Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Action mechanisms and biocontrol of *Purpureocillium lilacinum* against green mould caused by *Penicillium digitatum* in orange fruit

E.A. Elsherbiny[1], M.A. Taher[2], M.H. Abd El-Aziz[3] and S.Y. Mohamed[4]

1 Plant Pathology Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt
2 Agricultural Chemistry Department, Faculty of Agriculture, Mansoura University, Mansoura, Egypt
3 Department of Genetics, Faculty of Agriculture, Mansoura University, Mansoura, Egypt
4 Horticulture Research Institute, Agricultural Research Center, Cairo, Egypt

Keywords
biological control, defence-related enzymes, fungal metabolites, gene expression, orange green mould, *Purpureocillium lilacinum*.

Correspondence
Elsherbiny A. Elsherbiny, Plant Pathology Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt.
E-mail: sherbiny@mans.edu.eg; sherbiny44@hotmail.com

2021/2497: received 20 November 2020, revised 20 January 2021 and accepted 21 January 2021

doi:10.1111/jam.15016

Abstract

Aims: The present study evaluated, for the first time, the inhibitory effects of the filtrate of *Purpureocillium lilacinum* against *Penicillium digitatum*.
Methods and Results: No direct contact between *P. lilacinum* and *P. digitatum* was observed during the dual culture test and the inhibition zone was 6·1 mm. The filtrate of *P. lilacinum* completely inhibited *P. digitatum* growth and spore germination at the concentration of 64%. The filtrate increased the permeability of the cell membrane and the content of MDA in *P. digitatum*. The ergosterol content in *P. digitatum* was strongly inhibited at 32% by 81·1%. The green mould incidence and severity in filtrate-treated fruit at 64% were 71·7 and 80·7% lower than in the control, respectively. The filtrate enhanced the activity of PAL, PPO and POD enzymes in orange fruit. The POD and PAL gene expression levels were significantly upregulated in the fruit treated with the filtrate.
Conclusions: This study indicated that the antifungal mechanism of *P. lilacinum* filtrate against *P. digitatum* is mainly by the damage of the fungal cell membrane and its components.
Significance and Impact of the Study: This work provides the pioneer evidence on the application of *P. lilacinum* filtrate as a novel biocontrol agent for orange green mould.

Introduction

Green mould is the most economically significant postharvest disease of orange caused by *Penicillium digitatum* in several regions of the world and it is responsible for 90% of orange losses during storage, transport and marketing (Costa *et al.* 2019). *Penicillium digitatum* is a filamentous ascomycete that infects the citrus fruit during harvesting and postharvest handling and does not cause the mould symptoms as a natural infection in any other fresh fruit (López-Pérez *et al.* 2015). This fungal pathogen produces abundant amounts of conidia as asexual spores about 1–2 billion on one fruit during 3–5 days as a disease cycle at 25°C, which readily spread by air currents or water (Holmes and Eckert 1999). Synthetic fungicides such as imazalil, fludioxonil, thiabendazole, pyrimethanil or different mixtures of these chemicals are the main components used for controlling the green mould in citrus fruit (Youssef *et al.* 2014; Costa *et al.* 2019). However, the prolonged and excessive applications of these fungicides are becoming increasingly restricted because of the toxicity on human health and the environmental hazards as well as the development of resistant strains to many fungicides (Youssef *et al.* 2014). Therefore, alternative approaches and effective strategies are needed for controlling the postharvest diseases, including physical methods, generally regarded as safe compounds, and biological control as well as improving the natural defence efficiency of the fruit either as alone or combined treatments (Talibi *et al.* 2014; Palou *et al.* 2016).

Several biological control agents such as yeasts, bacteria and fungi are implemented to control *P. digitatum* in

citrus fruit (Borras and Aguilar 1990; Benhamou 2004; Calvo *et al.* 2017; Ma *et al.* 2019). Although yeasts and bacteria have received more attention as biocontrol agents against *P. digitatum* in postharvest citrus, only a very few fungal strains were evaluated as biocontrol agents in inhibition of *P. digitatum* on citrus fruit, including *Aureobasidium pullulans*, *Muscodor albus* and *Verticillium lecanii* (Talibi *et al.* 2014). For the fungus *Purpureocillium lilacinum*, formerly named as *Paecilomyces lilacinus*, there is no obvious line of evidence regarding its efficacy as a fungal antagonist on controlling *P. digitatum* in citrus fruit until this date. *P. lilacinum* is mainly considered as a biocontrol agent against plant-parasitic nematodes, especially economically significant species of *Meloidogyne incognita* (Hajji *et al.* 2017). This fungus has been also evaluated for the biological control of different species of insects, including cotton aphid, greenhouse whitefly, glasshouse red spider mite, leaf-cutting ant and western flower thrips (Fiedler and Sosnowska 2007; Goffré and Folgarait 2015; Elbanhawy *et al.* 2019). Besides, *P. lilacinum* was reported as a promising agent in the biocontrol of some fungal plant pathogens such as *Sclerotinia sclerotiorum* on oilseed rape (Yang *et al.* 2015) and common bean (Elsherbiny *et al.* 2019), *Verticillium dahliae* in eggplant (Lan *et al.* 2017), *Phytophthora capsici* in pepper (Hu *et al.* 2020) and *P. infestans* (Wang *et al.* 2016). Moreover, *P. lilacinum* is capable of producing different types of bioactive secondary metabolites such as leucinostatins, named as paecilotoxins (Park *et al.* 2004; Prasad *et al.* 2015), and hydrolysing enzymes, including serine protease, chitinase, lipase and esterase (Khan *et al.* 2003; Dahlin *et al.* 2019).

Consequently, this study is the first report on applying the fungus *P. lilacinum* and its culture filtrate as a novel and promising biocontrol agent against *P. digitatum* on orange fruit. The specific objectives were (i) to investigate the biocontrol activity of *P. lilacinum* and its filtrate on *P. digitatum in vitro* conditions and for controlling the orange green mould, (ii) to determine the changes in the biochemical properties of *P. digitatum* after treatment with the filtrate and (iii) to estimate the induction of the defence-related enzymes activity such as phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenoloxidase (PPO) with the changes of gene expression level after application of the filtrate on orange fruit.

Materials and methods

Pathogen

The fungal pathogen *P. digitatum* was isolated from naturally infected orange fruit with typical green mould symptoms and maintained on potato dextrose agar (PDA). The pathogen identification was confirmed based on morphological properties of cultures, conidia and conidiogenous as well as the growth features after inoculation on healthy orange fruit according to Pitt and Hocking (2009). Spores were gathered in sterile distilled water with 0·01% Tween 80 from 7-day-old culture grown at 25°C. The spore concentration was adjusted to 1×10^6 conidia per ml with the aid of a haemocytometer.

Dual culture test

A 5-mm disk of *P. lilacinum* (AUMC 8824, Assiut University Mycological Centre) was placed at a distance of 1 cm from the edge of the PDA plates. After 4 d, a disk of *P. digitatum* (5 mm diameter) was transferred to the opposite side of the PDA plates due to the slow growth of the fungus *P. lilacinum*. After 10 days at 25°C, the inhibition zone (IZ) was determined at the shortest distance between *P. lilacinum* and *P. digitatum* cultures. The growth inhibition of *P. digitatum* was calculated according to Royse and Ries (1978) as mycelial growth inhibition (%) = [100 × (longest growth of *P. digitatum* − shortest growth of *P. digitatum*)/longest growth of *P. digitatum*]. To explore the effect of the metabolites produced by *P. lilacinum* on the growth inhibition of *P. digitatum*, disks (5 mm diameter), from the interaction region between both fungi, were put on the front of *P. digitatum* culture. Two PDA disks were then placed at opposite sides of *P. digitatum* culture as a control (Benhamou and Brodeur 2000).

Preparation of *Purpureocillium lilacinum* filtrate

Four 5-mm diameter disks of *P. lilacinum* were transferred to potato dextrose broth (PDB) (200 ml). All flasks were incubated in the dark for 30 days at 25°C. The culture filtrate was separated from fungal mycelium and the filtrate was centrifuged at 12 000 ***g*** for 30 min at 4°C. The solution was sterilized using a 0·2-μm pore size filter. The sterile filtrate was considered as 100% concentration and stored at 4°C.

Antifungal activity *in vitro*

Culture filtrate was added to the plates containing PDA (9 cm diameter) to obtain 16, 32 and 64% concentration. The control plates were used without culture filtrate. 5-mm diameter disks of *P. digitatum* were put in the centre of the Petri dishes for 7 days at 25°C. The fungal growth inhibition was calculated as follows: Growth inhibition (%) = [(fungal growth (mm) in control−fungal growth (mm) in treatment) / fungal growth (mm) in control] × 100.

For spore germination, aliquots of 100 µl spore suspension of *P. digitatum* (1×10^6 spores per ml) were added to PDB with culture filtrate at the final concentrations of 16, 32 and 64% in the glass tubes. The tubes contained PDB and spore suspension without filtrate were served as a control. All tubes were incubated at 25°C for 15 h on a rotary shaker at 100 rev min^{-1}. The germination rate and germ tube length were measured in approximately 200 spores within each replicate per treatment. The inhibition of spore germination was measured as follows: Spore germination inhibition (%) = [(number of germinated spores in control−number of germinated spores in treatment)/number of germinated spores in control)] × 100. Conidia were considered germinated when the length of a germ tube exceeded half of the small-end diameter of the spore (Elsherbiny *et al.* 2017). The inhibition of germ tube elongation was measured as a percentage in relation to the control.

Measurement of cell membrane permeability

The electric conductivity of *P. digitatum* mycelia was measured according to the method of Shao *et al.* (2013) with minor modification. The fungus was added into PDB (100 ml). After the flasks were shaken at 140 rev min^{-1} and 25°C for 2 days, the flasks were supplemented with the culture filtrate at the ultimate concentration of 64%. The control flasks were used without culture filtrate. After the flasks were shaken for an additional 32 h, samples were collected at 4-h intervals from the suspensions, following centrifugation for 10 min at 4000 ***g***. After 0, 4, 8, 12, 16, 20, 24, 28, 32 and 36 h, the electrical conductivity was determined in the supernatants with a conductivity meter (Model Jenco 3173; Jenco Technologies, San Diego, CA). After 36 h, the supernatants were boiled for 5 min, and the final conductivity was recorded. The relative conductivity was calculated as: Relative conductivity (%) = conductivity/final conductivity × 100.

Measurement of lipid peroxidation

The lipid peroxidation degree was measured by the production levels of malondialdehyde (MDA) according to Ritter *et al.* (2008). The collected mycelia (3 g) were suspended in 100 ml of sterile double-distilled water containing the culture filtrate at 64%. The control flasks were used without culture filtrate. After 1, 2, 3 and 4 days in incubator shaker at 140 rev min^{-1} and 25°C, mycelia (0·5 g) was homogenized with 0·2% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA). The mixture was heated at 95°C for 30 min, and then cold in an ice bath for 10 min. The solution was centrifuged for 20 min at 12 000 ***g***, and the distilled water was used to dilute the supernatant to 40 ml. Absorbance of the supernatant at 532 nm (A532) was measured. Malondialdehyde concentration was estimated by referring to a standard 1,1,3,3-tetramethoxypropane. The results were defined as micromoles of MDA per kilogram of fresh mycelia.

Measurement of cellular leakage

The pathogen was cultured in PDB (100 ml) at 25°C and 140 rev min^{-1}. Mycelia were collected after incubation for 3 days and washed thoroughly thrice with sterile double-distilled water. Mycelia of *P. digitatum* were resuspended in sterile distilled water and culture filtrate at the final concentration of 64% (100 ml). The sterile distilled water without culture filtrate was used as a control. After 1, 2, 3 and 4 h, the solution was filtrate to remove mycelia using a 0·2-µm pore size membrane, and the filtrate was used for determination of the total soluble protein and total soluble sugar. The Lowry assay, with bovine serum albumin as the standard, was applied to quantify the release of proteins (Lowry *et al.* 1951). The sugar content was determined using glucose as the standard by the phenol–sulphuric acid method of Dubois *et al.* (1956). The value for soluble protein or soluble sugar was expressed as gram per kilogram of mycelia.

Measurement of ergosterol content

The content of ergosterol in the fungal samples was measured using the method described by Zhou *et al.* (2018). One hundred µl of *P. digitatum* spores (1×10^6 spores per ml) was added to PDB medium containing culture filtrate at the concentrations of 16, and 32% for 4 days at 25°C. The control containing PDB medium without culture filtrate. After being washed twice with sterile distilled water, the mycelia (1 g) were added to 5 ml of 25% alcoholic potassium hydroxide solution and incubation for 4 h at 85°C. Ergosterol was extracted from each sample by adding 2 ml sterile double-distilled water and 5 ml petroleum ether. The mixture was then mixed for 2 min allowing the layers to separate at room temperature for 1 h. The absorbance of the petroleum ether layer was measured between 200 and 300 nm. The content of ergosterol was calculated as a percentage of the wet weight of mycelia and the absorbance as follows: Ergosterol content (%) = [(A280/290)−(A230/518)]/mycelia weight.

Development of orange green mould

Oranges fruit (*Citrus sinensis* cv. Washington) were dipped in 2% sodium hypochlorite solution for 2 min

followed by washing with sterile distilled water and then air-dried before wounding. Each fruit was wounded (3 mm depth × 3 mm wide) on the outer surface of each fruit in four locations with a sterile borer. The wounds were treated with 20 µl of culture filtrate at the concentrations of 16, 32 and 64%. The control was treated with the same volume of sterile distilled water. After 4 h at 25°C, 10 µl spore suspension (1×10^6 spores per ml) was injected into each wound. Different treatments were sealed in separated plastic boxes and stored at 25°C for 7 days with a high relative humidity (95–100% RH). Each treatment consisted of three replicates (10 fruit per replicate). The disease incidence and disease severity were calculated as follows:

$$\text{Disease incidence (\%)} = [(\text{number of rotten wounds}/\text{number of total wounds})] \times 100$$

$$\text{Disease severity (\%)} = [(\text{average lesion diameter of treatment}/\text{average lesion diameter of control})] \times 100$$

Measurement of defence-related enzyme activities

Oranges fruit were wounded (3 mm deep and 3 mm wide) on four locations at the outer surface. Twenty microliters of the culture filtrate at the concentration of 64% were added to each wound. The wounds treated with 20 µL of sterile distilled water were served as a control. The fruit were placed on plastic boxes at 25°C. Peel samples were taken from the entire wound for enzyme assays at 1, 2, 3 and 4 days after treatment.

For polyphenoloxidase (PPO) and peroxidase (POD), 2 g of sample was mixed with 10 ml of sodium phosphate buffer (100 mmol l^{-1}, pH 6·4) and 0·2 g of polyvinylpolypyrolidone (PVPP). The homogenate was centrifuged at 12 000 ***g*** for 30 min at 4°C and the supernatant was collected for enzymes analysis. The POD activity was performed according to the method of Fu *et al.* (2017), by mixing 100 µl crude enzyme extract, 100 mmol l^{-1} sodium phosphate buffer (pH 6·4) and 2 ml guaiacol (8 mmol l^{-1}). After 30 min at 30°C, reaction production was measured spectrophotometrically at 470 nm for 4 min after adding 1 ml H_2O_2 (24 mmol l^{-1}). The activity of POD was expressed in units (U), where one unit was defined as the change in absorbance at 470 nm per mg of protein per minute. Lowry assay, with bovine serum albumin (BSA) as the standard, was applied to determine the protein concentration (Lowry *et al.* 1951). PPO activity was measured as described by Chen *et al.* (2000). The assay was carried out by mixing 100 µL of enzyme extract, 0·5 ml catechol (500 mmol l^{-1}) and 2 ml of 0·05 mol l^{-1} phosphate buffer (pH 7·0). After 2 min of incubation at 24°C, the absorbance was spectrophotometrically assayed at 398 nm for 4 min, and expressed in units (U).

The PAL activity was analysed using the method of Zhang *et al.* (2013). One gram of sample was mixed with extracting buffer consisted of 10% (w/v) PVPP, 200 mmol l^{-1} boric acid buffer (pH 8·8), 50 mmol l^{-1} β-mercaptoethanol and 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA). The mixture of 1 ml L-phenylalanine (20 mmol l^{-1}) and extracting buffer (2 ml) was added to the supernatant (200 µl) after centrifugation at 12 000 ***g*** for 30 min (4°C). After 2 min at 24°C, the absorbance was measured at 290 for 4 min, and expressed in units (U).

Expression analysis of defence-related gene

Orange fruit were prepared and wounded as described above to evaluate the effects of culture filtrate at 64% on the expression level of genes involved in peroxidase (POD), and PAL. Total RNA was extracted from orange peel tissues using TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. RNA was examined on 1% agarose gel. The RNA purity was checked at wavelengths of 260, and 280 nm with a T80 UV/VIS spectrophotometer. DNase I (RNase-Free) was used to removing genomic DNA from RNA preparations. RNA samples were reverse-transcripted into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA). Reverse transcription reaction (25 µl) was performed using oligo 10 pmol (dT) primer and 2·5 µl RNA (2 mg ml^{-1}) in Techni TC-512 PCR system using a thermal cycler programmed at 42°C for 1 h. RibolockRNase inhibitor was added, and then terminated by heating at 72°C for 10 min and stored at −80°C until expression analysis. The specific primers of the genes used for the assay and β-tubulin as a reference gene (Table 1) were derived according to Youssef *et al.* (2014). RT-PCR was performed using SensiFAST SYBR® No-ROX protocol in 36-well reaction plates on Rotor-Gene-6000-system (Corbett Robotics, Brisbane, Australia). The reaction conditions were the following: 95°C for 5 min, and 40 cycles at 94°C for 20 s, 60°C for 20 s and 72°C for 20 s. PCR programme dissociation was carried out through slowly heating the PCR products from 72 to 95°C and continuous recording of the reduction in SYBR Green fluorescence following the dissociation of dsDNA on Melting Curve for each targeted gene. The peak of the curve for all samples was approximately the same Tm for each amplicon. All relative change in gene expression levels was calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Each treatment was performed in three biological replicates, and the experiment was conducted two times.

Table 1 Primers targeted in the genes involved in peroxidase (POD) and phenylalanine ammonia lyase (PAL)

Gene Name	Primer	Primer sequence	Accession number	Tm (°C)	Size (bp)
Peroxidase 1	POX F	5-AGGGTGATACGATCAGCTCTT-3	XM_006492610	82	131
	POX R	5-AGCAGGATGCGAACAAACAAA-3			
Phenylalanine ammonia lyase 1	PAL1 F	5-AGGCAGACTGTTGAGAATGGA-3	XM_006481430	86	287
	PAL1 R	5- CCATTTAGTCACATCGGCAAT-3			
β-Tubulin	β-Tub F	5-CCTTACAACGCTACTCTCTCTGT-3	XM_006481335	79	101
	β-Tub R	5-AGAGTGCGGAAACAGATGTCG-3			

Tm = optimal melting temperature for each primer pair. Size = product length of the PCR in base pairs (bp).

Statistical analysis

All the experiments were repeated twice. The results were analysed by one-way analysis of variance and comparison of means using the Tukey's HSD test at the level $P < 0{\cdot}05$. The statistical analysis was performed using SAS (ver. 9.1; SAS Institute, Cary, NC).

Results

Dual culture test

No direct contact was observed between *P. lilacinum* and *P. digitatum* during dual culture assay at 25°C for 10 days on PDA plates (Fig. 1a). The percentage of the mycelial growth inhibition of *P. digitatum* by the antagonistic fungus *P. lilacinum* was 68·2%. In addition, the inhibition zone (IZ) was 6·1 mm by *P. lilacinum* towards the plant pathogen *P. digitatum*. The mycelial growth of *P. digitatum* was stopped, when the disks taken from the interaction region between two fungi were put on the front of the pathogen cultures (Fig. 1b-3). However, the mycelium of *P. digitatum* was fully overgrown on control PDA disks (Figs 1b-1 and 2).

Antifungal activity *in vitro*

Mycelial growth of *P. digitatum* was significantly ($P < 0{\cdot}05$) prevented by the culture filtrate of *P. lilacinum* at all concentrations (Table 2). *P. lilacinum* filtrate completely suppressed *P. digitatum* growth at 64%. Also, spore germination of the fungal pathogen was prevented by 100% at the same concentration. Moreover, the concentration of 32% of the culture filtrate caused a strong inhibition on the fungal growth, conidial germination and germ tube length by 77·4, 80·1 and 83·3%, respectively (Table 2).

Changes in cell membrane permeability

The values of relative conductivity, evidence of the alterations in the integrity of the fungal cell membrane, were increased gradually and continuously in the *P. digitatum* mycelia after exposure to the culture filtrate of *P. lilacinum* at 64% during the whole time (Fig. 2). The values of relative conductivity of *P. digitatum* in the control were slightly increased during 180 min. Generally, *P. digitatum* mycelia treated with culture filtrate were higher levels than control during 36 h.

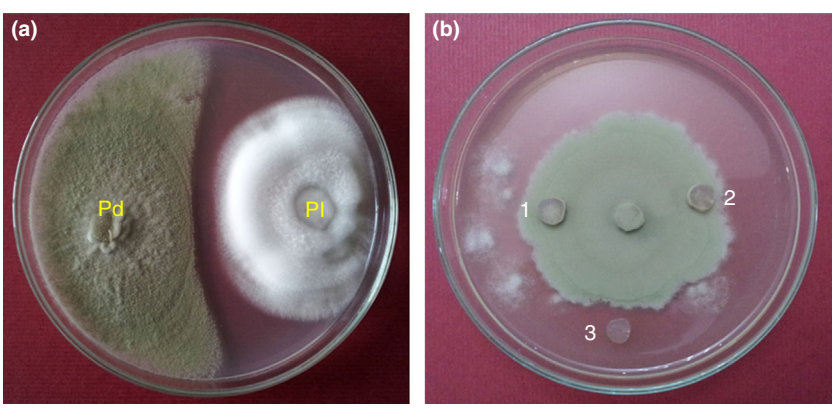

Figure 1 Effect of *Purpureocillium lilacinum* (Pl) and its metabolites on *Penicillium digitatum* (Pd) in a dual culture test (a). Growth of *P. digitatum* in the presence of an agar disk (3) taken from the interaction region between two fungi, no effect is observed with the PDA disks (1 and 2) taken from fresh PDA plates (b).

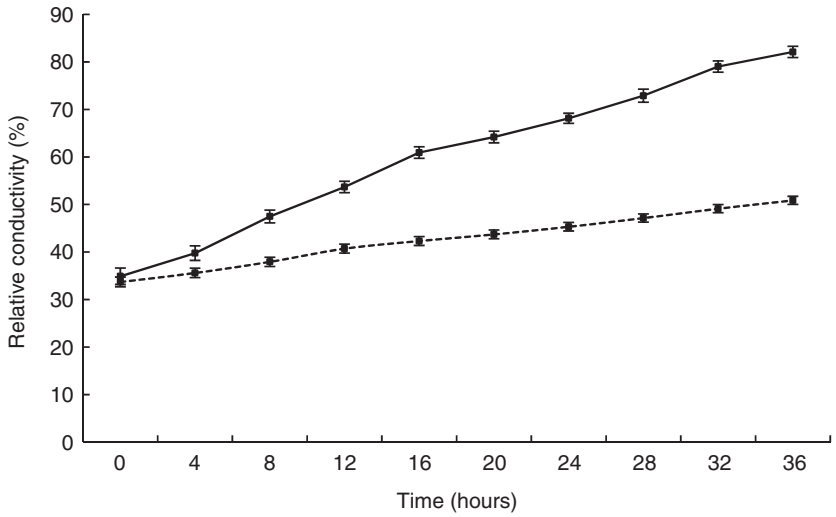

Figure 2 Relative conductivity of *Penicillium digitatum* mycelia with or without the culture filtrate of *Purpureocillium lilacinum*. Results represent mean ± standard error (SE). (**-----**) Control; (——) filtrate.

Table 2 Effect of *Purpureocillium lilacinum* culture filtrate at different concentrations on mycelial growth, spore germination and germ tube elongation of *Penicillium digitatum*

Concentration (%)	Mycelial growth inhibition (%)	Spore germination inhibition (%)	Germ tube elongation inhibition (%)
0	0·0 a*	0·0 a	0·0 a
16	54·4 ± 0·3 b	51·8 ± 0·9 b	55·6 ± 0·4 b
32	77·4 ± 0·3 c	80·1 ± 0·8 c	83·3 ± 0·3 c
64	100 ± 0·0 d	100 ± 0·0 d	100 ± 0·0 d

*Results represent mean ± standard error (SE). Different letters in the same column are significantly different according to Tukey's HSD test at $P < 0{\cdot}05$.

Changes in lipid peroxidation

The effect of *P. lilacinum* culture filtrate on lipid peroxidation of *P. digitatum* mycelia, measured as MDA production, was determined by TBA method. The results showed that the culture filtrate treatment at the concentration of 64% significantly ($P < 0{\cdot}05$) increased the MDA content in *P. digitatum* mycelia during the whole time compared with the untreated mycelia (Fig. 3). The maximum value of MDA content was 15·33 mmol l^{-1} kg^{-1} of *P. digitatum* mycelia on the fourth day compared to the control by 0·44 mmol l^{-1} kg^{-1}.

Changes in protein and sugar leakage

The leakage of soluble protein and soluble sugar in the mycelia of *P. digitatum* treated with 64% of *P. lilacinum* filtrate was significantly ($P < 0{\cdot}05$) higher than of the control mycelia over 4 h at 25°C (Fig. 4). The leakage of protein from the mycelia treated with the filtrate was increased slightly during the first 3 h and decreased in the fourth hour by 0·3 g kg^{-1}, whereas the leakage of sugar was almost stable during the whole experiment period.

Changes in ergosterol content

The ergosterol content in *P. digitatum* mycelia was dramatically reduced after the treatment by the culture filtrate at 16 and 32% (Fig. 5). The content of ergosterol was decreased by 49·5 and 81·1% when the pathogen mycelia exposed to the culture filtrate at 16 and 32%, respectively, compared with that of the control.

Development of orange green mould

The disease incidence in orange fruit was significantly ($P < 0{\cdot}05$) lower in all concentrations of culture filtrate compared to untreated control fruit after 7 days at 25°C (Fig. 6). Disease incidence in the filtrate-treated fruit at 64% was 78·3% lower than that in the untreated fruit. Also, the disease severity in the filtrate-treated fruit was significantly ($P < 0{\cdot}05$) lower than that in the control (Fig. 6). After 7 days incubation at 25°C, the filtrate at 64% caused a voluminous reduction in the severity of orange green mould by 80·7% compared with the control.

Changes in defence-related enzymes activity

The fruit treated with culture filtrate at 64% were higher in the activity of POD than untreated fruit during storage at 25°C for 4 days (Fig. 7a). POD activity increased

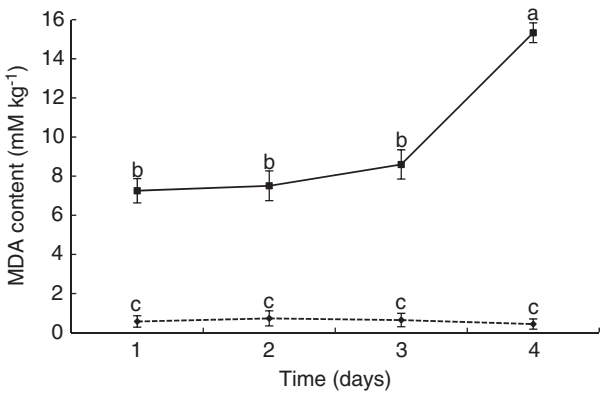

Figure 3 Malondialdehyde (MDA) content in the mycelia of *Penicillium digitatum* after treatment with the culture filtrate of *Purpureocillium lilacinum* at 64%. Results represent mean ± standard error (SE). Different letters indicate significant differences according to Tukey's HSD test at $P < 0{\cdot}05$. (------) Control; (——) filtrate.

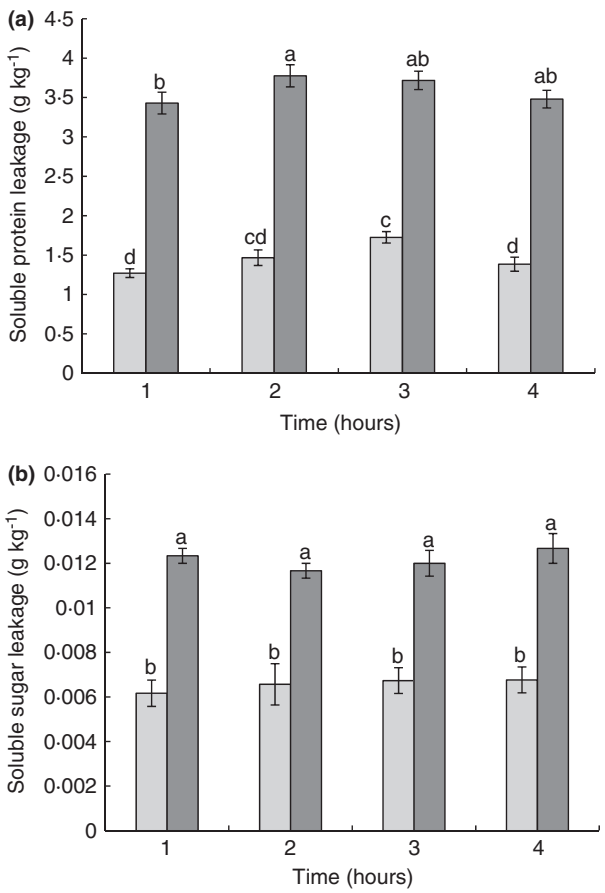

Figure 4 Effect of *Purpureocillium lilacinum* filtrate at 64% on soluble protein leakage (a) and soluble sugar leakage (b) of *Penicillium digitatum* mycelia. Results represent mean ± standard error (SE). Bars with different letters are significantly different according to Tukey's HSD test at $P < 0{\cdot}05$. (□) Control; (■) Filtrate.

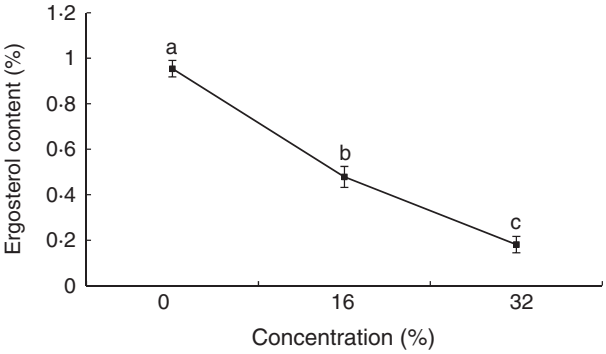

Figure 5 Effect of *Purpureocillium lilacinum* filtrate at different concentrations on ergosterol content in the plasma membrane of *Penicillium digitatum*. Results represent mean ± standard error (SE). Different letters indicate significant differences according to Tukey's HSD test at $P < 0{\cdot}05$.

in the first and second days and then slightly decreased during the following 2 days. Orange fruit treated with *P. lilacinum* filtrate induced significantly ($P < 0{\cdot}05$) higher PPO activity during the entire storage period compared with the control fruit (Fig. 7b). The activity of PPO reached its peak on the second day and then showed a progressive diminution in the other 2 days. The activity of PAL was increased in the filtrate-treated fruit as compared with the untreated group during 4 days of storage (Fig. 7c). The PAL activity reached a peak value by 6·5 times higher than that in the control fruit on the second day and then decreased sharply on the third day.

Changes in defence-related genes expression

The results indicated that the orange fruit treated with the culture filtrate of *P. lilacinum* at 64% significantly ($P < 0{\cdot}05$) impacted on the relative gene expression levels of POD and PAL compared to the control. The expression level of POD in the fruit treated with 64% culture filtrate significantly ($P < 0{\cdot}05$) upregulated in the first 2 days after incubation at 25°C, and then downregulated during the third and fourth days (Fig. 8a). A maximum increase in the POD relative gene expression level was observed on the second day compared to the control. Also, the PAL relative expression level in the fruit treated with the culture filtrate at 64% was higher than that of the control group during the first and second days, and reached a peak on the 2nd day (Fig. 8b). Whereas the concentration of 64% caused a low induction in the PAL gene expression on the third and fourth days.

Discussion

Up to now, there is no unequivocal evidence about the efficacy of the fungus *P. lilacinum* and its metabolites for

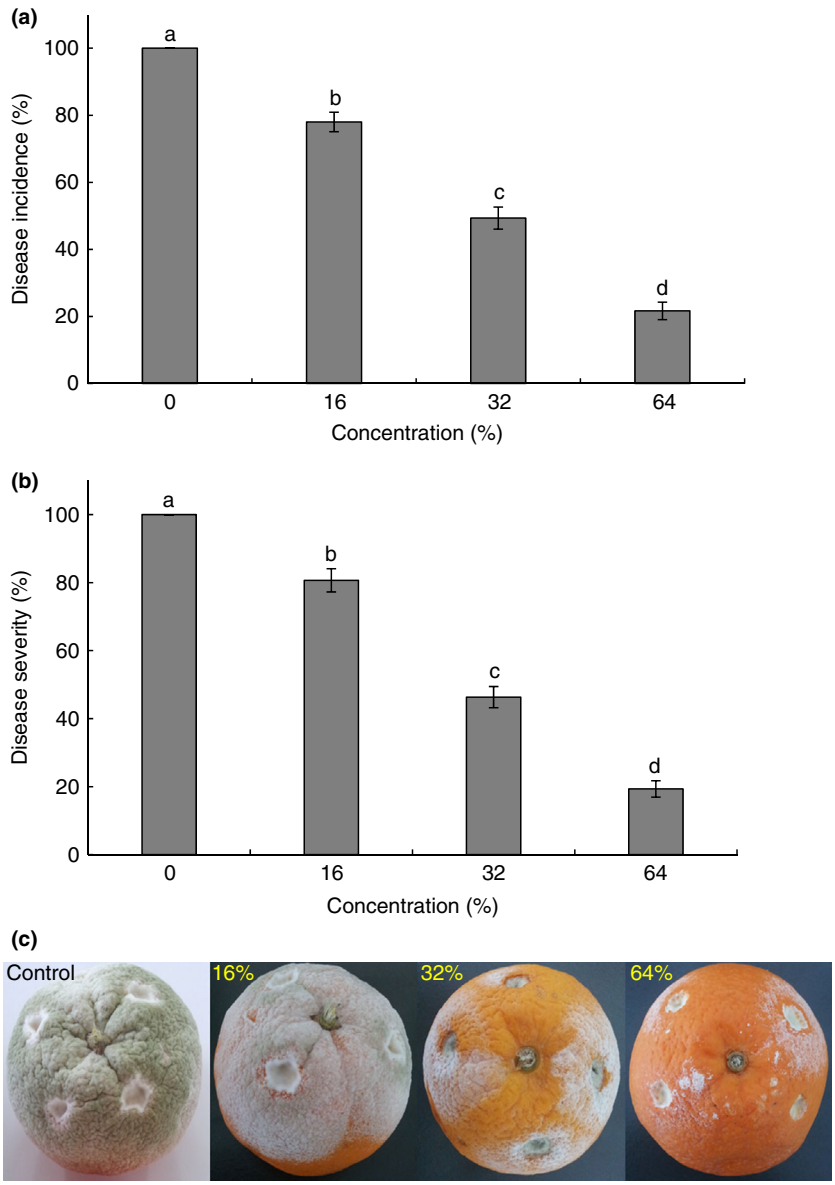

Figure 6 Effect of *Purpureocillium lilacinum* culture filtrate at different concentrations on incidence and severity of green mould in orange fruit inoculated with *Penicillium digitatum*. Results represent mean ± standard error (SE) for two experiments. Bars with different letters are significantly different according to Tukey's HSD test at $P < 0{\cdot}05$.

the control of *P. digitatum* in citrus fruit. Therefore, this is the first study to investigate the application of *P. lilacinum* culture filtrate as a novel agent to control green mould and enhanced the defence mechanisms in orange fruit. Our results showed that *P. lilacinum* was able to inhibit the growth of *P. digitatum* by 68·2% in the dual culture test with no physical contact between both. This inhibition indicates the presence of fungistatic metabolites produced by *P. lilacinum* grown on PDA. In general, antagonism can occur between fungi without physical contact when one of the opponents produces diffusible organic compounds (DOCs), including bioactive metabolites, toxins and enzymes as well as the emission of volatile organic compounds (VOCs) such as alcohols, aldehydes, alkenes, acids, esters, ketones, terpenes, benzenoids and pyrazines. Chemical signalling by these compounds (VOCs and DOCs) plays a major role in fungal recognition systems and antagonistic effects (Howell 2003; Elsherbiny *et al.* 2020). Moreover, the mycelial growth, spore germination and germ tube elongation of *P. digitatum* were completely inhibited by the culture filtrate of *P. lilacinum* at the concentration of 64%. We

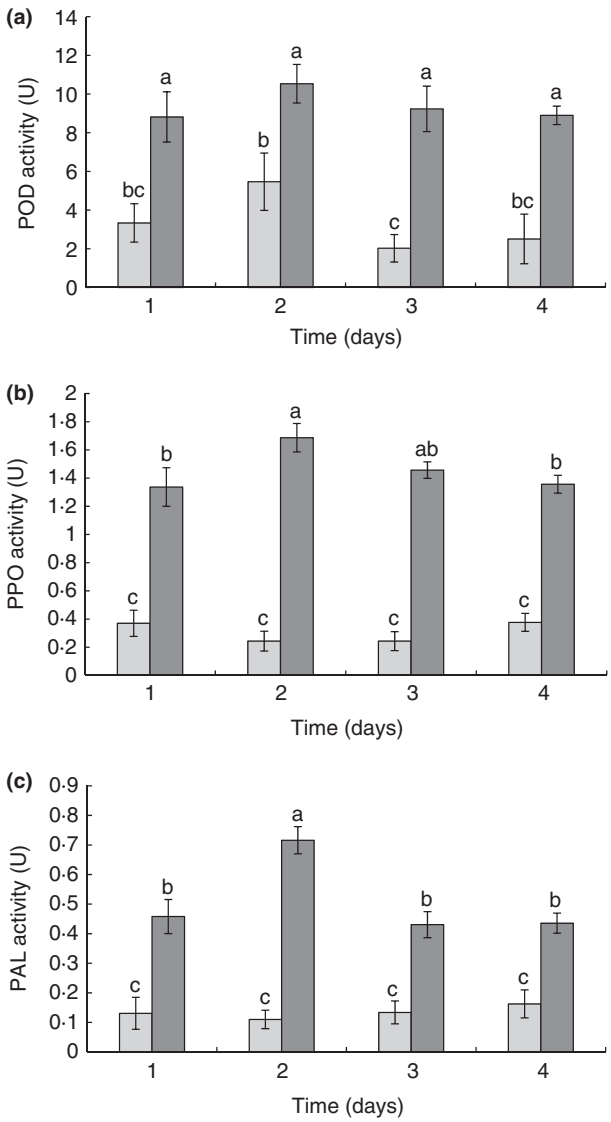

Figure 7 Effect of *Purpureocillium lilacinum* culture filtrate at the concentration of 64% on defence-related enzymes activity of orange fruit stored at 25°C for 4 days after treatment. (a) Peroxidase (POD), (b) polyphenoloxidase (PPO) and (c) phenylalanine ammonia lyase (PAL). Results represent mean ± standard error (SE). Bars with different letters are significantly different according to Tukey's HSD test at $P < 0{\cdot}05$. (□) Control; (■) Filtrate.

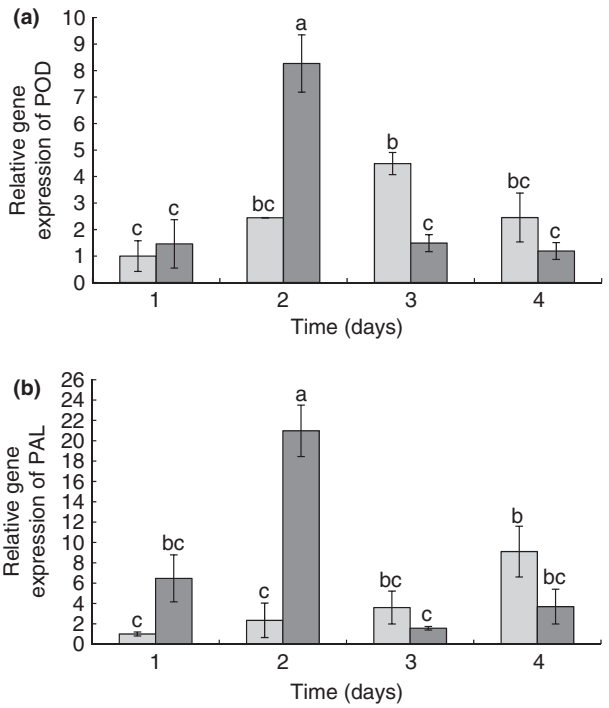

Figure 8 Effect of *Purpureocillium lilacinum* culture filtrate at the concentration of 64% on the expression of (a) peroxidase (POD) and (b) phenylalanine ammonia lyase (PAL) genes in orange fruit during storage at 25°C for 4 days after treatment. Results represent mean ± standard error (SE). Bars with different letters are significantly different according to Tukey's HSD test at $P < 0{\cdot}05$. (□) Control; (■) Filtrate.

have previously shown that this culture filtrate at 75% suppressed the fungal growth and sclerotia formation of *S. sclerotiorum* by 100%, with inhibition of myceliogenic and carpogenic germination by 93·5 and 90%, respectively (Elsherbiny *et al.* 2019). Also, the cell-free culture filtrate of *Paecilomyces lilacinus* (pt361) caused a strong inhibition ranging from 60·3 to 100% on the mycelial growth of *S. sclerotiorum* (Yang *et al.* 2015).

The antifungal activity of *P. lilacinum* might be due to the presence of bioactive secondary metabolites, including leucinostatins known as paecilotoxins. Leucinostatins group is a class of nonpeptides and nonribosomal peptides (Kuwata *et al.* 1992). *P. lilacinum* is known for the production of seven congeners of leucinostatins: A, B, C, D, F, H and K (Wang *et al.* 2016). Recently, a new peptaibiotic called leucinostatin Y was isolated from the culture broth of *P. lilacinum* (Momose *et al.* 2019). In a previous independent study, we also determined leucinostatins H and K as the major compounds in the culture filtrate of *P. lilacinum* based on the NMR studies (Elsherbiny *et al.* 2019). Several studies have demonstrated that leucinostatins were very effective against various species of fungi and bacteria (Radics *et al.* 1987; Yang *et al.* 2015; Wang *et al.* 2016).

The plasma membrane is vital for the maintenance of important cell constituents such as sugar and protein, cellular morphology, exchange of the materials, and transfer the energy and information in the fungal cell (Liang *et al.* 2016). Therefore, many fungicide compounds target the fungal cell membrane or its components by the direct or indirect way to increase the cell membrane permeability and cellular leakage (Wang *et al.* 2019). In the current

work, the permeability of the cell membrane and cellular leakage of *P. digitatum* were significantly increased after treatment with the filtrate of *P. lilacinum* at 64% during the whole time of the experiment. In concordance with this study, Chen *et al.* (2019) evaluated the inhibitory effects of 7-demethoxytylophorine (DEM), an alkaloid isolated from the rhizomes of *Cynanchum polipid*, on *P. italicum*. Similarly, the antifungal activity of pinocembrin-7-glucoside (P7G), a flavanone glycoside isolated from *Ficus hirta* on *P. italicum* (Chen *et al.* 2020).

Moreover, we found another disrupt in the fungal cell membrane was associated with oxidative damage by increasing the MDA content in filtrate-treated mycelia of *P. digitatum* at 64%. MDA is the major product of lipid peroxidation as a decomposition product of polyunsaturated fatty acid hydroperoxides, and it increases following exposure to reactive oxygen species. MDA plays a critical role in the damage of phospholipids, enzymes, proteins, nucleic acids and biofilms (Elsherbiny and Taher 2018; Ju *et al.* 2020). This was supported by Liu *et al.* (2017) who found that epsilon-poly-lysine (e-PL), a polycationic peptide used as a natural antimicrobial food additive, significantly causes lipid peroxidation in *P. digitatum* at all tested concentrations from 12·5 to 200 mg l^{-1}. In this context, the lack of ergosterol biosynthesis can typically reflect irreversible damage to the fungal cell membrane because ergosterol is responsible for the maintaining of membrane fluidity, cellular integrity and transport of the cellular materials (Tian *et al.* 2012). Our results indicated that the treatment of the culture filtrate at 16 and 32% strongly decreased the ergosterol content in *P. digitatum* compared with the control group. This result was consistent with a previous study on the effect of antofine, the alkaloid that identified from *Cynanchum atratum* BUNGE, on the content of ergosterol in *P. digitatum* (Xin *et al.* 2019).

The results from the present study also showed that the high ability of the culture filtrate of *P. lilacinum* at all tested concentrations to reduce both the incidence and severity of orange green mould during 7 days of incubation at 25°C, which was consistent with the results of *in vitro* assays. Our results agree with previous studies that confirm the potential of *P. lilacinum* and its culture filtrate in biocontrol of plant diseases, such as stem rot in oilseed rape (Yang *et al.* 2015), *Verticillium* wilt in eggplant (Lan *et al.* 2017), white mould in common bean (Elsherbiny *et al.* 2019) and *Phytophthora* blight in pepper (Hu *et al.* 2020). In addition, *P. lilacinum* has remarkable effects on plant growth promotion. For example, Lopez and Sword (2015) found a significant increase in the growth of cultivated cotton (*Gossypium hirsutum*) after treatment by the fungus in the greenhouse trials. *P. lilacinum* also promotes plant growth in *Capsicum chinense*, including stem diameter, fresh and dry weight of shoots and roots under greenhouse conditions (Moreno-Salazar *et al.* 2020). On the other hand, no previous studies have addressed the biocontrol efficacy of *P. lilacinum* against postharvest diseases. However, very few reports investigated the biological control of postharvest diseases using entomopathogenic fungi. For instance, Sarven *et al.* (2020) studied the effect of culture filtrate, crude extract and volatile organic compounds of entomopathogenic fungus *Metarhizium anisopliae* on grey mould caused by *Botrytis cinerea* in tomato fruit.

Generally, the activity of POD, PPO and PAL enzymes plays a crucial role in the induction of systemic resistance in citrus fruit. The present study showed that the *P. lilacinum* filtrate at 64% enhanced the activity of PAL, PPO and POD in orange fruit compared with the control fruit, which might be answerable for reducing both the incidence and severity of the disease. Moreover, POD and PAL genes were upregulated and reached a maximal expression level at 48 h in orange fruit treated with *P. lilacinum* filtrate as compared to the control. Therefore, the expression of POD and PAL genes confirmed that they are important to the activation of the defence system in the fruit. POD belongs to multifunctional enzymes that implicated in the biosynthesis pathway of lignin to counteract pathogen penetration of host plant cells. In addition, POD oxidizes phenols to quinones in the presence of hydrogen peroxide, which are strongly toxic to fungal pathogens (Zhu *et al.* 2019). PPO is a copper-containing enzyme, which oxidizes phenolic compounds to highly toxic quinones as antimicrobial compounds. Besides, PPO is involved in the lignification of the plant cells and suberin formation at the fungal invasion (Chen *et al.* 2000). PAL is a key enzyme in the shikimate and phenylpropanoid pathways and is directly associated with the biosynthesis of active metabolites such as phenols, phytoalexins, tannin, lignins and salicylic acid, that enhance the disease resistance in plants (Zhang *et al.* 2013). Various investigations have shown that several chemical or biological elicitors caused the activation of the defence-related enzymes and their gene expression levels, which largely involved in the defence mechanisms of citrus fruit against *P. digitatum* (Youssef *et al.* 2014; Zhu *et al.* 2019).

In conclusion, the present study provides the first evidence on the application of *P. lilacinum* and its metabolites as a novel biocontrol agent for green mould caused by *P. digitatum* in orange fruit. Our results supported that the fungal cell membrane is the major target of *P. lilacinum* filtrate to inhibiting the growth of *P. digitatum* by increasing the cell membrane permeability, MDA content, and protein and sugar leakage in *P. digitatum* as well as decreasing the ergosterol synthesis in the pathogen. The results showed that the culture filtrate was

strongly effective in controlling orange green mould. In addition, the filtrate induced the activity of POD, PPO and PAL enzymes and enhanced the relative expression of the defence enzymes genes in orange fruit.

Conflict of Interest

The authors declare no conflict of interest.

References

Benhamou, N. (2004) Potential of the mycoparasite, *Verticillium lecanii*, to protect citrus fruit against *Penicillium digitatum*, the causal agent of green mold: a comparison with the effect of chitosan. *Phytopathology* **94**, 693–705.

Benhamou, N. and Brodeur, J. (2000) Evidence for antibiosis and induced host defense reactions in the interaction between *Verticillium lecanii* and *Pencillium digitatum*, the causal agent of green mold. *Phytopathology* **90**, 932–943.

Borras, A.D. and Aguilar, R.V. (1990) Biological control of *Penicillium digitatum* by *Trichoderma viride* on postharvest citrus fruits. *Int J Food Microbiol* **11**, 179–183.

Calvo, H., Marco, P., Blanco, D., Oria, R. and Venturini, M.E. (2017) Potential of a new strain of *Bacillus amyloliquefaciens* BUZ-14 as a biocontrol agent of postharvest fruit diseases. *Food Microbiol* **63**, 101–110.

Chen, C., Bélanger, R., Benhamou, N. and Paulitz, T.C. (2000) Defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. *Physiol Mol Plant Pathol* **56**, 13–23.

Chen, C., Chen, J. and Wan, C. (2020) Pinocembrin-7-glucoside (P7G) reduced postharvest blue mold of navel orange by suppressing *Penicillium italicum* growth. *Microorganisms* **8**, 536.

Chen, C., Qi, W., Peng, X., Chen, J. and Wan, C. (2019) Inhibitory effect of 7-demethoxytylophorine on *Penicillium italicum* and its possible mechanism. *Microorganisms* **7**, 36.

Costa, J.H., Bazioli, J.M., de Moraes Pontes, J.G. and Fill, T.P. (2019) *Penicillium digitatum* infection mechanisms in citrus: What do we know so far? *Fungal Biol* **123**, 584–593.

Dahlin, P., Eder, R., Consoli, E., Krauss, J. and Kiewnick, S. (2019) Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. *Crop Prot* **124**, 104874.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric methods for the determination of sugars and related substances. *Anal Chem* **28**, 350–352.

Elbanhawy, A.A., Elsherbiny, E.A., Abd El-Mageed, A.E. and Abdel-Fattah, G.M. (2019) Potential of fungal metabolites as a biocontrol agent against cotton aphid, *Aphis gossypii* Glover and the possible mechanisms of action. *Pestic Biochem Physiol* **159**, 34–40.

Elsherbiny, E.A., Amin, B.H., Aleem, B., Kingsley, K.L. and Bennett, J.W. (2020) *Trichoderma* volatile organic compounds as a biofumigation tool against late blight pathogen *Phytophthora infestans* in postharvest potato tubers. *J Agric Food Chem* **68**, 8163–8171.

Elsherbiny, E.A., Safwat, N.A. and Elaasser, M.M. (2017) Fungitoxicity of organic extracts of *Ocimum basilicum* on growth and morphogenesis of *Bipolaris* species (teleomorph *Cochliobolus*). *J Appl Microbiol* **123**, 841–852.

Elsherbiny, E.A. and Taher, M.A. (2018) Silicon induces resistance to postharvest rot of carrot caused by *Sclerotinia sclerotiorum* and the possible of defense mechanisms. *Postharvest Biol Technol* **140**, 11–17.

Elsherbiny, E.A., Taher, M.A. and Elsebai, M.F. (2019) Activity of *Purpureocillium lilacinum* filtrates on biochemical characteristics of *Sclerotinia sclerotiorum* and induction of defense responses in common bean. *Eur J Plant Pathol* **155**, 39–52.

Fiedler, Z. and Sosnowska, D. (2007) Nematophagous fungus *Paecilomyces lilacinus* (Thom) Samson is also a biological agent for control of greenhouse insects and mite pests. *Biocontrol* **52**, 547–558.

Fu, D., Sun, Y., Yu, C., Zheng, X., Yu, T. and Lu, H. (2017) Comparison of the effects of three types of aminobutyric acids on the control of *Penicillium expansum* infection in pear fruit. *J Sci Food Agric* **97**, 1497–1501.

Goffré, D. and Folgarait, P. (2015) *Purpureocillium lilacinum*, potential agent for biological control of the leaf-cutting ant *Acromyrmex lundii*. *J Invertebr Pathol* **130**, 107–115.

Hajji, L., Hlaoua, W., Regaieg, H. and Horrigue-Raouani, N. (2017) Biocontrol potential of *Verticillium leptobactrum* and *Purpureocillium lilacinum* against *Meloidogyne javanica* and *Globodera pallida* on potato (*Solanum tuberosum*). *Am J Potato Res* **94**, 178–183.

Holmes, G.J. and Eckert, J.W. (1999) Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. *Phytopathology* **89**, 716–721.

Howell, C.R. (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis* **87**, 4–10.

Hu, J., Hou, S., Li, M., Wang, J., Wu, F. and Lin, X. (2020) The better suppression of pepper *Phytophthora* blight by arbuscular mycorrhizal (AM) fungus than *Purpureocillium lilacinum* alone or combined with AM fungus. *J Soil Sediment* **20**, 792–800.

Ju, J., Xie, Y., Yu, H., Guo, Y., Cheng, Y., Zhang, R. and Yao, W. (2020) Synergistic inhibition effect of citral and eugenol against *Aspergillus niger* and their application in bread preservation. *Food Chem* **310**, 125974.

Khan, A., Williams, K., Molloy, M.P. and Nevalainen, H. (2003) Purification and characterization of a serine

protease and chitinases from *Paecilomyces lilacinus* and detection of chitinase activity on 2D gels. *Prot Expr Purif* **32**, 210–220.

Kuwata, S., Nakaoishi, A., Yamada, T. and Miyazawa, T. (1992) Total synthesis of leucinostatin D. *Tetrahedron Lett* **33**, 6995–6998.

Lan, X., Zhang, J., Zong, Z., Ma, Q. and Wang, Y. (2017) Evaluation of the biocontrol potential of *Purpureocillium lilacinum* QLP12 against *Verticillium dahliae* in eggplant. *BioMed Res Int* **2017**, 4101357.

Liang, H.-J., Lu, X.-M., Zhu, Z.-Q. and Zhu, F.-X. (2016) Effect of organic solvent on fungicide toxicity to *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Eur J Plant Pathol* **146**, 37–45.

Liu, K., Zhou, X. and Fu, M. (2017) Inhibiting effects of epsilon-poly-lysine (e-PL) on *Pencillium digitatum* and its involved mechanism. *Postharvest Biol Technol* **123**, 94–101.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Lopez, D.C. and Sword, G.A. (2015) The endophytic fungal entomopathogens *Beauveria bassiana* and *Purpureocillium lilacinum* enhance the growth of cultivated cotton (*Gossypium hirsutum*) and negatively affect survival of the cotton bollworm (*Helicoverpa zea*). *Biol Control* **89**, 53–60.

López-Pérez, M., Ballester, A.-R. and González-Candelas, L. (2015) Identification and functional analysis of *Penicillium digitatum* genes putatively involved in virulence towards citrus fruit. *Mol Plant Pathol* **16**, 262–275.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

Ma, J., Hong, Y., Deng, L., Yi, L. and Zeng, K. (2019) Screening and characterization of lactic acid bacteria with antifungal activity against *Penicillium digitatum* on citrus. *Biol Control* **138**, 104044.

Momose, I., Onodera, T., Doi, H., Adachi, H., Iijima, M., Yamazaki, Y., Sawa, R., Kubota, Y. *et al.* (2019) Leucinostatin Y: a peptaibiotic produced by the entomoparasitic fungus *Purpureocillium lilacinum* 40-H-28. *J Nat Prod* **82**, 1120–1127.

Moreno-Salazar, R., Sanchez-Garcia, I., Chan-Cupul, W., Ruiz-Sanchez, E., Hernandez-Ortega, H.A., Pineda-Lucatero, J. and Figueroa-Chavez, D. (2020) Plant growth, foliar nutritional content and fruit yield of *Capsicum chinense* biofertilized with *Purpureocillium lilacinum* under greenhouse conditions. *Sci Hortic* **261**, 108950.

Palou, L., Ali, A., Fallik, E. and Romanazzi, G. (2016) GRAS, plant-and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. *Postharvest Biol Technol* **122**, 41–52.

Park, J.O., Hargreaves, J.R. and McConville, E.J. (2004) Production of leucinostatins and nematicidal activity of Australian isolates of *Paecilomyces lilacinus* (Thom) Samson. *Lett Appl Microbiol* **38**, 271–276.

Pitt, J.I. and Hocking, A.D. (2009) *Fungi and Food Spoilage*, 3rd Edition. New York, USA: Springer Science & Business Media.

Prasad, P., Varshney, D. and Adholeya, A. (2015) Whole genome annotation and comparative genomic analyses of bio-control fungus *Purpureocillium lilacinum*. *BMC Genom* **16**, https://doi.org/10.1186/s12864-015-2229-2.

Radics, L., Kajtar-Peredy, M., Casinovi, C.G., Rossi, C., Ricci, M. and Tuttobello, L. (1987) Leucinostatins H and K, two novel peptide antibiotics with tertiary amine-oxide terminal group from *Paecilomyces marquandii* isolation, structure and biological activity. *J Antibiot* **40**, 714–716.

Ritter, A., Goulitquer, S., Salaun, J.P., Tonon, T., Correa, J.A. and Potin, P. (2008) Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp *Laminaria digitata*. *New Phytol* **180**, 809–821.

Royse, D.J. and Ries, S.M. (1978) The influence of fungi isolated from peach twigs on the pathogenicity of *Cytospora cincta*. *Phytopathology* **68**, 603–607.

Sarven, M., Hao, Q., Deng, J., Yang, F., Wang, G., Xiao, Y. and Xiao, X. (2020) Biological control of tomato gray mold caused by *Botrytis cinerea* with the entomopathogenic fungus *Metarhizium anisopliae*. *Pathogens* **9**, 213.

Shao, X., Cheng, S., Wang, H., Yu, D. and Mungai, C. (2013) The possible mechanism of antifungal action of tea tree oil on *Botrytis cinerea*. *J Appl Microbiol* **114**, 1642–1649.

Talibi, I., Boubaker, H., Boudyach, E.H. and Aoumar, A.B. (2014) Alternative methods for the control of postharvest citrus diseases. *J Appl Microbiol* **117**, 1–17.

Tian, J., Ban, X., Zeng, H., He, J. and Chen, Y. (2012) The mechanism of antifungal action of essential oil from dill (*Anethum graveolens* L.) on *Aspergillus flavus*. *PLoS One* **7**, e30147.

Wang, B., Liu, F., Li, Q., Xu, S., Zhao, X., Xue, P. and Feng, X. (2019) Antifungal activity of zedoary turmeric oil against *Phytophthora capsici* through damaging cell membrane. *Pestic Biochem Physiol* **159**, 59–67.

Wang, G., Liu, Z., Lin, R., Li, E., Mao, Z., Ling, J., Yang, Y., Yin, W.-B. *et al.* (2016) Biosynthesis of antibiotic leucinostatins in bio-control fungus *Purpureocillium lilacinum* and their inhibition on *Phytophthora* revealed by genome mining. *PLOS Pathog* **12**, e1005685.

Xin, Z., OuYang, Q., Wan, C., Che, J., Li, L., Chen, J. and Tao, N. (2019) Isolation of antofine from *Cynanchum atratum* BUNGE (Asclepiadaceae) and its antifungal activity against *Penicillium digitatum*. *Postharvest Biol Technol* **157**, 110961.

Yang, F., Abdelnabby, H. and Xiao, Y. (2015) A mutant of the nematophagous fungus *Paecilomyces lilacinus* (Thom) is a novel biocontrol agent for *Sclerotinia sclerotiorum*. *Microb Pathog* **89**, 169–176.

Youssef, K., Sanzani, S.M., Ligorio, A., Ippolito, A. and Terry, L.A. (2014) Sodium carbonate and bicarbonate treatments induce resistance to postharvest green mould on citrus fruit. *Postharvest Biol Technol* **87**, 61–69.

Zhang, Z., Yang, D., Yang, B., Gao, Z., Li, M., Jiang, Y. and Hu, M. (2013) β-Aminobutyric acid induces resistance of mango fruit to postharvest anthracnose caused by *Colletotrichum gloeosporioides* and enhances. *Sci Hortic* **160**, 78–84.

Zhou, D., Wang, Z., Li, M., Xing, M., Xian, T. and Tu, K. (2018) Carvacrol and eugenol effectively inhibit *Rhizopus stolonifer* and control postharvest soft rot decay in peaches. *J Appl Microbiol* **124**, 166–178.

Zhu, H., Zhao, L., Zhang, X., Foku, J.M., Li, J., Hu, W. and Zhang, H. (2019) Efficacy of *Yarrowia lipolytica* in the biocontrol of green mold and blue mold in *Citrus reticulata* and the mechanisms involved. *Biol Control* **139**, 104096.