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Evaluation of the synergy between *Schwanniomyces vanrijiae* and propolis in the control of *Penicillium digitatum* on lemons

Kamal A. M. Abo-Elyousr[1,2*], Adel D. Al-Qurashi[1] and Najeeb M. Almasoudi[1]

Abstract

Background: Green mold disease on citrus caused by *Penicillium digitatum* is the most serious and destructive disease. It is causing 90% of production losses during post-harvest handling.

Results: In this study, the activity of seven yeast isolates from lemons against *P. digitatum*, a fungal pathogen that causes the green mold disease in lemons, was isolated and examined. In vitro experiments showed that isolate three significantly reduced pathogen growths and were later identified as *Schwanniomyces vanrijiae*. In addition, 3% ethanolic extracts of propolis (EEP) caused a strong mycelial growth inhibition with inhibition halos of 1.4 cm. The use of *S. vanrijiae* treatments to protect lemon fruits from green mold has been reported (55%); however, reports describing the application of EEP are limited (40%). Thus, the effectiveness of the combination of *S. vanrijiae* and 3% EEP in an antagonistic mixture for protecting lemon fruits from *P. digitatum* was examined. EEP and *S. vanrijiae* treatments were applied alone and in combination in both in vitro and in vivo conditions. The combined application of 3% EEP + *S. vanrijiae* on lemon fruits significantly reduced the severity and incidence of green mold (80 and 93.7%, respectively) with much higher efficacy than either treatment alone. Lemon fruits treated with both *S. vanrijiae* and 3% EEP showed increased levels of antioxidants, peroxidase (POD), polyphenol oxidase (PPO), and phenol than the untreated control.

Conclusion: The results indicated that the combination of *S. vanrijiae* + 3% EEP can strongly protect lemon fruits from green mold compared with the sole application of either bioagent.

Keywords: *Penicillium digitatum*, Lemon, Biocontrol, Antioxidants, Propolis, Synergy

Background

Citrus fruits are one of the most economically important fruits in Saudi Arabia and have additional worldwide importance, being a major source of vitamin C and carotenoids. The most serious postharvest diseases of lemons are the blue and green mold caused by *Penicillium* sp. (Wang et al. 2018), with the green mold disease caused by *Penicillium digitatum* being the most serious and destructive (Eckert and Ogawa 1988). It is responsible for 90% production losses during postharvest handling (Bagy et al. 2020). *P. digitatum* enters mature citrus fruits through wounds; this results in the appearance of white fungal mycelia exhibiting green-colored growths. This disease then spreads throughout the fruit, resulting in changes in color, texture, and taste, and the development of a large olive-green surface surrounded by mycelial growth. Synthetic fungicides are the most common method for controlling these types of plant diseases due to their speed and efficacy (Palou et al. 2008). However,

* Correspondence: kaaboelyousr@agr.au.edu.eg
[1]Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah 80208, Saudi Arabia
[2]Department of Plant Pathology, Faculty of Agriculture, University of Assiut, Assiut 71526, Egypt

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post-harvest disease control with synthetic fungicides has resulted in the evolution of resistant strains, environmental pollution, and harmful effects on human health. Therefore, other methods for disease control besides synthetic fungicides must be developed.

The best current alternative to synthetic fungicides is bioagents application (Parafati et al. 2015). Yeast antagonists, including *Pichia guilliermondii*, *Clavispora lusitaniae*, and *Cryptococcus laurentii* (Zhang et al. 2020), have been shown to repress the growth of *P. digitatum* and *P. italicum* that affect citrus fruits. However, the biocontrol performance of antagonistic microorganisms is less effective than conventional fungicides under semi commercial circumstances (Liu et al. 2013). The efficacy of such antagonists in controlling postharvest diseases should thus be enhanced (Mahunu et al. 2016).

Propolis is a natural product from honeybees that exhibits multiple biological activities and has recently been described as a health supplement suitable for consumers. Water and ethanolic extracts of propolis (EEP) showed antifungal, antibacterial, and antioxidant behavior (Abo-Elyousr et al. 2017). The biological activities exhibited by and chemical compounds contained in propolis depend on the collection time, geographical distribution, and nearby plant species (Hegazi et al. 2014). Propolis is composed of approximately 10% essential oils, 30% wax, 50% resin, 5% pollen, and 5% of other organic compounds (Falcao et al. 2010). Propolis has been used to delay fugal decay in different fruits and extend lifetimes of stored fruits (Ozdemir et al. 2010). In addition, 3% EEP was used to reduce the decay of oranges caused by *Penicillium* spp. (El-Badawy et al. 2012), and 10% EEP completely repressed *Penicillium* spp. mycelial growth on potato dextrose agar (PDA) (Ayhan et al. 2013).

The study on the combined application of yeast + propolis bioagents to control the green mold disease of lemon has not been conducted before. Thus, this study was undertaken to examine the in vitro antagonistic activity of *S. vanrijiae* and EEP alone and in combination against *P. digitatum*, the pathogen responsible for green mold of lemon fruits. Also, in vivo studies to test the efficacy of the combination on *P. digitatum* in lemon fruits were performed.

Methods

Isolation and characterization of endophytic yeasts and green mold

Samples of healthy and infected lemon fruits that showed typical symptoms of the green mold disease were collected from a storage house located in Jeddah, Saudi Arabia, and seven isolates of endophytic yeasts and *P. digitatum* were obtained. Green mold pathogens were morphologically identified based on mycelial growth, colony color, and spore structure as described by Kurtzman et al. (2000), with identities confirmed by ITS region sequencing.

In vitro inhibition of *P. digitatum* by yeast

The in vitro inhibition of *P. digitatum* by seven yeast isolates was tested. A loop containing 24-h-old yeast was horizontally streaked near the edges of PDA; dextrose, 20 g; agar, 15 g; potato, 200 g) plates. A 5-mm mycelial plug of a 3-day-old *P. digitatum* colony grown on PDA was placed upside down on each streaked plates 3 cm from each streak. PDA plates containing only *P. digitatum* plugs were used as controls. Plates were incubated at 25 °C for 5 days, and the reduction in *P. digitatum* growth was calculated using the following formula:

$$I\% = (A - B/A) \times 100$$

Where $I\%$ is the percent mycelial growth inhibition, A represents the growth diameter of the control, and B represents the growth diameter (cm) of the pathogen for each treatments. Each experiment was performed twice with four replicates per experiment. The yeast isolates that most significantly reduced *P. digitatum* mycelial growth were selected for additional experiments.

Preparation of EEP

EEP were prepared by grinding 200 g of frozen propolis that was placed for 3 h prior to grinding. For grinding, 69% ethanol was used with an extraction ratio of 3:1 ethanol/propolis, and mixtures were shaken for 2 days at 150 rpm. Extracts were centrifuged at 5000 rpm for 10 min and filtered using filter paper. The resulting supernatant was collected and kept at room temperature for 3days to allow for evaporation, and the remaining resin was collected for future use. To prepare 1, 2, and 3% EEP solutions, equivalent weights of propolis were dissolved in the required volume of 70% ethanol. Then, EEP solutions were incubated in the refrigerator for further use Abdel-Rahim and Abo-Elyousr (2017).

In vitro inhibitory activity of EEP

To evaluate the in vitro inhibitory activity of EEP, conidia were collected from 1-week-old *P. digitatum* cultures grown on PDA plates incubated at 25 °C. Briefly, spore suspensions were prepared by addling distilled water to growing colony plates to collect spores, the concentration of each spore suspension was adjusted to 10^4 spore/mL, and 1 mL spore suspension was spread on the surface of a fresh PDA plate. Next, 0.5-cm-diameter wells were prepared in each plate in which 50 μL of 1%, 2%, or 3% EEP were poured, with 100 μL SDW plus 3% ethanol poured into wells of the control plates. Plates were then incubated at 25 °C for 5 days in the dark, and the inhibition zone (cm) around each well was

measured. Four replicates for each treatment were used, and the experiment was conducted twice.

Molecular identification of bioagents

The selected bioagents were identified by PCR analysis of their ITS regions, except for *P. digitatum*, was identified based on morphology. For PCR amplification, DNA was extracted from 24-h-old yeast cultures using a DNeasy plant extraction kit (Qiagen, CA, USA) according to the manufacturer's instructions. The primer pair ITS1 F (GCATCGATGAAGAACGCAGC) and ITS4 R (TCC TCC GCT TAT TGA TATGC) was used to amplify partial ITS gene sequences (White et al. 1990). Standard PCR was performed with reaction mixtures containing 20 μL DNA template, 4 μL 10 mM dNTPs, 5 μL 5× PCR buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase (Promega), 25 pmol of each primer, and sterilized distilled water up to a final volume of 50 μL. Reactions were performed in a thermal cycler using the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles consisting of 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s, followed by a final extension of 72 °C for 7 min. PCR products (10 μL aliquots) were then analyzed on 1% agarose gels run for 75 min in TAE buffer (Tris, 40 mM; sodium acetate, 20 mM; EDTA, 1 mM; pH 7.2), with bands detected using a UV illuminator. Ribosomal ITS PCR products were identified based on their relative amounts of electrophoretic migration, then eluted from the gel, and purified using a purification kit (SolGent, Daejeon, South Korea), following the manufacturer's instructions. PCR products were confirmed by sequencing (SolGent). Partial sequences of PCR products were compared with the whole yeast genome sequence obtained from NCBI, and the isolated sequences were 100% identical to the sequence obtained from NCBI.

In vitro effects of *S. vanrijiae* PHYTSV1 and EEP

In vitro inhibitory activity

To examine the in vitro inhibition of *P. digitatum* by EEP and *S. vanrijiae*, spore suspensions of *P. digitatum* were prepared from 3-day-old colonies by adding 5 mL distilled water to Petri plates containing 10^4 spores/mL. The resulting spore suspension (200μL) was then spread over the surface of a PDA plate. After the suspension had been absorbed by the plate, 0.5-cm punches were made into the agar and then filled with a 50-μL mixture of *S. vanrijiae* PHYTSV1 (1×10^9 CFU mL^{-1}) and 3% EEP (1:1). Next, plates were incubated at 27 °C for 5 days, and the reduction in mycelial growth (*I*%) was calculated. Four replicates were used per experiment, and each experiment was conducted twice.

Effects on spore germination

Effects of EEP and *S. vanrijiae* PHYTSV1 on spore germination were determined. Glass tubes were filled by potato dextrose broth (PDB), and 100 μL *P. digitatum* conidial suspension (1×10^6 conidia/mL) was transferred to tubes containing *S. vanrijiae* PHYTSV1, EEP, or both. Control treatment tubes were also prepared with only PDB. Tubes were then incubated at 25 °C on a rotary shaker at 100 rpm for 15 h. To observe the germination rate for each treatment, at least 200 spores per replicate were examined under a microscope, and spore germination was determined using the criteria described by Elsherbiny et al. (2021). Germination inhibition of spores was calculated using the equation presented above. Three replicates were performed for each condition, and the experiment was conducted twice.

In vivo activity of *S. vanrijiae* PHYTSV1 and EEP

Preparation of pathogen and antagonist inoculum

To prepare the inoculum, *P. digitatum* was transferred onto PDA plates and incubated at 25 °C for 7 days. After germination, 10 mL distilled water was added to each plate, and spores were scraped into solution using a sterilized bacterial rod. The spore suspension was then filtered through a layer of sterile muslin cloth. Using a hemocytometer, the concentration of the conidial suspension was counted and adjusted to 24×10^6 CFU/mL (Abdel-Rahim and Abo-Elyousr 2018). Erlenmeyer flasks were filled with 50 mL yeast malt broth medium, inoculated with *S. vanrijiae* PHYTSV1, and then incubated at 25 °C with shaking at 200×*g* for 48 h. Tubes containing the resulting broth culture were then centrifuged at 6000 rpm for 10 min. The density of the re-suspended cells was then adjusted to 2×10^7 cells/mL using a hemocytometer.

Effects of S. vanrijiae and EEP on stored lemons

Effects of *S. vanrijiae* PHYTSV1 and EEP on lemon fruits (*Citrus limon* L. Burm) were examined using protocols described by Sallam et al. (2012) with minor modifications. Briefly, healthy lemons of the seedless lime variety were purchased, and the surface was sterilized by dipping the fruits in 2% NaClO for 2 min. After surface sterilization, fruits were rinsed 3 times with sterilized distilled water and then placed on sterile filter paper to dry. Next, 2-mm-deep and 5-mm-wide wounds were made on the surface of the peel of each fruit in opposing directions. Each wound was covered with 50 μL of *S. vanrijiae* (1×10^9 CFU/mL), 3% EEP, or a 1:1 v/v mixture of each. For control samples, 20 μL of sterile distilled water was added to each wound. Fruits were then allowed to sit at for 1 h, and then, 20 μL *P. digitatum* conidial suspension (10^4 CFU/mL) was injected into each fruit. The fruits were then incubated at 25 °C and

90% humidity for 7 days in (25 × 30 cm) plastic boxes. The resulting lesion diameter on each fruit was measured according to Madbouly et al. (2020), and the disease incidence and severity determined as follows:

$$\%\text{Disease incidence} = (\text{No.of infected lemons}/\text{Total no.of lemons}) \times 100$$

Disease severity was evaluated using the following rating scale: 0 = no infection observed; 1 = a quarter of the fruit decayed; 2 = half the fruit decayed; 3 = three-quarters of the fruit decayed; and 4 = the entire fruit decayed.

$$\%\text{Disease severity} = 100 \times \Sigma\,(\text{No.of infected lemons} \times \text{No.scale}) / \text{Total no.of lemons} \times \text{highness no.scale}$$

Four replicates were used to perform each treatment with 20 lemons per treatment.

Effects of yeast and propolis on peroxidase (POD) and polyphenol oxidase (PPO) activities in lemon tissues

Enzyme extraction

To extract enzymes from lemons, 2 g of tissue from each fruit was collected. Tissues were placed in 20 mM Tris-HCl buffer (pH 7.2) and homogenized using a homogenizer. The homogenate was poured into glass tubes and centrifuged at 10,000 rpm at 4 °C for 10 min. Next, the supernatant was designated as crude extract and stored at − 20 °C for later determination of PPO and POD activities.

POD activity

POD activity was measured according to Bereika et al. (2020) with some modifications. Each prepared reaction mixture was 1 mL in total and contained 0.25 mL (0.2 M) sodium acetate (pH 5.5), 8 μL (0.97) M H_2O_2, 0.08 mL (0.5 M) guaiacol, and least amount of enzyme preparation. The change in absorbance at 470 nm reflecting guaiacol oxidation was then followed for 1 min using a spectrophotometer. Under standard assay conditions, the amount of enzyme that caused an increase in absorbance of at 1.0 O.D per min was defined as one unit of enzyme. Extraction buffer alone served as a blank reference sample. Three replicates were used for each treatment.

PPO activity

PPO activity was determined, following protocols from Batra and Kuhn (1975) using catechol as a substrate. A catechol solution (20 mM) was prepared using 0.01 M Na_3PO_4 (pH 6.8); then, 0.2 mL extract was added as quickly as possible to 2.8 mL catechol. Using a spectrophotometer, the change in absorbance at 400 nm was recorded for 2 min. An enzyme activity unit was defined as the amount of enzyme that caused an absorbance change of 0.1 in 1 min under standard assay conditions. Extraction buffer alone served as a blank reference. Three replicates were measured for each treatment.

Non-enzymatic assays

Sample preparation

For flavonoid and phenolic compound extraction, 1.0 g sample was suspended in 10 mL 70% ethanol (v/v). The suspension was then placed at 30 °C on a shaker at 120 rpm for 2 h and then centrifuged at 1013×*g* for 5 min. The resulting supernatant was used for further analysis.

Total phenol content

The total phenol content of each sample was measured by the method established by Malik and Singh (1980). Methanol extracts (50 μL) of each sample or standard (gallic acid) were mixed with 850 μL methanol, 100 μL Folin-Ciocalteu reagent was added, and samples were left at room temperature for 5 min. Next, samples were mixed with 500 μL 20% Na_2CO_3 and incubated at room temperature for 30 min to react. The absorbance of each sample at 750 nm was then measured, and the total phenol content was calculated based on a standard curve. The standard curve was prepared using gallic acid, and the phenol content in each extract was expressed as mg of gallic acid/g of fresh weight. Phenol content measurements were performed twice with three replicates.

Total flavonoid content

Using the modified colorimetric assay reported by Zhishen et al. (1999), flavonoid concentrations in each sample were determined. A 250-μL aliquot of standard (catechin) or methanol extract was mixed with 75 μL 5% $NaNO_2$ and 1.25 mL sterile distilled water. The reaction mixture was left for 6 min and then mixed with 150 μL 10% $AlCl_3$, 0.5 mL (1 M) NaOH, and 275 μL sterile distilled water was added to solution. The absorbance of each solution was measured at 510 nm, and the total flavonoid content was calculated using a standard curve. Known concentrations of catechin were used to generate a standard curve, and the results were described as mg of catechin equivalent/g.

Statistical analysis

Experimental data were analyzed using the statistical package Statistix (ver. 8.1), and two-way ANOVA was used. The least significant difference test at $P = 0.05$ was performed for disease severity and disease incidence means to identify meaningful differences between the means of results of various treatments. Data were expressed as mean ± SE.

Results

Isolation of endophytic yeasts and the causal pathogen of green mold of lemons

Fungal pathogens of green mold were isolated from infected lemons, while seven isolates of endophytic yeasts isolates were collected from healthy lemons.

In vitro antagonistic activity of yeast against *P. digitatum*

All isolates were able to inhibit the mycelial growth of *P. digitatum* pathogens with varying levels of efficacy. Isolate 3 reduced *P. digitatum* growth by a significantly higher level than other isolates, with mycelium growth reductions of over 75% than the control (Table 1). Thus, three isolates were selected for further experiments.

Molecular identification of bioagents and yeast species

Yeast isolate 3 was identified as *S. vanrijiae* based on BLAST searches of the NCBI Nucleotide Collection Database for similar ITS sequences. A phylogenetic tree was also assembled using ITS sequences from *S. vanrijiae* and close homologues (Fig. 1) by using MEGAX. The ITS sequences of the pathogenic and yeast isolates we obtained have been added to the GenBank database under accession no. MT523046.

In vitro inhibition of *P. digitatum* by EEP

The in vitro antimicrobial effect of 1, 2, and 3% EEP against *P. digitatum* was tested. The 3% EEP solution was the most effective one for inhibiting the mycelium growth of *P. digitatum*, with inhibition halos of 2.4 cm observed. Although lower, inhibitory activity was also observed for 1% and 2% EEP solutions, with inhibition halos of 1.3 cm and 1.4 cm, respectively.

In vitro inhibitory activity of *S. vanrijiae* PHYTSV1 and 3% EEP mixtures

The growth reduction of fungal pathogens was significantly higher when mixtures of *S. vanrijiae* and 3% EEP were used compared with the application of either component alone, with mixtures producing 3.3-cm inhibition halos.

Table 1 Percentage of *Penicillium digitatum* growth inhibition caused by different yeast strain in vitro

Isolates no.	Inhibition zone (mm)
1	31.0 ± 2.0 b
2	11.0 ± 3.0 d
3	75.0 ± 1.1 a
4	22.0 ± 2.0 c
5	33.0 ± 2.0 b
6	32.0 ± 2.0 b
7	13.0 ± 3.0 d
Control	0.0 ± 0.0 e

Values in the column followed by different letters indicate significant differences among treatments according to lease significant differences test ($P = 0.05$)

Impact of the *S. vanrijiae* PHYTSV1 and 3% EEP mixture on spore germination

A mixture of *S. vanrijiae* and 3% EEP caused significantly higher inhibition of *P. digitatum* spore germination ($P < 0.05$) than other individual treatments. The combination of *S. vanrijiae* + 3% EEP reduced *P. digitatum* spore germination by 80.5%, while 3% EEP and *S. vanrijiae* caused 65 and 75% reductions in spore germination, respectively (Fig. 2).

In vivo activity of *S. vanrijiae* PHYTSV1 and 3% EEP

The activities of *S. vanrijiae* PHYTSV1 and 3% EEP were tested in vivo on lemon fruits. Artificial wounds were treated with *S. vanrijiae* PHYTSV1 and EEP either alone or as part of a mixture. After 1 h of incubation of wounds with *S. vanrijiae* PHYTSV1 and/or EEP, each wound was inoculated with a conidial suspension of *P. digitatum*. Results showed a significantly (*P* 0.05) greater reduction in disease incidence on fruits treated with *S. vanrijiae* PHYTSV1 and EEP mixture compared with fruits treated with either component individually. Furthermore, disease severity was also reduced in fruits receiving combined treatments than in untreated control (Table 2).

Effect of *S. vanrijiae* PHYTSV1 and EEP alone and in combination on levels of defense-related enzymes, phenolic compounds, and flavonoids in lemons

Effects on enzyme activity

During fruit storage, PO activity had significantly increased ($P < 0.05$) in all treated lemons compared with control lemons, 2 days post treatment. The maximum PO activity was reached 4 days post-treatment (Fig. 3). In addition, a similar pattern was observed for PPO activity, which began to decline 4 days post-treatment (Fig. 4).

Total phenolic content

The total phenol content was determined at multiple times post treatment (Fig. 5). The three tested treatments increased the total phenol content at all times, except day 1. The total phenol content was highest in lemons treated with both *S. vanrijiae* PHYTSV1 and 3% EEP, followed by lemons treated with either control and with untreated lemons containing the lowest amounts. After 4 days post-treatment, total phenolic levels began to decline.

Total flavonoid content

The total flavonoid content was measured up to 8 days post-treatment (Fig. 6). All treatments caused significant

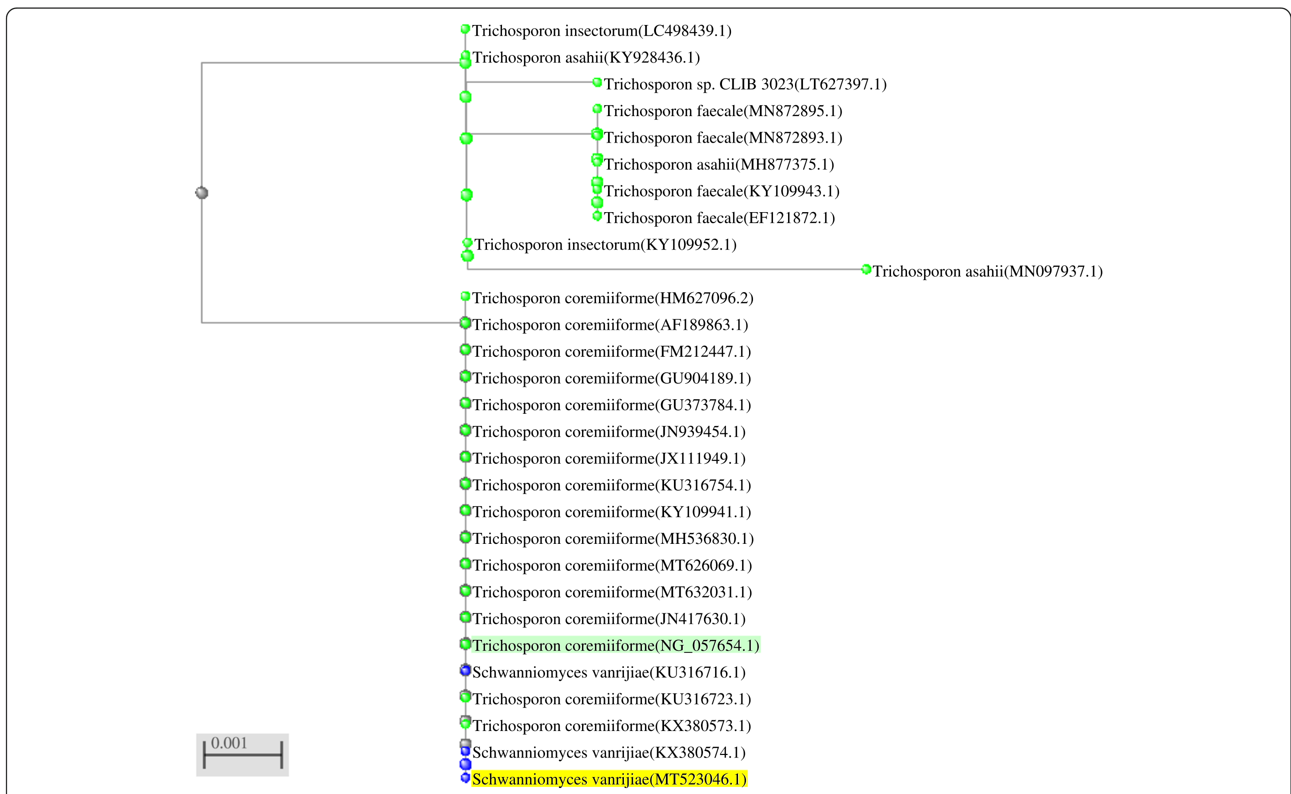

Fig. 1 Phylogenetic analysis of yeast isolate on ITS gene. This analysis was performed using the neighbor-joining method in BLAST pairwise alignments

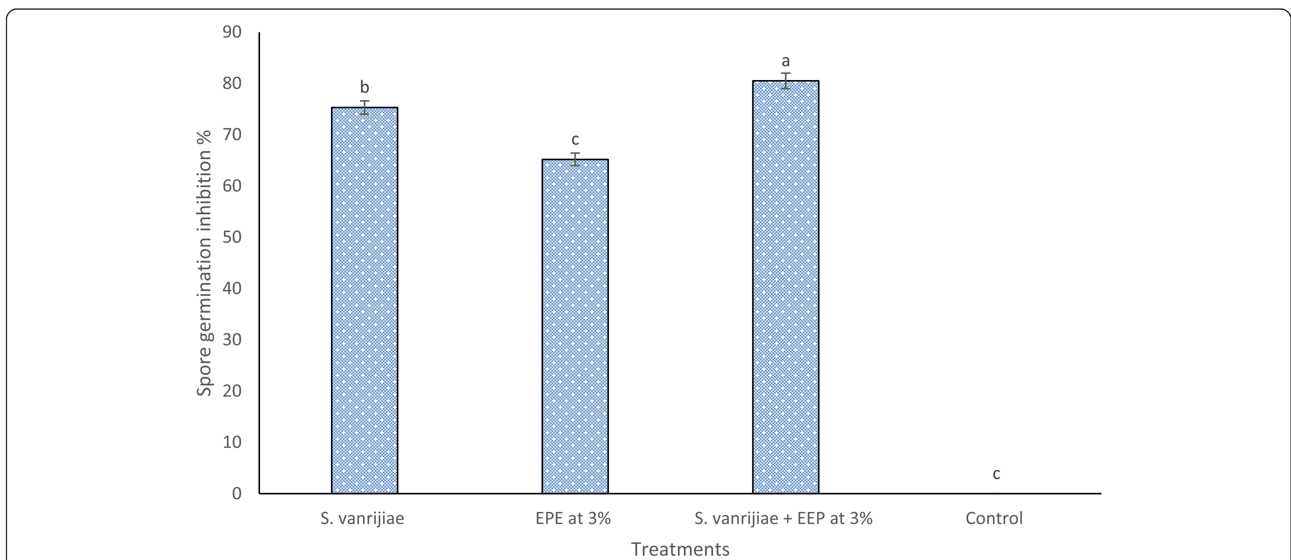

Fig. 2 Effect of *S. vanrijiae*, 3% EPE, and mixture on spore germination. Values in the column followed by different letters indicate significant differences among treatments according to lease significant differences test (P = 0.05)

Table 2 Effect of *S. vanrijiae* and ethanolic extract of propolis (EPE) alone and in combination on inhibiting *Penicillium digitatum* on lemon fruits 7 days post-incubated at 25 °C

Treatments	Incidence (%)	Severity (%)	Lesion diameter (cm)
S. vanrijiae	45 ± 0.6 c	10 ± 0.3 c	1.0 ± 0.03 c
EPE	60 ± 0.6 b	15 ± 0.5 b	1.9 ± 0.02 b
S. vanrijiae ± EPE	20 ± 1.1 d	5 ± 0.5 d	0.5 ± 0.04 d
Infected control	100 ± 0.5 a	80 ± 0.2 a	5.0 ± 0.02 a

Values in the column followed by different letters indicate significant differences among treatments according to lease significant differences test ($P = 0.05$)

increases in the total flavonoid content than the controls. At 4 and 6 days post-treatment, flavonoid levels reached their maximum values, with flavonoid levels trending downward in lemons treated with a mixture of *S. vanrijiae* PHYTSV1 and EEP, EEP alone, and *S. vanrijiae* PHYTSV1 alone. After day 6, the flavonoid content began decreasing in all samples.

Discussion

In this study, seven yeast isolates from lemons had significant in vitro antifungal activity against *P. digitatum*, the causal pathogen of the green mold disease in citrus fruits. The antifungal potency of biocontrol agents likely arises due to the competition between antagonists and pathogens for nutrients, which can result in the production of antifungals, antibiotics, or mycoparasitism via cell wall hydrolysis (Madbouly et al. 2020). A previous study on the use of the yeast (*P. guilliermondii*) as treatment for green mold pathogens provides additional evidence that competition between pathogens and biocontrol antagonists for nutrients likely plays a key role in the biological control of *P. digitatum* (Winiewski et al. 1991). The production of metabolites by yeast may significantly affect the ability of other fungi to produce cell wall-degrading enzymes and may hydrolyze the conidia of competing fungi (Li et al. 2019).

In the present study, 3% EEP caused the highest reduction of mycelia growth of *P. digitatum* in vitro than 1 and 2% EEP, potentially due to the higher levels of antimicrobial and antioxidant activities of phenolic compounds resulting from increased EEP. Other researchers have reported a positive correlation between the antioxidant and antimicrobial characteristics of propolis and the polyphenols and flavonoid content of (Chaillou and Nazareno 2009). Moreover, previous work by Yang et al. (2009) showed that EEP contains more flavonoids and phenols than propolis extracts and exhibits more promising antifungal activity against *P. italicum*.

Spore germination and mycelial growth of *P. digitatum* was significantly reduced by *S. vanrijiae* and EEP. The discrepancy might be related to the levels of phenolic compounds in EEP or metabolites in *S. vanrijiae* (Agirman and Erten 2020).

Madbouly et al. (2020) reported that *S. vanrijiae* can be used to control disease in apple fruits under storage conditions. Using a separate metric for treatment success, Mattiuz et al. (2015) reported that propolis extracts can decrease the weight loss of citrus, apples, tomatoes,

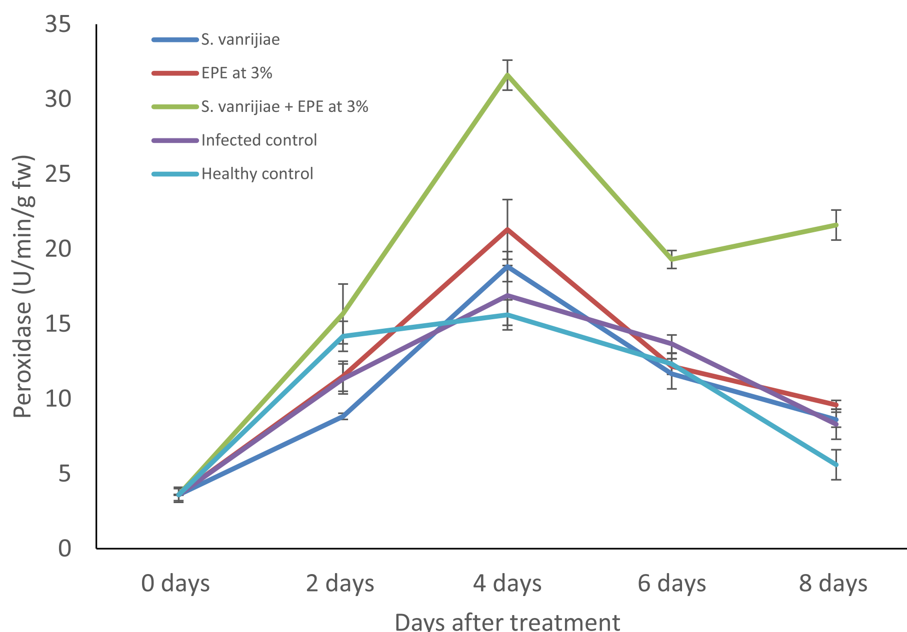

Fig. 3 Time course of peroxidase activity (U/min/g fw) in extracts from lemon cv seedless limes tissues treated or not with *S. vanrijiae* and 3% EPE in mixture. All the values are the means of four replicates ± SE

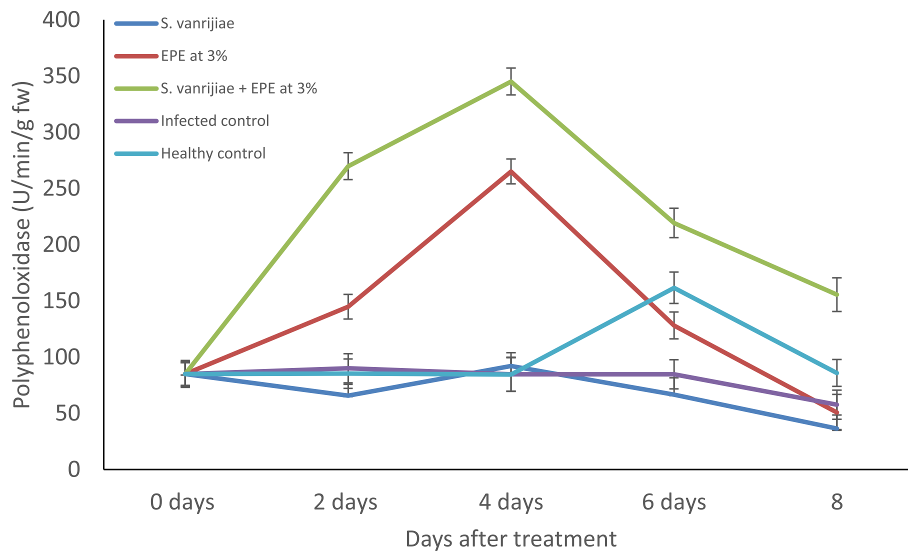

Fig. 4 Time course of polyphenoloxidase activity (U/min/g fw) in extracts from lemon cv seedless limes tissues treated or not with *S. vanrijiae* and 3% EPE in mixture. All the values are the means of four replicates± SE

and cherries during storage. When fruits are treated with propolis extracts, their surfaces develop coatings of beeswax, which can minimize water loss, gas exchange, and microbial infestations; extend shelf life; and maintain tissue firmness. In addition, the postharvest treatment of citrus fruits with propolis extracts was found to protect against natural disease incidence. For example, treatment with 1000 mg/L propolis reduced fruit decay from 30 to 13% after 22 days of storage. Moreover, numerous additional studies have described the broad activity of propolis against yeast, bacteria, and fungi, including phytopathogenic fungi such as *Colletotrichum gloeosporioides*, *Botrytis*, and *P. italicum*.

PO has a key role in lignification and suberization of host cell walls, which restricts disease development (Bereika et al. 2020). PO also helps strengthen plant cell walls at attachment sites by promoting lignification or cross-linking of specific proteins (Abo-Elyousr et al.

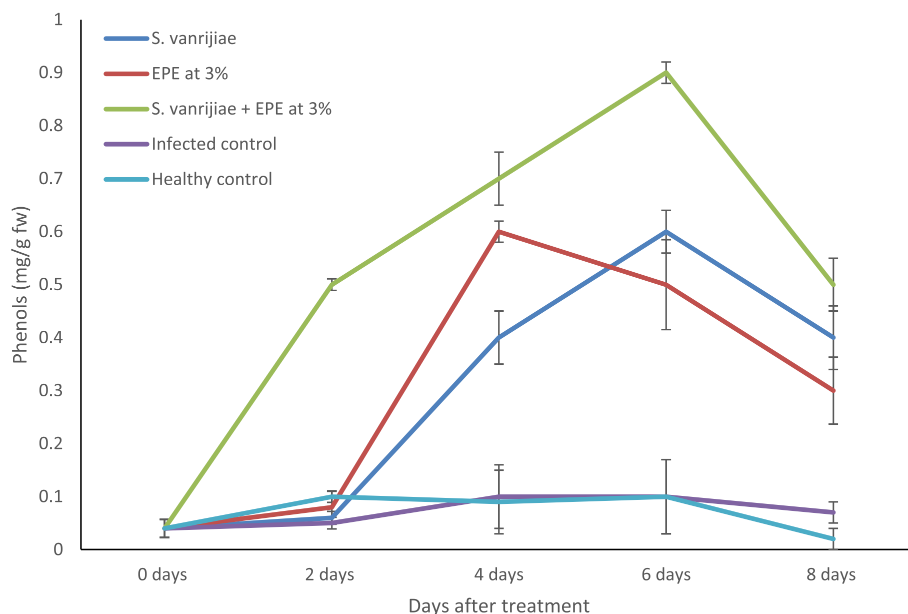

Fig. 5 Time course of phenol content (phenols (mg/g fw) in extracts from lemon cv seedless limes tissues treated or not with *S. vanrijiae* and 3% EPE in mixture. All the values are the means of four replicates ± SE

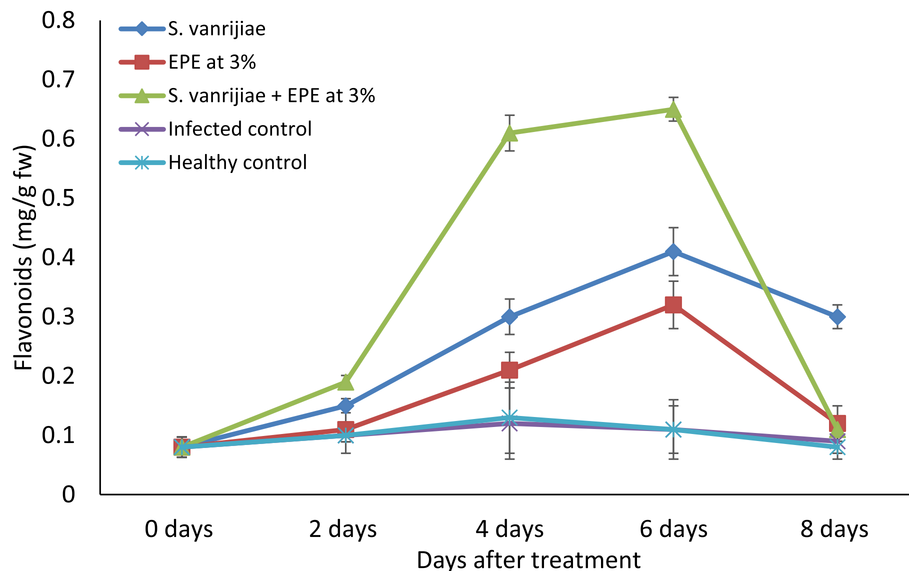

Fig. 6 Time course of flavonoids (mg/g fw) in extracts from lemon cv seedless limes tissues treated or not with *S. vanrijiae* and 3% EPE in mixture. All the values are the means of four replicates ± SE

2008). In the present experiments, PPO activity was higher in lemon fruits treated with yeast and EEP than in control fruits, with such activity increasing from 2 to 3 days after application.

In terms of total flavonoid and phenol content, this study demonstrated that a mixture of yeast and EEP caused a greater increase in phenol content and total flavonoids in lemons than application of either treatment on its own. These results are in agreement with previous studies that measured higher POD activity in apple fruits treated with *Aureobasidium pullulans* compared with control fruit (Youssef et al. 2020). *S. vanrijiae* and 3% EEP application either alone or in combination exhibited strong in vitro reduction of *P. digitatum* radial growth and in vivo reductions in disease development on lemon fruits. Moreover, these treatments showed promise in conidia germination inhibition. Furthermore, the enhancement of PO and PPO activities, as well as levels of flavonoids and phenolic compounds, may have a direct relationship with the process by which yeast and EEP induce resistance against green mold in lemon fruits. Intensification of natural host resistance significantly correlated with augmentation of antioxidant systems.

Abbreviations
EPE: Ethanol propolis extracts; POD: Peroxidase activity; PPO: Polyphenol oxidase; LSD: Least significant difference

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Authors' contributions
All authors contributed equally in the manuscript. AKAM suggested the idea of the work and contributed to data curation and their validation as well as writing original draft. ADQ contributed to the formal analysis of the data. NMM contributed to the reviewing and editing the manuscript. All authors reviewed and approved the final version of the manuscript.

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Declarations

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