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Full Length Article

Biological Management of Southern Blight of Chili by *Penicillium oxalicum* and Leaves of *Eucalyptus citriodora*

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Abstract

Southern blight is a devastating disease of chili (*Capsicum annuum* L.) that causes significant yield losses in the crop in many parts of the world. In the present study, leaf dry biomass of *Eucalyptus citriodora* (Hook) Hill & Johnson and a biological control agent *Penicillium oxalicum* Currie & Thom were evaluated for management of southern blight disease of chili. In a pot trial, soil was made sick with inoculum of *Sclerotium rolfsii* Sacc., the cause of southern blight disease. Soil was amended with dry leaf biomass of *E. citriodora* (1, 2 and 3%) and *P. oxalicum* alone or in combination. Soil amendment with 3% leaf biomass of *E. citriodora* significantly reduced plant mortality by 65% over positive control. Consequently, root and shoot growth, fruit yield as well as chlorophyll content were significantly enhanced. The effect of *P. oxalicum* alone was less pronounced and there was 32% reduction in plant mortality over positive control. However, this biological control agent in combination with 3% leaf biomass significantly reduced plant mortality by 54% and enhanced yield by 386% over positive control. Protein content in *P. oxalicum* inoculated treatments was also significantly higher than control. *S. rolfsii* inoculation significantly enhanced peroxidase (POX), phenylalanine ammonia-lyase (PAL), catalase (CAT) and polyphenole oxidase (PPO) activities in leaves of chili while these parameters were drastically reduced by application of *P. oxalicum* and leaf biomass of *E. citriodora.* In laboratory bioassay, different concentrations of methanolic leaf extract of *E. citriodora* viz. 1, 2, 5% were assessed against *in vitro* growth of *S. rolfsii.* There was 3–51% reduction in fungal biomass over control due to different concentrations of the extract. The effect of 3% and higher concentrations was significant as compared to control. In methanolic leaf extract, compounds namely 2-furancarboxaldehyde, 5-(hydroxymethyl) (**1**); eucalyptol (**2**); cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl (**3**); pentadecanoic acid, 14-methyl, methyl ester (**4**); and 14, 17-octadecadienoic acid, methyl ester (**5**) were identified through GC-MS analysis. The present study concludes that 3% leaf biomass of *E. citriodora* can significantly reduce plant mortality due to *S. rolfsii* and enhance plant growth and yield in chili.

Keywords: Chili; Biological control; Disease management; *Eucalyptus citriodora*; *Penicillium oxalicum*; Southern blight

Introduction

Chili (*Capsicum annuum* L.), family Solanaceae, is the fourth most important vegetable of the world and the first important cash crop of Asia (Osuna-Garcia *et al.* 1998). Its fruit is rich source of nutrition containing proteins, fats, carbohydrate and minerals, vitamins A, C and E (Bosland and Votava2000; Ismail *et al.* 2011). It is extensively used as spices in many national cuisines. Apart from its nutritional values, chili also has several pharmacological properties such as antioxidant (Park *et al.* 2012), antimicrobial (Yamasaki *et al.* 2011), anti-inflammatory (Luo *et al.* 2011), cardio-protective and anti-carcinogenic (Alonso-Castro *et al.* 2011). Southern blight disease of chili caused by *Sclerotium rolfsii* is a very important fungal disease of warm and moist climate accounts for an economic loss of 10–30% worldwide (Yaqub and Shahzad 2005; Chen *et al.* 2013; Ji *et al.* 2019). Disease has a huge significance in Pakistan as well (Hausbeck and Lamour 2004; Sana *et al.* 2017).

S. rolfsii is a well-known polyphagous soil-borne fungal pathogen causes diseases in a variety of crop plants, including 500 host species in 100 families of plants (Remesal *et al.* 2013). The diseases caused by *S. rolfsii* are severe in tropical, sub-tropical and warm temperate regions (Al-Askar *et al.* 2013). Mustard seed like sclerotia produced by pathogen are very resistant to degradation in soil and serve as inoculum for the next season and also help in spreading of the disease to other plants (Rekha *et al.* 2012). Sclerotia are produced by mass of hyphae and can survive in soil more than 7 years depending upon the conditions of environment (Yaqub and Shahzad 2008). Control of *S. rolfsii* has a limited success because of wide host range of the pathogen, extensive growth, and ability of the pathogen to produce plenty of resistant sclerotia (Sennoi *et al.* 2013). Generally, cultural practices and application of fungicides

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are applied for its management (Edmunds *et al.* 2003). Cultural practices are labour-demanding, expensive and some are not environment friendly such as residue burning (Edmunds *et al.* 2003). Fungicides such as thiram, oxicarboxin, mencozeb, quitozene, pentachloronitrobenzene (PCNB), captan, tebuconazole, carbendazin, benomyl and triadimenol are used to control *S. rolfsii* in many crops (Edmunds 2000; Zamora *et al.* 2008; Khan and Javaid 2015). However, fungicide application to the soil is not convenient in many landscape locations. Moreover, it is studied that in peanut fields the fungal isolates were tolerant to PCNB (Woodward *et al.* 2010). The use of extensive and repeating fungicides has posed severe hazards to human health and to the existing human ecogeographical environment (Awla *et al.* 2017).

Various recent studies have shown that natural products from plants and biological control agents such as *Trichderma harzianum* and *Penicillium oxalicum* can control fungal diseases both *in vitro* and *in vivo*, and can be used as alternatives to fungicides (Villarino *et al.* 2015; Javaid *et al.* 2017; Akhtar and Javaid 2018). Ethyl acetate fraction of methanolic shoot extract of *Coronopus didymus* completely arrested the *in vitro* growth of *S. rolfsii* and soil amendment with 3% dry biomass of the weed reduced incidence of southern blight of chili by 50% (Javaid and Iqbal 2014). Likewise, there was a significant reduction in disease incidence in chili and chickpea due to soil amendments with leaves of *Eucalyptus camaldulensis* and *Azadirachta indica*, respectively (Javaid and Khan 2016; Sana *et al.* 2016). It has also been found that plant dry biomass in combination with biological control agents such as *Trichoderma harzianum* gave better results in management of plant diseases than soil amendment with plant materials alone (Javaid *et al.* 2017; Munir *et al.* 2018). Most of the previous studies regarding biological control potential of *P. oxalicum* were carried out against wilt pathogens of tomato (Cal *et al.* 1997; 2000; Larena *et al.* 2001 2003; Sabuquillo *et al.* 2009), or against various isolates of *Fusarium oxysporum* (Cal *et al.* 2009). Studies about its antagonistic effect against *S. rolfsii* are lacking. The present study was, therefore, carried out to control southern blight disease of chili by extracts and dry biomass of *E. citriodora* and *P. oxalicum.*

Materials and Methods

Pot trial

For fungal inoculum preparation, 1 kg pearl millet seeds [*Pennisetum glaucum* (L.) R. Br.] were soaked in water for 2 h, mildly boiled and autoclaved for 50 min at 121^{o}C. After cooling at room temperature, inoculation was done aseptically using an actively growing *S. rolfsii* culture and incubated at 27^{o}C for 10 days. Same procedure was followed to prepare the inoculum of *P. oxalicum*, a biological agent.

For soil fumigation, formalin dipped cotton plugs were buried at different places in a soil heap and covered with plastic sheet for one week. After that plastic sheet and cotton plugs were removed from soil and left for two days for complete removal of formalin from the soil.

Earthen pots were filled with fumigated soil at 2 kg pot^{-1}. Pearl millet seeds based pathogen inoculum was thoroughly mixed in pot soil (10 g pot^{-1}) in all treatment pots except negative control where 10 g pot^{-1} autoclaved pearl millet seeds were mixed. After irrigation, pots were left for one week under natural environmental conditions for the establishment of fungal inoculums.

P. oxalicum was procured from Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan. It was sub-cultured on 2% malt extract agar and its mass culture was prepared on pearl millet seeds. After one week of pathogen inoculation, soil of respective pots was inoculated with *P. oxalicum* inoculum (10 g pot^{-1}). Pots were left for one week and watered. Dried leaf material of *E. citriodora* was mixed in soil of respective pots at 1, 2 and 3% (w/w). In positive control only *S. rlfsii* was inoculated while negative control treatment was without *S. rolfsii* inoculum, *P. oxalicum* inoculum and dry leaf amendment. Pots were left for one week and irrigated with tap water as per requirement (Javaid *et al.* 2017). There were following 9 treatments which were arranged in a completely randomized design using four replications:

T_1 Negative control
T_2 Positive control [only *S. rolfsii* (SR) was added]
T_4 SR+ 1% leaves of *E. citriodora*
T_4 SR+ 2% leaves of *E. citriodora*
T_5 SR+ 3% leaves of *E. citriodora*
T_6 SR+ *P. oxalicum*
T_7 SR+ *P. oxalicum* + 1% leaves of *E. citriodora*
T_8 SR+ *P. oxalicum* + 2% leaves of *E. citriodora*
T_9 SR+ *P. oxalicum* + 3% leaves of *E. citriodora*

Certified seeds of chili var. Tatapuri were procured from Ayub Agriculture Research Institute, Faisalabad. Healthy seed were sorted out carefully, surface sterilized with 1% NaOCl solution for 2 min followed by washing with sterilized water. Surface-sterilized seeds were sown in pots and chili nursery was raised. Eight seedlings with 4–5 leaves were transplanted in each pot, which were thinned to 6 seedlings per pot after establishment. Plants were harvested at maturity and data about plant mortality, shoot and root growth, and number and biomass of fruits were recorded.

Physiological tests of leaves of chili plants were done at flowering stage. These tests included total chlorophyll content (Arnon 1949), total protein content (Lowry *et al.* 1951), peroxidase (Kumar and Khan 1982), phenylalanine ammonia-lyase (Dickerson *et al.* 1984), catalase (Chance and Machly 1967) and polyphenole oxidase (Mayer *et al.* 1965).

Bioassays with methanolic leaf extract

Two hundred grams of dry powder of *E. citriodora* leaves were soaked in 1000 mL methanol in air tight plastic jars for two weeks at room temperature. After two weeks, soaked material was filtered first through cheese and then by filter paper. Filtrate was evaporated under vacuum in a rotary evaporator and 10.4 g crude methanolic extract of *E. citriodora* leaves were obtained (Javaid and Rauf 2015).

In vitro bioassay was carried out with methanolic extract of *E. citriodora* leaves. Crude methanolic extract (9 g) of leaves was dissolved in 5 mL DMSO (dimethyl sulfoxide) and adequate amount of autoclaved distilled water was added to prepare 15 mL of the stock solution. In a similar way, a control solution was prepared by adding 5 mL DMSO in 10 mL autoclaved distilled water. In conical flasks (250 mL), malt extract broth (55 mL) was autoclaved at 121^{o}C for 30 min. After cooling the autoclaved medium at room temperature, six concentrations *viz.*, 0, 1, 2, 3, 4, 5% were prepared by adding 0, 1, 2, 3, 4, 5 mL stock solution and 5, 4, 3, 2, 1, 0 mL control solution, respectively, to each flask to make total volume 60 mL that was then divided into three equal portions. Control treatment contained 5 mL of control solution in 55 mL of growth medium. Inoculation was done aseptically using 5 mm fungal plug. After one week incubation at 28^{o}C, fungal biomass was filtered, dried at 70^{o}C and weighed (Javaid and Akhtar 2015).

GC-MS analysis

Perkin Elmer Turbo Mass Spectrophotometer (Norwalk, CTO6859, and USA) was used for GC-MS analysis. The instrument comprised of a Perkin Elmer Auto sampler XLGC with Perkin Elmer Elite -5 capillary column (measuring 30 m × 25 mm with film thickness of 0.25 mm) that was composed of 95% dimethyl polysiloxane. Helium was used as a carrier gas (flow rate of 0.5 mL min^{-1}). The instrument was set to inlet temperature at 250^{o}C. Oven was preset as 110^{o}C for 4 min, rose up to 280^{o}C and run time was finished in 90 min. The temperature of MS transfer line was kept at 200^{o}C and that of the source was maintained at 180°C. Volume of sample that was used in injection 1 μL. For compound identification electron impact ionization (70 eV) was utilized and data was assessed through total ion count (TIC). The acquired spectrums of the components were examined with the databank of known components spectrum. Turbo-Mass-OCPTVS-Demo SPL software was used to measure peak areas and data processing (Aneesh *et al.* 2013).

Statistical analysis

All the data were analyzed by applying ANOVA followed by LSD test to delineate treatment means at $P \leq 0.05$ using Statistix 8.1 software.

Results

Effect of pathogen and soil amendments on plant mortality

No plant mortality was found in negative control treatment. The highest mortality (37%) was recorded in positive control where *S. rolfsii* was inoculated without any soil amendment. Application of different doses of dry leaf biomass of *E. citriodora* reduced mortality to 13–21% that was significantly lower than positive control by 43–65%. In general, plant mortality was gradually reduced by increasing dose of leaf biomass as soil amendment. The effect of application of biological control agent *P. oxalicum* on plant mortality was less pronounced than application of *E. citriodora* leaf biomass. In *P. oxalicum* inoculated treatment, plant mortality was 25% that was reduced to 21 and 17% when *P. oxalicum* inoculation was done in combination with 2 and 3% leaf biomass, respectively (Fig. 1).

Effect of pathogen and soil amendments on plant growth

The effect of the pathogen inoculation on shoot length was not significant. However, shoot fresh and dry biomasses were significantly reduced by 13 and 30%, respectively, over negative control due to *S. rolfsii* inoculation. Application of *E. citriodora* leaf biomass significantly enhanced different shoot growth parameters over positive control. The effect of dry leaf amendment was dose dependant. The highest shoot dry biomass (18.5 g pot^{-1}) was recorded in treatment where 3% leaf biomass was added to *S. rolfsii* inoculated soil that was 164 and 85% higher than positive and negative control treatments, respectively. Inoculation of *P. oxalicum* significantly increased length, fresh weight and dry weight of shoot by 7, 23 and 57%, respectively, over positive control. *P. oxalicum* in combination with different doses of leaf biomass of *E. citriodora* further improved shoot growth. However, in general, the effect of combined application of *P. oxalicum* and *E. citriodora* leaf biomass was less pronounced than sole application of leaf biomass (Fig. 2 A&B).

S. rlfsii had nonsignificant effect on root biomass. Application of different doses of *E. citriodora* leaf biomass markedly enhanced root fresh and dry biomass. The effect of 3% leaf amendment significantly enhanced ($P \leq 0.05$) root dry biomass by 15 and 19% as compared to negative and positive control treatments, respectively. The effect of *P. oxalicum*, either alone or in combination with different doses of *E. citriodora* leaf biomass on root biomass was nonsignificant (Fig. 2C).

Effect of pathogen and soil amendments on yield

S. rolfsii markedly reduced number, fresh weight and dry weight of chili fruits by 55, 67 and 44%, respectively, over negative control treatment. Application of *E. citriodora* leaf biomass markedly alleviated the biotic stress of *S. rolfsii* and improved chili yield. The highest increase in number of fruit

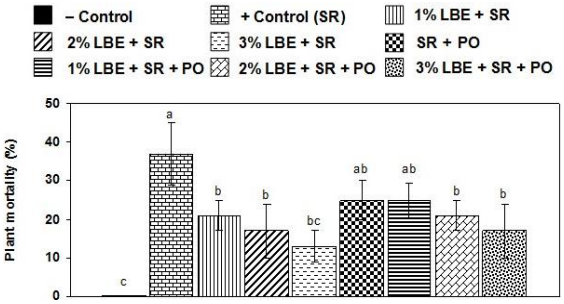


Fig. 1: Effect of *S. rolfsii* (SR), dry leaf biomass of *E. citriodora* (LBE) and *P. oxalicum* (PO) on mortality of chili plants. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

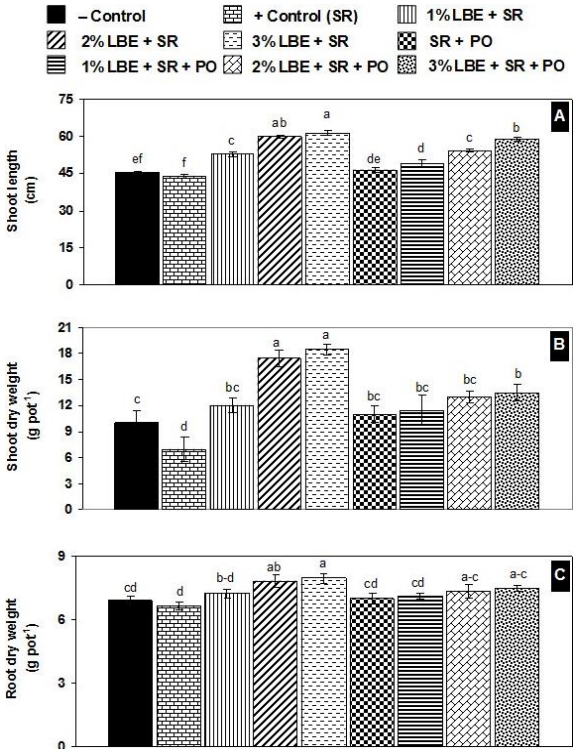


Fig. 2: Effect of *S. rolfsii* (SR), dry leaf biomass of *E. citriodora* (LBE) and *P. oxalicum* (PO) on shoot and root growth of chili. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

over positive control (343%) was recorded due to 3% leaf amendment. However, maximum increase in fresh (277%) and dry biomass (240%) of chili over positive control was recorded due to 2% leaf amendment. Application of *P. oxalicum* had no effect on fruit yield. *P. oxalicum* in combination with 2 and 3% leaf amendment significantly improved fruit yield over positive control. However, the effect of combined application was less pronounced as compared to leaf amendment alone (Fig. 3).

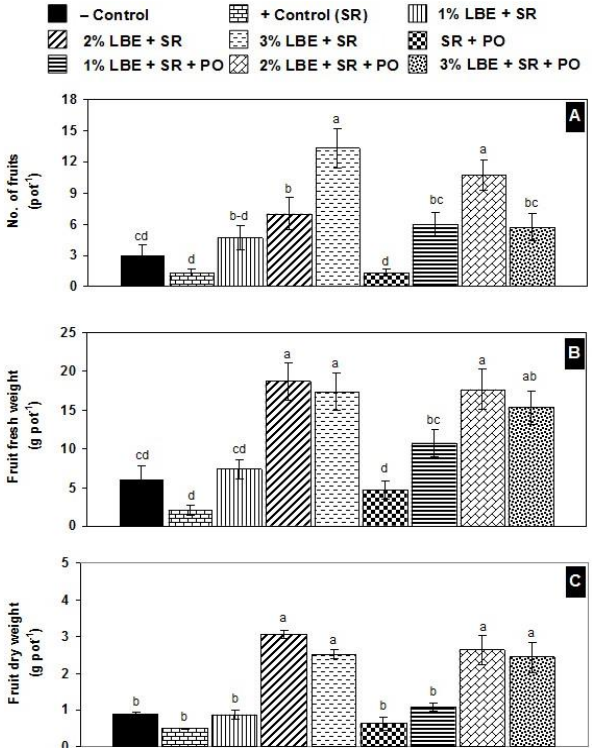


Fig. 3: Effect of *Sclerotium rolfsii* (SR), dry leaf biomass of *Eucalyptus citriodora* (LBE) and *Penicillium oxalicum* (PO) on yield of chili. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

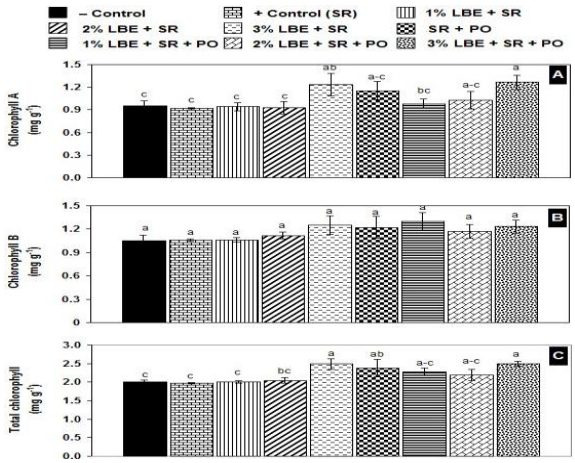


Fig. 4: Effect of *S. rolfsii* (SR), dry leaf biomass of *E. citriodora* (LBE) and *P. oxalicum* (PO) on chlorophyll content of chili. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

Effect of pathogen and soil amendments on plant physiology

Chlorophyll A content remained unaffected due to inoculation of *S. rolfsii.* Application of 1 and 2% *E.*

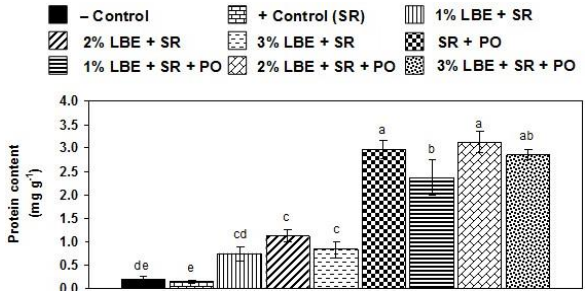


Fig. 5: Effect of *S. rolfsii* (SR), dry leaf biomass of *E. citriodora* (LBE) and *P. oxalicum* (PO) on protein content of chili. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

citriodora leaf biomass or *P. oxalicum*, either alone or in combination, also exhibited nonsignificant effect on this studied parameter. On the other hand, 3% leaf biomass alone or in combination with *P. oxalicum* significantly enhanced chlorophyll A content by 30 and 33% over negative control, and 35 and 38% over positive control, respectively (Fig. 4A). The effect of different treatments on chlorophyll B was nonsignificant (Fig. 4B). The effect of various soil amendment treatments on total chlorophyll content was generally similar to their effect on chlorophyll A (Fig. 4C).

The lowest protein content was recorded in negative (0.206 mg g^{-1}) and positive (0.144 mg g^{-1}) control treatments. Different doses of *E. citriodora* leaf biomass enhanced protein content by 418–681% over positive control. *P. oxalicum* inoculation significantly enhanced protein content by 1956% over positive control. Likewise, combined application of *P. oxalicum* and different doses of leaf biomass also enhanced protein content by 1538–2070% as compared to positive control (Fig. 5).

In negative control, PPO activity was 0.0285 units min^{-1} mg^{-1} protein that was significantly increased to 0.0358 units min^{-1} mg^{-1} protein due to *S. rolfsii* inoculation in positive control. Application of different doses of *E. citriodora* leaf biomass significantly lowered this parameter to 0.0040–0.0087 units min^{-1} mg^{-1} protein. *P. oxalicum* inoculation further reduced PPO activity to 0.0011 units min^{-1} mg^{-1} protein. In a similar way, PPO activity was also very low in treatments where *P. oxalicum* inoculation was done in combination with different doses of *E. citriodora* leaf biomass and was in the range of 0.0005–0.0019 units min^{-1} mg^{-1} protein (Fig. 6A).

The effect of different treatments on POX activity was similar to the effect of these treatments on PPO activity. It was high in negative control (25.183 units min^{-1} mg^{-1} protein) and the highest in positive control (37.14 units min^{-1} mg^{-1} protein). Application of *E. citriodora* leaf biomass either alone or in different combinations drastically lowered POX activity to 1.777–7.990 units min^{-1} mg^{-1} protein (Fig. 6B).

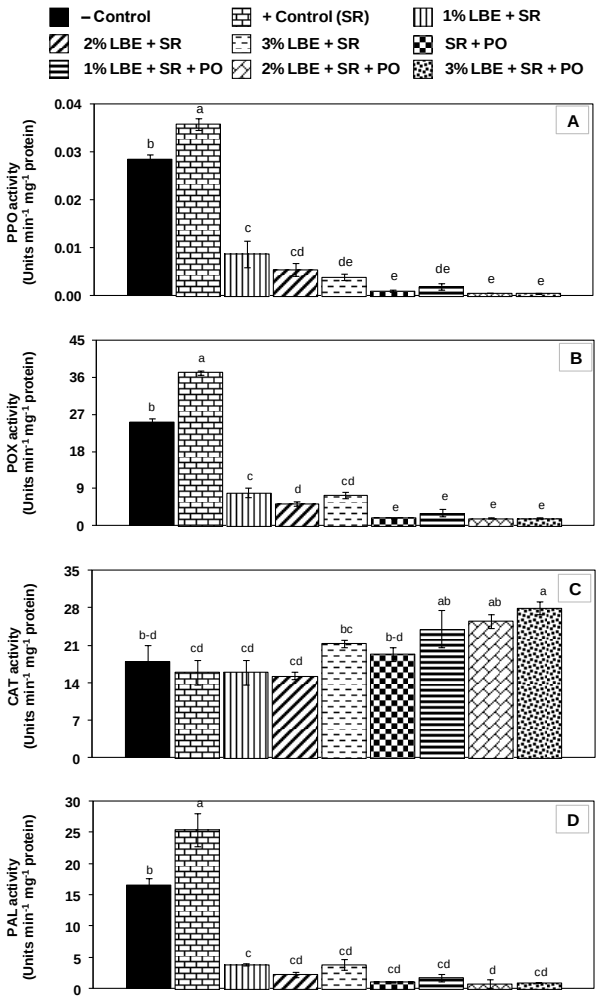


Fig. 6: Effect of *S. rolfsii* (SR), dry leaf biomass of *E. citriodora* (LBE) and *P. oxalicum* (PO) on polyphenol oxidase (PPO), peroxidase (POX), catalase (CAT) and polyphenol ammonia lyase (PAL) activities of chili. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

CAT activity in negative control was 16.58 units min^{-1} mg^{-1} protein that was significantly increased to 25.47 units min^{-1} mg^{-1} protein due to *S. rolfsii* inoculation. The effect of *E. citriodora* leaf biomass and *P. oxalicum* on CAT activity was similar to that of PPO and POX (Fig. 6C).

The effect of *S. rolfsii*, *E. citriodora* leaf biomass and *P. oxalicum* on PAL activity was in line with their effects on POX, PPO and CAT activity where *S. rolfsii* significantly enhanced PAL activity over negative control, while rest of the treatments drastically reduced this parameter (Fig. 6D).

In vitro antifungal activity of methanolic leaf extract of *E. citriodora*

The highest fungal biomass (0.188 g) was recorded in

control. All the extract concentrations variably reduced fungal biomass over control. The effect of 3% and higher concentrations was significant as compared to control (Fig. 7A). There was 3 to 51% reduction in fungal biomass over control due to different concentrations of methanolic leaf extract of *E. citriodora.* In general, fungal biomass was gradually decreased as the concentration of methanolic leaf extract was increased from 1 to 5%. There was a linear relationship between extract concentration and fungal biomass with $R^2 = 0.9749$ (Fig. 7 B&C).

GC-MS analysis of methanolic leaf extract of *E. citriodora*

Data regarding GC-MS analysis of methanolic leaf extract of *E. citriodora* are presented in Fig. 8 and Table 1. Five compounds namely 2-furancarboxaldehyde, 5-(hydroxymethyl) (**1**); eucalyptol (**2**); cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl (**3**); pentadecanoic acid, 14-methyl, methyl ester (**4**); and 14, 17-octadecadienoic acid, methyl ester (**5**) were identified at retention times 8.870, 9.833, 10.237, 16.112 and 17.833 min; having peak areas 21.929, 7.859, 15.763, 4.378 and 50.070%, respectively. Structures of these compounds are presented in Fig. 9.

Discussion

In laboratory bioassay, 3% and higher concentration of methanolic leaf extract of *E. citriodora* significantly suppressed growth of *S. rolfsii.* Methanolic leaf extract of *E. citriodora* also inhibited growth of *Macrophomina phaseolina* (Javaid and Rehman 2011), *Alternaria alternata* (Javaid and Samad 2012), *Fusarium oxysporum* f. sp. *capsici* (Shafique *et al.* 2015), *Aspergillus fumigatus, A. flavus, A. nidulans* and *A. terreus* (Javed *et al.* 2012). Likewise, aqueous leaf extract of *E. citriodora* markedly declined growth of *Ascochyta rabiei* and *A. flavus* (Jabeen and Javaid 2008; Iram *et al.* 2018). In pot trial, different doses of dry leaf biomass of *E. citriodora* reduced plant mortality by 43–65% over positive control. Consequently, there was an increase in plant growth and yield. Essential oils of *E. citriodora* leaves are generally considered to be responsible for *in vitro* and *in vivo* antifungal activity (Abdul-Majeed *et al.* 2017). Major components of essential oil are citronellal, citronellol, Terpinen-4-ol and eucalyptol (Morcia *et al.* 2012; Tolba *et al.* 2015; Salem *et al.* 2018), which could be responsible for antifungal activity.

In the present study, *P. oxalicum* inoculation reduced plant mortality by 32% over positive control. It is one of the significant biocontrol agents that have the ability to control a large number of fungal plant pathogen (Villarino *et al.* 2015). It successfully controlled vascular wilt disease of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium* spp., both under field conditions and glass house (Larena *et al.* 2003), Fusarium wilt of watermelon and melon caused by *F. oxysporum* f. spp. *niveum* and *F. oxysporum* f. spp. *melonis,* respectively (Cal *et al.* 2009).

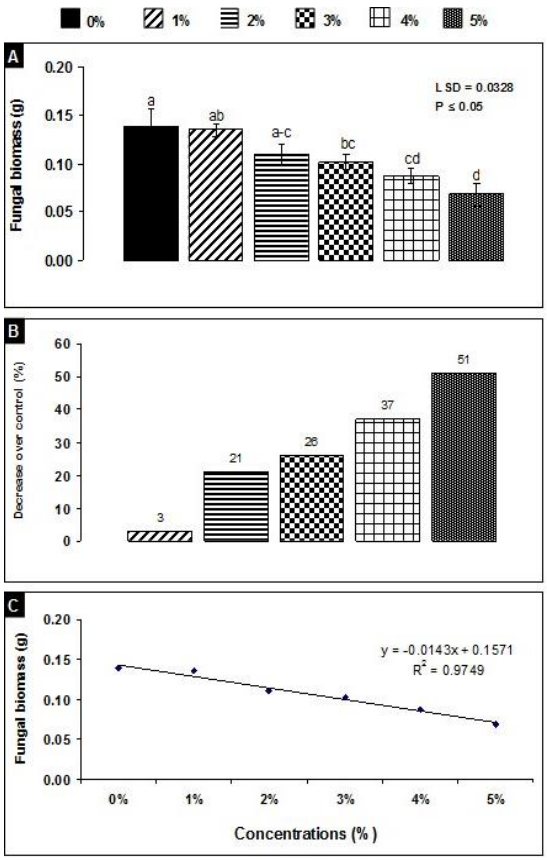


Fig. 7: A: Effect of different concentrations of methanolic leaf extract of *Eucalyptus citriodora* on biomass of *Sclerotium rolfsii.* **B:** Percentage reduction in fungal biomass due to extracts over control. **C**: Linear regression between extract concentration and fungal biomass. Vertical bars show standard errors of means of four replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by LSD Test

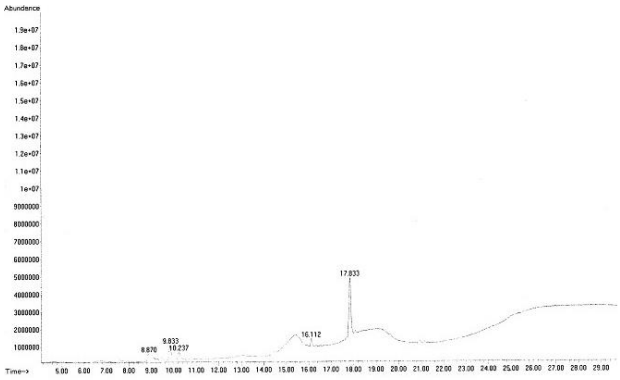
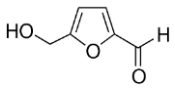


Fig. 8: GC-MS chromatogram of methanolic leaf extract of *Eucalyptus citriodora*

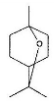
Similarly, application of *P. oxalicum* significantly reduced number of conidia of *Colletotrichum coccodes,* the cause of black dot disease of potato (Farber *et al.*

Table 1: Compounds identified from methanolic leaf extract of *E. citriodora* through GC-MS analysis

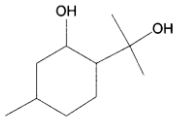
Comp. No.	Names of compounds	Retention time (min)	Molecular formula	Molecular weight	Peak area (%)
1	2-Furancarboxaldehyde, 5-(hydroxymethyl)	8.870	$C_6H_6O_3$	126	21.93
2	Eucalyptol	9.833	$C_{10}H_{18}O$	154	7.859
3	Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl	10.237	$C_{10}H_{20}O_2$	172	15.76
4	Pentadecanoic acid, 14-methyl, methyl ester	16.112	$C_{17}H_{34}O_2$	270	4.38
5	14, 17-Octadecadienoic acid, methyl ester	17.833	$C_{19}H_{34}O_2$	294	50.07



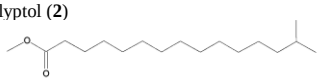
2-Furancarboxaldehyde, 5-(hydroxymethyl) (**1**)



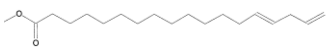
Eucalyptol (**2**)



Cyclohexanol, 2-(2-hydroxy-2-propyl)-5-methyl (**3**)



Pentadecanoic acid, 14-methyl, methyl ester (**4**)



14, 17-Octadecadienoic acid, methyl ester (**5**)

Fig. 9: Structures of compounds identified from methanolic leaf extract of *E. citriodora* through GC-MS analysis

2018). The chief mechanism of action of *P. oxalicum* against Fusarium wilt pathogen was the induction of resistance in the tomato plants (Cal *et al.* 1997). Later on, Larena *et al.* (2001) established that *P. oxalicum* induces a generalized response instead of conferring specific resistance against a specific pathogen when applied to tomato plants. Induction of resistance was linked with renewed or prolonged cambial activity that resulted in the development of extra secondary xylem (Cal *et al.* 2000).

Inoculation of pathogen had insignificant effect on chlorophyll content. However, application of 3% leaf biomass and *P. oxalicum* either separately or in combination enhanced this studied parameter significantly over control. Variations in total content of chlorophyll after application of biofungicides could be result of altered stomatal conductance, source-sink balance and rubisco activity (Kasai *20*08). The chief mechanism of action of *P. oxalicum* against pathogens is the induction of resistance in the host plant (Cal *et al.* 1997).

Low protein content in infected plants after 45 days of inoculation revealed high level of susceptibility in chili. It might be ascribed to denaturation or breakdown of proteins, as well as polypeptide chains and bound amino acids due to negative consequences of pathogen on plant (Chatterjee and Ghosh 2008). Increase in the total protein content after application of biofungicides is supported by the results of El-Khallal (2007). This increase could be the result of positive action of biofungicides on DNA-RNA synthesizing protein machinery at transcriptional and/or translocational levels (El-Bahay and Moursy 2003). Protein synthesis could be related with the increase of the demand for substrates, necessary to the production of plant defense mechanisms induced by biofungicides treatment.

Plants respond to pathogen attack through preexisting physical and chemical barriers and inducible defense responses that interfere with pathogen establishment (Vanitha *et al.* 2009). Inducible defense responses include many defense related enzymes like POX, CAT, PPO and PAL. POX and PAL are associated with induction of systemic resistance in plant. POX decomposes indole-3-acetic acid (IAA) and is involved in the lignification, suberification, polymerization of hydroxy-proline-rich glycoproteins, regulation of cell wall elongation, wound healing and resistance against pathogens in plants by consuming hydrogen peroxide in different cell components (Anuradha *et al.* 2015). PAL activity catalyze conversion of phenylalanine to transcinnamic acid, a key intermediate in the synthesis of salicylic acid, general response associated with resistance against pathogen attack (Ramamoorthy *et al.* 2002). CAT is an important oxygen-scavenging enzyme that helps in specific peroxidative against toxic hydrogen peroxide (Hameed and Iqbal 2014). In the present research, enzymes activities were increased in inoculated treatments (positive control), which indicated increased stress conditions in plants due to production of reactive oxygen species (ROS). The POX and PAL activities were improved in pepper roots during interactions with *Verticillium dahliae* (Idoia *et al.* 2006). Likewise, activities of many defense related enzymes including POX and CAT were found to increase in soybean after inoculation with fungus *Corynespora cassiicola* (Fortunato *et al.* 2015). Application of different doses of *E. citriodora* leaf biomass and *P. oxalicum* inoculation deceased enzymes activities, which may suggested optimizing the protein synthesis and plant resistance response. *P. oxalicum* combined with *E. citriodora* leaf biomass treatment

however resulted in the lowest plant mortality with improved growth and yield could be ascribed to induction of desirable level of enzymes activity in chili plant.

The most abundant compound **5** as well as compound **4** identified in methanolic leaf extract are fatty acid methyl esters. Compounds of this group are known to possess antifungal activity (Agoramoorthy *et al.* 2007; Ali *et al.* 2017). Fatty acid methyl ester extract of *Salicornia brachiata*, rich in lauric acic (61.85%), showed remarkable antifungal activity against *Candida parapsilosis* and *Candida albicans* (Chandrasekaran *et al.* 2007). Sunflower fatty acid methyl esters showed antifungal activity against *Candida parapsilosi* and *C. glabrata* with MIC of 31.2 μg mL^{-1} and 15.6 μg mL^{-1}, respectively (Pinto *et al.* 2017). Mixtures of fatty acid methyl esters from soybean oil showed potent antifungal activity against *Blumeria graminis* f. spp. *hordei*, the cause of powdery mildew in barley (Choi *et al.* 2010). Likewise, fatty acid methyl esters present in *Annona cornifolia* seeds and *Excoecaria agallocha* have been reported to suppress growth of fungi namely *Paracoccidioides brasiliensis*, Candida *parapsilosis*, *C. krusei* and *C. albicans* (Agoramoorthy *et al*. 2007; Lima *et al*. 2011). The second most abundant compound 2-furancarboxaldehyde, 5-(hydroxymethyl) (**1**) is present in sugar containing processed foods and drinks such as juice, cookies, dried fruits, honey, beverages, coffee and bread (Gao *et al.* 2015). This compound is known to inhibit fermentation process of *Saccharomyces cerevisiae* (Akıllıoglu *et al.* 2011). Eucalyptol, identified as compound **2** in the present study, is the major component of Eucalyptus oil and is known to possess antifungal activity against many fungi includng *Fusarium subglutinans, F, sporotrichioides, F. cerealis, F. proliferatum Alternaria alternata, Penicillium* spp. *Aspergillus tubingensis*, and *A. carbonarius* (Morcia *et al.* 2012)

Conclusion

The present study concludes that application of 3% leaf biomass of *E. citriodora* can significantly reduce plant mortality due to *S. rolfsii* and enhance crop growth and yield in *S. rolfsii* inoculated soil. Sole inoculation of *P. oxalicum* reduced plant mortality by 32%. Methanolic leaf extract of *E. citriodora* has antifungal potential against *S. rolfsii*. Fatty acid methyl esters and eucalyptol may be responsible for antifungal activity against the pathogen.

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