 10 January 2022).

2.5. Preparation of Plant Extracts

To conduct this study, we used the leaves of *C. procera* as a source of the available plant extract because this plant is a wild plant widely spread in Saudi Arabia, and the extract is an alternative to chemical pesticides and an eco-friendly substance; we sought to evaluate its effect on the Fwd control. Fresh leaves of *C. procera* were collected, washed, and oven dried (50 °C) for three days. After that, in a laboratory blender, 10 g of dried samples were blended for 1.5 min (Waring, commercial, USA), and 100 mL of sterilized distilled

water was added and blended again. The mixture was placed in falcon tubes (50 mL) and centrifuged (3220× *g*) at 20 °C for 30 min. Once again, the supernatant was recuperated and centrifuged (20,817× *g*) at room temperature for 30 min. Then, the supernatant was filtered through 0.65 µm, and 0.45 µm membrane filters to obtain an aqueous solution free of particle waste [26]. The final supernatant was sterilized with 0.22 µm membrane filter and the AE was then considered ready to use for bioassays.

Further concentrations of extracts were prepared by diluting the 100% stock solution and stored for further application in a petri dish and greenhouse experiment.

2.6. Isolation of Phytochemical Compounds from C. procera Leaves

After collection, the fresh leaves were immediately frozen in the laboratory on dry ice. After that, the frozen leaves were ground in cryogenic grinding for three cycles (one minute each), after which the plant material (50 g) was soaked directly in 100 mL of cold water. Then, the solution was shaken at 200 rpm for 24 h at 37 °C [27,28].

Subsequently, the extract was transferred and divided into several centrifugal bottles with screw cap (100 mL utofil®PP) and centrifuged (5000 rpm) for 10 min at 4 °C to eliminate plant debris. Then, the upper layers of the extract were transferred to new storage bottles (250 mL), 3 mL of each extract was pumped into three new glass tubes (1.5 mL with caps) to reduce the loss of some volatile matter, with each glass tube (1.5 mL) containing 1 mL of the extract. Then, all tubes were covered with parafilm after closing them in screw-top vials with silicone/PTFE septum lids and stored at −20 °C until GC–MS analysis [27,28].

To separate the active components from leaf extracts, a Trace GC-ISQ quantum mass spectrometer system was used for phytochemical analysis.

Samples (one µL) were injected into a GC–MS equipped with a TG–5MS column (30 m × 0.25 mm ID, 0.25 µm film thickness), and helium gas was used as a carrier with a constant flow (1.0 mL min^{-1}). The mass spectra were detected between 50–500 m/z.

A temperature at 50 °C was used initially for 10 min, then increased at a rate of 5 °C every minute until it reached 250 °C; the isothermal temperature was kept at 300 °C (for 2 min), and at the end, isothermal was kept at 350 °C for 10 min. Libraries of NIST, Adams, Terpenoids, and VOCs were used to identify phytochemical components by comparing known mass spectra for each compound with data stored in those libraries. The relative percentage of each component was estimated by RT and the average peak area compared with the total peak areas [28].

2.7. In Vitro Antifungal Activity

A total of 10 mL of the pre-prepared aqueous methanol extract of *C. procera* was placed with 50 mL of PDA (5, 10, or 15%), then 15 mL of the former were placed in sterile Petri dishes and left to solidify. Then, the plates were seeded with a 9 mm disc of 10-day-old *F. oxysporum* f. sp. *lycopersici* (FOL7) cultured on PDA and incubated at room temperature (27 °C). The percentage of radial growth inhibition of the pathogen was measured. Four replicates were used for each concentration, and each replicate consisted of five plates. The percentage decrease (Mr) in colony diameter resulting from each concentration from the extract was calculated using the technique used by Alamri et al. [29]:

$$\mathrm{Mr} = (\mathrm{M1} - \mathrm{M2}) \,/\, \mathrm{M1} \times 100. \quad (2)$$

where Mr = % reduction in colony diameter, M2 = colony diameter in the treated medium, and M1= colony diameter in the untreated medium (control).

2.8. Effects of C. procera on Seeds Germination and Seedling Vigor In Vitro

The effect of pretreatment of seeds with *C. procera* was examined in three treatments in vitro. Three groups of tomato seeds were treated as follows: first group had 50 seeds soaked in each concentration of *C. procera*, the second group had 50 seeds soaked in suspension of the isolate of *Fusarium* KAUK7 as the infected control and the third group had the seeds soaked in sterile distilled water along with a healthy control. Each treatment

(50 seeds) was repeated three times and the treated seeds were then placed on wet filter paper and incubated for one week at 28 °C. Then, germinated seeds (%), mean shoot length (MSL), and root length (MRL) were recorded. Moreover, the seedling strength index (VI) was determined using the following equation [30]:

$$\text{VI} = (\text{MSL} + \text{MRL}) \times \text{germination \%} \quad (3)$$

2.9. Effects of C. procera on Disease Severity under Greenhouse Condition

The *F. oxysporum* f. sp. *lycopersici* KAUK7 fungal inoculum was prepared as stated in the pathogenicity test. Plastic pots (30 cm) were sterilized seven days before planting and planted with sterile soil inoculated with the KAUK7 vaccine at 3% (*w*/*w*). Tomato seedlings were soaked in AE (5, 10 and 15%) of *C. procera* (best pathogen reduction and best treatment for VI %) for 30 min [31], then planted in infected pots (2 seedling/pot). Three replicates were used, each replicate consisted of 5 posts. The seedlings of the control treatment were also treated with water instead of the aqueous extract of *C. procera* at the same time. The wilt intensity (%) was registered after 90 days of planting.

Plants from different treatments were removed at the end of the experiment, cleaned thoroughly with running water, blotted with tissue paper, weighed to determine fresh weights, and then oven dried for 72 h to determine dry weights.

2.10. Assay Total Phenol Contents

After 15, 20, and 25 days of treatment, tomato leaves were collected and immersed in N_2 liquid, then 1 g of plant material was placed in methanol (80%), and then centrifuged at 4 °C at 1000 rpm (for 20 min). Then, the pellet were discarded after adding ascorbic acid (0.1 g per 5 mL), and the homogenates were evaporated in a rotary evaporator at 65 °C and repeated three times for 5 min. In 5 mL of 80 percent methanol, the residue was dissolved. Each treatment has four replicates.

The following procedure was used to estimate phenols, as mentioned by Zhishen et al. [32]: 0.02 methanol extract, 0.5 mL Folin–Ciocalteu reagent, 0.75 mL Na_2CO_3 solution (20%), and 8 mL water made up the reaction mixture. In a water bath, the mixture was incubated for one hour at 37 °C. Water was used as a control. Total phenol content was measured as mg g^{-1} plant fresh weight using a spectrophotometric method at 767 nm. A standard curve of Gallic acid (0–5 mg) was used. Total phenol was calculated as mg Gallic acid/g of plant material.

2.11. Total Flavonoid Content

To measure the flavonoid content in each sample, a modified chromatographic method was used as determined by Zhishen et al. [32].

2.12. Antioxidant Enzyme Activity Analysis

Ten days after *C. procera* treatment with *F. oxysporum*, antioxidant enzyme activity levels were assessed, with plants inoculated only with *F. oxysporum* serving as controls. Next, 1 g tomato leaves were collected, washed, dried, put into a precooled mortar, mixed with 8.0 mL of 0.05 M (pH 7.8) phosphate buffer, ground into a homogenate, and centrifuged (10,000 rpm) for 15 min at 4 °C. After that, the supernatant was transferred to a new test tube, and the crude enzyme extract from tomato leaves was obtained. The activity of peroxidase (POD) was measured by guaiacol colorimetry, and the amount of enzyme required for a 0.01 increase (represented by A470) per minute was defined as one unit of POD. PPO activity was determined as mentioned by Batra and Kuhn [33].

2.13. Statistical Analysis

All tests were set up in a completely randomized design. Each experiment consisted of three replicates and was repeated twice as mentioned in each experiment. Using the

statistical analysis method, data were subjected to ANOVA [34]. The LSD test was used to compare the means at $p < 0.05$ values.

3. Results

3.1. Isolation of the Causal Pathogen and Pathogenicity Tests

Ten isolates of diseased tomato plants that showed signs of wilting were isolated. It was found that all these isolates showed typical symptoms of tomato wilt, but varied in their virulence. Isolate No. 5 caused the highest severity of the disease, followed by isolates No. 6, 10, and 9 while isolates No. 2, 1, and 3 showed less severity than the other isolates Figure 1. For all the following experiments, isolate No. 5 was used after being identified by ITS sequencing where it caused the highest disease severity on tomato plants.

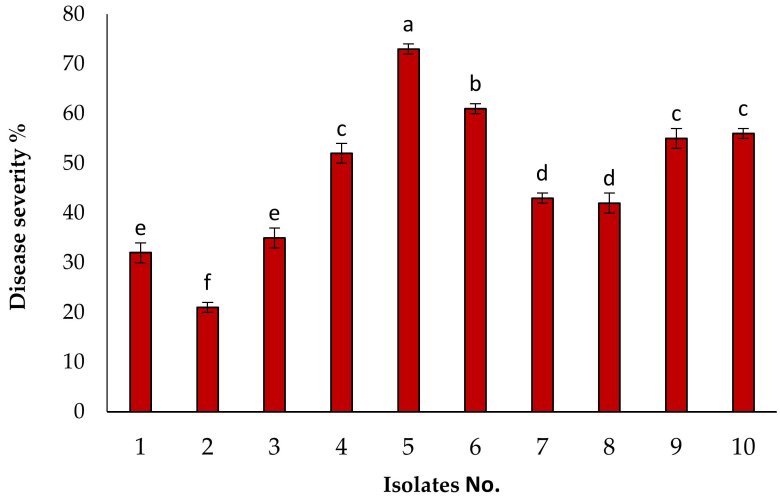

Figure 1. Pathogenicity tests of 10 isolates on tomato plants. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test ($p = 0.05$). Bars indicate the standard error.

3.2. Identification of KENF7 by Using ITS Sequencing

The ITS sequence was used to characterize the FOL isolate KENF7 (ITS1 and ITS2). A BLAST search of NCBI data libraries for ITS sequence similarities yielded the highest results, with 100% sequence similarity to the *F. oxysporum* strain ATCC 48112 (GenBank accession No. MW165780.1). The ITS sequence for the FKAU1 isolate has been lodged in the GenBank sequence database under accession number OM201310.1 (Figure 2).

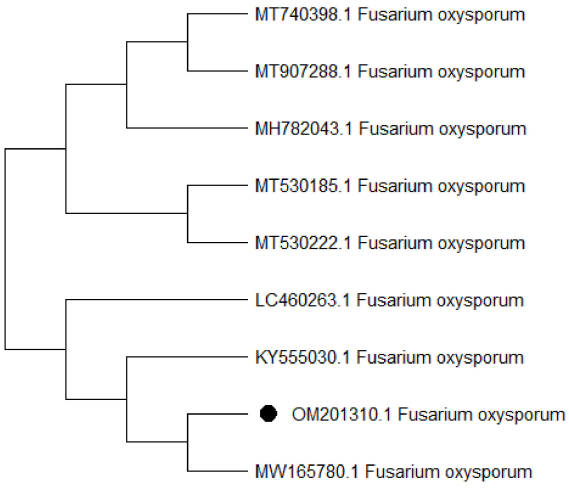

Figure 2. Phylogenetic analysis of *Fusarium oxysporium* isolate on the ITS gene. This analysis was performed using the neighbor-joining method in BLAST pairwise alignments.

3.3. In Vitro Antifungal Activity

The results in Figure 3 show that all tested levels (5, 10, and 10%) of the aqueous extract inhibited the growth of *F. oxysporum* f. sp. *lycopersici* KENF7 with different percentages. Aqueous extracts of *C. procera* at concentration 15% inhibited the growth of the pathogen (70.2%) relative to the control. All concentrations of *C. procera* extracts also suppressed growth of the pathogen but this was lower than in the aqueous extract. The least inhibition of the pathogen was shown by the lowest concentration of the extract used.

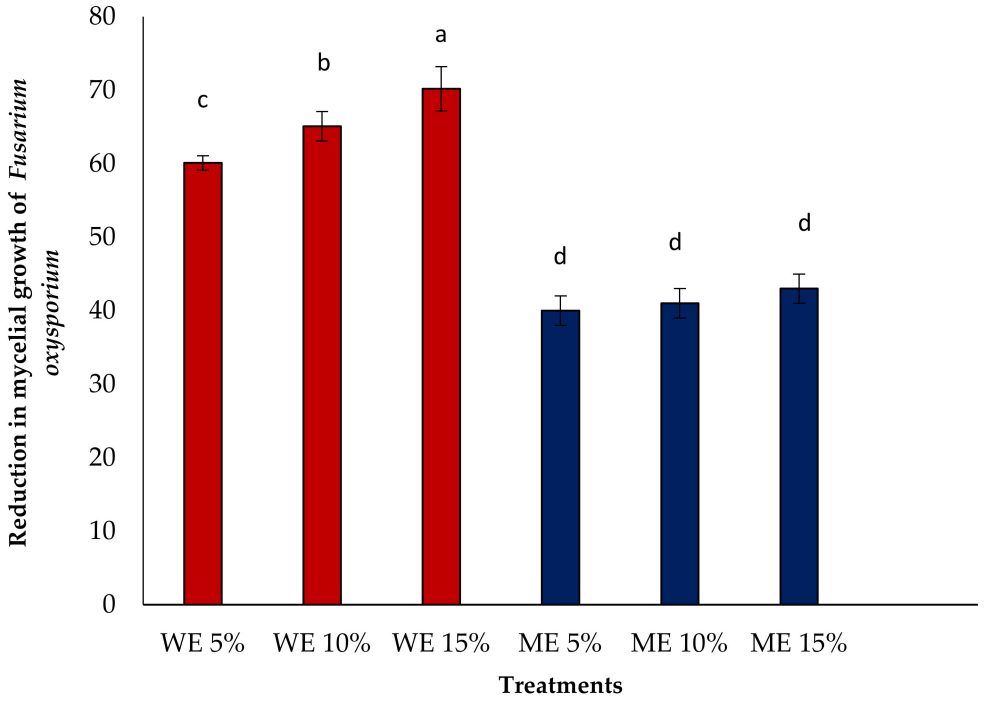

Figure 3. Percentage of *F. oxysporum* KENF7 reduction in mycelial growth caused by different concentrations (5, 10, and 15%) of *C. procera* in vitro. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test (p = 0.05). WE means aqueous and ME: means ethanol extract. Bars indicate the standard error.

3.4. Effect of Aqueous Extract on Seed Germination and Seedling Vigor Index

The data included in Table 1 show that treatment with *C. procera extract* at 15% gave the highest impact on germination (+25%) and seedling vigor (569.5%) relative to the other concentrations or control treatment. MSL and MRL were also increased by *C. procera* and showed the least improvement in seed germination and MSL in seeds infected with the fungus *F. oxysporum* f.s. *lycopersici* KENF7.

Table 1. Effect of different concentration of aqueous extract of *C. procera* (5, 10, and 15%) on seed germination and seedling vigor under Fwd infection.

Concentration	Germination (%)	MSL (cm)	MRL (cm)	Vigor Index (VI %)
C. procera 5%	75 ^{b} (+10.3)	1.2 (±0.4) ^{b}	4.5 (±0.9) ^{b}	427.5
C. procera 10%	75 ^{b} (+10.3%)	1.3 (±0.2) ^{b}	5.2 (±0.7) ^{a}	487.5
C. procera 15%	85 ^{a} (+25%)	1.5 (±0.1) ^{a}	5.2 (±0.5) ^{a}	569.5
Infected control	68 ^{c} (-)	1.1 (±0.2) ^{b}	3.1(±0.7) ^{c}	285.6
Healthy control	85 ^{a} (+25%)	1.5 (±0.1) ^{c}	5.1 (±0.4) ^{a}	561.0

Mean shoot length (MSL), mean root length (MRL), vigor index (VI). Means (±SE) followed by different lettering indicates differences ($p \leq 0.05$) between treatments determined by ANOVA LSD.

3.5. Effect of Aqueous Extract on Disease Reduction under Greenhouse

The results shown in Figures 4 and 5 showed that the tested aqueous extract of *C. procera* at each concentration significantly reduced the incidence of Fwd disease in tomatoes under greenhouse conditions. Aqueous extract of *C. procera* at 15% also resulted in the highest reduction in disease severity followed by other levels.

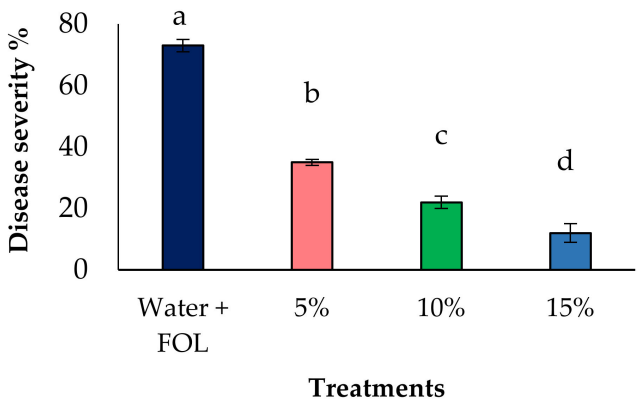

Figure 4. Effect of different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on Fusarium wilt caused by *F. oxysporum* KENF7 on tomato plants under greenhouse conditions. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test ($p = 0.05$). Bars indicate the standard error.

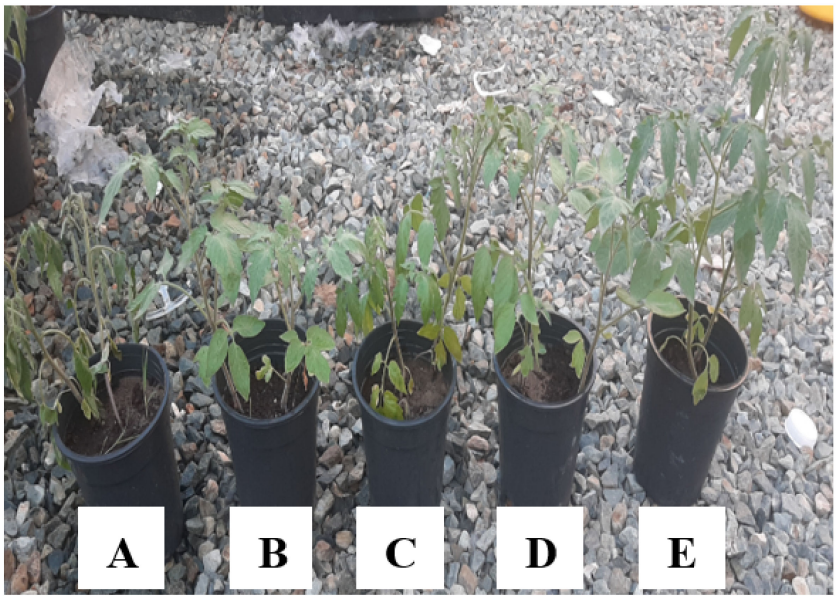

Figure 5. Effect of treatment with different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on Fusarium wilt caused by *F. oxysporum* KENF7 on tomato plants under greenhouse conditions. (**A**) Infected control, (**B**) healthy control, (**C**) soaked tomato seedling with *C. procera* at 5%, (**D**), *C. procera* at 10%, and (**E**) *C. procera* at 15%.

3.6. Vegetative Growth

The data illustrated in Figure 6 indicate that the highest fresh and dry weights achieved was by the aqueous extract of *C. procera* at 15%, and there was a significant enhancement in the fresh and dry weight of tomato plants, followed by the rates of 10 and 5%, the lowest fresh and dry weights in the infected plants.

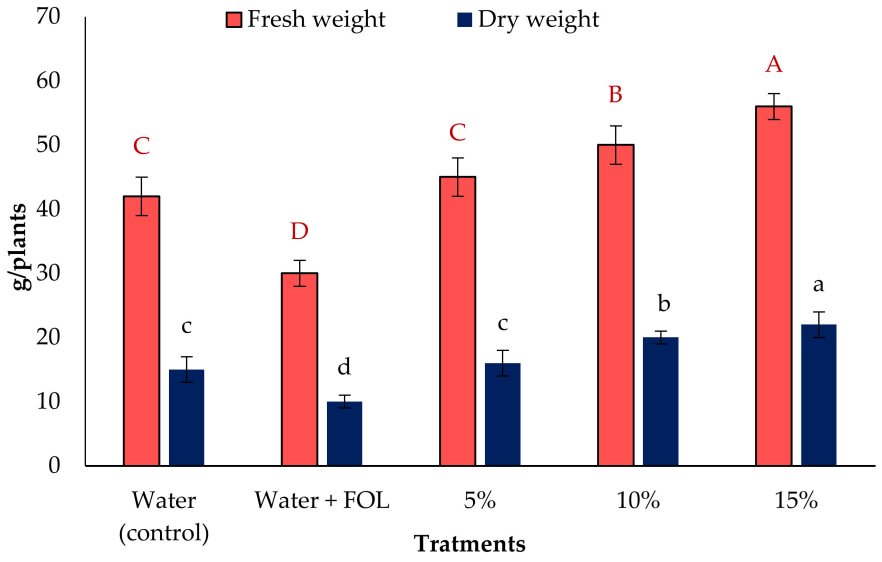

Figure 6. Effect of different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on fresh and dry weight of whole plants (g plant^{-1}) under greenhouse conditions. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test (p = 0.05). Bars indicate the standard error.

3.7. Effect of Aqueous Extract on Enzymatic Activities

3.7.1. Activity of POD in Inoculated Plants

The results in Figure 7 show that the POD activity of treated tomato plants with the extract of *C. procera* was significantly ($p \leq 0.05$) higher than that of untreated plants after 10 days of application. Moreover, the enzyme activity was higher when using the highest level of the extract (15%) compared to the other treatments. In general, the POD activity in the treated plant was highest after 10 days followed by after 5 days of treatment.

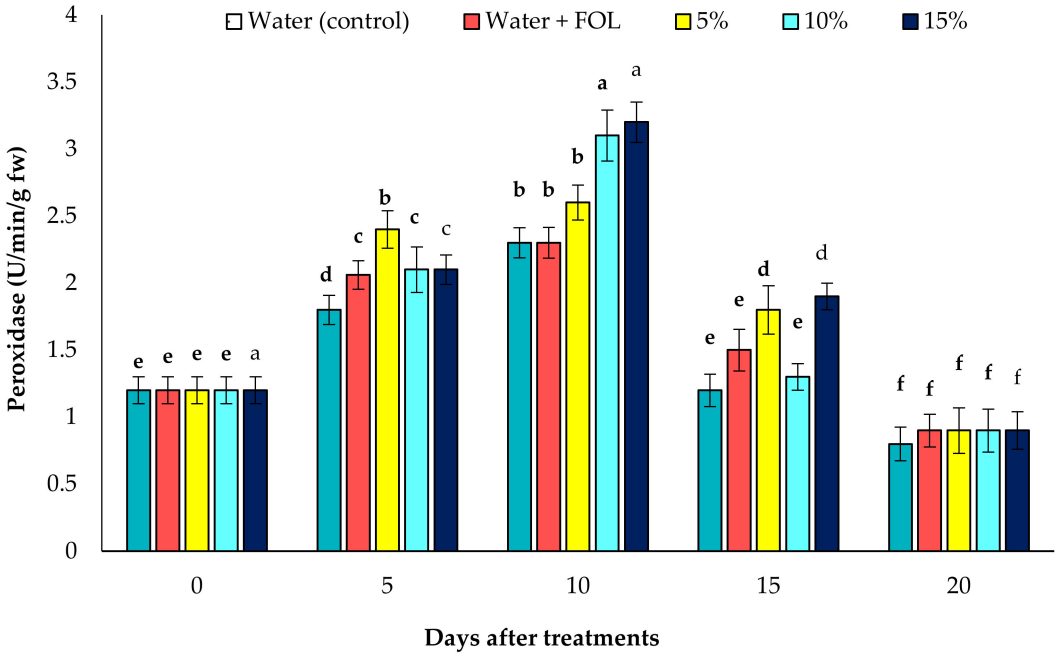

Figure 7. Effect of different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on peroxidase activity (U/min g^{-1} FW) in leaf extracts of tomato plants after treatment with *C. procera* and inoculation with *F. oxysporum* KENF7. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test (p = 0.05).

3.7.2. PPO Activity in Inoculated Plants

In comparison to control plants, *C. procera* induced a significant increase in the activity of the PPO enzyme at all time points, as shown in Figure 8. The largest increase in PPO activity was shown in tomato plants treated with *C. procera*. In general, PPO activity was highest at 10 days from *C. procera* application followed by 10 and 5%; the infected and healthy plant nearly gave the same activity of PPO.

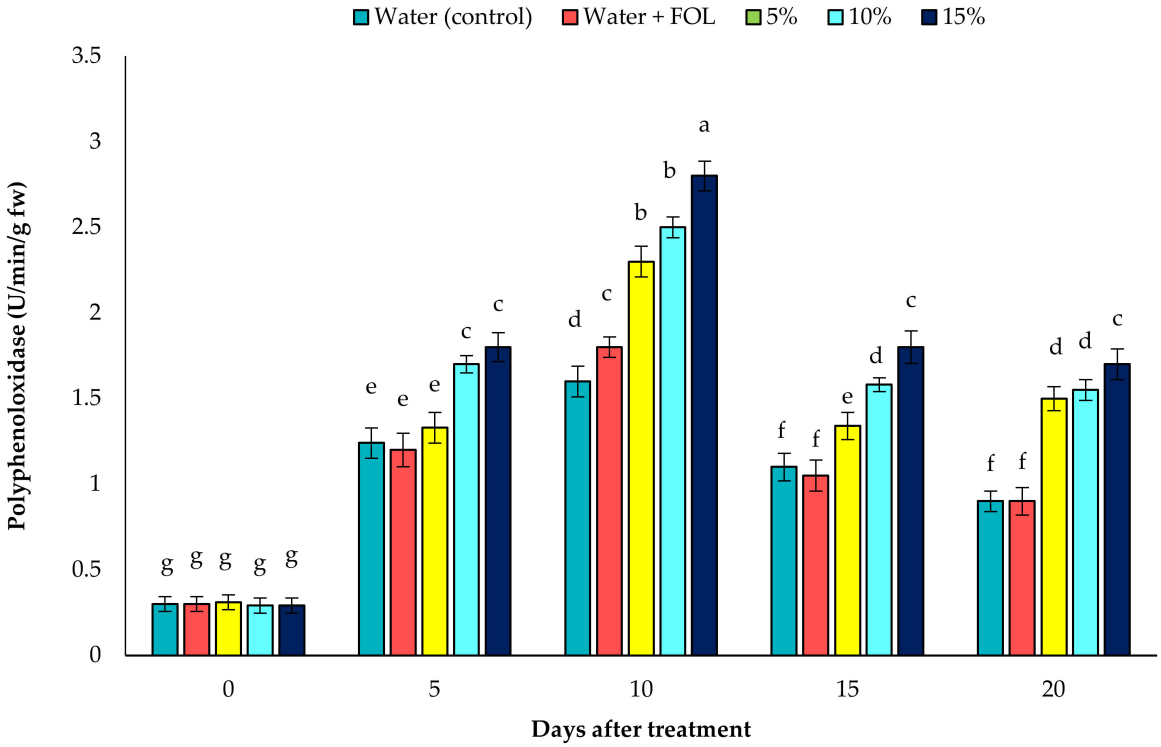

Figure 8. Effect of different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on polyphenoloxidase activity (U/min g^{-1} FW) in leaf extracts of tomato plants after treatment with *C. procera* and inoculation with *F. oxysporum* KENF7. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test ($p = 0.05$).

3.8. Effect of C. procera on Phenol and Flavonoid Contents

It was found from the data illustrated in Figures 9 and 10 that treatment of tomato plants with *C. procera* extract gave the highest of flavonoids and phenol contents in inoculated or un-inoculated plants. The other treatment also increased the flavonoid and phenol contents relative to the untreated plants.

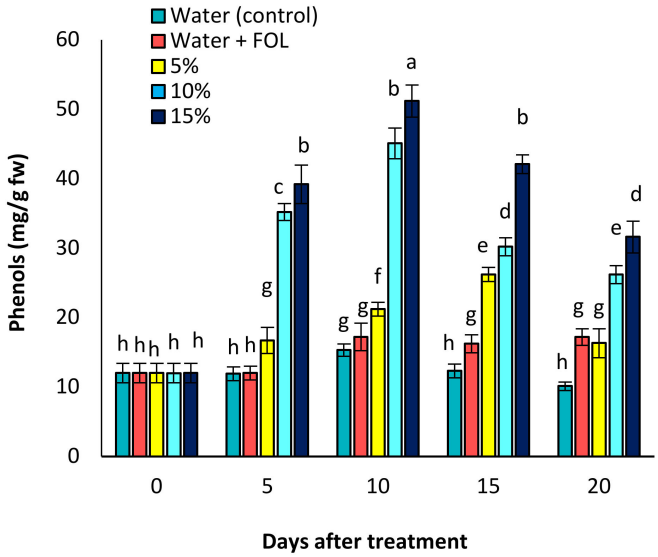

Figure 9. Effect of different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on phenol content (phenols; mgg^{-1} FW] in leaf extracts of tomato plants after treatment with *C. procera* and inoculation with *F. oxysporum* KENF7. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test (p = 0.05).

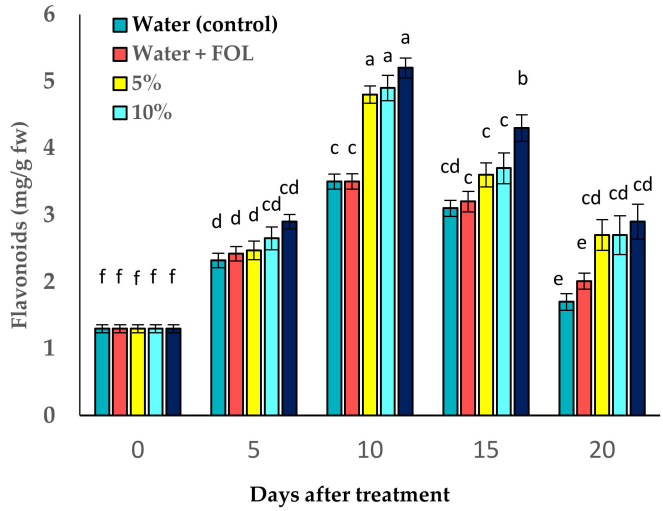

Figure 10. Effect of different concentrations of aqueous extracts (5, 10, and 15%) of *C. procera* on flavonoids (mg g^{-1} FW) in leaf extracts of tomato plants after treatment with *C. procera* and inoculation with *F. oxysporum* KENF7. Values in the column followed by different letters indicate significant differences among treatments according to a least significant difference test (p = 0.05).

3.9. Identification of Phytochemical Components of C. procera AE

By GC–MS analysis, 31 bioactive compounds were identified in the AE of *C. procera* leaves. The results were recorded for the qualitative and quantitative analyses of all phytochemicals in the three extracts, shown in Table 2. In AE, among the compounds identified in AE, N-Benzylideneisopropylamine was recorded as the main compound (16.53%), followed by (+)-delta-Cadinene (12.24%), Biocytin (9.89%), Palmitic acid (6.87%), and 1, 3-Dipalmitin, TMS derivative (5.98%).

Table 2. GC–MS profile of aqueous extract from *C. procera* leaves.

No.	Compound Name	R.T	Formula	M.W	Peak Area (%)
1.	1,3-Dipalmitin, TMS derivative	4.27	$C_{38}H_{76}O_5Si$	640	5.98
2.	Palmitoyl glycerol	4.62	$C_{19}H_{38}O_4$	330	1.36
3.	Palmitic acid	5.23	$C_{16}H_{32}O_2$	256	6.87
4.	Ethyl iso-allocholate	6.14	$C_{26}H_{44}O_5$	436	2.65
5.	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, cis	8.53	$C_{19}H_{36}O_3$	312	1.12
6.	Astaxanthin	9.18	$C_{40}H_{52}O_4$	596	1.32
7.	α-D-mannopyranoside	9.96	$C_{15}H_{28}B_2O_6$	326	1.84
8.	Biocytin	11.79	$C_{16}H_{28}N_4O_4S$	372	9.98
9.	Ascaridole	13.08	$C_{10}H_{16}O_2$	168	2.57
10.	(+)-delta-Cadinene	13.89	$C_{15}H_{24}$	204	12.24
11.	N-Benzylideneisopropylamine	14.57	$C_{10}H_{13}N$	147	16.53
12.	2-(3,4-dimethoxyphenyl)-3,5-dihydroxy-7-methoxy-4H-1-Benzopyran-4-one	15.69	$C_{18}H_{16}O_7$	344	1.24
13.	5,8,11-Eicosatrienoic acid, (Z)-, TMS derivative	16.84	$C_{23}H_{42}O_2Si$	378	1.67
14.	All-trans-beta-Carotene	18.24	$C_{40}H_{56}$	536.4	1.27
15.	1-Monooleoylglycerol, 2TMS derivative	18.69	$C_{27}H_{56}O_4Si_2$	500	1.64
16.	Tristrimethylsilyl ether derivative of 1,25-dihydroxy vitamin D2	18.89	$C_{37}H_{68}O_3Si_3$	644	0.69
17.	5,8,11-Eicosatriynoic acid,tert-butyldimethylsilyl ester	19.36	$C_{26}H_{42}O_2Si$	414	2.26
18.	Octadecanoic acid,9,10-epoxy-18-(trimethylsiloxy)-, methyl ester, cis-	19.92	$C_{22}H_{44}O_4Si$	400	0.59
19.	10,12,14-Nonacosatriynoic acid	20.34	$C_{29}H_{46}O_2$	426	0.42
20.	9,12-Octadecadienoic acid(Z,Z)-, 2,3-bis[(trimethylsilyl)oxy]propyl ester	20.67	$C_{27}H_{54}O_4Si_2$	498	2.18
21.	Rhodopin	21.13	$C_{40}H_{58}O$	554	1.45
22.	Palmitic acid, methyl ester	21.76	$C_{17}H_{34}O_2$	270	0.82
23.	Methyl 2-O,3-O-bis(trimethylsilyl)-4-O,6-O-(methylboranediyl)-β-D-glucopyranoside	22.79	$C_{14}H_{31}BO_6Si_2$	362	3.69
24.	Methyl 2-O,3-O-bis(trimethylsilyl)-4-O,6-O-(methylboranediyl)-α-D-glucopyranoside	23.68	$C_{14}H_{31}BO_6Si_2$	362	2.73
25.	Glycodeoxycholic acid	24.07	$C_{26}H_{43}NO_5$	449	0.54
26.	à-D-Glucofuranose, 6-O-(trimethylsilyl)-, cyclic 1,2:3,5-bis(butylboronate)	24.25	$C_{17}H_{34}B_2O_6Si$	384	0.56
27.	1,25-Dihydroxyvitamin D3, TMS derivative	24.58	$C_{30}H_{52}O_3Si$	488	0.56
28.	Oleic acid, methyl ester	24.79	$C_{19}H_{36}O_2$	296	3.94
29.	Glyceryl 2-linoleate	25.18	$C_{27}H_{52}O4Si_2$	496	1.14
30.	Stigmasterol	26.18	$C_{32}H_{56}OSi$	484	1.09
31.	Trilinolein	31.64	$C_{57}H_{98}O_6$	878	1.47

4. Discussion

Tomato plants are attacked by several soil fungi that infect them during their different growth stages, resulting in diseases, root rot, and wilt. This study focused on wilt disease, which causes reduced production of tomato plants in all countries that grow them, and it is known that the cause of this disease is the fungus *Fusarium oxysporium* f. s. *lycopersici*. Ten isolates were isolated from tomato plants in Jeddah city; all these isolates were able to

cause wilt disease on tomato plants to various different degrees, and these results are in agreement with these obtained by Abo-Elyousr and Mohamed [2] who determined that fungal isolates differ in their ability to cause disease.

The main method for control of such disease is the use of fungicides [35], but the chemical control is harmful to the environment and human health, and the control effects decrease gradually due to the drug resistance of pathogens. Therefore, it was necessary to search for alternatives to chemicals through the use of natural alternatives, especially as many previous studies have recommended using plant extracts to control fungal, bacterial, and helminthic diseases [36,37]. In our study, the aqueous extract of *C. procera* with different levels suppressed growth of *F. oxysporum* f. sp. *lycopersici* in vitro to different degrees. AE (15%) showed the highest effect in reducing radial growth of the pathogen compared with the other levels. This was in the same context with the report of Mousa et al. [1] on *F. oxysporum* f. sp. *lycopersici*. Similarly, Kareem et al. [38] found an antimicrobial activity of the leaf extracts of *C. procera* against *F. oxysporum*. Moreover, Nikam et al. [39] used the stem, leaves, and flowers of *C. procera* extracts to test the bioassays for antimicrobial activities against *F. oxysporum*. Otherwise, Olufolaji et al. [40] reported that phytochemicals, i.e., alkaloids, glycoside, tannins, saponin, steroids, flavonoids, medicagenic acid, and phenols present in the several plants and our plant could be toxic to the fungus, thereby causing its growth inhibition. In addition, Doshi et al. [41] analyzed extracts of buds, stems, flowers, and mature leaves of *C. procera.* They found a number of biologically active compounds, such as triterpenoids, glycosides, tannins, alkaloids, phenolics, and saponin, that may cause toxic effects on several pathogens as our data recorded. Along the same line, several authors reported that the plant extracts affected the growth of phytopathogenic fungal as well as reduced the diseased severity, and this may be due to secondary metabolites (e.g., phenolic, flavonoids, terpenoids, and alkaloids [42–44]. The results of our present study showed that aqueous extract of *C. procera* significantly reduced wilt diseases in all tested concentrations. When testing the aqueous extract on seed germination and seedling vigor in vitro, the treatments increased seed germination, MSL, and MRL when compared to the infection control. At 15%, *C. procera* gave the highest seed germination and seedling vigor followed by the other levels. Many researchers have noted that plant extracts increased the seed germination as well as vigor index among neem, garlic, and ginger [45–47]. Our results revealed that *C. procera* extracts increased POD activity in inoculated tomato plants compared to untreated inoculated plants after 5, 10, and 15 days of application. Several authors have demonstrated that enhanced POD activity was related to the systemic induced resistance in plants to fungi [48,49]. In many plants, plant extracts have been used and documented to induce systemic resistance, reduce disease incidence, and thus improve plant growth and production [20,50].

Plant defense genes that are dormant in healthy uninoculated plants can induce systemic disease resistance when activated by various factors. Increased POD, PPO, and PAL enzyme activity has been shown to be effective in the treatment of a variety of fungal illnesses [51,52]. POD is involved in several plant defense processes, including lignin production and oxidative cross-linking of plant cell walls, as well as pathogen infection-induced formation of active oxygen species [53]. The increase in resistance against disease can be attributed to the increased activities of peroxidase and chitinase because both enzymes are known to increase the plant's ability to resist diseases. In agreement with this, similar results have been reported by Shabana et al. [54], who mentioned that plant extract application (neem, cloves, and garden quinine) to wheat plant significantly reduced leaf rust infection compared to a control. In this context, phytosterols, a group of steroidal alcohols and phytochemicals naturally present in plants, have been found to induce antifungal activity against *Aspergillus*, *Penicillium*, and *Fusarium* [55].

The treatment of plants with *C. procera* at all concentrations were found to significantly increase total phenol and flavonoid content compared to an untreated control. Other studies support our findings, because phenolic compounds are toxic to pathogens and the accumulation of these bioactive ingredients at infection sites was associated with pathogen

development restriction [56]. Moreover, changes in the pH of plant cell cytoplasm due to the increase of phenolics may also promote resistance, inhibiting pathogen development [57].

5. Conclusions

Tomato seedlings treated with aqueous extracts of *C. procera* at different concentrations reduced *F. oxysporum* f. sp. *lycopersici* radial growth in a laboratory environment as well as when used as seedling pre-treatment for tomatoes, promoting seed germination and seeding vigor. In addition, treatment with aqueous extracts increased the vegetative growth of tomatoes under greenhouse conditions. The treatment of tomato seedlings with aqueous extracts of *C. procera* increased the antioxidant enzymes (peroxidase and polyphenol oxidase) and increased flavonoid and phenol contents in seedlings after inoculation. Our results are promising for the use of *C. procera* as eco-friendly material against *F. oxysporum* under greenhouse conditions.

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