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PHYTOCHEMICAL-INDUCED DEFENSE ACTIVATION IN CHICKPEA BY *CALOTROPIS PROCERA* L. EXTRACT TO CONTROL *FUSARIUM* WILT

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ABSTRACT

Fungicide application offers a solution for crop protection, but synthetic fungicides pose greater harm to the environment. This study explores the potential of *Calotropis procera* L. leaf extracts as a nature-friendly alternative to combat *Fusarium* wilt in chickpea. Methanol (MeOH) extracts were tested at various concentrations (0-6.5%) against *Fusarium oxysporum*, with complete growth suppression observed at 5% and 6.5%. Bioassay-guided fractionation of the crude extract was performed using n-hexane, dichloromethane (DCM), ethyl acetate (EtOAc), acetone (Ace), and methanol (MeOH). The results indicated that EtOAc and Ace fractions at 0.6% completely inhibited fungal growth. Crude extracts (3.5%, 5%, and 6%) along with EtOAc (0.2% and 0.6%) and Ace (0.2% and 0.6%) fractions were further subjected to phytotoxicity analysis on chickpea seedlings. Among these, the EtOAc-0.6% fraction exhibited the least phytotoxicity. In a pot experiment, the group inoculated with *F. oxysporum* and treated with the botanical extract (T6) showed a lower disease incidence of 41.7%, compared to 50% in the *F. oxysporum*-inoculated group treated with carbendazim (T5). T6 also exhibited improved root elongation (124%), seed germination (88.89%), catalase activity (52 U/mg protein), and guaiacol peroxidase activity (26.63 U/mg protein). The highest carotenoid content (1.6 µg/mL) was observed in T5, while the uninoculated group treated with the botanical extract (T4) recorded the highest SOD activity (78.24 U/mg protein). Overall, these findings suggest that the EtOAc-0.6% fraction of *C. procera* leaf extract could serve as a safer and effective candidate for managing *Fusarium* wilt in chickpea.

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INTRODUCTION

The agriculture industry contributes 4% to the global GDP and has the potential to reduce food insecurity by up to 80%. However, its growth is threatened by several factors, the most significant being pest attacks, which are responsible for 10-15% of annual yield losses worldwide,

resulting in substantial economic losses. Fungi account for a major proportion (70-80%) of plant pathogens (Peng et al., 2021). To control the diseases caused by these fungal pathogens and to maximize crop yields, fungicides are widely used (Leong et al., 2020).

However, fungicides can adversely affect not only the

target plants but also other organisms that consume them. Resistance to fungicides has also been observed in both target and non-target species. For instance, the agricultural use of azole fungicides has contributed to antifungal drug resistance in human pathogens. Furthermore, fungicides negatively impact biodiversity; for example, tebuconazole has been shown to reduce soil microbial populations and activity. Fungicide residues can also be toxic to humans, potentially causing health issues such as dermatitis, hormonal imbalances, fetal abnormalities, and cancer (Xu et al., 2020). According to the World Bank, the overuse of pesticides in Pakistan is projected to reduce agricultural yields by up to 50% by 2050 (Raja, 2023).

Plant extracts offer a safer alternative to synthetic fungicides, providing effective pest control with faster decomposition and lower environmental impact (Kayani et al., 2012; Mukhtar et al., 2013; Leong et al., 2020; Saeed et al., 2021). Bio-fungicides are classified into three categories: microbial, biochemical, and plant-incorporated protectants (PIPs) (Kumar and Singh, 2014). Numerous studies highlight the role of microbes in promoting plant growth. For example, *Aspergillus niger*, *Bipolaris* sp., *Curvularia lunata*, *Penicillium* sp., and *Trichoderma harzianum* have been shown to help plants withstand various stresses (Aizaz et al., 2023). However, microbial performance is often inconsistent due to biotic and abiotic factors, including competition with native species, poor adaptability to the plant environment, and changing environmental conditions (Bonaterra et al., 2012). PIPs also have limitations, such as risks of gene flow, resistance development in pests, and adverse effects on non-target organisms (Kumar and Singh, 2014).

Biochemical fungicides, such as plant extracts, are cost-effective, eco-friendly, biodegradable, and less likely to induce resistance in pathogens. Their effectiveness is attributed to the secondary metabolites present in botanicals, which possess various biological activities, including the ability to combat plant diseases. Based on global research, botanicals are considered safer options for plant protection (Deresa and Diriba, 2023).

Chickpea is an essential legume cultivated across almost every continent. India is the largest producer, followed by Pakistan, while China has recently emerged as a major producer. Chickpea is a low-cost protein source, making it vital for meeting nutritional needs in developing countries. The crop is cultivated on 13.2 million hectares globally, producing 13.1 million tons annually, with an average

yield of 1 ton per hectare. Under optimal conditions, yields can reach up to 6 tons per hectare. However, various factors, including pest infestations, salinity, drought, and temperature stress, contribute to underproduction (Zhang et al., 2024). In Pakistan, chickpea is grown on 830,000 hectares, yielding 238,000 tons. To meet the national demand of 825,000 tons, the country imports approximately 600,000 tons annually (Raja, 2023).

Fusarium oxysporum f. sp. *ciceris* (FOC) is the causative agent of chickpea wilt, capable of causing yield losses of up to 90%. This soil- and seed-borne disease is prevalent in many Asian countries. The pathogen consists of eight races, although only two are known to be highly virulent. Some strains can even colonize the plant asymptotically. FOC invades the xylem tissues of the roots and spreads throughout the plant via the transpiration stream. Wilt symptoms typically appear 10-20 days after inoculation, with dark brown discoloration of the xylem serving as a key diagnostic feature (Jiménez-Díaz et al., 2015).

There is ongoing debate regarding the efficacy of botanicals as alternatives to synthetic fungicides. *Calotropis procera*, a plant from the Asclepiadaceae family, is native to Asia and parts of Africa. It thrives in sandy, nutrient-poor soils and is traditionally used for fuel and medicinal purposes in regions such as northern Pakistan, Saudi Arabia, and India. Rather than eliminating such vegetation, which may lead to environmental degradation and contribute to climate change, plants like *C. procera* should be utilized for human benefit. In Pakistan, 84% of the population uses home remedies, relying on 400-600 known wild plant species out of the more than 6,000 native species (Siraj and Khan, 2021).

In this study, we evaluated the efficacy of *C. procera* as a seed priming agent for chickpeas infected with *F. oxysporum*. Plant extracts were initially tested *in vitro* against *F. oxysporum*. Various solvent extracts at different concentrations were assessed, and the most effective extract, based on complete inhibition of fungal growth and minimal phytotoxicity, was selected for *in vivo* trials. The aim of the study was to evaluate the defense responses of *C. procera*-primed chickpea seeds against *F. oxysporum* infection. The specific objectives were to: (1) identify the most effective concentration and extract of *C. procera* against *F. oxysporum*, (2) assess the phytotoxicity of *C. procera* extracts on chickpea seedlings, (3) perform morpho-physiological profiling of *C. procera*-primed chickpea seedlings and (4) evaluate

the antioxidant defense system of the plants under treatments with carbendazim and *C. procera* extracts.

MATERIAL AND METHODS

Acquisition of biological material

Seeds of the *Fusarium*-susceptible *Cicer arietinum* variety NIAB-16 were obtained from the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad. A pure culture of *F. oxysporum* (Accession No. 1573) was acquired from the First Culture Bank of Pakistan at the University of the Punjab, Lahore. The fungus was re-cultured on 2% malt extract agar (MEA). Pots and garden soil were purchased from a local nursery in Lahore. Healthy leaves of *C. procera* were collected from the Pakistan Technocrats Housing Cooperative Society (PTHCS), Lahore, for plant extract preparation.

Preparation of plant extracts

Solvent extraction was performed using a Soxhlet extractor (Reihenheizgerät 4, Spannung 230V/50Hz), following the method described by Ali et al. (2023). Shade-dried leaves (25 g) were placed in a thimble and fixed in the extraction chamber. Methanol (MeOH) was used to prepare the crude extract. For fractionated extracts, sequential extraction was carried out using hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), acetone (Ace), and MeOH. The extraction process continued until the solvent became colorless. After extraction, the solvents were evaporated at room temperature (RT) under sterile conditions. Fresh stock solutions were prepared in autoclaved distilled water (ADW).

Antifungal activity of crude plant extracts

The agar dilution method described by Zubairi et al. (2021) was used to evaluate the antifungal activity of the crude leaf extract. A 2% MEA medium served as the artificial growth medium. A 20% stock solution of each extract was used to prepare five concentrations: 0.5%, 2%, 3.5%, 5%, and 6.5%. These dilutions were made using ADW and the formula $M_1V_1 = M_2V_2$. The required volume of stock solution was mixed with 2% MEA. A plain medium served as the negative control. Carbendazim 50 WP (Methyl benzimidazol-2-yl carbamate, 50% w/w) from Solex Chemicals (Pvt.) Ltd. was used as the positive control at the same concentrations as the plant extracts.

All prepared solutions were simmered for 10 min. Then, 250 mg of amoxicillin was added to each sample, and the flasks were gently swirled to mix. The mixtures were poured into 9 cm petri dishes. After solidifying at room

temperature, a 5 mm disc from a 10-day-old fungal culture was placed at the center of each plate. All treatments were performed in triplicate. The experiment was conducted in a UV-equipped laminar flow cabinet (LFC) from Streamlined Lab Products. All equipment was sterilized using dry heat at 90°C for 3 h, followed by UV exposure for 30 min.

The plates were incubated at 28°C for one week. Fungal colony diameters were measured at three different points per plate, and the average was calculated for each replicate. Growth inhibition (GI%) was calculated using the formula:

$$GI (\%) = \frac{GC - GT}{GC} \times 100$$

Bioassay-guided fractionation

Based on the significant antifungal activity of the crude extract, the plant material was subjected to bioassay-guided fractionation as described by Zubairi et al. (2021). Sequential extraction was performed to obtain organic fractions of varying polarities. Each plant fraction was tested for antifungal activity against *F. oxysporum*. Two concentrations (0.2% and 0.6%) were prepared from a 10% stock solution of each fraction as well as the synthetic fungicide (used as a positive control). The remaining steps followed the procedure described in the previous section. A 3 mm inoculum disc was used in this assay.

Phytotoxicity analysis of selected concentrations

Extracts showing the most effective antifungal activity were selected for phytotoxicity analysis. The selected plant extracts and their concentrations were as follows: *C. procera* crude 5% (CPCr 5%), *C. procera* crude 3.5% (CPCr 3.5%), *C. procera* ethyl acetate 0.2% (CPEA 0.2%), *C. procera* ethyl acetate 0.6% (CPEA 0.6%), *C. procera* acetone 0.2% (CPAce 0.2%), and *C. procera* acetone 0.6% (CPAce 0.6%). A negative control was maintained by hydropriming seeds with distilled water.

The top-of-paper method (Yago et al., 2011) was used to germinate the treated seeds. Briefly, surface-sterilized seeds (2% sodium hypochlorite) of the NIAB-16 chickpea variety were soaked separately in each treatment solution for 10 min. The experiment was conducted using moist filter paper placed in sterilized 9 cm Petri dishes. The seeds were incubated at 22°C in a seed germinator (Sanyo, MIR-153) for 7 days. Data were recorded for radicle length, plumule length, and seed germination. These data were used to calculate root elongation percentage (RE%), seed germination

percentage (SG%), and seed germination index (SGI), following the method of Zucconi et al. (1981).

$$RE\% = \frac{Trl}{Crl} \times 100$$

Where Trl = Mean root length in the treatment, Crl = mean root length in the control

$$SG\% = \frac{ST}{SC} \times 100$$

Where ST = No. of germinated seeds in treatments, SC = No. of germinated seeds in control

$$SGI = \frac{SG\% \times RE\%}{100}$$

Experimental site for pot experiment

The experiment was conducted in pots under natural conditions at a local nursery in Lahore (31.37°N, 74.18°E), from October 20, 2023, to November 3, 2023. The soil was sterilized by placing formaldehyde-soaked cotton balls in soil sacs for two weeks, followed by solarization for two days. Afterward, the soil was sieved, and each pot was filled with 3 kg of soil.

Research design

The following experimental groups were used:

T1 = No inoculation + no treatment (Healthy control)

T2 = Inoculation + no treatment (Inoculated control)

T3 = Carbendazim-treated + not inoculated

T4 = CP-treated + not inoculated

T5 = Carbendazim-treated + inoculated

T6 = CP-treated + inoculated

The experiment was conducted using a completely randomized design (CRD) with four replicates per treatment. Six seeds were initially sown in each pot.

Seed treatment

A 0.6% ethyl acetate extract of *C. procera* was used for seed treatment. The extract was diluted in ADW to obtain a 0.6% solution using the following equation:

$$\text{Percentage (\%)} = \frac{\text{Weight of extract}}{\text{Volume of water}} \times 100$$

The surface-sterilized seeds (both CP-treated inoculated and uninoculated) were soaked in the freshly prepared extract for 10 min. For the carbendazim treatment, both inoculated and uninoculated seeds were treated similarly. The seeds were then dried on sterilized filter paper under a laminar air flow chamber.

Preparation of inoculum and inoculation

A 7-day-old liquid culture of *F. oxysporum* was used to prepare the inoculum. The culture flasks were shaken on an orbital shaker at 150 rpm for 30 min to loosen the conidia. The culture was then filtered through steam-

sterilized muslin cloth to remove fungal hyphae and mycelia, allowing only the microconidia (the infectious units) to pass through. The conidial concentration was determined using a hemocytometer and adjusted to 2×10^4 CFU/ml by dilution with ADW, using the equation: $M_1V_1 = M_2V_2$.

Seeds for the inoculated control (T2), as well as the fungicide- and CP-treated inoculated groups (T5 and T6), were soaked separately in 25 ml of the inoculum for 10 min and then immediately sown into the soil. The soil was watered to maintain adequate moisture. Sampling for biochemical assays was conducted on the 14th day of the experiment.

Disease incidence percentage (DI%)

To calculate DI%, the number of germinated seeds was recorded for each sample. The number of non-germinated seeds was obtained by subtracting the number of germinated seeds from the total (6). DI% was calculated using the following formula:

$$DI(\%) = \frac{\text{Number of nongerminated seeds}}{\text{Total number of seeds}} \times 100$$

Morphological parameters

Young seedlings were carefully removed from the soil, and their roots were washed with ADW to remove any adhering soil particles. Morphological parameters such as radicle length, plumule length, number of leaves per plant, and seed germination were recorded. These parameters were then used to calculate the seed germination percentage and root elongation percentage, as described by Zucconi et al. (1981).

Physiological parameters

The pigment content was measured to assess the photosynthetic activity of the plants. Total pigments from the leaves were extracted using a slightly modified method of Hiscox and Israelstam (1979). Briefly, 0.05 g of leaf tissue from each treatment was cut into small pieces and soaked in 10 ml of dimethyl sulfoxide (DMSO) in sterilized vials. The vials were incubated at 70°C for 2 h until the leaves lost their color. The solvent was then cooled to room temperature, and absorbance was measured at 480 nm, 649 nm, and 665 nm using a UV-Vis spectrophotometer. Pigment concentrations (µg/ml) were calculated using the equations provided by Lichtenthaler (1987).

$$\text{Chl. a} = 12.47 \times A_{665.1} - 3.6 \times A_{649.1}$$

$$\text{Chl. b} = 25.06 \times A_{649.1} - 6.5 \times A_{665.1}$$

$$\text{Carotenoids} = \frac{1000 \times A_{480} - 1.29 \times \text{Chl. a} - 53.7 \times \text{Chl. b}}{220}$$

Carbohydrate content estimation

To estimate carbohydrate content, 0.2 g of leaf tissue from each treatment was homogenized in ADW and the volume was adjusted to 10 ml. The homogenate was filtered through Whatman No. 4 filter paper. The resulting filtrate was mixed with anthrone reagent in a 1:4 ratio and boiled for 10 min. After cooling to RT, the absorbance was measured at 620 nm (Morsy, 2014). A standard curve prepared using glucose was used to calculate the carbohydrate content, which was expressed as mg/g of fresh weight (FW). The equation obtained for quantification is:

$$y = 0.0062x - 0.0191$$

Antioxidant profile of the seedlings

To assess the antioxidant activity of the seedlings under different treatments, enzyme extracts were prepared following the method of Ali and Ashraf (2011), with slight modifications. Briefly, 0.2 g of root tissue was homogenized in 1.2 ml of 0.2 M potassium phosphate buffer (KPB) containing 1 mM EDTA (pH 7.6). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The resulting pellet was resuspended in 0.8 ml of KPB and centrifuged again. The supernatant was collected and kept on ice for further analysis.

The estimation of hydrogen peroxide (H₂O₂) scavenging activity, as well as the activities of catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPx), was performed according to the methodology described by Haida and Hakimian (2019).

Hydrogen peroxide (H₂O₂) scavenging activity

A 40 mM H₂O₂ solution was prepared in 50 mM KPB (pH 7.4). Plant extracts were added to the solution in a 2:1 ratio, and the absorbance was measured at 230 nm. The H₂O₂ scavenging activity was calculated using the following formula:

$$\text{Scavenging (\%)} = \left[\frac{\text{Control} - \text{Treatment}}{\text{Control}} \right] \times 100$$

Where, control and treatment refer to absorbance values.

Catalase (CAT) activity

Enzyme extracts (66 µl) were mixed with 2 ml of reaction mixture, and the absorbance was recorded at 240 nm for 2 min. CAT activity was calculated using the extinction coefficient of H₂O₂ (34.9 mM⁻¹ cm⁻¹ at 240 nm). The activity was expressed in Units·mg⁻¹ protein using the following equation:

$$\text{CAT Activity} = \frac{\text{Absorbance change} \times \text{Vt}}{\epsilon \times d \times \text{Vs} \times \text{Ct} \times \text{Pc} \times 0.001}$$

Whereas d = optical path length of the cuvette (1 cm); 0.001 = absorbance change caused by 1 unit of enzyme per min at 240 nm; Pc = protein concentration (mg/ml).

Superoxide dismutase (SOD) activity

An aliquot of 20 µl of enzyme extract was added to 2 ml of the reaction mixture, and the absorbance was recorded at 560 nm. The reaction mixture consisted of 0.05 M potassium phosphate buffer (KPB, pH 7.6), 0.002 M EDTA, 0.0099 M L-methionine, 0.000055 M nitroblue tetrazolium (NBT), and 0.025% Triton X-100. SOD activity was expressed in Units per mg of protein (Units·mg⁻¹ protein) using the following formula:

$$\text{SOD} = \left[\left(\frac{\text{Sabs}}{\text{Cabs}} \right) - 1 \right] \times \frac{\text{df}}{\text{Pc}}$$

Where Sabs = absorbance of the sample; Cabs = absorbance of the control; df = dilution factor (100); Pc = protein concentration (mg/ml).

Guaiacol peroxidase (GPx) assay

To perform the assay, 20 µl of enzyme extract was added to 2 ml of assay mixture, and the absorbance was measured at 420 nm for 2 min, with readings recorded every 30 sec. The assay mixture was prepared by combining 40 µl of H₂O₂, 1.8 ml of guaiacol, 8 ml of potassium phosphate buffer (KPB, pH 6), and 1 ml of distilled water (DW). The following formula was used:

$$\text{GPx Activity} = \frac{\text{Absorbance change} \times \text{Vt}}{\epsilon \times d \times \text{Vs} \times \text{Ct} \times \text{Pc} \times 0.001}$$

Where ϵ = 26.1.

Statistical analysis

The results are presented as means $\pm$ standard error (SE). Two-way ANOVA was applied to the bioassay-guided fractionation, while the remaining experiments were analyzed using one-way ANOVA. Tukey's Honestly Significant Difference (HSD) test was used for mean separation at the 95% confidence level. Data were compiled in MS Excel (2021), and CSV files were generated using the same platform. Statistical analyses and graphical representations were performed in RStudio (R 4.4.1) using the agricolae, effectsize, and ggplot2 packages.

RESULTS

Antifungal activity of crude extracts

The results showed a significant difference ($P < 2e-16$, $\eta^2 = 1.00$) in inhibiting fungal growth at various doses. Higher concentrations (5% and 6.5%) completely suppressed fungal growth (Figure 1), while lower concentrations inhibited fungal growth to varying extents.

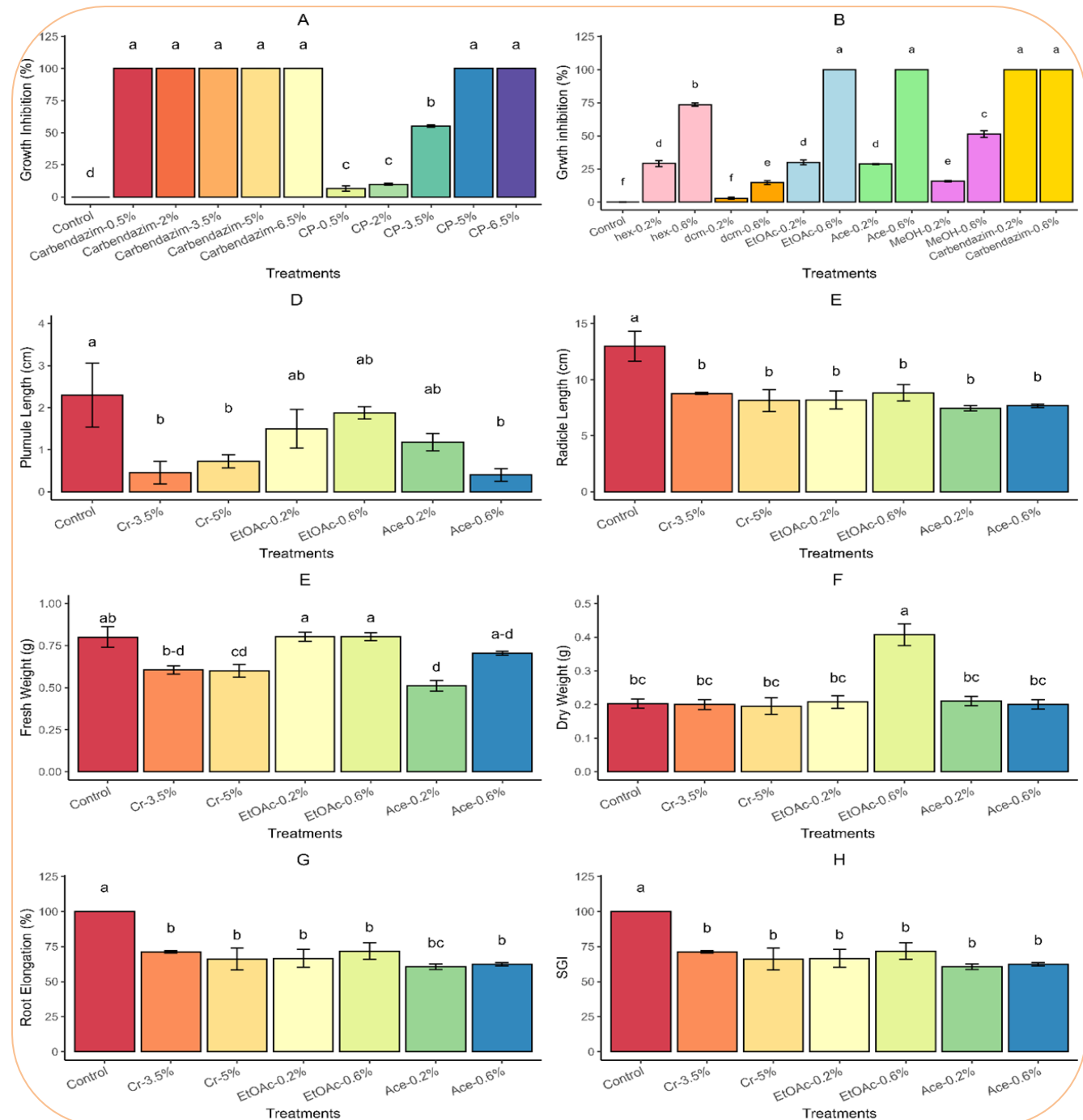


Figure 1. The figure presents data on the percentage of fungal growth inhibition and the phytoxic effects of selected extracts. (A) Growth inhibition (%) of the fungus by crude extracts; (B) Growth inhibition (%) of the fungus by organic fractions of *C. procera*; (C) Plumule length (cm) of chickpea seedlings exposed to different extracts; (D) Radicle length (cm) of chickpea seedlings exposed to different extracts; (E) Fresh weight of chickpea seedlings exposed to different extracts; (F) Dry weight of chickpea seedlings exposed to different extracts; (G) Relative elongation percentage (REP) of chickpea seedlings exposed to different extracts; (H) Seed germination index (SGI) of chickpea seedlings exposed to different extracts.

Data are presented as mean $\pm$ SE. Means with the same letters are not significantly different at $P = 0.05$. Two-way ANOVA was applied to panel B, while one-way ANOVA was used for the remaining datasets.

Bioassay-guided fractionation

All tested fractions effectively inhibited fungal growth. Among them, EtOAc and Ace at 0.6% completely suppressed the fungus (Figure 1). The differences were statistically significant ($P < 2e-16$, $\eta^2 = 1.00$).

Phytotoxic analysis

For the phytotoxic analysis, seed germination and seedling growth parameters of treated *C. arietinum* were carefully observed (Figure 2). All parameters showed statistically significant differences: plumule length ($P = 0.01$, $\eta^2 = 0.52$), radicle length ($P = 0.001$, $\eta^2 = 0.64$), fresh

weight ($P = 7.7e-6$, $\eta^2 = 0.77$), dry weight ($P = 1.25e-6$, $\eta^2 = 0.81$), REP ($P = 7.85e-5$, $\eta^2 = 0.71$), and SGI ($P = 7.85e-5$, $\eta^2 = 0.71$). Among all the extracts, seedlings treated with EtOAc-0.6% showed the best performance in terms of radicle and plumule length, fresh weight, dry weight, SGP, and REP. However, the healthiest growth overall, except for fresh and dry weight, was observed in the untreated control (Figure 1). Based on all parameters, EtOAc-0.6% exhibited the least phytotoxicity to *C. arietinum* while completely suppressing *F. oxysporum* growth, and was therefore selected for the pot experiment.



Figure 2. Phytotoxic analysis of chickpea seedlings exposed to different *C. procera* extracts.

Percent disease incidence

The lowest DI% was observed in T6, at 41.6%, followed by the T5 group, which showed 50% (Figure 3). In contrast, the inoculated control group exhibited the highest DI% at 70.8%. Statistical analysis indicated a significant difference among groups ($P = 2.98e-7$, $\eta^2 = 0.87$).

Morphological profile of treatments

Morphological parameters such as shoot length, root length, and number of leaves per plant were evaluated as seedling parameters (Figure 4). Additional seedling parameters included germination index (GI), seed germination percentage (SG%), and root elongation (RE). All treatments performed better than the inoculated control (T2). The CP-treated + inoculated group showed the best results, followed by the fungicide-treated + inoculated group (Figure 3). All tested parameters showed statistically significant differences, including plumule length ($P = 2.11e-6$, $\eta^2 = 0.93$), radicle length ($P = 0.003$, $\eta^2 = 0.75$), number of

leaves ($P = 0.0001$, $\eta^2 = 0.85$), REP ($P = 0.002$, $\eta^2 = 0.76$), and SGP ($P = 3.7e-6$, $\eta^2 = 0.92$).

Photosynthetic parameters (PG) of the plants

Statistical analysis revealed significant differences across all tested parameters, including carotenoid content ($P < 2e-16$, $\eta^2 = 0.99$), total chlorophyll ($P = 7.26e-16$, $\eta^2 = 1.00$), chlorophyll a ($P < 2e-6$, $\eta^2 = 1.00$), chlorophyll b ($P = 5.07e-9$, $\eta^2 = 0.97$), and carbohydrate content ($P = 1.05e-11$, $\eta^2 = 0.99$).

T3 (fungicide-treated), followed by T5 (fungicide-treated + inoculated), showed higher chlorophyll content, with T5 exhibiting the highest carotenoid content. Photosynthetic pigment levels were lowest in T2 (inoculated control) (Figure 5). Chlorophyll and carotenoid contents were highest in T3 and T5. Regarding carbohydrate content, there were minimal differences among the treatments. However, the uninoculated groups (T1, T3, and T4) exhibited slightly higher carbohydrate content compared to the inoculated groups (T2, T5, and T6) (Figure 6).

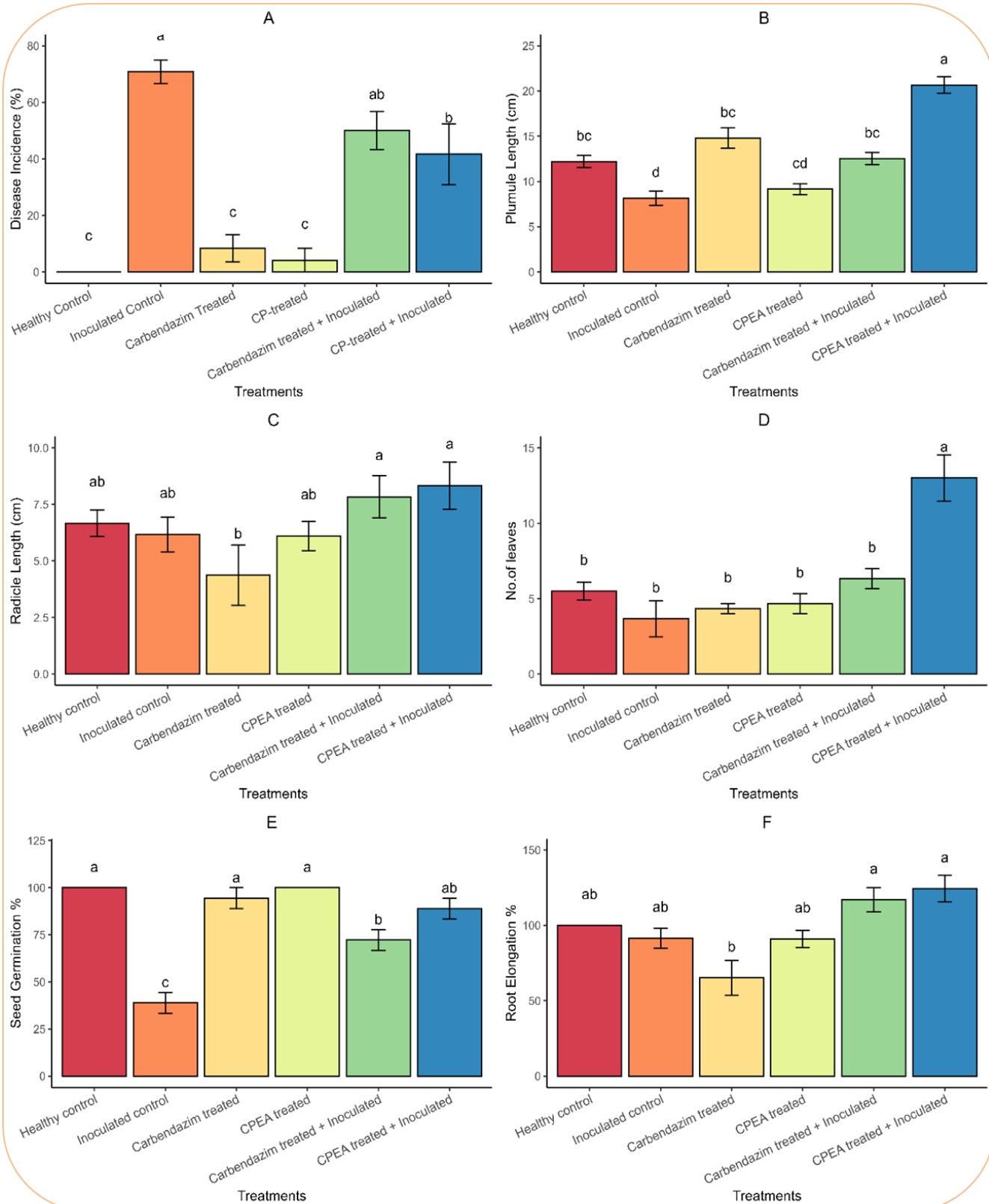


Figure 3. The figure presents (A) DI% and (B-F) morphological parameters of seedlings under various treatments. The morphological parameters are: (B) plumule length, (C) radicle length, (D) number of leaves, (E) SGP, and (F) REP. Data are presented as mean $\pm$ SE in bar graphs. Means sharing the same letters are not significantly different ($P = 0.05$).



Figure 4. Fourteen-day-old chickpea seedlings showing morphology under various treatments.

Antioxidant activity of the plants

Overall, CAT content was higher compared to SOD and GPx. In this study, the inoculated groups (T2, T5, and T6) showed greater CAT activity than their respective uninoculated counterparts (T1, T3, and T4). A similar pattern was observed for GPx. However, the opposite pattern was observed for SOD. For GPx, the highest activity was seen in T6, but there was no significant difference between T2 and T5. Regarding H_2O_2 scavenging, the uninoculated groups performed better (Figure 5). Statistical analysis showed significant differences in all assays: CAT ($P = 3.6e-12$), GPx ($P = 2.31e-12$), SOD ($P = 2.41e-14$), and H_2O_2 scavenging activity ($P = 2.41e-10$).

DISCUSSION

There is extensive literature on the efficacy of plant extracts against pests (Khan et al., 2021; Jabbar et al., 2022; Azeem et al., 2025). However, these plants still require testing for their effectiveness under natural conditions (Tembo et al., 2018). When exposed to stress, plants produce reactive oxygen species (ROS) to regulate pathogen defense. Low levels of ROS are essential for normal biological functions, but elevated ROS levels are harmful, causing DNA damage and ultimately programmed cell death. Plants possess enzymatic and non-enzymatic systems to scavenge ROS. An increased antioxidant level indicates a stressed plant (Huang et al., 2019). Exposure to biological agents activates the plant's defense mechanisms, and antioxidants help plants cope with stress, either fully or partially (El-Esawi et al., 2019; Nagpal et al., 2021). Therefore, in this study, phytopriming was employed to evaluate the plant's

defense response. Morphological and photosynthetic parameters were measured to assess plant health.

C. procera extracts effectively inhibited fungal growth *in vitro*. For example, Cr 5%, Cr 6.5%, EtOAc 0.6%, and Ace 0.6% showed 100% growth inhibition of the fungus. Previous studies have reported various extracts of this plant inhibiting the growth of different *F. oxysporum* subspecies, possibly due to the plant's toxicity against the fungus. The efficacy of CPEA 0.6% in controlling *Fusarium* wilt *in vivo* aligns with earlier studies where wilt was suppressed using aqueous extracts and nanoparticles prepared from *C. procera* (Hussain et al., 2011; Abo-Elyousr et al., 2022). *C. procera* is known to contain various phytochemicals that may inhibit fungal growth (Doshi et al., 2011).

Phytotoxicity analysis showed that uninoculated phytoprimed seeds exhibited good germination and seedling growth. The best results were observed with EtOAc 0.6%, followed by Cr 3.5%. These results are consistent with previous findings, where aqueous leaf extracts of *C. procera* promoted seed germination and growth in different plants (Radwan et al., 2019; Abo-Elyousr et al., 2022). However, suppressive effects of the extract priming on seeds of *Vigna radiata*, *V. mungo*, *Hordeum vulgare* L., *Triticum aestivum* L., *Cucumis sativus* L., *Solanum lycopersicon* L., and *S. melongena* have also been reported (Ghasemi et al., 2011; Bhattacharya et al., 2022). These differences may be due to varying extract concentrations and differential allelopathic interactions between the botanical and the treated plant (Radwan et al., 2019).

Reduced chlorophyll levels were observed in the inoculated groups compared to their uninoculated counterparts, consistent with findings by Akram et al. (2024) in *S. lycopersicon* under *F. oxysporum* attack. Improved chlorophyll and carotenoid levels were noted in bio-primed plants in both inoculated and uninoculated groups. The higher carotenoid content suggests a protected photosynthetic system, whereas reduced chlorophyll in inoculated groups may be a stress response, as plants tend to conserve resources under unfavorable conditions. Therefore, the combination of better carotenoid content and lower chlorophyll levels does not indicate impaired photosynthesis in the treatments studied. Moreover, improved morphological traits further support this conclusion. Carril et al. (2025) also reported reduced chlorophyll content but good morphological traits in tomato plants treated with bacterial biofertilizers, similar to our findings.

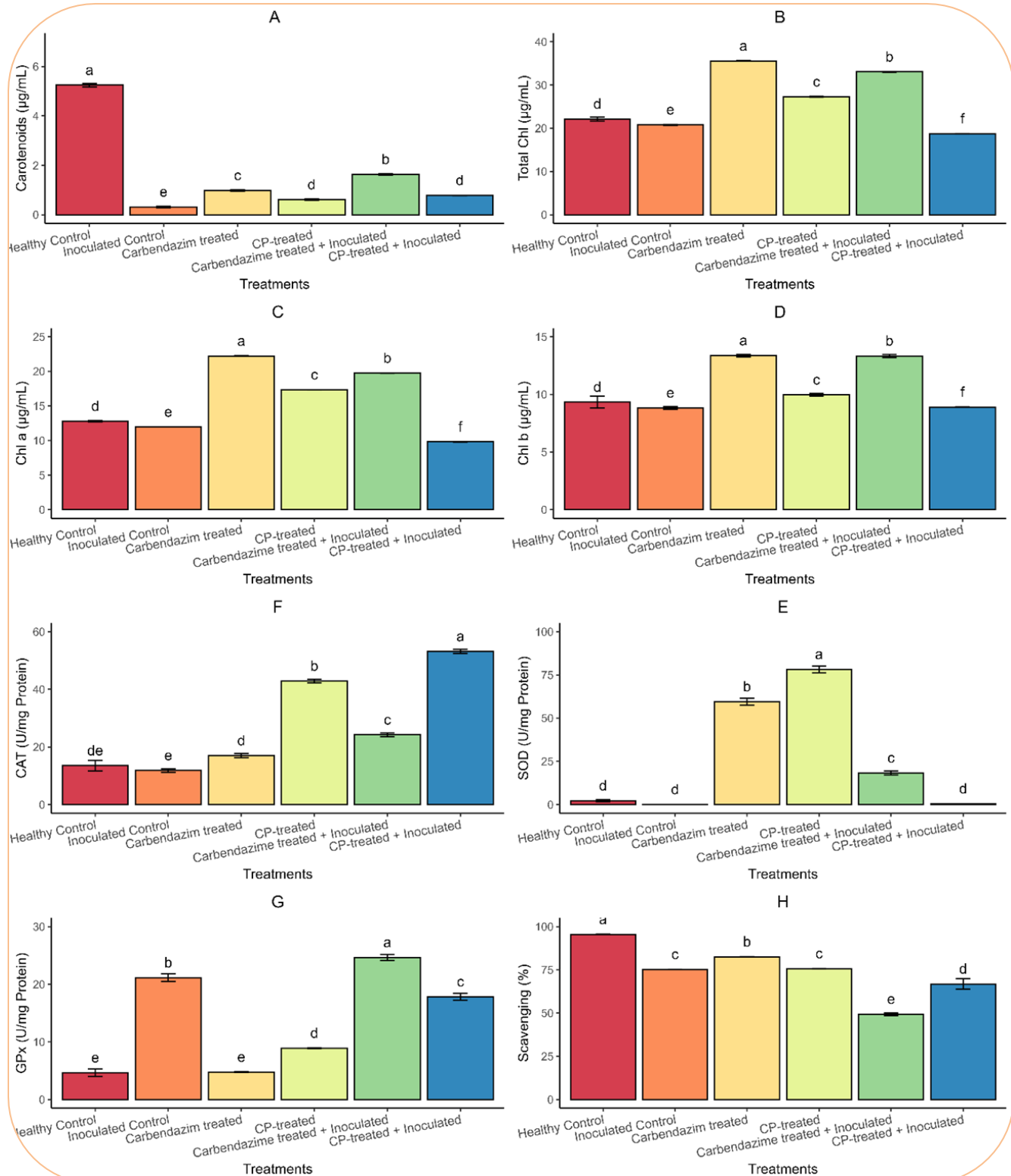


Figure 5. The figure shows photosynthetic pigment content and antioxidant activities in the treated plants: (A) Carotenoid content, (B) Total chlorophyll content, (C) Chlorophyll a content, (D) Chlorophyll b content, (E) SOD activity, (F) CAT activity, (G) GPx activity, and (H) H_2O_2 scavenging activity. Data are presented as mean $\pm$ SE. Means with the same letters are not significantly different ($P = 0.05$).

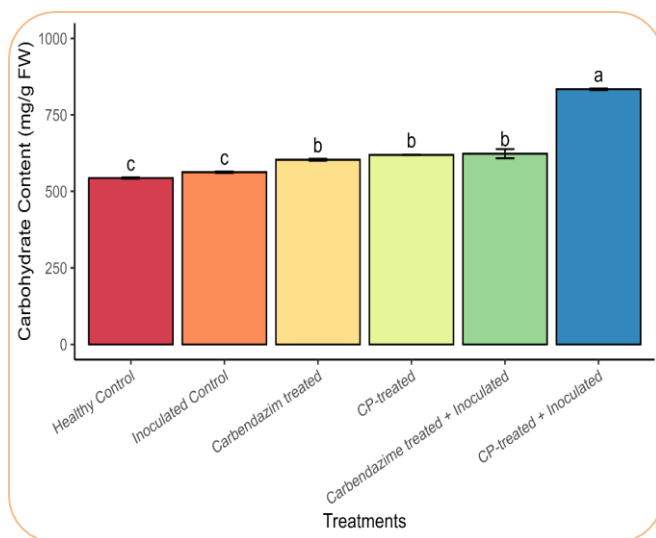


Figure 6. Carbohydrate contents in *C. arietinum* seedlings subjected to different treatment groups are presented. Data are shown as Mean $\pm$ SE in bar graphs. Means sharing the same letters are not significantly different ($P = 0.05$).

Our study showed higher carbohydrate content in the control groups compared to all treatments; however, all treatments had better carbohydrate levels than the inoculated untreated group (T2). Enhanced sugar content in PGPR-treated saline-stressed chickpea plants has also been reported (El-Esawi et al., 2019). Roots showed increased soluble sugar levels under stress at all concentrations, possibly because roots act as sugar sinks and are less affected by stress in terms of sugar production (Kumar et al., 2021).

Antioxidant profiling was conducted to assess the chickpea defense system under *F. oxysporum* infection and phytopriming. Elevated CAT activity was observed in all treatments compared to the control, with the highest activity in T6, indicating a robust defense system mitigating wilt disease effects. Increased activities of CAT, SOD, and GPx under PGPR treatment in chickpea exposed to *F. oxysporum* stress have been reported previously (El-Esawi et al., 2019; Nagpal et al., 2021; Abo-Elyousr et al., 2022).

Seed priming with botanicals induces systemic acquired resistance (ISR), a plant defense mechanism similar to systemic acquired response. ISR, triggered by biocontrol agents, is a novel plant protection strategy (Nagpal et al., 2021). SOD levels were lower in inoculated controls, possibly due to an initial ROS burst causing a temporary decline in SOD as it is consumed to neutralize ROS (Wang

et al., 2021). This is supported by the role of H_2O_2 , a redox-active ROS that reversibly oxidizes proteins and interacts with phytohormones to regulate stress responses. H_2O_2 cleavage produces hydroxyl radicals, the most reactive ROS, capable of oxidizing cell wall polysaccharides and damaging DNA (Huang et al., 2019). In our study, the healthy control (T1) showed the highest H_2O_2 scavenging activity without any stimulus, consistent with findings by Thite et al. (2013), who observed similar patterns in healthy leaves of *Xanthium strumarium* compared to powdery mildew-infected leaves. However, they reported a reverse trend in *Dalbergia sisso* leaves, concluding that fungal infection differently affects radical scavenging activity depending on the plant species. Furthermore, H_2O_2 also plays a role in normal developmental processes such as seed germination and metabolic regulation (Li et al., 2010; Wojtyla et al., 2016). Thus, H_2O_2 serves a dual function depending on the context. Multiple antioxidant assays are necessary to draw reliable conclusions. To our knowledge, this is the first report of H_2O_2 content measurement in plants under fungal inoculation.

CONCLUSION

The present study demonstrates the practical application of the leaf ethyl acetate extract of *C. procera* in activating the antioxidant system of chickpea plants under *F. oxysporum* inoculation. This activation enables the plant to better mitigate the harmful effects of the disease through enhanced defense responses. Due to its low phytotoxicity and strong antifungal efficacy *in vitro*, CPEA 0.6% shows promise as a seed priming agent to combat pathogens and could serve as an eco-friendly alternative to synthetic fungicides for managing *Fusarium* wilt in chickpea. The phytochemicals present in the extract likely play a key role in activating the plant's antioxidant defense system.

AUTHORS' CONTRIBUTIONS

TZ and ZS designed the study; TZ prepared the materials, collected and analyzed the data; AA and KJ provided biological material; ZS, MU and KJ supervised the studies; TZ and ZS wrote the manuscript; All the authors proofread and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUSTAINABLE DEVELOPMENT GOALS TARGETED

SDG 2: Zero Hunger

SDG 3: Good Health and Well-being

SDG 12: Responsible Consumption and Production

REFERENCES

- Abo-Elyousr, K.A.M., Ali, E.F., Sallam, N.M.A., 2022. Alternative control of tomato wilt using the aqueous extract of *Calotropis procera*. Horticulturæ 8, 197.
- Aizaz, M., Ahmad, W., Asaf, S., Khan, I., Saad Jan, S., Salim Alamri, S., Bilal, S., Jan, R., Kim, K.-M., Al-Harrasi, A., 2023. Characterization of the seed biopriming, plant growth-promoting and salinity-ameliorating potential of halophilic fungi isolated from hypersaline habitats. International Journal of Molecular Sciences 24, 4904.
- Akram, W., Waqar, S., Hanif, S., Anjum, T., Aftab, Z.-H., Li, G., Ali, B., Rizwana, H., Hassan, A., Rehman, A., Munir, B., Umer, M., 2024. Comparative effect of seed coating and biopriming of *Bacillus aryabhattai* Z-48 on seedling growth, growth promotion, and suppression of *Fusarium* wilt disease of tomato plants. Microorganisms 12, 792.
- Ali, F., Saddiqe, Z., Shahzad, M., Rafi, A., Javed, M., Haq, F., Saleem, S., Kusar, S., 2023. *Crotalaria medicaginea* Lamk.: An unexplored source of anticancer, antimicrobial and antioxidant agents. European Journal of Integrative Medicine 58, 102226.
- Ali, Q., Ashraf, M., 2011. Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: growth, photosynthesis, water relations and oxidative defence mechanism. Journal of Agronomy and Crop Science 197, 258-271.
- Azeem, W., Mukhtar, T., Haq, M.I., Khan, M.A., Ibrahim, M.S., Hassan, A., Regmi, H., Duncan, L.W., 2025. The nematicidal potential of *Moringa oleifera* extracts and rhizobacteria against *Meloidogyne incognita* in tomato. Frontiers in Plant Science 16, 1562074. <https://doi.org/10.3389/fpls.2025.1562074>
- Bhattacharya, S., Saha, T., Debnath, S., Krishna, S.A., 2022. Allelopathic potential of weed extracts on seed germination and seedling growth of *Vigna radiata* L. Wilzek and *Vigna mungo* L. International Journal of Life Sciences Research 10, 13-20.
- Bonaterre, A., Badosa, E., Cabrefiga, J., Francés, J., Montesinos, E., 2012. Prospects and limitations of microbial pesticides for control of bacterial and fungal pomefruit tree diseases. Trees 26, 215-226.
- Carril, P., Ghorbani, M., Azarnejad, N., Anselmi, S., Renzi, M., Loppi, S., 2025. Promoting early growth in tomato (*Solanum lycopersicum* L.) by co-application of biochar and beneficial bacteria. Journal of Soil Science and Plant Nutrition 25, 1493-1503.
- Deresá, E.M., Diriba, T.F., 2023. Phytochemicals as alternative fungicides for controlling plant diseases: A comprehensive review of their efficacy, commercial representatives, advantages, challenges for adoption, and possible solutions. Heliyon 9, e13810.
- Doshi, H., Satodiya, H., Thakur, M.C., Parabia, F., Khan, A., 2011. Phytochemical screening and biological activity of *Calotropis procera* (Ait). R. Br. (Asclepiadaceae) against selected bacteria and *Anopheles stephansi* larvae. International Journal of Plant Research 1, 29-33.
- El-Esawi, M.A., Al-Ghamdi, A.A., Ali, H.M., Alayafi, A.A., 2019. *Azospirillum lipoferum* FK1 confers improved salt tolerance in chickpea (*Cicer arietinum* L.) by modulating osmolytes, antioxidant machinery and stress-related genes expression. Environmental and Experimental Botany 159, 55-65.
- Ghasemi, S., Ghasemi, M., Moradi, N., 2011. Effect of *Calotropis procera* leaf extract on seed germination of some plants. Journal of Ornamental and Horticultural Plants 2(1), 27-32.
- Haida, Z., Hakimian, M., 2019. A comprehensive review on the determination of enzymatic assay and nonenzymatic antioxidant activities. Food Science and Nutrition 7, 1555-1563.
- Hiscox, J.D., Israelstam, G.F., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Canadian journal of botany 57(12), 1332-1334.
- Huang, H., Ullah, F., Zhou, D.X., Yi, M., Zhao, Y., 2019. Mechanisms of ROS regulation of plant development and stress responses. Frontiers in plant science 10, 800.
- Hussain, M.A., Mukhtar, T., Kayani, M.Z., 2011. Efficacy evaluation of *Azadirachta indica*, *Calotropis procera*, *Datura stramonium* and *Tagetes erecta* against root-knot nematodes *Meloidogyne incognita*. Pakistan Journal of Botany 43 (Special

- Issue), 197-204.
- Jabbar, A., Tariq, M., Gulzar, A., Mukhtar, T., Zainab, T., 2022. Lethal and sub-lethal effects of plant extracts and green silver nanoparticles against *Culex pipiens* mosquitoes. Pakistan Journal of Zoology 54(3), 1259-1267.
- Jiménez-Díaz, R.M., Castillo, P., Jiménez-Gasco, M.M., Landa, B.B., Navas-Cortés, J.A., 2015. *Fusarium* wilt of chickpeas: Biology, ecology and management. Crop Protection 73, 16-27.
- Kayani, M.Z., Mukhtar, T., Hussain, M.A., 2012. Evaluation of nematicidal effects of *Cannabis sativa* L. and *Zanthoxylum alatum* Roxb. against root-knot nematodes, *Meloidogyne incognita*. Crop Protection 39, 52-56.
- Khan, H.S., Tariq, M., Mukhtar, T., Gulzar, A., 2021. Insecticidal toxicity of plant extracts and green silver nanoparticles against *Aedes albopictus*. Pakistan Journal of Zoology 53(6), 2123-2128.
- Kumar, S., Li, G., Yang, J., Huang, X., Ji, Q., Liu, Z., Ke, W., Hou, H., 2021. Effect of salt stress on growth, physiological parameters, and ionic concentration of water dropwort (*Oenanthe javanica*) cultivars. Frontiers in plant science, 12, 660409.
- Kumar, S., Singh, A.J.J.B.B., 2014. Biopesticides for integrated crop management: environmental and regulatory aspects. Journal of Biofertilizers and Biopesticides 5, e121.
- Leong, W.-H., Teh, S.-Y., Hossain, M.M., Nadarajaw, T., Zabidi-Hussin, Z., Chin, S.-Y., Lai, K.-S., Lim, S.-H.E., 2020. Application, monitoring and adverse effects in pesticide use: The importance of reinforcement of Good Agricultural Practices (GAPs). Journal of Environmental Management 260, 109987.
- Li, X., Yang, Y.Q., Zhang, M., Wang, X.F., 2010. Effect of PEG priming on plasma membrane H⁺-ATPase activities and mitochondrion function in soybean seeds. Seed Science and Technology 38, 49-60.
- Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, in: Methods in Enzymology, Plant Cell Membranes. Academic Press, pp. 350-382.
- Morsy, N., 2014. Phytochemical analysis of biologically active constituents of medicinal plants. Main Group Chemistry 13, 7-21.
- Mukhtar, T., Kayani, M.Z., Hussain, M.A., 2013. Nematicidal activities of *Cannabis sativa* L. and *Zanthoxylum alatum* Roxb. against *Meloidogyne incognita*. Industrial Crops and Products 42, 447-453.
- Nagpal, S., Sharma, P., Sirari, A., 2021. Induction of antioxidant response with compatible combination of *Mesorhizobium* sp. and *Pseudomonas* sp. against *Fusarium oxysporum* sp. *ciceris* in chickpea. Bangladesh Journal of Botany 50, 359-364.
- Peng, Y., Li, S.J., Yan, J., Tang, Y., Cheng, J.P., Gao, A.J., Yao, X., Ruan, J.J., Xu, B.L., 2021. Research progress on phytopathogenic fungi and their role as biocontrol agents. Frontiers in Microbiology 12, 670135.
- Radwan, A.M., Alghamdi, H.A., Kenawy, S.K.M., 2019. Effect of *Calotropis procera* L. plant extract on seeds germination and the growth of microorganisms. Annals of Agricultural Sciences 64, 183-187.
- Raja, R., 2023. Pulses outlook (Agricultural Situation, Market Development Reports No. PK2023-0015). United States Department of Agriculture Foreign Agricultural Service, Pakistan.
- Saeed, M., Mukhtar, T., Haq, M.I., Khan, M.A., 2021. Assessment of nematicidal potential of *Cannabis sativa* and *Azadirachta indica* in the management of root-knot nematode (*Meloidogyne javanica*) on peach. Pakistan Journal of Agricultural Sciences 58, 1555-1561.
- Siraj, Khan, N., 2021. Quantitative ethnobotany of *Calotropis procera* and associated vegetation: a step forward for conservation and management practice in northern areas of Pakistan. Ethnobotany Research and Applications 22, 1-17.
- Tembo, Y., Mkindi, A.G., Mkenda, P.A., Mpumi, N., Mwanauta, R., Stevenson, P.C., Ndakidemi, P.A., Belmain, S.R., 2018. Pesticidal plant extracts improve yield and reduce insect pests on legume crops without harming beneficial arthropods. Frontiers in Plant Science 9, 1425.
- Thite, S., Aparadh, V., Kore, B., 2013. Effect of powdery mildew infection on DPPH radical scavenging activity and ferric-reducing antioxidant power of plants. World Journal of Pharmaceutical Research 2, 1-6.
- Wang, Q., Pokhrel, A., Coleman, J.J., 2021. The extracellular superoxide dismutase Sod5 from *Fusarium oxysporum* is localized in response to external stimuli and contributes to fungal pathogenicity. Frontiers in Plant Science 12, 608861.
- Wojtyla, L., Lechowska, K., Kubala, S., Garnczarska, M., 2016. Different modes of hydrogen peroxide

- action during seed germination. *Frontiers in Plant Science* 7, 66.
- Xu, J., Xiong, H., Zhang, X., Muhayimana, S., Liu, X., Xue, Y., Huang, Q., 2020. Comparative cytotoxic effects of five commonly used triazole alcohol fungicides on human cells of different tissue types. *Journal of Environmental Science and Health, Part B* 55, 438-446.
- Yago, J., Roh, J., Bae, S., Yoon, Y., Kim, H., Nam, M., 2011. The effect of seed-borne mycoflora from sorghum and foxtail millet seeds on germination and disease transmission. *Mycobiology* 39, 206-218.
- Zhang, J., Wang, J., Zhu, C., Singh, R.P., Chen, W., 2024. Chickpea: its origin, distribution, nutrition, benefits, breeding, and symbiotic relationship with *Mesorhizobium* species. *Plants* 13, 429.
- Zubairi, T., Jabeen, K., Khalid, S., Iqbal, S., 2021. Isolation and molecular characterization of causal agent of blue mold on *Allium cepa* L. and its control by *Pennistum flaccidum* Griseb. *Saudi Journal of Biological Sciences* 28, 6774-6781.
- Zucconi, F., Pera, A., Forte, M., DeBertolli, M., 1981. Evaluating toxicity of immature compost. *Biocycle*, 22, 54-57.