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AMELIORATIVE EFFECTS OF *Calotropis procera* AMENDED SOIL ON *Fusarium* WILT DISEASE, ENHANCEMENT IN GROWTH AND NUTRITIONAL QUALITIES IN PEA (*Pisum sativum*)

Muhammad AKBAR[1], Tayyaba KHALIL[1], Nasim Ahmad YASIN[2], Waheed AKRAM[3], Aqeel AHMAD[4], Muhammad Sajjad IQBAL[1]

[1]Department of Botany, University of Gujrat, Gujrat, 50700, Pakistan
[2]SSG, University of the Punjab, Lahore, Pakistan
[3]Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan
[4]Guangdong Key Laboratory for New Technology Research of Vegetables, Guangdong Academy of Agricultural Sciences, Guangzhou, 510640, China

Corresponding author email: makbarpu@gmail.com

Abstract

Commercial fungicides are effective to control fungal pathogens in agriculture but all are associated with ill effects. In multi-years pot and field trials, we investigated the disease suppressing efficacy of Calotropis procera against Fusarium oxysporum, the causal agent of wilt disease in pea. C. procera reduced negative effects of disease and resulted in 41.1 and 52.8% increase in shoot dry weight besides 94.8 and 84% improvement in root dry weight of pea plant, during years 1 and 2, respectively, in pot bioassays. Similarly, C. procera amendment increased 25.5% and 17.4% green pod yield under field conditions, in years 1 and 2, respectively. Incorporation of C. procera mulches in pea also improved proteins up to 64% and carbohydrates content up to 37.2%. Moreover, iron, calcium and potassium also showed an increased concentration in response to C. procera addition. The present study concluded that C. procera mulches can be used to manage Fusarium wilt disease and to improve nutritional traits of pea.

Key words*: nutritional, calotropis, fusarium, pisum, wilt.*

INTRODUCTION

Fungi that spoil foods or infect crops can have major socioeconomic impacts, posing threats to food security (Davies et al., 2021). Major food safety issues are related to fungal pathogens (Fisher et al., 2012). Pea (*Pisum sativum*) is a member of family fabaceae. Proteins and amino acid lysine are abundant in pea (Nawab et al., 2008). Higher amount of proteins, vitamins, dietary fibers, antioxidant and carbohydrates have made pea an excellent food source. Pea is among the major cultivated crops in Pakistan with 71,792 tons production over 10478 hectares, due to which it is considered as third most important crop in Pakistan (Achakzai et al., 2006). In Pakistan, pea production is facing many biotic and abiotic threats (Khan et al., 2016). Wilt is a crucial disease of peas in Pakistan (Nawab & Rashid, 2014). Fungal problems in pea are crucial and yield decline ranges between 50-75% (Fisher et al., 2012; Amian et al., 2011). Especially, the root diseases severely impede grain legume cultivation worldwide (Wille et al., 2019). *Fusarium oxysporum* is responsible for causing vascular wilt in many crops (Dean et al., 2012). *Fusarium* wilt, caused by *F. oxysporum* is a pervasive disease of pea in all pea cultivation fields resulting in complete failure of crop under conducive environmental conditions for the pathogen (Hafez et al., 2014; Aslam et al., 2019).

It is one of the most common fungal threats to pea production in Pakistan leading to substantial economic losses (Hafez et al., 2014).

Disease can be controlled by the use of several management schemes including biological, cultural, and chemical as well as by planting resistant varieties. The quickest and active method to control *Fusarium* wilt disease is with application of synthetic fungicides (Khan et al., 2016). The need of current increased food production to feed a fast-growing human populace is creating a pressure on the extreme use of pesticides but these agrochemicals are contaminating the environment and poisoning food products. Use of synthetic chemicals and

fungicides has been reported to cause serious threats to human life. Moreover, there are reports of fungal resistance against these fungicides. Environmental pollution and toxicity in food products posed by these chemical fungicides is another serious issue.

Use of weeds to overcome plant disease caused by fungi seems the economical and very promising strategy. In an investigation, compost tea and a commercial fungicide (Vitavax-T) was evaluated on controlling soil borne diseases such as wilt of pea plants (cv. Master-B) caused by *F. solani, F. moniliform, F. oxysporum* and *Macrophomina phaseolina*. These treatments significantly enhanced yield in greenhouse and field assays and protected the pea plants from fungal wilt diseases (Taha et al., 2017). *Calotropis procera* (Aiton) (Vern. apple of sodom, calotrope, and giant milkweed), family Apocynaceae, is an evergreen, perennial shrub, mainly found in arid and semi-arid regions. It is a multipurpose plant, which can be utilized for medicine, and phytoremediation. It has been widely used in traditional medicines (Kaur et al., 2021; Oliveira et al., 2021). Cysteine peptidases from *C. procera* latex were inhibitory (IC_{50} of 50 µg/mL) to *F. oxysporum* spores. These peptidases enhanced membrane permeabilization, changes in morphology, leakage of cellular contents, and induction of reactive oxygen species (ROS) in *F. oxysporum* spores (Freitas et al., 2020). *C. procera* contains glycosides (mostly cardenolides), flavonoids, triterpenes, alkaloids, steroids, saponins, proteins and enzymes. These phytochemicals have antioxidant, wound healing and antimicrobial activities (Amini et al., 2021).

The *n*-hexane extract of *C. procera* controlled *M. phaseolina*, the cause of charcoal rot in *Vigna radiata*. Spectroscopic analysis of the extract of *C. procera* revealed the presence of chlorocarbon, aromatic hydrocarbon, azocompounds, aromatic carboxylic acids and fatty acids (Waheed et al., 2016). In another investigation, plant extract of *C. procera* at 25% concentration inhibited the mycelium growth of *F. oxysporum* to 87% (Nasrin et al., 2018). There are number of reports describing the antifungal activity of *C. procera* against *F. oxysporum* under *in vitro* conditions, but, there is no report available that describes *C. procera* disease suppressing ability under pot and field conditions as well as its impact on nutritional values of pea grains. Therefore, the present study was planned to evaluate of disease suppressing ability of *C. procera* under pot and field conditions as well as nutritional enhancement in pea by an eco-friendly method.

MATERIALS AND METHODS

Test plant, fungal pathogen and crop

C. procera, member of family Asclepiadaceae was selected to evaluate the antifungal activity against selected fungal species, *F. oxysporum.* One pea variety "Meteor Faisalabad" was selected as test crop to evaluate the disease suppressing ability of *C. procera* as well as impact on the nutritional quality of pea grains.

Culturing of fungal isolate, storage and preparation of inoculum

The fungal culture was sub cultured on Potato Dextrose Agar (PDA) medium and kept in a refrigerator at 4°C till further use. Fungal inoculum was prepared on dried peas. These peas were washed in running tap water to remove dust or any other impurity with a final rinse with dH_2O. After washing, these peas were packed in plastic bags at 1 kg/bag. These peas were then soaked in water overnight and then autoclaved at 121°C for 20 minutes. Each bag of chickpea was inoculated with bits of fungal mycelia and spores and mixed well. The inoculated peas were left at 25°C for 21 days.

Processing of allelopathic weed

The *C. procera* plants growing wildly were uprooted at flowering stage. After collection, *C. procera* plants were cleaned under tap water to remove soil and other impurities and then put under fan to remove extra moisture. Then these plants were cut into smaller pieces (~ 3-4 cm) and sun dried for 1 week and then stored in paper bags for further use in pot and field experiments.

Pot experiments

Five treatments were made as T_1 (negative control; where neither fungal inoculum nor *C. procera* powder was added), T_2 (positive control; where only fungal inoculum was added), treatment T_3 comprised dead inoculum of *F. oxysporum*, while T_4 and T_5 comprised fungal inoculum along with 0.75% and 1.5%

(w/w) mulches of *C. procera,* respectively. Total 15 pots were made and for every treatment there were three replicates arranged in a Completely Randomized Design (CRD). 10 kgs of loam soil was put into each pot. Pots were frequently watered and left for two weeks before sowing of pea with hand hoeing twice. 10 seeds of pea were sown in each pot at equal distance from each other. After germination, 3 uniform seedlings were kept in each pot for growth and data collection and 10 chickpea seeds coated with fungal inoculum were agitated with 250 mL of autoclaved water for 5 minutes and this spore suspension was mixed with pot soil yielding ~ 2.1 x 10^4 colony forming units g^{-1} of soil as determined by dilution method.

Field experiments

Field experiments were carried out in two growing seasons. For field experiments, area with loam soil was chosen. Plots were prepared adopting all agronomic procedures. Seeds of pea were sown on hills of each plot at 2 seeds/hill. After germination, thinning was carried out for 1 pea plant/hill and total 25 seedlings in each plot were maintained. After maintaining equal number of pea seedlings in each plot, *F. oxysporum* inoculum at 1 kgs chickpeas inoculated with pathogen, *F. oxysporum* were agitated with 10 liters of autoclaved water for 5 minutes and this spore suspension was mixed with soil yielding approximately 3.6 x 10^4 colony forming units g^{-1} of pathogen as determined by dilution method. 2 kg of fungal inoculated soil was introduced in each plot and *C. procera* mulches were also gently mixed in soil through hand hoeing. There were 3 treatments: T_1 (negative control; where neither fungal inoculum nor *C. procera* was added), T_2 (positive control; where only fungal inoculum was added), and T_3 comprised fungal inoculum along with mulches of *C. procera* at 400 grams/plot. Treatments were arranged in Randomized Complete Block Design (RCBD) and each treatment was replicated thrice. Plots were watered as per requirement by visual observations. No fertilizer was added in any plot and weeds were removed by hand hoeing.

Data harvesting

Data were computed for after removing soil particles, rinsing in tap water and evaporation of excessive moisture. After this, all the plants were dried in sunlight for 3 days followed by final drying in an electrical oven at 65°C till constant weight and then dry biomass was recorded. Following parameters were recorded in pot experiments; shoot length (cm), root length (cm), shoot fresh weight (g), shoot dry weight (g), root fresh weight (g), and root dry weight (g); while in field experiments, following parameters were recorded viz. plant height (cm), Green pod yield (Mt. ha^{-1}), and 100 seed weight. The chemical composition of wheat grains was calculated following AOAC (1970). Carbohydrate content was determined by using the protocol as described by Watson et al. (1975). Concentrations of mineral elements were calculated by atomic absorption spectrophotometer. At harvest, composite soil samples for analysis were taken from every replicate/treatment.

Confirmation of Koch's postulates

From pathogenicity tests conducted in pot and field experiments, pathogen was reisolated and identified again with the help of colony morphology, texture, color as well as conidial size and shape.

Statistical analysis

Statistical analyses (ANOVA & Tukey's Test) were computed by using Minitab-19.

RESULTS AND DISCUSSIONS

Pot experiments

Shoot and root length

There was 37.5 and 39.4% decline in shoot length of pea in pot experiments during year 1 and 2, respectively. Soil amendment with *C. procera* plant powder significantly overcome the fungal attack and subsequently increased the shoot length of both pea varieties. There was a significant increase of 53, 62% increase in shoot length of pea by the introduction of *C. procera* mulches. There were non-significant effects by the application of dead *F. oxysporum* inoculum. However, the higher conc. of *C. procera* was found inhibitory to the growth of pea plants (Figure 1 A).

Effect of different treatments on root length of pea plant was also found significant as there was reduction of 40.1 and 40.8% in pots where

fungal inoculation was not accompanied by soil amendment with powder of *C. procera.* However, soil incorporation with different concentrations of *C. procera* significantly increased the growth parameter of pea. There was a significant increase of 69.3 and 78.3% in shoot length of pea plants during the year 1 and 2, respectively. While, inhibitory effects were encountered where higher concentration of *C. procera* mulches was investigated (Figure 1 B).

Shoot fresh and dry weight

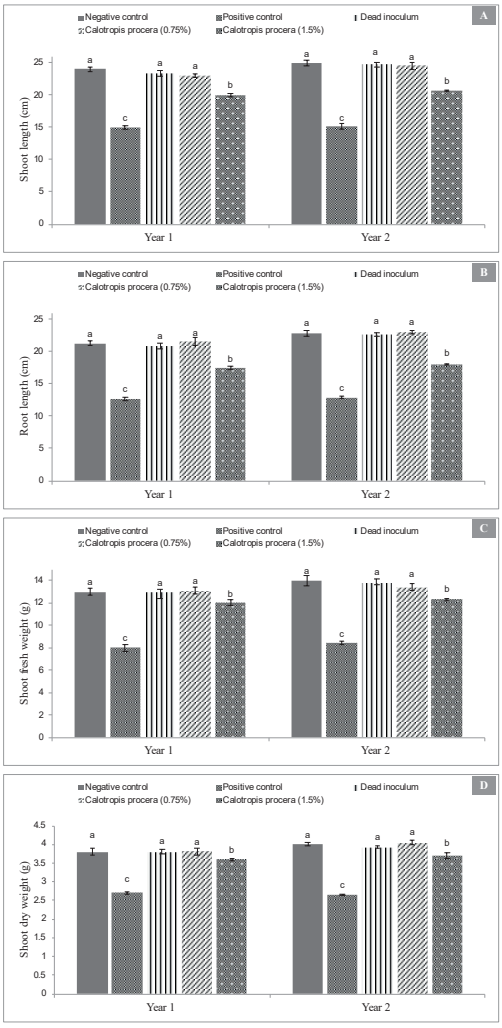
There was significant decrease of 38.5 and 40% in shoot fresh weight of pea in years 1 and 2, respectively. Soil treatment with *C. procera* significantly controlled *F. oxysporum* and ultimately increased the shoot fresh weight by 63.7 and 59.5% in pea plants, in study years 1 and 2, respectively. On the contrary, higher concentrations of *C. procera* were found inhibitory to the growth of pea plants (Figure 1 C).

Similarly, shoot dry weight also decreased in fungal inoculated pots where no *C. procera* treatment was employed and this decrease was 28.9 and 33.7% in years 1 & 2, respectively. Application of *C. procera* significantly increased the growth parameter of pea crop by 41 and 52.8%, in experiment years 1 & 2, respectively. The higher concentrations were found inhibitory to the growth of pea variety as there was a decrease of 5.6 and 8% in the shoot dry weight of pea against 1.5% *C. procera* conc. in years 1 & 2, respectively (Figure 1 D).

Root fresh and dry weight

There was significant decrease of 51.8 and 43% in root fresh weight of pea plants by the introduction of *F. oxysporum* inoculum, in years 1 & 2, respectively. Soil treatment with *C. procera* powder significantly controlled *F. oxysporum* and ultimately increased the root fresh weight by 111.3 and 72% in pea plant against conc. of 0.75% *C. procera* mulches. On the other hand, higher concentrations of *C. procera* were found inhibitory to the root growth of both pea plants. There was 22 and 41.2% decrease in root fresh weight of pea where 1.5% concentration of mulches was investigated and compared with negative control (Figure 1 E).

Likewise root dry weight of pea plants was reduced because of fungal inoculation. There was significant decrease of 48.9 and 46.8% in root dry weight in fungal inoculated pea plants, when compared with negative control. The harmful effect in pea plants was improved by soil application of *C. procera* plant powder. 0.75% *C. procera* significantly controlled wilt disease in both pea varieties and enhanced root dry weight by 94.8 and 84%, during years 1 and 2, respectively, but inhibitory trend was noted at higher employed concentrations of 1.5% where this concentration resulted in 24.4 and 19.1% decline in root dry weight, when compared with negative control (Figure 1 F).



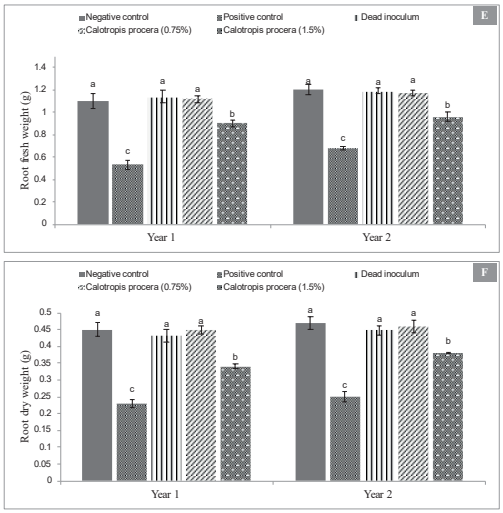


Figure 1 (A-F). Effect of inoculation of *Fusarium oxysporum* and different concentrations of *Calotropis procera* on pea plants, in pot experiments. Bars with similar letters show insignificant difference ($P \leq 0.05$), as computed by Tukey's test using, Minitab 19. Y error bars represent standard errors of means of 3 replications

Field experiments

Plant height

F. oxysporum significantly reduced the plant height of pea crop up to 35 and 31.3%, during field experiments of year 1 and 2, respectively. Application of *C. procera* mulches overcome the deleterious effects caused by fungal pathogen in pea crop. There was significant rise of 43.2 and 37.5% increase in plant height by the application of *C. procera* mulches, during the year 1 and 2, respectively (Figure 2 A).

Green pod yield

F. oxysporum significantly reduced the green pod yield of pea crop up to 21.6 % and 17.3%, in study years 1 and 2, respectively. Application of *C. procera* mulches overcome the deleterious effect caused by fungal pathogen in pea crop. There was significant increase of 25.5 and 17.4% in green pod weight of pea crop by the application of *C. procera,* during investigation years 1 and 2, respectively (Figure 2 B).

100 seed weight

F. oxysporum significantly reduced the 100 seed weight of pea crop up to 21.9 % and 15.8%, in study years 1 and 2, respectively. Application of *C. procera* mulches overcome the deleterious effect caused by fungal pathogen in pea crop. There was significant increase of 23.3 and 28.3% in 100 seed weight of pea crop by the application of *C. procera*, during investigation years 1 and 2, respectively (Figure 2 C).

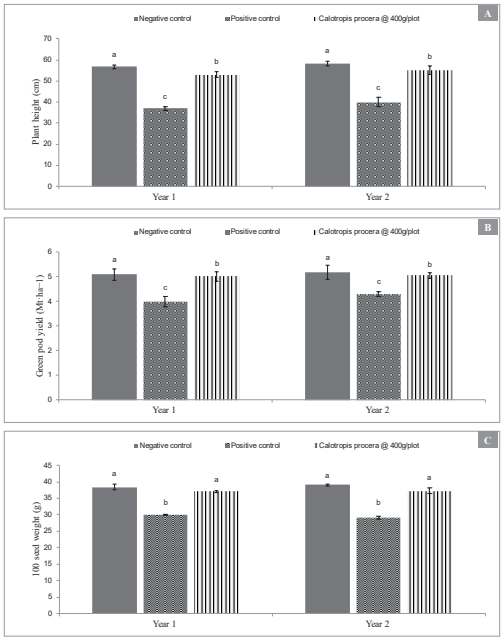


Figure 2 (A-C): Effect of inoculation of *Fusarium oxysporum* and *Calotropis procera* on pea crop, in field experiments. Bars with similar letters show insignificant difference ($P \leq 0.05$), as determined by Tukey's test using, Minitab 19. Y error bars represent standard errors of means of 3 replications

Nutritional analysis of pea grains

In this study, incorporation of *C. procera* mulches in pea also improved the nutritional characteristics of pea grains. Protein content was significantly increased to 55.8 and 64% when compared with plots having inoculation of *F. oxysporum*, during year 1 and year 2, respectively. Carbohydrate content also depicted a significant rise due to amendment of *C. procera* mulches in pea fields. There was 37.2 and 35.8% increase in the carbohydrate contents when compared with positive control plots, in years 1 and 2, respectively. Iron, calcium and potassium also showed an increased concentration in response to mycorrhizal inoculation. There was 20.3% and 15.4% rise in the concentration of iron and 44.4 and 48.3% rise in the concentration of calcium while there was 25 and 25.5% enhancement in the concentration of potassium, at site 1 and site 2,

respectively. The above mentioned values were calculated by comparing the rise in concentrations of proteins, carbohydrates, iron, calcium and potassium obtained in plots where *C. procera* was amended with diseased plant samples of positive control. However, the increase in concentrations of proteins, carbohydrates, iron, calcium and potassium of grains obtained from plots with *C. procera* amendments was less pronounced when compared with grains obtained from plants in negative control treatments, where neither *C. procera* amendments were made neither these plots were inoculated with *F. oxysporum* pathogen (Table 1).

Table 1. Nutritional analysis of pea grains in different treatments in field experiments

Treatments	Proteins (g/100 g)		Carbohydrates (g/100 g)		Iron (mg/100 g)		Calcium (mg/100 g)		Potassium (mg/100 g)	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
Negative control	5.1± 0.09b	5.3± 0.07b	13.6± 0.37b	14.2± 0.9b	1.39± 0.09a	1.43± 0.08a	22± 1.7b	23± 2.2b	232± 5.7c	240±5.0c
Positive control	4.3± 0.09c	4.2± 0.07c	11.3± 0.17b	12.0± 0.7c	1.23± 0.08b	1.3± 0.07b	18± 1.3c	18.2± 1.2c	212± 7.2b	216±9.1b
***Calotropis procera* at 400 g/plot**	6.7± 0.11a	6.9± 0.14a	15.5± 0.81b	16.3± 1.1a	1.48± 0.05a	1.5± 0.1a	26± 1.3a	27± 1.2a	265± 13.5bc	271±8.3b

Abbreviations used in Table 1: g. grams, mg. milli grams.

Note: Values are means of 3 replicates ± standard error. Standard error values were rounded off up to second decimal. Values sharing same letter do not differ at $P \leq 0.05$ as computed by ANOVA & Tukey's Test, using Minitab-19.

Fungal disease caused a significant decrease in shoot length of pea plants. Our results are in agreement with the findings of Abdel-Monaim et al. (2011) showing that the organic solvent extracts had reduced damping off and wilt disease in lupine plant. Likewise, the mulches of *C. procera* had improved the plant shoot and root length. Shoot fresh weight decreased significantly in pea plants, during the years 1 and 2, respectively. Lower concentration (0.75%) of *C. procera* increased the shoot fresh weight in years 1 and 2, respectively. On the other hand, higher concentration of *C. procera* negatively affected pea plants. Similar behavior was recorded in case of shoot dry weight of pea plants in both study years. This decline in growth of pea plants at higher concentration of *C. procera* can be attributed to allelopathic inhibition by *C. procera* and this effect was also reported by other workers.

Fungal inoculation also decreased root fresh and dry weight in pea plants. The allelopathic inhibition was observed at the highest concentration of 1.5% as this concentration significantly decreased the root growth as compared to fungal inoculated control. In pot experiments, a significant reduction in the growth of pea plants was recorded by the application of higher concentrations of *C. procera*. Gulzar and Siddiqui (2017) also reported that *C. procera* extracts reduced germination and growth of *Brassica oleracea*. The inhibitory effects increased with the increase in the concentration of extract. Similarly, another study showed that 7% and 10% aqueous extract of *C. procera* significantly declined seed germination and early seedling growth of *Triticum aestivum* under laboratory conditions. In contrast, the extract had no inhibitory effect on seed germination of *Hordeum vulgare* but significantly repressed its early seedling growth (Radwan et al., 2019).

In field assays, plant height of pea crop was significantly declined due to inoculation with *F. oxysporum*. In another study, it was observed that *Solidago canadensis* L. suppressed tomato crop pathogens like *Pythium ultimum* and *Rhizoctonia solani* (Zhang et al., 2009). Our result is in agreement with the findings of Abdel-Monaim et al. (2011) who revealed that organic solvent extracts had reduced damping off and wilt disease in lupine plants. *Lycium arabicum* is a source of antifungal compounds which suppressed *F. oxysporum* f. sp. *radicis-lycopersici.* Besides this, *L. arabicum* extracts enhanced tomato growth. Equally, water and organic solvent extracts of *L. arabicum* also reduced mycelial growth of pathogen (Nefzi et al., 2017). Root dry weight of pea plants probably reduced because of fungal inoculation.

In a previous study, crude water, ethanol and acetone extracts of *Adhatoda vasica*, *Jatropha curcas*, *Sapindus emarginatus* and *Vitex negundo* are reported to inhibit mycelial growth of *F. oxysporum* during *in vitro* experiments. Amongst these, *A. vasica* at 40% conc. exhibited complete inhibition of mycelial growth. Likewise, in pot experiments, water extract of six plant species reduced the disease symptoms of the eggplant and consequently improved the root and shoot growth of eggplant (Siva et al., 2008). Lupine (*Lupinus termis* Forsik) seeds treated with extracts of different plant species including *C. procera* overcome the effect of pathogens causing damping-off and wilt diseases. Moreover, under field conditions, the extracts of *Nerium oleander, Eugenia jambolana* and *Citrullus colocynthis* significantly reduced wilt disease as well as improved crop growth parameters (Abdel-Monaim et al., 2011). Peptide fraction from *C. procera* (PepCp) latex showed a low effect against the spore germination of both fungi *Colletotrichum gloeosporioides* and *Fusarium solani*. However, PepCp (1.25 mg mL^{-1}) inhibited the mycelial growth of *C. gloeosporioides* by 80% (Amaral et al., 2021). In another study, α-helical propeptides (SnuCalCpIs) from *C. procera* are antifungal against yeasts. The peptide, SnuCalCpI15, exhibited minimum inhibitory activity of 0.20±0.01, 0.20±0.01, 0.26±0.01, and 0.26±0.01 mM against *Candida albicans*, *Saccharomyces cerevisiae*, *Pichia anomala*, and *Rhodotorula mucilaginosa*, respectively. SnuCalCpI15 initially bound to yeast cell surfaces and then enter the cells and cause increased cell membrane permeability and alter cell wall thickness of yeast cells (Han et al., 2022). Similarly, the essential oils of *C. procera* exhibited significant antifungal activity against fungal species, *Trichophyton shoenlenii* and *Aspergillus fumigatus* (Al-Rowaily et al., 2020). *C. procera* extract is used as an alternative to fungicides due to its effectiveness on several pathogens, including *Fusarium oxysporum* f. sp. *lycopersici*. Ten isolates of the pathogens were obtained from diseased tomato plants. These isolates exhibited tomato wilt disease symptoms to variable extents, and isolate 5 exhibited the highest disease severity of 73%. Aqueous extract of *C. procera* was effective against fusarium wilt disease of tomato. This extract exhibited antifungal activity and also induced systemic resistance in tomato plants. All concentrations of *C. procera* extracts suppressed the growth of *F. oxysporum*. The 15% aqueous extract exhibited antifungal activity of 70%. In greenhouse experiments, the aqueous *C. procera* extract at 15% significantly reduced fusarium wilt disease of the tomato by 83.6%. This conc. of extract also significantly increased fresh and dry weight of tomato plants (g $plant^{-1}$) compared to inoculated plants to 86.6 and 120%, respectively. Treatment with extracts of *C. procera* also enhanced total phenolics, flavonoids and antioxidant enzymes in inoculated and non-inoculated tomato plants (Abo-Elyousr et al 2022).

CONCLUSIONS

Fusarium wilt disease substantially decreased the growth of pea plants. However, soil amendment with *C. procera* ameliorated the adverse effects of fungal attack resulting enhanced growth of applied plants. *C. procera* amendment increased 25% and 17.4% green pod yield under field conditions. Incorporation of *C. procera* mulches in pea also improved the nutritional characteristics of pea grains as proteins were significantly increased up to 64% and carbohydrates content also depicted a significant rise 37.2%. Moreover, iron, calcium and potassium also showed an increased concentration in response to *C. procera* addition.

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