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Propolis extract and oregano essential oil as biofungicides for garlic seed-cloves: *in vitro* assays and synergistic interaction against *Penicillium allii*

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Running title: Propolis and oregano as biofungicides

Abstract

Aims: This study aimed to evaluate *in vitro* individual and combined antifungal activity of propolis extract (PE) and oregano essential oil (OEO) against *Penicillium allii*, causal agent of blue mold disease. The chemical characterization of both products was also included.

Methods and Results: Chromatographic analysis of PE and OEO confirmed the presence of bioactive compounds. The antifungal susceptibility assays showed that PE and OEO were highly active against the mycelial growth and conidial germination of *P. allii*. PE and OEO MICs were 12.5 μL mL^{-1} and 1.5 μL mL^{-1}, respectively. The MFCs of these products were 50 μL mL^{-1} and 3.1 μL mL^{-1}, respectively. PE acted mainly through diffusion, while OEO acted by a mixed contribution of vapor and diffusion. Synergism and additive effect between both products were found in some combination ratios.

Conclusion: PE and OEO, both natural products with different chemical composition, have a strong antifungal activity against *P. allii* and show a favorable interaction causing synergism.

Significance and Impact of the Study: The results of this study indicated the potential use of PE combined with OEO as a non-conventional strategy towards the formulation of a biofungicide to control blue mold disease in garlic seed-cloves.

Keywords: antifungal activity; biofungicide; blue mold disease; essential oil; natural products; plant pathology; propolis; synergy testing

Introduction

Blue mold disease caused by *Penicillium allii* produces significant economical loss worldwide. At present it can only be preventively controlled using synthetic fungicides in seed-cloves (Valdez *et al*. 2006). It has been reported that this practice does not guarantee an effective control, posing toxicity risks to the operator and the environment, and tending to generate pathogen resistance (Salinas and Cavagnaro 2020). Thus, alternative treatments are required and the interest in natural bioactive compounds has been growing rapidly, especially in those of plant origin as they could be used as biofungicides (Abbey *et al*. 2018; Zhang *et al*. 2018).

Propolis is a complex substance elaborated by honey bees (*Apis mellifera* L.) by mixing plant resins with their saliva and wax (Ghisalberti 1979). It comprises around 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% organic compounds. This composition is quite variable depending on the origin of the samples, as it is related to the flora surrounding the hives (Wagh 2013). The antimicrobial activity is the most commonly and extensively investigated attribute of propolis (Burdok 1998; Bankova *et al*. 2014). However, the use of propolis to control phytopathogens has been poorly studied. According to Yang *et al*. (2011), propolis ethanolic extract has a strong antifungal activity against *Penicillium italicum*, causal agent of citrus blue mould. Matny *et al*. (2014) found that propolis inhibits *Sclerotinia sclerotiorum* growth on culture media, and protects snap bean against white rot disease under storage conditions. Various compounds like phenols and flavonoids have been reported as responsible for propolis antifungal activity by affecting the permeability of the cytoplasmic membrane, leading to the total leakage of the cellular constituents such as nucleic acids, proteins and inorganic ions, causing complete cell death (Petruzzi *et al*. 2020).

Oregano essential oil is an aromatic liquid obtained from the aerial vegetative and reproductive organs of *Origanum vulgare* plants. About 500 compounds have been reported to constitute this essential oil, including terpenes, terpenoids, aliphatic and aromatic compounds. Its composition depends on many factors including environmental and growing conditions, collection season and extraction method (Sakkas and Papadopoulou 2017). Previous studies have demonstrated that oregano essential oil has a strong antimicrobial activity against several phytopathogens such as *Fusarium verticillioides* (Velluti *et al*. 2004), *Phytophthora infestans* (Soylu *et al*. 2006)*, Sclerotinia sclerotiorum* (Soylu *et al*. 2007) and *Plasmopara viticola* (Rienth *et al*. 2019). It is generally believed that the mechanism of action of essential oils is principally performed against microorganism cytoplasmic membrane. Essential oil components as well as hydrophobicity accumulate in cell membranes, disturbing their structures and increasing permeability. Leakage of intracellular constituents and impairment of microbial enzyme systems can then occur, and extensive loss of the cell content will cause the death of the cell (Lv *et al*. 2011).

Our previous reports showed that propolis has antimicrobial activity against *Didymella bryoniae*, *Rhizoctonia solani* (Gallez *et al*. 2014), *Penicillium* sp. (Cibanal *et al*. 2019) and several phytopathogenic bacteria (Cibanal *et al*. 2020). Based on those results and given the present context, we carried out a detailed study of the antifungal activity of propolis extract (PE) and oregano essential oil (OEO) against *Penicillium allii*. Different *in vitro* antimicrobial tests were performed for each product, and their antifungal interaction was assayed for the first time. The main chemical characteristics of both products were also described in this work.

Materials and methods

Natural products

Propolis characterization

A representative sample of *Apis mellifera* propolis was collected in Río Colorado, Argentina (36°09′02″S and 70°23′47″W). Many cultivated tree species grow near the apiary such as *Populus* sp., *Salix* sp., *Malus domestica*, *Prunus persica* and *Pyrus communis*. The chemical characterization of PE included total polyphenol, flavonoid content and final concentration of soluble compound, previously described in Cibanal *et al*. (2020). The chemical polyphenol profile was also determined with high performance liquid chromatography equipped with a diode array detector (HPLC-DAD). An HPLC Prominence LC-20A series (Shimadzu Corporation, Japan), equipped with auto-sampler, quaternary pump and diode array detector (SPD-M20A) was used for this purpose. Separation of each EH sample was carried out on a Phenomenex C-18 column (250 x 4.6 mm i.d., particle size 5 μm) using as a mobile phase water with 0.1% TFA (A) and acetonitrile with 0.1% TFA (B). The injected volume was 10 μl. The following gradients of mobile phase were used at a flow rate of 0.7 mL/min: 35% B, 0 min; 50% B, 10 min; 50% B, 15 min; 80% B, 40 min; 35% B, 50 min; 35% B, 55min. The main components were identified by comparing retention times and UV-spectrum with those of the standards (Cibanal *et al. 2020)*.

OEO characterization

Stems and leaves of *Origanum vulgare* cv. Alpa Sumaj FCA-INTA were collected from an experimental plot at INTA (National Institute of Agricultural Technology), Hilario Ascasubi, Argentina (39°23'33"S and 62°37'44"W). The sample was identified and deposited at the Herbario del Departamento de Agronomía, Universidad Nacional del Sur (voucher specimen BB 4975). The essential oil was obtained by hydrodistillation in a Clevenger-type apparatus heating during 4 h (Rodriguez and Murray 2010). The oil yield was recorded, and the chemical composition was determined by gas chromatography-mass spectrometry (GC-MS). These analyses were performed with a Hewlett-Packard 6890 chromatograph connected to a Hewlett-Packard 5972A mass spectrometer equipped with a capillary column (HP-5, 30 m × 0.25 mm, 0.25 μm film thickness). The carrier gas was helium with flow 1 mL/min. The GC oven temperature was held at 50 °C for 3 min, programmed at 5 °C/min to 200 °C, and then held at this temperature for 1 min and increased again at 50 ° C / min to 280 ° C. Mass spectra were recorded at 70 eV. Mass range was from m/z 35–350 amu. The temperature of the injection block was 250 °C. GC analyses were performed on a Shimadzu G14B chromatograph with a flame ionization detector on a DB-5 column (30 m × 0.25 mm, 0.25 μm film thickness) with the same analytical conditions used for GC-MS analyses. The compounds were identified by comparing their retention indices (Kovats Indices) with those of known compounds, their retention times with authentic samples when available and their MS with those stored in the MS database (NBS75K.L MS DATA) (Sparkman 2005). Relative percentage amounts were obtained directly from GC peak areas.

Plant pathogenic fungus: *Penicillium allii*

Penicillium allii was isolated from highly sick garlic cloves by taking direct smear of spores from the surface of the bulbs. This fungus was morphologically characterized by micromorphology and its pathogenicity was confirmed. *P. allii* was maintained in potato dextrose agar medium (PDA) at 20 ± 2 °C in the collection of Laboratorio de Estudios Apícolas (LabEA), Universidad Nacional del Sur (Argentina). Monosporic isolates were grown for each trial in fungi and yeast agar medium (HyL, Britania®, Argentina), at 25 ± 2 °C during 7 days. When needed, spore suspensions were prepared by rubbing spores from one-week-old cultures and re-suspended in sterile saline solution (0.85%). The number of spores was calculated using a haemocytometer and adjusted to a final concentration of 10^5 spores mL^{-1}.

Antifungal susceptibility assays

Agar dilution method

PE, OEO and three two-fold dilutions of each product (PE$_2$, PE$_3$, PE$_4$, OEO$_2$, OEO$_3$ and OEO$_4$) were tested by the agar dilution method to determine their effect over mycelial growth. PE dilutions were made in sterile distilled water, while OEO dilutions were made in sterile distilled water with 50% (*v*/v) of Tween 20 (Biopack, Argentina). We used a treatment without aggregates as a positive control (C), methodological controls of hydroalcohol as a PE solvent and Tween 20 as an OEO diluent (TW and HA) and Carbendazim as a synthetic fungicide (SC 50%, Gleba, Argentina) (F).

The trial was conducted according to Gallez *et al.* (2014) with minor modifications. Briefly, 100 μL of each treatment were poured into Petri plates (90 mm in diameter) and mixed with 20 mL of HyL medium (40 °C). Once the agar was solid, a fungal plug of 6 mm in diameter was placed in the center of each dish. Plates were incubated in the dark for 30 days at 25 ± 2 °C. The colony growth was measured daily and the percentage of inhibition (I%) was calculated as:

$$I\% = [(dC - dT)/dC] \times 100$$

where dC is the diameter of the control colony (C) and dT is the diameter of the treatment colony. The experimental design was completely randomized with three replicates per treatment, and the trial was repeated twice.

Disc diffusion method

The same treatments and controls described for the agar dilution assay were performed to determine PE and OEO effect over conidial germination, except for the synthetic fungicide (Sawaya *et al*. 2002). Hence, 100 μL of spore suspension were homogeneously spread thoroughly the surface of HyL agar plates. Four filter paper discs (6 mm in diameter) per plate were equidistantly placed on the agar surface. Discs were impregnated with 10 μL of the corresponding treatment, and plates were incubated in the dark for 30 days at 25 ± 2 °C. The zones of inhibition around the discs were measured in millimeters every 48 h of incubation.

The percentage of inhibition (I%) was calculated as:

$$I\% = \{1 - [(dM - dT)/dM]\} \times 100$$

where dM is the diameter of the maximum inhibition halo, and dT is the diameter of the inhibition halo in the treatment. The experimental design was completely randomized with three replicates per treatment, and the trial was repeated twice.

Volatile phase assay

The contribution of the volatile phase in the antifungal activity of PE and OEO was assayed. Four treatments were tested: PE, OEO, hydroalcohol as a control of PE solvent (HA) and sterile distilled water as a positive control (C). Three types of devices were set: inverted Petri dishes with a paper disc placed in the center of the upper plate (vapor test); Petri dishes with a paper disc placed in the center of the agar with a small plastic bell over (vapor-inhibitory test); Petri dishes with a paper disc placed in the center of the agar (diffusion test). All Petri dishes contained 20 mL of HyL agar and were inoculated with 100 μL of spore suspension. Paper discs (6 mm in diameter) were impregnated with 10 μL of the corresponding treatment. Plates were sealed with Parafilm and incubated at 25 ± 2 °C in darkness. The inhibition zone diameter was measured after 9 days. The proportions of the vapor and diffusion activity were estimated according to the contribution index (δ) (Inouye *et al*. 2006). The experimental design was a complete randomized block, considering a block as the type of device with three replicates of each treatment.

MIC and MFC determinations

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of PE and OEO were determined by the broth macrodilution method (Sawaya *et al*. 2002). Eight arithmetical dilutions of each product were tested. PE concentrations ranged from 5% to 0.019% (*v*/v) while OEO concentrations ranged from 1.25% to 0.009% (*v*/v). Growth control (C+), sterility control (C-) and solvent control of PE hydroalcohol (HA, 5%) were also included. This assay was performed in wide-neck conical flasks (25 mL of capacity) with microbiological stopper. Flasks contained 20 mL of HyL broth (Britania®, Argentina), were inoculated with 100 μL of spore suspension and incubated at 25 ± 2 °C in an orbital shaker (80 rpm) for 5 days. At the end of the trial, treatments with visible fungus development were filtered through two gauzes and dried to constant weight to determine fungal biomass.

MICs were defined as the lowest concentration of PE and OEO completely inhibiting fungus growth. MFCs were determined as the lowest concentration that killed 99.9% of the final inoculum, after subculturing, in PDA plates, 10 μL from the MIC determination treatments with no visible fungal growth. The whole trial was performed three times.

Synergy testing

The antifungal interaction of PE with OEO was determined by the checkerboard method (Orhan *et al*. 2005). The assay was developed as follows: PE dilutions were displayed horizontally, while OEO dilutions were shown vertically. The concentrations of PE and OEO were prepared according to 1/2, 1/4 and 1/8 of their MIC values. One growth control (C+), one sterile control (C-) and one solvent control of PE hydroalcohol (HA, 53 μL mL^{-1}) were also included. The assay conditions and procedures were the same as in MIC and MFC determinations. To assess the interaction of both compounds, the fractional inhibitory concentration index (FICI) was calculated (Orthan *et al*. 2005):

$$FICI = FIC_{PE} + FIC_{OEO}$$

where FIC is the fractional inhibitory concentration of each natural product calculated as the quotient of the minimum inhibitory concentration (MIC) in the combination and the MIC alone. The results were regarded as synergy (FICI<0.5), addition (0.5≤FICI≤1), indifference (1<FICI≤4) and antagonism (FICI>4). The whole trial was carried out three times.

Statistical analysis

ANOVA analyses were applied to the results of the agar dilution method, disc diffusion method and MIC and MFC determinations. When a significant F-value was detected, the diameter means were compared with Fisher's Least Significant Difference (LSD) test ($P \leq 0.05$). The association strength between the percentages of inhibition (I%) in the agar dilution method and disc diffusion method was determined by the Spearman correlation coefficient. Infostat software was used for all statistical analyses.

Results

Natural product characterization

The chemical characterization of PE was previously described by Cibanal *et al*. (2019, 2020). These determinations revealed a phenolic content of 28.64 ± 0.17 mg eq gallic acid g^{-1} and a total flavonoid concentration of 15.88 ± 0.42 mg eq quercetin g^{-1}. The dry extract was 6.9% and the oxidation index was 2.33 ± 0.57 s. The analyses of the polyphenol profile by HPLC-DAD (Fig. 1) confirmed the presence of caffeic acid, quercetin, 1,5,7-trihydroxy-flavanone, apigenin, pinobanksin, chrysin, pinocembrin and galangin.

The average yield of OEO was 1.43% on dry mass. The chromatographic analysis (Table 1) showed a high percentage of hydrocarbon monoterpenes (46.60%), followed by oxygenated monoterpenes (36.15%) and aromatic monoterpenes (14.98%). The dominant compound was ʊ-terpinene (26.62%), followed by cis-p-menth-2-en-1-ol (14.93%), terpinen-4-ol (11.79%), thymol (11.17%) and α-terpinene (6.25%). The amounts of the other twenty components found in the sample were in the range of 0.37 to 4.5%.

Antifungal susceptibility assays

Table 2 shows the results of the agar dilution and disc diffusion assays. All treatments based on PE and OEO exhibited, in different degrees, antifungal activity against *P. allii* in both assays. Dose-dependent activity was observed mainly for PE, the most concentrated treatments being the most effective ones (LSD $P \leq 0.05$). The antifungal activity of all treatments declined over time. Neither of the controls showed a significant inhibitory effect in these trials.

In the agar dilution assay (Table 2a and Fig. 2), OEO presented the maximum antifungal activity and remarkably stood out, as it totally inhibited the growth of *P. allii* during 9 days. OEO_2 also showed an excellent performance reaching an I% of more than 95%. PE and its dilutions showed less inhibitory effect than the equivalent

OEO-based treatments. F showed less effect than PE and OEO treatments, but it was statistically different from C. In the agar diffusion assay (Table 2b), OEO was the most effective treatment with an I% of 100% until day 6. PE, PE$_2$, OEO$_2$ and OEO$_3$ reached an I% over 30%, remaining almost constant during the entire trial.

Significant favorable correlations were found by the Spearman's rank, between the inhibition percentages of the agar dilution and the disk diffusion assays. The correlation coefficient ($P \leq 0.001$) was 0.95 after 3 days, 0.97 after 6 days and 0.93 at the end of the trials (9 days).

The δ values in the volatile phase assay against *P. allii* were 13.85 for PE and 50.0 for OEO. These results revealed a major contribution of diffusion in PE activity and a mixed contribution of vapor and diffusion in OEO activity. Indeed, no halo was observed in PE treatment during the vapor test, while a considerable inhibition halo of 20.67 ±7.5 mm was measured in OEO treatment.

Note that in all these assays, OEO-based treatments induced morphological changes in growing colonies regarding the controls (C, HA, TW). The coloration of colonies under OEO treatments was considerably subdued: white or light green in OEO-based treatments versus dark green in the controls.

MIC and MFC determinations

Both natural products, PE and OEO, were highly effective against *Penicillium allii*. PE MIC and MFC were 12.5 μL mL^{-1} (8.6 mg mL^{-1} of dry propolis) and 50 μL mL^{-1} (34.4 mg mL^{-1} of dry propolis), respectively. OEO MIC and MFC were 1.5 μL mL^{-1} and 3.1 μL mL^{-1}, respectively.

HA did not show a significant inhibitory effect compared to C+. We observed statistical differences (LSD $P \leq 0.05$) between the fungal biomass developed in the treatments with the lowest doses of the bioactive products and C+, as PE and OEO treatments significantly reduced fungus growth.

Synergy testing

The FICI for PE and OEO combinations are shown in Table 3. Synergism against *Penicillium allii* was statistically proved in the combination composed of 3.12 μL mL^{-1} of PE and 0.375 μL mL^{-1} of OEO (FICI = 0.5). Four combinations of PE with OEO showed useful additive effect. Although other low concentrations of PE and OEO combined showed visible fungus growth, they did not reach the growth of C+. No antagonism was observed in the combinations evaluated.

Discussion

In recent years, the interest in biofungicides has increased rapidly due to pressing needs for effective and safe tools in crop protection (Chandler *et al*. 2011). Accordingly, our research focused on PE and OEO. The first step of this study was to evaluate and compare the individual antifungal activity of PE and OEO against *Penicillium allii*. Subsequently, we assayed the antifungal interaction between both products. The chemical characterization of PE and OEO was included, since the antimicrobial activity of propolis and essential oils depends, to a large extent, on their

composition and helped us to explain outcomes from our antimicrobial trials (Sforcin 2016; Rostro-Alanis *et al*. 2019).

Several reports have attributed the biological activity of propolis to a wide diversity of plant-derived substances (Salatino *et al*. 2005). The major bioactive constituents of this bee product are phenols and flavonoids, such as chrysin, galangin, pinocembrin and pinobanksin. In addition, caffeic and ferulic acids have also been cited as antimicrobial components of propolis (Ghisalberti 1979). The phenol and flavonoid content determined in the propolis sample used in this study was within the values reported by Bedascarrasbure *et al*. (2004) and Cahillou *et al*. (2004) who compared the quality of different PE samples from Argentina. In addition, chromatographic analysis (Fig. 1) confirmed the presence of the main bioactive compounds of propolis (Yang *et al*. 2011; Cibanal *et al*. 2019).

The antimicrobial prowess of OEO is mainly attributed to carvacrol and thymol, two phenols that usually represent up to 85% of this oil composition (Sakkas and Papadopoulou 2017). However, several studies have demonstrated that OEO could contain a major concentration of γ-terpinene or p-cymene, which have also been associated with antimicrobial activity (Quiroga *et al*. 2013; Olmedo *et al*. 2014). In this study, monoterpenes were the major constituents of OEO, γ-terpinene being predominant over thymol and carvacrol (Table 1). It has been reported that the increase of γ-terpinene is accompanied by a decrease of thymol and vice-versa (Tibaldi *et al*. 2011). Moreover, Bussata *et al*. (1998) reported that hydrodistillation may raise the concentrations of γ-terpinene and 4-terpineol, as observed in this study. The remarkably low concentration of carvacrol in our sample matched those reported by other Argentinean OEO compositions (Quiroga *et al*. 2013; Olmedo *et al*. 2014). According to Dambolena *et al*. (2010), Argentinean OEOs present low concentration of carvacrol due to the genotype of the original material introduced into the country and to environmental conditions.

In the initial screening of antifungal activity for PE and OEO, both products had inhibitory effect in a dose-dependent way over the mycelial growth and conidial germination of *P. allii* (Table 2 and Fig. 2). Although prior studies reported antifungal activity of these products against phytopathogens of the genus *Penicillium* (Daferera *et al*. 2000; Chaillou *et al* 2004), this work showed excellent and consistent results in all antifungal susceptibility assays. In general, OEO treatments presented an efficacy higher than that shown by their equivalent PE treatments. The differences in performance may result from the bioactive components of each product. The outcomes of this trial could also indicate that *P. allii* is more sensitive to OEO compared to PE. As none of the control treatments showed antifungal activity, the results of PE and OEO treatments can be attributed exclusively to bioactive compounds present in these products (Arcila-Lozano *et al*. 2004; Cibanal *et al*. 2020). Treatment F showed less antifungal effect in relation to PE and OEO treatments. This may be accounted for on the basis that the action of synthetic products such as Benzimidazole fungicides like Carbendazim has been reduced by the emergence of resistant strains (Tashiro *et al*. 2012).

Regarding the vapor test, OEO showed a significant inhibition against *P. allii*. This confirms that volatiles make a major contribution to OEO antifungal activity. Indeed, γ-terpinene, the main component of the sample, is considered a highly volatile substance with antimicrobial properties (Rostro-Alanis *et al*. 2019). The delta value of OEO was comparable with those from other essential oils (Bouaziz *et al*. 2009; Inouye *et al*. 2009). On the other hand, the most abundant bioactive compounds of propolis are not volatile, *i.e.* flavonoids and phenolic acids, which is consistent with the lack of bioactivity recorded in this assay. It has been suggested that propolis, beyond the

compounds mentioned above, show some volatile molecules that contribute to its antimicrobial activity (Bankova *et al*. 2014). The most suitable method to isolate propolis volatiles is simultaneous distillation-solvent extraction (Bracho *et al*. 1996). In the present study propolis was extracted using hydroalcohol at 40 °C during 24 h. This temperature is not considered to alter the main bioactive compounds of propolis; however, it might evaporate its volatile molecules.

The decrease observed in the inhibitory action of both products over time, especially in OEO dilutions, might be due to the fact that certain bioactive components of these natural products are sensitive to oxygen, light and moisture, hence they could be degraded (Chouhan et al. 2017). Therefore, in treatments with fungistatic action, the fungal colony may slightly resume some growth. Treatments with higher concentrations of PE and OEO would generate a fungicidal action, there would be no viable remains of the phytopathogen to restart the growth of the colony.

A large range of MICs have been reported for PE and OEO against several fungi, as their effectiveness depends on their composition which varies significantly worldwide. Pobiega *et al*. (2019) found that Poland PE had MIC ranking between 4 and 32 mg mL^{-1}. Moreno *et al*. (2020) reported MIC values from 0.14 to 0.58 mg mL^{-1} for northern Argentinean propolis against *Penicillium* species. In the case of OEO, MIC values ranging between 20 and 0.6 μL mL^{-1} were reported (Souza *et al*. 2007). The MFCs of both products were higher than their MICs. These findings support our previous results, indicating that *P. allii* is more sensitive to OEO than to PE. In this study MICs and MFCs significantly differed from PE and OEO, which was expected given the large difference in the nature of their active molecules. It should be noted that, in practice, other variables such as availability and cost of these products are important so as to select one from the other.

It is remarkable that throughout all the comparative individual assays, OEO showed an excellent performance even when the concentration of thymol and carvacrol was low in the original sample (Table 2 and Fig. 2). Probably, the biological activity of OEO was enhanced by the combined action of all constituents (Rostro-Alanis *et al*. 2019) and by the considerable amount of γ-terpinene (Couto *et al*. 2015). Bakkali *et al*. (2008) proposed that essential oils are complex mixtures of numerous molecules and their biological effects may result from the synergy between them. The morphological changes in *Penicillium* species observed in the treatments containing OEO were also reported in the literature (Tao *et al*. 2014; Lombardo *et al*. 2016). According to these authors, essential oils could affect spore germination, mycelial development and sporulation.

To explore the possibility of formulating a biofungicide for garlic seed-cloves based on the combination and improvement of different natural products, we extended our investigation to the study of a possible synergism between PE and OEO. Combined use of bioactive plant-derived products may have significant advantages associated with the synergistic interactions between them. These benefits include increased efficiency, reduced undesirable effects, improved stability or bioavailability of the free agents and adequate therapeutic effect with relatively small doses when compared with a synthetic product (Chanda *et al*. 2011). Even if synergy is not detected, its additive effect provides an interesting option for pest management. To date, few studies have investigated the association between PE and OEO (Probst *et al* 2011). In the present work, synergistic and additive effects were detected between PE and OEO (Table 3). The synergistic interaction allows reducing the use of PE and OEO up to four and six doses, respectively. This could be attributed to the combined targeting of the cytoplasmic membrane, since it seems that propolis and essential oil compounds act upon this structure on fungi (Chen *et al*. 2008). The results of this test are particularly

promising in view of the considerable decrease in the inhibitory concentration of PE and OEO, and the positive effects on the use of a combination between two different natural products. Moreover, by combining them, propolis would offer a long-lasting action given by phenolic and flavonoid compounds, while the highly volatile components of essential oil would cause intense, immediate and brief action.

The present study demonstrated that PE and OEO, both natural products whose chemical compositions markedly differ, have a strong antifungal activity against *P. allii*. The most prominent finding in this work is that a favorable interaction between PE and OEO is found in some given combination ratios, causing a synergistic antifungal effect against *P. allii*. The results indicated the likelihood of using propolis extract combined with oregano essential oil as a non-conventional strategy towards the formulation of a biofungicide to control blue mold in seed-cloves.

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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Authors contribution statement

All authors contributed to the study conception and design. Irene Cibanal performed the experiments, analyzed the results and wrote the manuscript; Leticia Andrea Fernández helped with the implementation of the research, the analysis of the results and the writing of the manuscript; Ana Murray performed part of the chemical characterization; Cecilia Pellegrini made substantial contributions revising the manuscript; Liliana Gallez revised the manuscript critically for important intellectual content.

Tables

Table 1 Chemical composition of *Origanum vulgare* cv. *Alpa Sumaj* essential oil (OEO) using gas chromatography-mass spectrometry (GC-MS).

N°	Compound Name *	R.I. [†]	[%]
1	α-thujene	924	0.93
2	α-pinene	939	0.77
3	sabinene	976	4.26
4	β-pinene	981	0.53
5	β-myrcene	991	1.12
6	α-felandrene	1004	0.37
7	α-terpinene	1017	6.25
8	p-cymene	1026	2.50
9	D-limonene	1028	1.80
10	γ-terpinene	1054	26.61
11	cis sabinene hydrate	1065	2.73
12	terpinolene	1086	1.34
13	cis-p-menth-2-en-1-ol	1118	14.93
14	trans-p-ment-2-en-1-ol	1136	4.54
15	cis-β-terpineol	1140	0.91
16	trans-β-terpineol	1159	0.40
17	terpinen-4-ol	1174	11.79
18	α-terpineol	1186	0.76
19	thymolmethylether	1232	1.48
20	isothymolmethylether	1244	1.52
21	thymol	1289	11.17
22	carvacrol	1298	0.72
23	trans-caryophyllene	1417	0.83
24	α-humulene	1452	0.87
25	germacrene D	1484	0.57

* All compounds were identified based on their Ri (retention index identical to bibliography) and MS spectra (comparison with database spectra). α-pinene, β-pinene, β-myrcene and thymol were also identified by their retention time identical to standard. Components are listed in order of elution in HP-5 column.

[†] Ki retention index in HP-5column.

Table 2 Antimicrobial activity of serial dilutions of propolis extracts (PE) and oregano essential oil (OEO) against *Penicillium allii* by the agar dilution method (a) and disc diffusion method (b). A control without aggregates (C) was included in both tests; Carbendazim as a synthetic fungicide (F) was also tested in the agar dilution method. The results of the agar dilution method are expressed as the inhibition percentage of the colony growth, while the results of the disc diffusion method are expressed as the diameter of the inhibition zones around the discs. Results are a mean of 3 determinations (mean ± standard error).

Treatment	72 h (3 days)	144 h (6 days)	216 h (9 days)
	a. Agar dilution method (%)*		
PE (100%)	71.77 ± 0.97 a	62.25 ± 1.26 a	56.91 ± 0.89 a
PE$_2$ (50%)	59.12 ± 1.69 b	52.69 ± 1.43 b	43.43 ± 3.09 b
PE$_3$ (25%)	37.72 ± 0.97 c	31.66 ± 1.26 c	27.94 ± 2.43 c
PE$_4$ (12.5%)	30.9 ± 0.97 d	22.10 ± 0.95 d	18.85 ± 0.89 d
OEO (100%)	100 ± 0 e	100 ± 0 e	100 ± 0 e
OEO$_2$ (50%)	100 ± 0 e	100 ± 0 e	96.97 ± 2.10 f
OEO$_3$ (25%)	95.13 ± 2.58 f	74.19 ± 3.31 f	68.69 ± 3.25 g
OEO$_4$ (12.5%)	60.09 ± 1.95 b	49.34 ± 0.95 b	42.42 ± 0.58 b
C	0 ± 0 g	0 ± 0 g	0 ± 0 h
F	10.46 ± 2.58 h	11.11 ± 2.19 h	10.10 ± 0.58 i
	b. Disc diffusion method (mm)*		
PE (100%)	16 ± 0.92 ab	13.67 ± 0.56 a	13.50 ± 0.62 a
PE$_2$ (50%)	13.83 ± 0.41 b	13.75 ± 0.30 a	13.75 ± 0.30 a
PE$_3$ (25%)	10.08 ± 0.69 c	8.92 ± 0.31 b	8.75 ± 0.43 b
PE$_4$ (12.5%)	9.58 ± 0.68 c	8.92 ± 0.31 b	8.58 ± 0.51 b
OEO (100%)	43 ± 0 d	38 ± 1.07 c	38 ± 1.07 c
OEO$_2$ (50%)	19.08 ± 2.18 a	15.58 ± 1.24 a	14.67 ± 1.14 a
OEO$_3$ (25%)	13.92 ± 1.11 b	13.75 ± 1.14 a	13 ± 0.88 a
OEO$_4$ (12.5%)	10 ± 0.78 c	9.33 ± 0.40 b	2.50 ± 1.10 d
C	0 ± 0 e	0 ± 0 d	0 ± 0 e

* Means with different letters in the same column for each day and for each antifungal susceptibility assay differed significantly at *P*≤0.05 according to Fisher´s LSD test.

Table 3 Combinations of propolis extract (PE) with oregano essential oil (OEO) showing synergism and additive effect against *Penicillium allii* determined by the checkerboard method. Results are mean of three independent experiments.

Combination	PE concentration (μL mL^{-1})	OEO concentration (μL mL^{-1})	FIC $_{PE}$ [a]	FIC $_{OEO}$ [a]	FICI [b]	Interpretation
1	6.25	0.75	0.5	0.5	1	Addition
2	6.25	0.375	0.5	0.25	0.75	Addition
3	6.25	0.18	0.5	0.13	0.63	Addition
4	3.12	0.75	0.25	0.5	0.75	Addition
5	3.12	0.375	0.25	0.25	0.5	Synergy

[a] FIC: fractional inhibitory concentration calculated as the quotient of the minimum inhibitory concentration (MIC) of the natural product in the combination and its MIC alone. PE and OEO MICs were 12.5 μL mL^{-1} and 1.5 μL mL^{-1}, respectively.
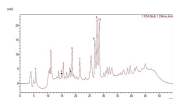
[b] FICI: fractional inhibitory concentration index (FIC$_{PE}$+FIC$_{OEO}$)

Figures

As required by the journal, figures (Fig. 1 and Fig. 2) are attached separately.

Figure 1 HPLC-DAD chromatogram of the hydroalcoholic extract (100 mg mL-1) of propolis (PE) collected in Río Negro Province, Argentina (Cibanal et al., 2020). Main components: 1. Caffeic Acid; 2. Coumaric Acid; 3. Ferulic Acid; 4. Quercetin; 5. 1,5,7-Trihydroxy-flavanone; 6. Apigenin; 7. Pinobanksin; 8. Chrysin; 9. Pinocembrin; 10. Galangin**.**

Figure 2 Effect of propolis extract (PE) and oregano essential oil (OEO) serial dilutions on the radial mycelia growth of *Penicillium allii,* during 9 days at 25 ± 2 °C, using the agar dilution assay. A control without aggregates (C), hydroalcohol as PE solvent (HA), Tween-20 as OEO diluent (TW), and a synthetic fungicide based on Carbendazim (F) were also tested. Symbols representing data series are the following: ♦ PE (100%), ■ PE_2 (50%), ···· PE_3 (25%), × PE_4 (12.5%), ● OEO (100%), ▪·▪· OEO_2 (50%), ▪▪▪▪ OEO_3 (25%), — OEO_4 (12.5%), ▲ C, ✳ F, ▪ ▪ ▪ HA, ＋ TW.

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Fig2

Fig2