Sub-lethal concentrations of *Perilla frutescens* essential oils affect phytopathogenic fungal biofilms

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ABSTRACT

The lack of deep knowledge of plant pathogenic fungal biofilms is reflected in the few existing environmental-friendly options for controlling fungal plant disease. Indeed, chemical fungicides still dominate the market but present-day concerns about their real efficacy, increasing awareness of the risk they pose to human health and the environment, and the incidence of fungicide resistance have all led to the current trend of near zero-market-tolerance for pesticide residues in fruit and vegetables. Here, essential oils (PK and PK-IK) from the edible leaves of two cultivars of *Perilla frutescens* are proposed as new, effective, non-toxic, eco-friendly pesticide-free options suitable for a preventive or integrative approach for sustainable crop protection and product preservation. PK and PK-IK were extracted and characterized, and their ability to affect the biofilm formation of the phytopathogenic model fungi *Colletotrichum musae, Fusarium dimerum* and *Fusarium oxysporum* was studied at non-lethal doses. Both essential oils at 1000 and 2000 mg l^{-1} showed excellent anti-biofilm performance: i) reducing conidia adhesion up to 80.3±16.2%; ii) inhibiting conidia germination up to 100.0±0.0%; iii) affecting biofilm structural development, with a reduction in dry weight of up to 100.0±0.0% and extracellular polysaccharides and proteins up to 81.4±8.0% and 51.0±6.1% respectively. In all cases PK-IK showed better activity than PK.

KEYWORDS

Anti-biofilm, essential oil, *Perilla frutescens*, sub-lethal concentration, phytopathogenic fungi, sustainable agriculture

1. INTRODUCTION

The application of pesticides in agricultural systems to control plant disease and safeguard crop yield and quality has increased in past decades (Liu et al., 2015; Pretty et al., 2015). Moreover, it has been predicted that pesticide application will increase substantially in the next few decades (Carvalho et al., 2017). However, the massive use of such chemicals is questionable because of draw-backs that include environmental persistence over a prolonged period of time and the harmful impact, via the food chain, on human health (Sousa et al., 2014). According to a survey made by the Human Rights Council of the United Nations, about 200,000 people are poisoned annually, and many across the world die because of toxic exposure to pesticides (HRCUN, 2017). Furthermore, in recent years, resistance to many of the most effective pesticides has emerged, and spread, throughout pathogen communities, lowering the availability of active substances (Luca et al., 2015).

Worldwide legislation has now adopted principles of integrated pest control to manage a more sustainable agriculture less dependent on pesticides (Barzman et al., 2015; EU, 2009). Specific measures include the use of disease- and pest-resistant crops, crop rotation, application of non-chemical control practices (thermic, mechanical, biological) and, as a last resort, the tactical use of agrochemicals (EU, 2009). However, local governments still struggle to put these principles into practice and chemical control often remains the simplest and most cost-effective way for controlling the presence of phytopathogens (Ehler and Bottrell, 2010; Parsa et al., 2014).

Phytopathogenic fungi account for most pest disease around the world, seriously affecting agricultural production and food availability. Recently, it has become evident that fungal phytopathogens do not interact with the plant as individual entities, but rather at the community level in the form of biofilm (Pandin et al., 2011; Villa et al., 2017). This seriously compromises the picture as microorganisms in the biofilm lifestyle display increased tolerance to conventional biocides, and a capacity to evade the host's defences, compromising disease control (Stewart, 2002).

The concept of biofilm in plant pathology offers an opportunity to exploit new effective non-toxic eco-friendly pesticide-free agricultural practices. Interference with the key-steps orchestrating the genesis of virtually every biofilm (e.g., attachment, cell-to-cell communication, dispersion) could provide a way for new preventive strategies that do not necessarily exert a lethal effect on cells, but rather sabotage the propensity for a biofilm lifestyle (Villa et al., 2017). As these substances do not act by killing the cells, they should not impose a selective pressure that would cause the onset of resistance (Villa and Cappitelli, 2013).

Terrestrial plants used as food offer a rich source of bioactive compounds with potential applications in the agri-food sector, as such plants are generally recognized as safe (Lucera et al., 2012).

Perilla frutescens is an annual short-day plant widely used in Asian traditional therapeutic medicine and food preparations (Laureati et al., 2010). It was recently found that some of the compounds of *P. frutescens* essential oil, namely perillaldehyde (PA), perillaketone (PK) and isoegomaketone (IK), activate the Transient Receptor Potential A1 ion channels involved in the perception of somatosensory sensations (Bassoli et al., 2009, Bassoli et al., 2013). Interestingly, compounds active on chemesthetic sensations, such as temperature (hot/cold) and pungency, have promising anti-biofilm activity (Villa et al., 2012a).

In this paper sub-lethal concentrations of two essential oils from *P. frutescens* leaves were tested for their anti-biofilm activity against the phytopathogenic fungi *Colletotrichum musae*, *Fusarium dimerum* and *F. oxysporum*, which are included in the Top 10 fungal plant pathogen list (Dean et al., 2012). These fungi affect the plant genus *Musa*, which includes bananas and plantains. *C. musae* causes anthracnose of banana (Zakaria et al., 2009) and, together with *F. dimerum* and *F. oxysporum*, is responsible for crown rot postharvest disease (Triest et al., 2016; Dita et al., 2018). Both anthracnose and crown root disease are major constraints in banana production worldwide, resulting in significant fruit loss every year (Kamel et al., 2016; Maryani et al., 2019). The pathogen inoculum is present in the water used to wash the bananas, and surface contamination and crown wounds mainly occur during the fruit's post-harvest handling, storage and marketing (Zakaria et al., 2009; Kamel et al., 2016). As the banana is one of the world's most important fruit crops in terms of production volume and trade (FAO, 2017), the ever-increasing demand for organic fruit makes the need for a strategy able to preserve organic banana production urgent (Padam et al., 2012). Indeed, the difficulties encountered in applying fungicides and the acceptance of principles of integrated pest management make natural compounds an attractive alternative for the biological control of banana diseases (Raza et al., 2017).

However, considering the huge amount of effort put into the use of natural products as fungicides (Al-Samarrai et al., 2012, El-Hossary et al., 2017), there are few papers dealing with molecules that show anti-biofilm activity against phytopathogenic fungi at non-biocidal concentrations.

In the present paper, the ability of *P. frutescens* oils to prevent biofilm formation through interference with conidia adhesion, germinative tube development and biofilm maturation was thoroughly investigated using, as models, phytopathogenic fungi of great economical relevance.

2. MATERIALS AND METHODS

2.1 *P. frutescens* plant material and extraction

Two green-leaf cultivars of *Perilla frutescens* (L.) Britt. ('Shiso Green' Koppert Cress and 'Korean perilla' Kitazawa Seed) were grown at the Fondazione Minoprio (Vertemate, Como, Italy, 45°

43’31’’08 N and 09°4’20’’40 E) as previously described (Martinetti et al., 2017). At the end of the flowering season the leaves were washed with water in the field the day before harvest, the following day they were cut and stored at -20 °C until distillation.

2.2 *P. frutescens* essential oil extraction

The extraction and distillation was done in a pilot plant consisting of an essential oil extractor (Albrigi 120 l), heated by a 2000 W electric plate, and a water chiller to recover the aromatic water (Fig. S1). The frozen leaves, sliced about 4 mm thick, were placed on 3 perforated stainless steel AISI 304 plates, the perforations favoring the diffusion of steam. The plates were set about 30 cm apart to simulate the normal conditions of fresh or dried vegetal material inside a distiller, and the leaves were arranged on the plates in such a way as to optimize their contact with the steam that extracted the essential oil. Under the bottom extractor plate we added 20 l of hot water, and the first aromatic water dropped about 45 min after starting the heating. The whole distillation lasted about 1.5 h.

After distillation, the aromatic water was extracted with dichloromethane and the organic phase was dried under vacuum to give the essential oils.

2.3 *P. frutescens* essential oil characterization

Essential oils were analysed on a Varian SD 200 liquid chromatograph with RP-column Alltima C18 (250 mm length, 4.6 mm ID, 5µ, Alltech). Twenty µl were injected and separated using methanol and water at 1 ml min^{-1} flow rate. Linear gradient elution was used for 15 min with methanol/water 70/30, followed by a gradient to 100 % methanol over 10 min. Signals were recorded with UV detention at 254 nm; Varian Galaxy software was used for the data processing. Pure PK was previously prepared by synthesis and characterized for its structure; pure IK was purified from ‘Shiso Green’ (Bassoli et al., 2013). In isocratic conditions the two compounds have retention times of 8.21 min (IK) and 10.42 min (PK). ^{1}H NMR spectra were recorded with Bruker AMX -300 (300 MHz) and Avance (600 MHz) instruments, using TMS as internal standard.

2.4 Fungal pathogens

Colletotrichum musae D128, *Fusarium dimerum* F30 and *Fusarium oxysporum* D221 (Department of Food, Environmental and Nutritional Sciences, University of Milan collection) were used as model systems for fungal biofilm. *C. musae* and *F. oxysporum* were grown on Potato Dextrose Agar (PDA, Difco Laboratories, USA) while *F. dimerum* was cultured on Czapek Agar (CA, Sigma Aldrich, USA). The fungi were maintained at 21 °C for 15 days until conidia collection. The conidia were collected in water and filtered through a double layered sterile gauze according to Kunova et al.

(2016), and the concentration determined by conidia counting using a light microscope (Leica DM4000 M, Leica Microsystems, Germany) and Thoma counting chamber.

2.5 Planktonic fungal growth in the presence of *P. frutescens* essential oils

The ability of the PK and PK-IK *P. frutescens* oils to affect fungal growth was assessed by disk diffusion assay (Baluoiri et al., 2016). Indeed, planktonic assay was preferred over biofilm assay, in accordance with the evidence that planktonic cells are more sensitive to biocides than their corresponding sessile counterparts (Stewart, 2002). Thus, any concentration not sub-lethal for planktonic cells is also not lethal for biofilm. Two hundred µL of 10^6 ml^{-1} conidia were distributed uniformly on Petri plates containing either PDA for *C. musae* or CA for *F. oxysporum* and *F. dimerum*. Filter-paper discs (6 mm diameter, Oxoid, United Kingdom) were imbibed with concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil with 3 % methanol (MetOH), and the discs were then placed at the center of the Petri plates.

Filter paper discs were also prepared with Phosphate Buffer Saline (PBS, Sigma Aldrich, USA) or 3 % MetOH as negative controls, and the antifungal Procloraz (0.01 %, Sportak, BASF, Germany) as positive control. The physiological pH value of each medium supplemented with *P. frutescens* oil was verified using a Jenway 3510 pH meter (pH 7.5). Fungi were maintained at 21 °C for 7 days. The essential oils diffused into the agar and i) inhibited fungal growth when toxic, showing a halo of inhibition around the disks, or ii) allowed undisturbed fungal growth if not toxic, with no visible halo of inhibition around the disks (Baluoiri et al., 2016). Notably, PDA and CA contain all the elements that fungi need for growth so the medium always allows fungal growth if no inhibition agents are added.

Three biological replicates were performed for each treatment and three technical replicates for each experiment.

2.6 *P. frutescens* essential oils as carbon and energy source for fungal models

The ability of fungi to grow with PK and PK-IK *P. frutescens* oils was evaluated by inoculating 200 µl of 10^6 ml^{-1} conidia on Petri plates containing a mineral medium agar (3.0 g l^{-1} sodium nitrate, 1.0 g l^{-1} di-potassium hydrogen phosphate, 0.5 g l^{-1} magnesium sulfate heptahydrate, 0.5 g l^{-1} potassium chloride, 0.01 g l^{-1} iron(II) sulfate heptahydrate, 15 g l^{-1} agar) with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oils supplemented with 3 % MetOH as unique carbon and energy source. Petri dishes were also prepared with PBS or MetOH as negative controls and with the addition of 30 g l^{-1} sucrose (Sigma Aldrich, USA) as a positive control. Fungi were maintained at 21 °C and, after 7 days the fungal growth in the presence of essential oils was

evaluated by comparison with controls. Essential oils were the only possible source of carbon and energy available for the fungal strains in the medium. Therefore, fungal growth was i) visible if fungi were able to use each essential oil as carbon and energy source, or ii) not visible if fungi were not able to use essential oils as carbon and energy source.

Three biological replicates were performed for each treatment and three technical replicates for each experiment.

2.7 Conidia adhesion

Conidia adhesion for each fungal strain was assessed in hydrophobic black-sided plates, as previously reported by Villa et al. (2010). Briefly, 200 µl of PBS containing 5 x 10^5 conidia with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil supplemented with 3 % MetOH were placed in microtiter plate wells and incubated at 21°C. Experiments were also performed with PBS and 3 % MetOH as negative controls. After 24 h, the wells were washed twice with 200 µl PBS, and adhered conidia were stained using Fluorescent Brightener 28 (Sigma Aldrich, USA) in PBS for 20 min in the dark at room temperature. Fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity was used to quantify the number of adhered conidia. The obtained data were normalized to the area, and the means reported. Three biological replicates were performed for each treatment and six technical replicates for each experiment.

2.8 Conidia germination

2.8.1 Germination

To assess the influence of PK and PK-IK *P. frutescens* oil against fungal conidia germination, 30 µL of 10^6 ml^{-1} conidia were plated on PDA (*C. musae* and *F. oxysporum*) or CA (*F. dimerum*) with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil and 3 % MetOH. Conidia were also plated with PBS and 3 % MetOH as negative controls. The plates were maintained at 21 °C and the proportion of germinated conidia was assessed by direct microscopic counts after 21 h of incubation. Counts were made in 10 random fields of view on each replicate. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

2.8.2 Fungicidal versus fungistatic activity

Concentrations of PK and PK-IK *P. frutescens* oils that inhibited fungal conidial germination were tested for their fungicidal rather than fungistatic activity. Petri dishes were prepared with PDA (*C. musae* and *F. oxysporum*) or CA (*F. dimerum*) and the addition of PK and PK-IK *P. frutescens* oil at concentrations found inhibiting conidia germination supplemented with 3% MetOH. A Cellophane© membrane was placed in the middle of each plate and uniformly inoculated with 30 µL of 10^6 ml^{-1}. After 21 h of incubation at 21 °C, the proportion of germinated conidia was assessed by direct microscopic counting (Leica DM4000 M, Leica Microsystems, Germany). Later, each membrane was transferred onto a new plate prepared with only PDA (*F. oxysporum* and *C. musae*) or CA (*F. dimerum*). The plates were maintained at 21 °C and after another 21 h of incubation the proportion of germinated conidia was assessed a second time by direct microscopic counts (Leica DM4000 M, Leica Microsystems, Germany). PK and PK-IK were i) fungicidal when the number of germinated conidia was equal in the first and second counts; ii) fungistatic when the number of germinated conidia in the first count was lower than that in the second count (Banihashemi and Abivardi, 2011). Microscopic counts were made in 10 random fields of view on each replicate. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

2.9 Biofilm structural development

2.9.1 Fungal biofilm growth

Two mL of 10^6/mL conidia in 10 % diluted Potato Dextrose Broth (PDB, Sigma Aldrich, USA; *C. musae* and *F. oxysporum*) or 10 % diluted Czapek Broth (CB, Sigma Aldrich, USA; *F. dimerum*) were inoculated in Petri dishes (35 mm diameter) with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil supplemented with 3 % MetOH. Biofilms were also grown with only PBS or 3 % MetOH as negative controls. Petri dishes were incubated at 21°C for 10 days allowing the development of mature biofilm. In order to avoid any change in the experimental conditions, and consequently of biofilm growth rate caused by the depletion of nutrients and the accumulation of metabolic products (Azeredo et al., 2017), the media were replaced every 48 h with fresh media consisting of 10 % diluted media and the addition of the corresponding concentrations of *P. frutescens* oil supplemented with 3 % MetOH. The obtained biofilms were recovered and analysed for their biomass abundance and extracellular polymeric substances (EPS) composition.

2.9.2 Biomass and extracellular polymeric substances (EPS) extraction

After 10 days of incubation, biomass and EPS within the biofilm were divided. The media were removed from the Petri dishes and the biofilm resuspended in 2 ml of 2 % ethylenediaminetetraacetic acid (EDTA) and transferred into new tubes. To remove biofilm aggregates, fungal suspensions were homogenized by a 30 s cycle at 14,500 rpm (T 10 basic Ultra-Turrax) followed by 15 s sonication (15 % amplitude, in water-bath; Branson 3510, Branson Ultrasonic Corporation, Dunburry, CT) and 30 s vortex mixing. Biofilm suspensions were incubated for 3 h at 4°C with gentle shaking (300 rpm) to extract EPS. Fungal suspensions were then centrifuged (11,000 g) for 30 min at 4°C. The supernatants containing soluble EPS were filtered through a 0.2 mm filter while the pellets were recovered for the biomass abundance assay.

2.9.3 Biomass abundance

Pellets obtained from the previous section were washed twice with distilled water to remove all EDTA traces and then incubated at 90 °C until complete exsiccation. Biomass abundance was obtained gravimetrically by fungal dry weight determination as previously described (Kunova et al., 2016). Obtained data were normalized to the area and the means are reported. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

2.9.4 EPS composition

Filtered soluble EPS were analysed for their polysaccharides and protein content. The phenol-sulfuric acid assay was applied for polysaccharide determination using glucose as the standard (Masuko et al., 2005) whereas the Bradford method (1976) was applied to analyse the protein concentrations. Absorbance was measured using a UV/VIS 7315 Spectrophotometer (Jenway). Obtained data were normalized to the area and the means are reported. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

2.10 Statistical analysis

The reduction percentage of conidia adhesion/conidia germination/biofilm structural parameters in comparison to the negative control prepared with 3 % MetOH was calculated as (PK and PK-IK *P. frutescens* oil data – negative control data) x 100 / negative control data. PK and PK-IK *P. frutescens* oil concentrations able to affect the above biofilm parameters by less than 20 %, with respect to the negative control, were considered to be without activity, between 20 % and 30 % with low activity, between 30 % and 40 % with moderate activity, and more than 40 % with excellent activity (Cattò et al., 2015).

Analysis of variance (ANOVA) via a software run in MATLAB (Version 7.0, The MathWorks Inc., Natick, USA) was applied to statistically evaluate any significant differences among the samples. The ANOVA analysis was carried out after verifying whether the data satisfied the assumptions of i) independence, ii) normal distribution and iii) homogeneity of variance. Tukey's honestly significant different test (HSD) was used for pairwise comparison to determine the significance of the data. Differences were considered significant for $p < 0.05$.

3. RESULTS

3.1 *P. frutescens* essential oils extraction and composition

The extraction procedure by the pilot plant gave essential oils in the following yields: 'Korean perilla' cultivar: 0.16%; 'Shiso Green' cultivar: 0.17 %.

The HPLC analysis revealed that the essential oil from 'Korean perilla' cultivar contained PK (1-(3-furyl)-4-methyl-1-pentanone) as the main component (> 97 %), confirming it to be a pure PK-chemotype. The essential oil from 'Shiso Green' cultivar contained a mixture of PK and IK (1-(3-furyl)-4-methyl-2-penten-1-one) in the proportion of 90 % and 10 % respectively. Both essential oils were also analysed by NMR to exclude the presence of other minor components not revealed by UV detection. (Fig. S2).

3.2 *P. frutescens* essential oils do not affect planktonic fungal growth

Experiments showed that both PK and PK-IK at all the tested concentrations did not affect C. *musae*, *F. dimerum* and *F. oxysporum* as no growth inhibition was appreciable around the disk. The same result was obtained for both the negative controls, indicating that the addition of 3 % MetOH to each essential oil did not disturb fungal growth. On the contrary, a significant growth inhibition diameter was found around the disk imbibed with the antifungal Procloraz used as positive control (Fig. S3).

3.3 *P. frutescens* essential oils are not a carbon source for fungal models

Experiments showed that both PK and PK-IK were not a carbon source for C. *musae*, *F. dimerum* and *F. oxysporum* as no fungal growth was visible when these essential oils were added to the mineral medium as sole carbon source. No fungal growth was obtained in the negative control plates. On the contrary, in the positive control, prepared with the addition of sucrose to the mineral medium, fungal growth was appreciable (Fig. S4).

3.4 *P. frutescens* essential oils affect conidia adhesion

The number of adhered conidia after 18 h of incubation in the presence of PK and PK-IK essential oils are reported in Figure 1.

F. oxysporum was the strain most affected by both essentials oils, followed by *F. dimerum*. Indeed, PK showed only a slightly anti-adhesion effect against *C. musae* at the maximum concentration tested. On the contrary, the same oil displayed an excellent anti-adhesion performance against both *F. dimerum* and *F. oxysporum*, at concentrations above 1000 ppm and 10 ppm respectively, with a reduction in the number of adhered conidia up to 82.3±9.7 % for *F. dimerum* and 73.4±16.9 % for *F. oxysporum*.

As regards PK-IK, concentrations above 0.1 ppm significantly reduced the number of adhered conidia in all the fungal strains. Indeed, the PK-IK effect was maximum against *F. oxysporum*, conidia adhesion being slightly affected by PK-IK at 0.01 ppm, moderately affected by PK-IK at 0.1 ppm and strongly affected by PK-IK at concentration above 1 ppm, with a maximum effect at 1000 ppm with a reduction in the number of adhered conidia of 61.6±15.2 %. Against *F. dimerum*, the same oil displayed low anti-adhesion performance between 0.1 and 10 ppm and maximum effect at 1000 and 2000 ppm with a maximum reduction in the number of adhered conidia up to 53.1±13.5 %. When tested against *C. musae*, the anti-adhesion performance of PK-IK was lower in comparison to both *Fusarium* strains. The optimal effect was reached only at the maximum concentration tested with a reduction in the number of adhered conidia up to the 44.4±6.2 %, whereas the anti-adhesion effect was low from 0.01 to 10 ppm and moderate at 100 and 1000 ppm.

As no significant differences were found between the negative controls prepared with PBS and 3 % MetOH, the anti-adhesion effect against all fungal strains was totally attributable to the essential oils.

3.5 *P. frutescens* essential oils affect conidia germination

The percentage of conidia germinated in the presence of each essential oil is shown in Figure S6.

The data show that both essential oils significantly affect conidia germination at concentrations above 1000 ppm. Indeed, germination was completely inhibited by both PK and PK-IK at 2000 ppm, with a reduction of up to 100.0±0.0 % compared to the negative control. At 1000 ppm, PK significantly decreased the number of germinated conidia but the percentage reduction of germinated conidia in comparison to the negative control was always lower than 20 %. On the contrary, at the same concentration, PK-IK massively reduced conidia germination in all the fungal strains, with a reduction of 84.5±3.1 % in *C. musae*, of 84.5±2.0 % *F. dimerum* and of 99.3±1.2 % in *F. oxysporum* (Fig. S6).

Samples treated with PK and PK-IK at 1000 and 2000 ppm were transferred onto a new medium without the addition of essential oils. After 21 h, all the fungal strains displayed 100 % of germinated conidia.

As no significant differences were found between the negative controls prepared with PBS and 3 % MetOH, the inhibition of germination was totally attributable to the essential oils.

3.6 *P. frutescens* essential oils affect biofilm structural development

3.6.1 Biomass abundance

As shown in Figures 2A-C and S7A-C, visible inhibition of biofilm growth was found for all the fungal strains in the presence of PK and PK-IK at 1000 and 2000 mg l^{-1}. The biofilm was collected and analysed for biomass and EPS polysaccharide and protein content.

The dry weight of biofilm grown in the presence of PK and PK-IK at the different concentrations tested is reported in Figure S7D-F . The experiments revealed that both essential oils significantly reduced biofilm biomass at the maximum concentration tested, with a reduction of up to 100.0±0.0 % compared to the negative control (Fig. 2D). The effects of PK and PK-IK at the same concentrations were comparable in *C. musae* and *F. oxysporum* whereas PK-IK displayed a more major effect than PK against *F. dimerum*. At 1000 mg l^{-1} PK-IK reduced biomass massively in *C. musae* and *F. dimerum* and moderately in *F. oxysporum*. At the same concentration, PK displayed a similar effect against *F. dimerum* and *F. oxysporum*, but showed no effect on *C. musae* at the same concentration. Overall, *F. dimerum* was the strain most affected by both essential oils, followed by *C. musae* and *F. oxysporum*.

No differences were found between the negative controls prepared with PBS and 3 % MetOH.

3.6.2 EPS polysaccharide and protein contents

Experiments showed that both essential oils significantly reduced the polysaccharides content in the EPS matrix at the maximum concentration tested, with a reduction of up to the 81.4±8.0 % compared to the negative control, and comparable values between PK and PK-IK for all fungal strains (Figure 2E, G-I). At 1000 mg l^{-1}, PK slightly reduced the polysaccharide content in the biofilm matrix of *C. musae* and *F. dimerum*, whereas no effect was recorded in *F. oxysporum*. On the contrary, at 1000 mg l^{-1} PK-IK displayed optimal performance against *C. musae*, a moderate effect toward *F. dimerum* and low effect in *F. oxysporum*. Comparing the effects against the different strains, *C. musae* resulted the most affected by the presence of both essential oils, followed by *F. dimerum* and *F. oxysporum*.

No differences were found between the negative controls prepared with PBS and 3 % MetOH.

The experiments showed that essential oils affect the EPS protein content depending on the fungal strains (Figure 2F, L-N). Indeed, *F. oxysporum* resulted the most affected, followed by *F. dimerum* and *C. musae.* The data showed no effect of either essential oil toward *C. musae* (Figure 2F). On the contrary, PK-IK significantly decreased the protein content in *F. dimerum* matrix up to the 40.7±3.4 % at 2000 mg l^{-1} (Figure 2F, M), whereas PK showed no activity against that strain. Both essential oils affected the EPS protein content in *F. oxysporum*: PK displayed a moderate effect at 100 and 1000 mg l^{-1} and excellent activity at 2000 mg l^{-1}, while PK-IK resulted active at 1000 and 2000 mg l^{-1} with a reduction in the EPS protein content of up to 51.0±6.1 % compared to the negative control (Figure 2F, N). At the same concentration, PK and PK-IK showed comparable activity in all the fungal strains, except for the effect at 1000 mg l^{-1} toward *F. dimerum*. In this case, PK-IK had better activity compared to PK. No differences were found between the negative controls prepared with PBS and 3 % MetOH.

4. DISCUSSION

While much is known about biofilm formed by yeast species in medical and industrial settings, there are few descriptions of biofilm formed by filamentous plant pathogenic fungi (Harding et al., 2009; Pandin et al., 2017). This study has investigated features of three filamentous phytopathogenic fungi of economical relevance, i.e. *C. musae, F. dimerum* and *F. oxysporum,* and contributes to filling the current gap in the literature. For the first time, there is the proposal of a kinetic mathematical modelling of spore adhesion, providing interesting details useful for the accurate management of crop treatments for pest prevention (Fang and Ramasamy, 2015). The kinetic parameters highlight that fungal conidia adhesion is a rapid process that occurs irreversibly after a few hours of contact with a suitable surface. Indeed, a small number of conidia was found irreversibly attached after 3 hours contact with the surface, while 18 hours was sufficient time for the complete adhesion of all the inoculated conidia. Once irreversibly adhered to a surface, the conidia took a maximum period of 15 hours to germinate. Furthermore, the investigation of mature biofilm highlighted that all the fungal strains were characterized by an extracellular matrix with a large polysaccharide amount and a low quantity of proteins.

The lack of a deep knowledge of plant pathogenic fungal biofilm is reflected in the few existing safe options for controlling fungal plant disease, therefore chemical fungicides still dominate the market (Villa et al., 2017). However, the current trend is to achieve near zero market-tolerance for pesticide residues in fruit and vegetables (Lamichhane et al., 2016).

In this research, chemical characterization allowed the identification of the main components of both *P. frutescens* essential oils, confirming the presence of PK as the main component of oil from the

‘Shiso Green’ cultivar, whereas a mixture of PK and IK was found in the oil from the ‘Korean type’ cultivar. Both PK and IK are two furyl ketone derivatives that had already been identified as major secondary metabolites in *P. frutescens* plants (Bassoli et al., 2009; Bassoli et al., 2013). The achievement of a detailed quali- and quantitative analysis of the mixture composition is an important step forward in the development of alternative natural-based treatments. In the past, a number of essential oils were proposed as anti-biofilm agents against both bacteria and fungi (Carezzano et al., 2010; Kocic-Tanackov et al., 2017; Manganyi et al., 2015; Nazzaro et al., 2017; Sarkhosh et al., 2018). However, in most of cases the active component in the oils was not identified due to the large number of different chemical compounds that interact with each other, making the antimicrobial action less reproducible.

The diffusion of essential oil compounds into the market requires a large amount of natural materials. Extraction facilities are now able to provide a constant supply and batch recovery of essential oils for organic agriculture. However, pilot scale plants require a notably high capital investment that small growers and farmers can generally ill-afford (Babu et al., 2002). Moreover, the building up and maintenance of a high-quality collection of such plants is not only expensive but also practically impossible for industrial scale quantities. In the case of *P. frutescens*, many chemotypes are described in the literature (Yu et al., 1997). However, this paper shows that the very simple composition of two selected essential oils, which are essentially mono- and bi-component oils, is an extremely rare and useful condition that makes recovery and analysis very simple and, most of all, allows reproducibility of the extracted material, an essential feature for developing industrial applications. Moreover, the use of a pilot plant and a full year’s harvest allowed us to obtain an average but stable content of each active principle, also in the case of the PK-IK chemotype. Another important feature of *P. frutescent* essential oil’s active components is their simple chemical structure, which offers the option of producing these natural drugs by chemical synthesis, with a satisfying level of purification and controlled concentration (Bassoli et al., 2013). Additionally, their relatively simple structure and the lack of stereogenic centers make them a good starting point to derive new synthetic analogues, many of which have already been synthesised and demonstrated to be also potent agonists of the TRPA1 channel (Bassoli et al., 2013). Compounds with improved anti-biofilm activity, and new derivatives, could be used to derive structure-activity relationships (Villa et al., 2013). Thus, the peculiar properties of both *P. frutescent* essential oils offer an advantage over other essential oils, making their real application more feasible.

P. frutescens was previously investigated for its antimicrobial properties against both bacteria (Choi et al., 2010; Kim et al., 2011; Yamamoto and Ogawa, 2002) and fungi. Tian et al. (2014) proved that the essential oil from *P. frutescens* (L.) Britt leaves exhibits antifungal activity against *Aspergillus*

flavus, *A. oryzae*, *A. niger* and *Alternaria alternata*, all involved in the spoilage of postharvest cherry tomatoes and grapes. The same authors further proved that 0.5 mL/L perillaldehyde completely inhibited mycelial growth of the same fungi. Additionally, a study by Ansari et al. (2016) demonstrated that planktonic growth of *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and C. *parapsilosis* was affected by the presence of perillyl alcohol, an active compound found in some cultivars of *P. frutescens*. However, these studies dealt with lethal concentrations, and experiments were carried out using shaken-liquid or solid media that favors fungal planktonic growth. Nowadays we know that biofilms are the major mode of microbial life in a natural environment (Costerton, 1999). Therefore, the biofilm lifestyle should be considered a relevant feature when studying microbial processes *in vitro* (Lerch et al., 2017; Pandin et al., 2017).

In this research the activity of PK and PK-IK essential oils was tested at sub-lethal concentrations, also taking into account that phytopathogenic fungi interact with plants in the form of biofilms. Indeed, a preventive approach was considered, following organic agriculture's general principle that dealing with the cause of a pest outbreak is preferable to treating the symptoms (Barzaman et al., 2015). Thus, each step essential to the development of fungal biofilm is a suitable target for intervention (De Vincenti et al., 2018). In line with the formation process of filamentous fungal biofilm (Harding et al., 2009), PK and PK-IK oils were tested for their ability to modulate conidial adhesion, germline formation and biofilm maturation.

The present results showed that PK and PK-IK essential oils are good candidates as anti-biofilm agents against phytopathogenic fungi, their activity depending on both fungal strain and concentration. Indeed, PK and PK-IK essential oils neither inhibited fungal growth nor were they utilized as a carbon energy source at the concentrations tested. Thus, the above anti-biofilm effects were exploited with a non-lethal mechanism, a must in the challenge to contrast the development of resistant fungal strains.

Essential oils have proved to exert an optimal anti-adhesion performance. Indeed, both the PK and PK-IK essential oils were very active against *F. oxysporum*, and just a little less so against *F. dimerum.* A minor effect was recorded against *C. musae*, the conidia adhesion being slightly affected in the presence of PK oil and reduced by up to 44.4±6.2 % in the presence of PK-IK oil at the maximum concentration tested. Notably, the PK-IK oil always displayed better activity than pure PK oil, becoming active at concentrations at least two orders of magnitude less than PK.

The trend showing PK-IK essential oil to be more active than PK oil also recurred in the germination experiments. At 1000 mg l^{-1} only PK-IK oil massively affected conidia germination. On the contrary, germ tube formation was completely inhibited by both essential oils at 2000 mg l^{-1}. Interestingly, once transferred to a medium lacking in oils, all the conidia reversed their status of inhibited

germination, indicating that the PK and PK-IK oils did not kill the spores but rather induced a transitory protection mechanism. Indeed, evidence has shown that pathogenic fungi are able to perceive multiple cues from the environment, and use these to influence the development of infection structures, e.g. germ tubes and appressoria (Bahn et al., 2007). In line with a multitude of other anti-biofilm natural compounds, it is possible to speculate that PK and PK-IK essential oils could act as a negative environmental signal, increasing the ability of fungal stains to express various protective mechanisms, like the ability to form infectious structures, to escape from adverse conditions (Villa et al., 2012b; Villa and Cappitelli, 2013).

PK and PK-IK essential oils at 1000 and 2000 mg l^{-1} were also able to modulate the biofilm maturation of all the fungal strains, PK-IK showing greater activity than PK. The dry weight of all the fungal strains was reduced by up to 100 % after treatment with the oils, and the effects were also visible to the naked eye. Additionally, changes in the extracellular polysaccharide and protein contents were found in all the treated strains. The polysaccharide content decreased significantly in comparison to the non-treated control, with a maximum reduction of up to 81.4±8.0 % in *C. musae*, followed by *F. dimerum* (up to 79.4±7.8 %) and *F. oxysporum* (up to 70.5±4.4 %) in the presence of both PK and PK-IK at 1000 and 2000 mg l^{-1}. The protein amount was less affected than the polysaccharide content: no effect was found in *C. musae* whereas in *F. dimerum* only PK-IK decreased the EPS proteins by 40.7±3.4 %. Instead, both oils affected the F. *oxysporum* EPS protein content, with a maximum decrease of 51.0±6.1 % with PK-IK at 1000 mg l^{-1}.

It is reported that EPS is a defining characteristic of filamentous fungal biofilms, providing the cells protection from hostile factors such as antifungal agents (Ramage et al., 2012). Treatments that affect biofilm matrix formation, i.e. PK and PK-IK essential oils, greatly enhance the efficacy of some biocidal treatments as the antimicrobial agents could penetrate the biofilm more easily, exerting their effect even at concentrations below those normally used in traditional applications and thus providing a more potent control against the development of drug-resistant strains. Indeed, treatments based on co-dosed PK and PK-IK essential oils and conventional pesticides might be potentially used to maximize the anti-biofilm effect on plants (Villa et al., 2012a).

An *in vitro* study in humans and various animals revealed that PK and IK activate the Transient Receptor Potential A1 (TRP A1) (Bassoli et al., 2013, Cattaneo et al., 2014). TRP channels allow individual cells and entire organisms to detect changes in the external environment, e.g. temperature, osmolarity and chemesthetic sensations (Clapham, 2003; Vrins et al., 2008; Wang and Siemens, 2015). Interestingly, several fungal genomes present genes encoding TRP homologue structures, which share a high degree of sequence similarity and topology to those of humans (Kaleta and Palmer, 2011; Lange et al., 2016; Palmer et al., 2001; Prole and Taylor, 2012; Zhou et al., 2005). It has been

speculated that modulators of the human TRP channel, e.g. PK and IK, may also have anti-fungal activity via effects on fungal TRP channel homologues (Prole and Taylor, 2012). Accordingly, in *Fusarium graminearum* and *Colletotrichum gramicola*, TRP homologues have been found to play an important role for hyphal growth and/or plant infection, being involved in the perception of, and response to, certain features of the host surface, e.g. the surface osmotic potential that needs to be sensed and tightly controlled to ensure successful colonization, appressorium development and penetration (Ihara et al., 2013; Lange et al., 2016). In this study, a massive reduction of adhered and germinated conidia was found after the treatment with both PK and PK-IK essential oils. Thus, in line with the previous considerations, these authors do not exclude an anti-biofilm mechanism by which PK and IK target and modulate the expression of TRP homologues, affecting the spore surface sensing mechanism and leading to a significant reduction of biofilm formation. Consistent with this chemo-sensory strategy, a number of food-derived molecules that are agonistic to TRP channels have also been found to display optimal anti-biofilm performance, e.g. cinnamaldehyde from cinnamon (Khan et al., 2017) and eugenol from clove oil (Khan and Ahmad, 2012).

In vitro studies have found that, in humans, IK is a more potent agonist of the TRP1 channel than PK, as the presence of a double bond in the IK chemical structure increases its ability to target TRP1 (Bassoli et al., 2013). Notably, in our fungal strains, PK-IK essential oil displayed better activity than pure PK essential oil, starting to be active at concentrations at least one order of magnitude less than PK, highlighting a major anti-biofilm activity of IK with respect to PK. These findings may further corroborate the hypothesis of a possible involvement of a TRP homologue channel in the anti-biofilm activity of *P. frutescens* essential oils. A possible reason for the major efficacy could be the presence of an extra double bond, conjugated to the carbonyl moiety in the IK structure. This hypothesis is consistent with the proposed mechanism for TRPA1 activation, where the increase in the molecule electrophilic properties makes it potentially more susceptible to undergo the attack of nucleophilic cellular sites, such as the thiol group of protein, and inhibiting activities within cells that are vital for the biofilm process, e.g. inactivating some enzymes important for biofilm formation. Accordingly, Worthington et al. (2012) designed a series of molecules bearing electrophilic functional groups to efficiently inhibit biofilm formation through their nucleophilic reaction with a cysteine residue present in the quorum sensing receptor LuxR.

With the perspective of spreading PK and PK-IK essential oils throughout the agri-food sector, their toxicological effect against beneficial microorganisms was part of a preliminary study (Supplementary materials S8). Microorganisms tested included i) two plant growth-promoting rhizobacteria, i.e. the Gram-negative nitrogen-fixing bacterium *Azotobacter vinelandii* and the Gram-positive bacterium *Bacillus subtilis* (Gambino et al., 2015); ii) an environmental bacterium, i.e. the

Gram-negative *Escherichia coli* K12 strain (Ishii et al., 2008); iii) two naturally occurring Gram-positive actinobacteria commonly found in soil and used as biocontrol agents against fungi, i.e. *Streptomyces lydicus* and *Streptomyces griseoviridis* (Sathya et al., 2017); and iii) a fungus used in a biocontrol strategy, i.e. *Trichoderma asperellum* (Wu et al., 2017). Experiments showed that all the bacteria and fungi were able to grow in the presence of both PK and PK-IK at all tested concentrations. Therefore, PK and PK-IK were not toxic against some beneficial microorganisms. Notably, the tested microorganisms are representative of the beneficial category, but are not representative of the total beneficial microbial communities in agroecosystems. However, biological control with sub-lethal concentrations of natural products provides a great alternative to the use of chemicals, given its eco-friendly nature, the low risk of adverse impacts on human health and the reduced probability of new resistance gene development among pathogens (Villa et al., 2017).

CONCLUSIONS

This research supports the incorporation of PK and PK-IK as alternative new, safe and eco-friendly products suitable for sustainable pest management applications, as well as being an attractive method for controlling post-harvest fungal decay in fruit and vegetables. Indeed, as they are natural ingredients of dietary plants, these compounds should be well-tolerated by humans. This is especially true in the light of the major challenges of efficiently increasing and protecting crop yield, maintaining economic profits but at the same time preserving human health and the environment. The simple PK and IK chemical structure also provides a positive foundation for their synthetic production at an industrial level at affordable cost. The challenge now is to apply this knowledge in the agricultural context.

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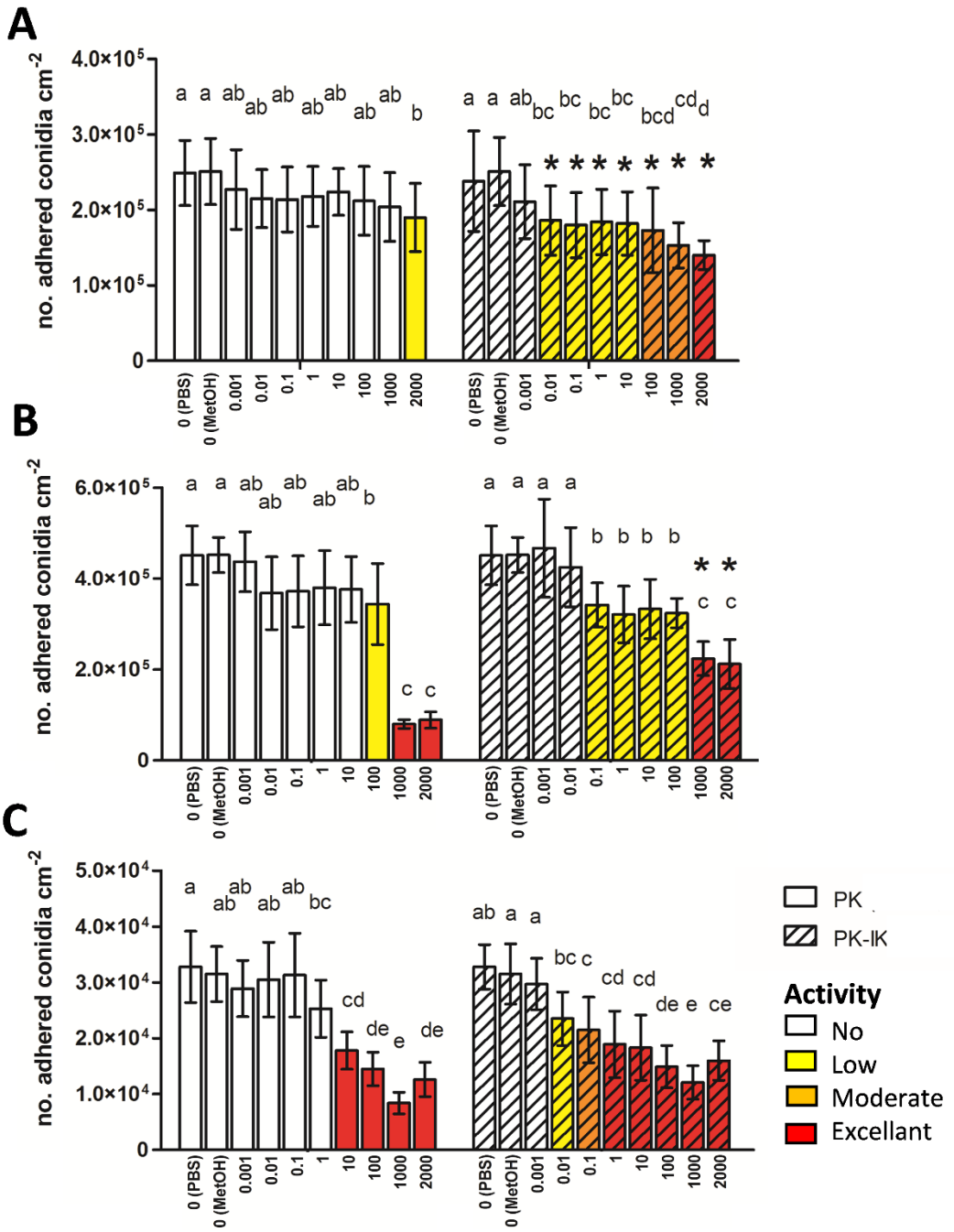
FIGURES

Figure 1. Conidia adhesion in the presence of *P. frutescens* essential oils. Number of adhered conidia of *C. musae* (A), *F. dimerum* (B) and *F. oxysporum* (C) in the presence of PK and PK-IK essential oils at different concentrations. Panel D shows the percentage reduction in comparison to the negative control prepared with the addition of 3 % MetOH. Data represent the mean ± standard deviation of at least four independent measurements. Different superscript letters statistically indicate significant differences (Tukey's HSD, $p \leq 0.01$) between the different concentrations, whereas an asterisk indicates a significant difference in the number of adhered conidia between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

Figure 2. Biofilm development in the presence of *P. frutescens* essential oils. Representative picture of *C. musae* (A), *F. dimerum* (B) and *F. oxysprum* (C) biofilms grown in semi-batch conditions in the presence of essential oils. A naked-eye visible difference is shown between the control and treated samples. Percentage reduction of dry weight (D), EPS polysaccharides (E) and EPS proteins (F) calculated in comparison to the negative control prepared with the addition of 3 % MetOH. EPS polysaccharide (G, H, I) and protein (L, M, N) content of *C. musae* (G, L), *F. dimerum* (H, M) and *F. oxysporum* (I, N) biofilms grown in the presence of PK and PK-IK essential oils at different concentrations. Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas an asterisk indicates a significative difference between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

in semi-batch

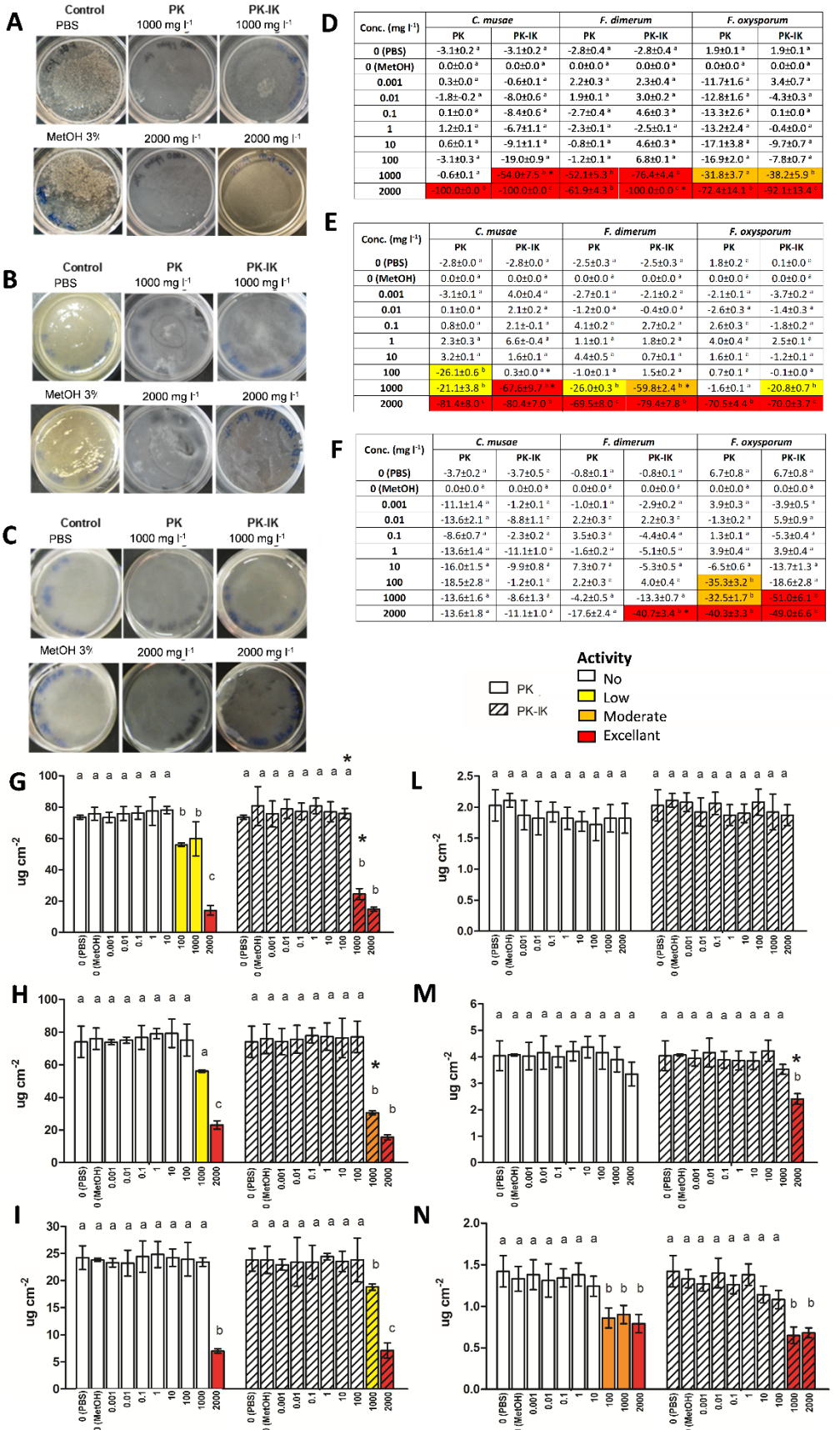
FIGURE 1



D

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-1.1±0.2 ^{a}	-1.1±1.5 ^{a}	-0.3±0.0 ^{a}	-0.3±0.0 ^{a}	4.1±0.8 ^{a}	4.1±0.8 ^{ab}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{ab}	0.0±0.0 ^{a}
0.001	-9.9±2.3 ^{ab}	-16.1±3.7 ^{ab}	-3.2±0.5 ^{ab}	3.4±0.8 ^{a}	-8.2±1.4 ^{ab}	-5.9±0.9 ^{a}
0.01	-14.3±2.6 ^{ab}	-26.0±6.4 ^{bc} *	-18.7±4.1 ^{ab}	-6.0±1.2 ^{a}	-3.4±0.7 ^{ab}	-25.5±5.2 ^{bc} *
0.1	-14.9±3.0 ^{ab}	-28.4±6.8 ^{bc} *	-15.8±3.4 ^{ab}	-24.3±3.4 ^{b}	-0.7±0.2 ^{ab}	-31.9±8.7 ^{c} *
1	-13.2±2.4 ^{ab}	-26.7±6.3 ^{bc} *	-17.8±3.7 ^{ab}	-29.0±5.6 ^{b}	-19.9±4.0 ^{bc}	-40.2±12.8 ^{cd} *
10	-10.8±1.5 ^{ab}	-27.4±6.3 ^{bc} *	-16.9±3.3 ^{ab}	-26.3±5.2 ^{b}	-43.4±8.1 ^{cd}	-42.0±13.4 ^{cd}
100	-15.7±3.4 ^{ab}	-31.2±8.2 ^{bcd} *	-23.9±6.2 ^{b}	-28.4±2.8 ^{b}	-54.1±11.2 ^{de}	-52.7±13.4 ^{de}
1000	-18.9±4.2 ^{ab}	-39.1±7.7 ^{cd} *	-82.3±9.7 ^{c}	-50.3±8.4 ^{c} *	-73.4±16.9 ^{e}	-61.6±15.2 ^{e} *
2000	-24.3±5.8 ^{b}	-44.4±6.2 ^{d} *	-80.3±16.2 ^{c}	-53.1±13.5 ^{c} *	-60.1±14.7 ^{de}	-49.2±10.9 ^{ce} *

FIGURE 2



D

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-3.1±0.2 ^{a}	-3.1±0.2 ^{a}	-2.8±0.4 ^{a}	-2.8±0.4 ^{a}	1.9±0.1 ^{a}	1.9±0.1 ^{a}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
0.001	0.3±0.0 ^{a}	-0.6±0.1 ^{a}	2.2±0.3 ^{a}	2.3±0.4 ^{a}	-11.7±1.6 ^{a}	3.4±0.7 ^{a}
0.01	-1.8±-0.2 ^{a}	-8.0±0.6 ^{a}	1.9±0.1 ^{a}	3.0±0.2 ^{a}	-12.8±1.6 ^{a}	-4.3±0.3 ^{a}
0.1	0.1±0.0 ^{a}	-8.4±0.6 ^{a}	-2.7±0.4 ^{a}	4.6±0.3 ^{a}	-13.3±2.6 ^{a}	0.1±0.0 ^{a}
1	1.2±0.1 ^{a}	-6.7±1.1 ^{a}	-2.3±0.1 ^{a}	-2.5±0.1 ^{a}	-13.2±2.4 ^{a}	-0.4±0.0 ^{a}
10	0.6±0.1 ^{a}	-9.1±1.1 ^{a}	-0.8±0.1 ^{a}	4.6±0.3 ^{a}	-17.1±3.8 ^{a}	-9.7±0.7 ^{a}
100	-3.1±0.3 ^{a}	-19.0±0.9 ^{a}	-1.2±0.1 ^{a}	6.8±0.1 ^{a}	-16.9±2.0 ^{a}	-7.8±0.7 ^{a}
1000	-0.6±0.1 ^{a}	-54.0±7.5 ^{b} *	-52.1±5.3 ^{b}	-76.4±4.4 ^{b}	-31.8±3.7 ^{a}	-38.2±5.9 ^{b}
2000	-100.0±0.0 ^{b}	-100.0±0.0 ^{c}	-61.9±4.3 ^{b}	-100.0±0.0 ^{c} *	-72.4±14.1 ^{b}	-92.1±13.4 ^{c}

E

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-2.8±0.0 ^{a}	-2.8±0.0 ^{a}	-2.5±0.3 ^{a}	-2.5±0.3 ^{a}	1.8±0.2 ^{a}	0.1±0.0 ^{a}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
0.001	-3.1±0.1 ^{a}	4.0±0.4 ^{a}	-2.7±0.1 ^{a}	-2.1±0.2 ^{a}	-2.1±0.1 ^{a}	-3.7±0.2 ^{a}
0.01	0.1±0.0 ^{a}	2.1±0.2 ^{a}	-1.2±0.0 ^{a}	-0.4±0.0 ^{a}	-2.6±0.3 ^{a}	-1.4±0.3 ^{a}
0.1	0.8±0.0 ^{a}	2.1±-0.1 ^{a}	4.1±0.2 ^{a}	2.7±0.2 ^{a}	2.6±0.3 ^{a}	-1.8±0.2 ^{a}
1	2.3±0.3 ^{a}	6.6±-0.4 ^{a}	1.1±0.1 ^{a}	1.8±0.2 ^{a}	4.0±0.4 ^{a}	2.5±0.1 ^{a}
10	3.2±0.1 ^{a}	1.6±0.1 ^{a}	4.4±0.5 ^{a}	0.7±0.1 ^{a}	1.6±0.1 ^{a}	-1.2±0.1 ^{a}
100	-26.1±0.6 ^{b}	0.3±0.0 ^{a} *	-1.0±0.1 ^{a}	1.5±0.2 ^{a}	0.7±0.1 ^{a}	-0.1±0.0 ^{a}
1000	-21.1±3.8 ^{b}	-67.6±9.7 ^{b} *	-26.0±0.3 ^{b}	-59.8±2.4 ^{b} *	-1.6±0.1 ^{a}	-20.8±0.7 ^{b}
2000	-81.4±8.0 ^{c}	-80.4±7.0 ^{c}	-69.5±8.0 ^{c}	-79.4±7.8 ^{b}	-70.5±4.4 ^{b}	-70.0±3.7 ^{c}

F

Conc. (mg l^{-1})	*C. musae*		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	-3.7±0.2 ^{a}	-3.7±0.5 ^{a}	-0.8±0.1 ^{a}	-0.8±0.1 ^{a}	6.7±0.8 ^{a}	6.7±0.8 ^{a}
0 (MetOH)	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
0.001	-11.1±1.4 ^{a}	-1.2±0.1 ^{a}	-1.0±0.1 ^{a}	-2.9±0.2 ^{a}	3.9±0.3 ^{a}	-3.9±0.5 ^{a}
0.01	-13.6±2.1 ^{a}	-8.8±1.1 ^{a}	2.2±0.3 ^{a}	2.2±0.3 ^{a}	-1.3±0.2 ^{a}	5.9±0.9 ^{a}
0.1	-8.6±0.7 ^{a}	-2.3±0.2 ^{a}	3.5±0.3 ^{a}	-4.4±0.4 ^{a}	1.3±0.1 ^{a}	-5.3±0.4 ^{a}
1	-13.6±1.4 ^{a}	-11.1±1.0 ^{a}	-1.6±0.2 ^{a}	-5.1±0.5 ^{a}	3.9±0.4 ^{a}	3.9±0.4 ^{a}
10	-16.0±1.5 ^{a}	-9.9±0.8 ^{a}	7.3±0.7 ^{a}	-5.3±0.5 ^{a}	-6.5±0.6 ^{a}	-13.7±1.3 ^{a}
100	-18.5±2.8 ^{a}	-1.2±0.1 ^{a}	2.2±0.3 ^{a}	4.0±0.4 ^{a}	-35.3±3.2 ^{b}	-18.6±2.8 ^{a}
1000	-13.6±1.6 ^{a}	-8.6±1.3 ^{a}	-4.2±0.5 ^{a}	-13.3±0.7 ^{a}	-32.5±1.7 ^{b}	-51.0±6.1 ^{b}
2000	-13.6±1.8 ^{a}	-11.1±1.0 ^{a}	-17.6±2.4 ^{a}	-40.7±3.4 ^{b} *	-40.3±3.3 ^{b}	-49.0±6.6 ^{b}

SUPPLEMENTARY INFORMATION

Supplementary S1: *P. frutescens* essential oil extraction

Fig. S1. Pilot plant used for *P.frutescens* essential oil extraction.

Supplementary S2: *P. frutescens* essential oil characterization

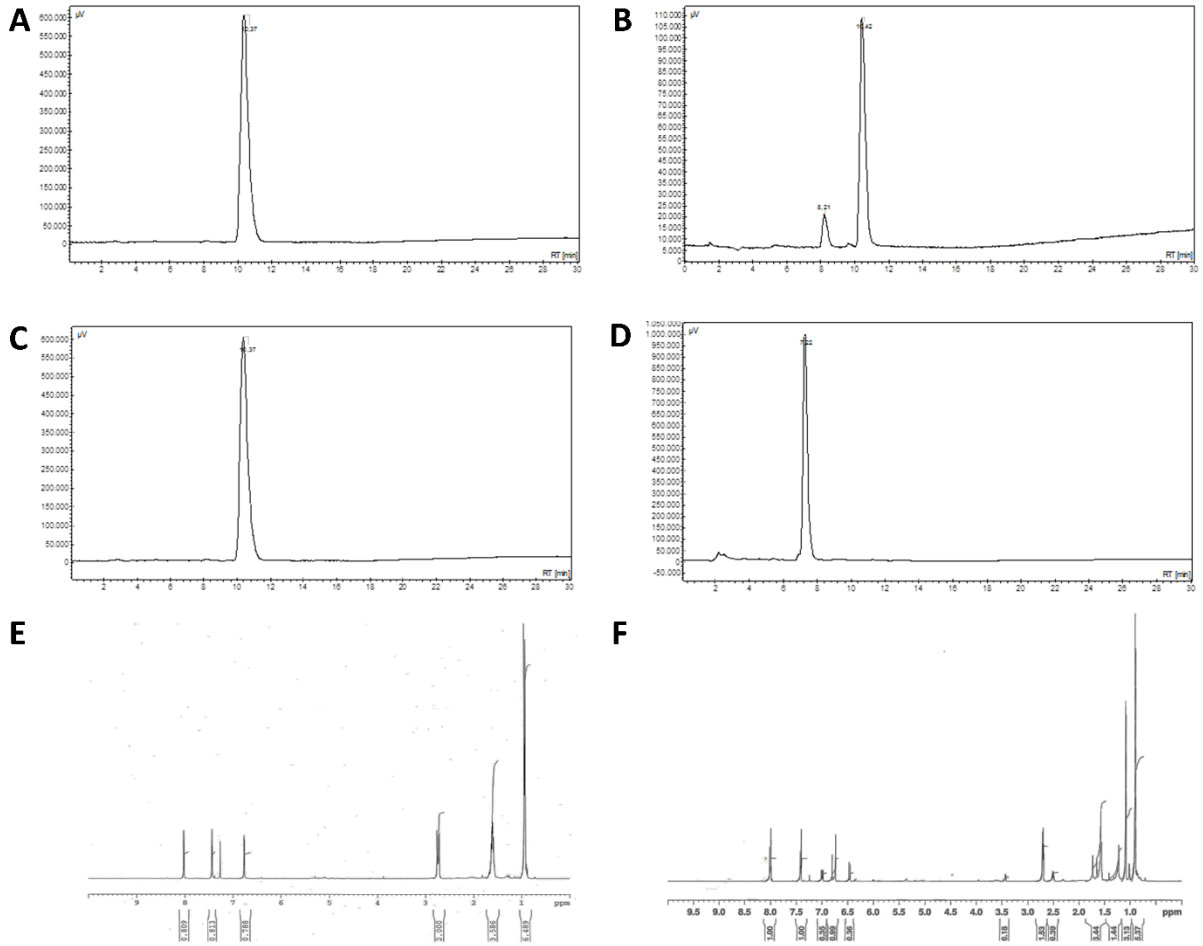


Fig. S2. *P. frutescens* essential oil characterization. HPLC chromatograms of PK (A) and PK-IK (B) *P. frutescens* essential oils; HPLC chromatograms of pure PK (C) and pure IK (D); x axis: absorbance (µV), y axis: retention time (min). ^{1}H NMR of PK (E) and PK-IK (F) *P. frutescens* essential oils in $CDCl_3$.

Supplementary S3: Planktonic fungal growth in the presence of *P. frutescens* essential oils

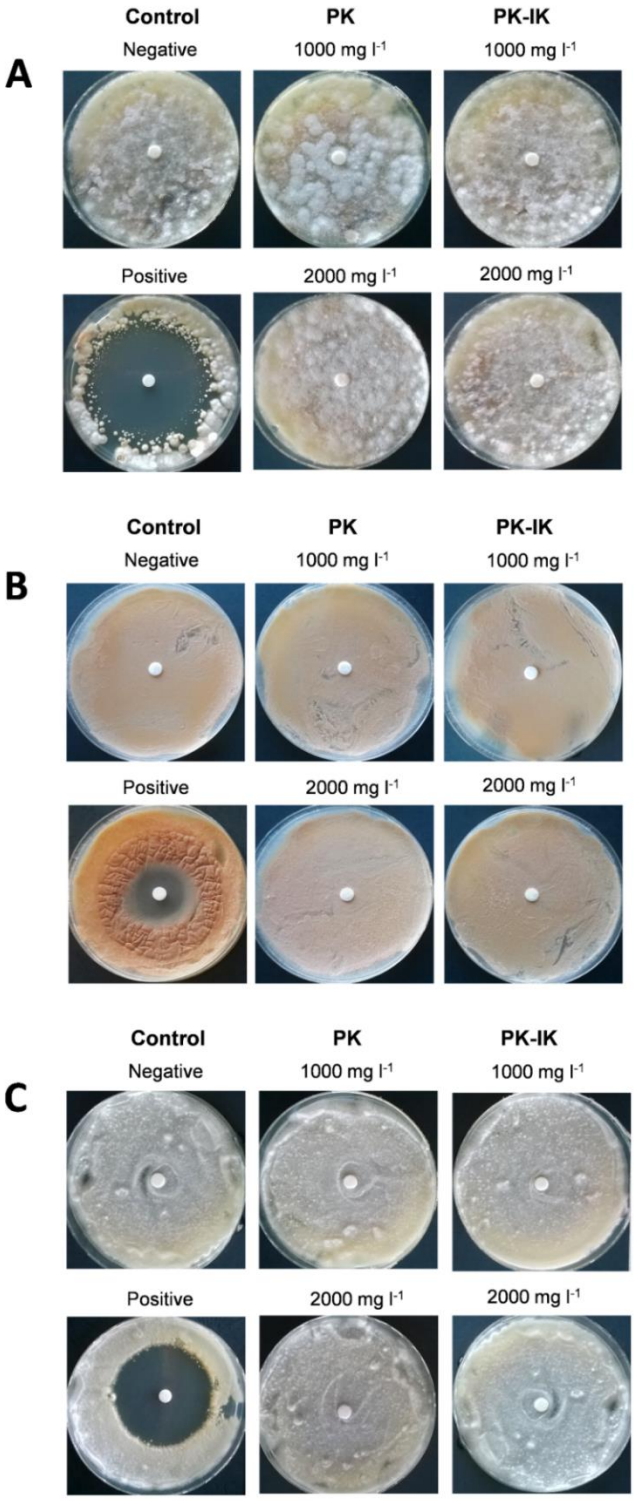


Fig. S3. Fungal growth in the presence of *P. frutescens* essential oils. Representative pictures of *C. musae* (A), *F. dimerum* (B) and *F. oxysprum* (C) grown in Petri plates with filter-paper discs at the center imbibed with PK or PK-IK oil. Filter paper discs were also prepared with PBS or 3 % MetOH as negative controls, and the antifungal Procloraz as positive control.

Supplementary S4: *P. frutescens* essential oils as sole carbon source

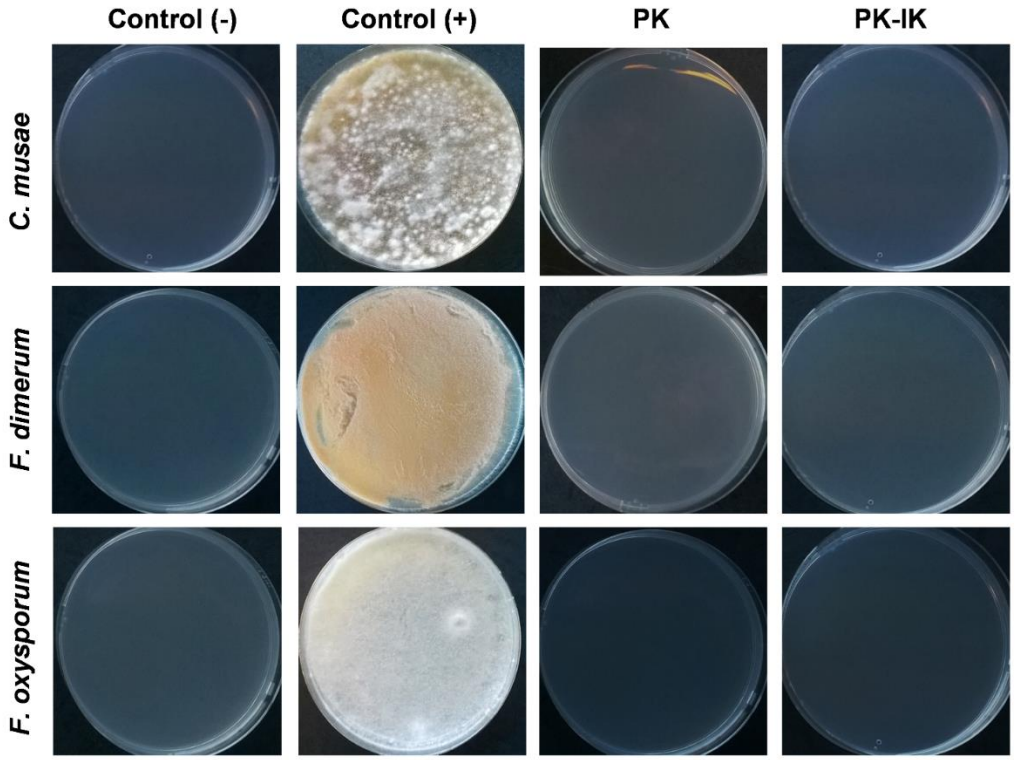


Fig. S4. Fungal growth in the presence of *P. frutescens* essential oils as sole carbon and energy source. Representative pictures of *C. musae*, *F. dimerum* and *F. oxysprum* grown in mineral medium agar with the addition of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK and PK-IK as sole carbon source. Petri dishes were also prepared with PBS or MetOH as negative controls and with the addition of sucrose as a positive control.

Supplementary S5: Adhesion kinetics

Material and methods

Conidia adhesion curves for each fungal strain were assessed in hydrophobic black-sided plates, as previously reported by Villa et al. (2010). Briefly, 200 µl of PBS containing 5 x 10^5 conidia were placed in microtiter plate wells and incubated at 21°C. After 0.25, 1 and then at 6 hourly intervals the microtiter plate wells were washed twice with 200 µl PBS, and the adhered conidia were stained using Fluorescent Brightener 28 (Sigma Aldrich, USA) in PBS for 20 min in the dark at room temperature. Fluorescence intensity was measured using the Infinite 200 PRO Microplate Reader (Tecan) at excitation wavelength of 335 nm and emission wavelength of 433 nm. A standard curve of fluorescence intensity was used to quantify the number of adhered conidia. Obtained data were normalized to the area and means were reported. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

The kinetics of conidia adhesion were determined according to Jopia et al. (2010) using the Gompertz growth model adapted for microbial growth by Zwietering (1990). The Neperian logarithm of relative growth ($N\ N_0^{-1}$) was used as the growth variable, where N_0 is the adhered conidia at the earliest time point and N is the adhered conidia at time t (in hours). The data were fitted to the model by non-linear regression using the software GraphPad Prism software (version 5.0, San Diego, CA, USA). Growth kinetic parameters were obtained: lag phase duration (λ; h), maximum specific growth rate (μ_m; conidia $cm^{-2}\ h^{-1}$) and the maximum number of adhered conidia in the stationary growth phase (A; conidia cm^{-2}).

Results

Figure S5 shows the adhesion curves for each fungal strain. The obtained data show that the number of adhered conidia of all the fungal strains increased until 18 h of incubation, after which the number of adhered conidia remained constant over time (Fig. S5A). Therefore, a 18 h-incubation time was used for the subsequent conidia adhesion assay in the presence of *P. frutescens* essential oils.

The interpolation of obtained data with the Gompertz growth model provided conidia adhesion parameters (Fig. S5A). *F. dimerum* displayed the longer lag phase, followed by *C. musae* and *F. oxysporum*. *F. dimerum* and *C. musae* showed the same maximum specific growth rate followed by *F. oxysporum* whereas *C. musae* and *F. oxysporum* reached the major number of adhered conidia during the stationary phase.

A

			C. musae	*F. dimerum*	*F. oxysporum*
Adhered conidia (conidia cm^{-2}) ($\times 10^5$)	Time (h)	0	0.00±0.00 ^{a}	0.00±0.00 ^{a}	0.00±0.00 ^{a}
		0.25	0.78±0.11 ^{ab}	0.84±0.15 ^{b}	0.15±0.02 ^{a}
		0.50	0.83±0.14 ^{ab}	0.88±0.17 ^{b}	0.14±0.01 ^{a}
		0.75	0.90±0.25 ^{ab}	0.84±0.23 ^{b}	0.14±0.02 ^{a}
		1	0.98±0.18 ^{b}	0.85±0.16 ^{b}	0.15±0.01 ^{a}
		6	1.46±0.92 ^{c}	0.96±0.38 ^{b}	0.52±0.09 ^{b}
		12	2.09±0.35 ^{d}	1.11±0.33 ^{bc}	2.00±0.37 ^{c}
		18	3.15±0.50 ^{e}	1.83±0.36 ^{d}	2.32±0.44 ^{cd}
		24	4.00±0.48 ^{e}	2.29±0.35 ^{e}	2.81±0.19 ^{d}
		30	3.89±0.45 ^{e}	2.16±0.46 ^{de}	3.04±0.25 ^{d}
		36	3.83±0.53 ^{e}	2.15±0.20 ^{de}	3.08±0.34 ^{d}
		42	3.96±0.49 ^{e}	2.11±0.33 ^{de}	3.15±0.53 ^{d}
		48	3.60±0.32 ^{e}	1.62±0.18 ^{cd}	3.00±0.14 ^{d}
λ (h)			4.01±0.20 ^{A}	6.20±0.21 ^{B}	3.29±0.33 ^{C}
μ_m (conidia cm^{-2} h^{-1}) ($\times 10^4$)			8.56±0.78 ^{AB}	9.43±0.85 ^{A}	7.24±0.15 ^{B}
A (conidia cm^{-2}) ($\times 10^5$)			4.04±0.80 ^{A}	2.02±0.94 ^{B}	2.94±0.61 ^{AB}
R^2			0.94±0.14	0.90±0.10	0.99±0.11

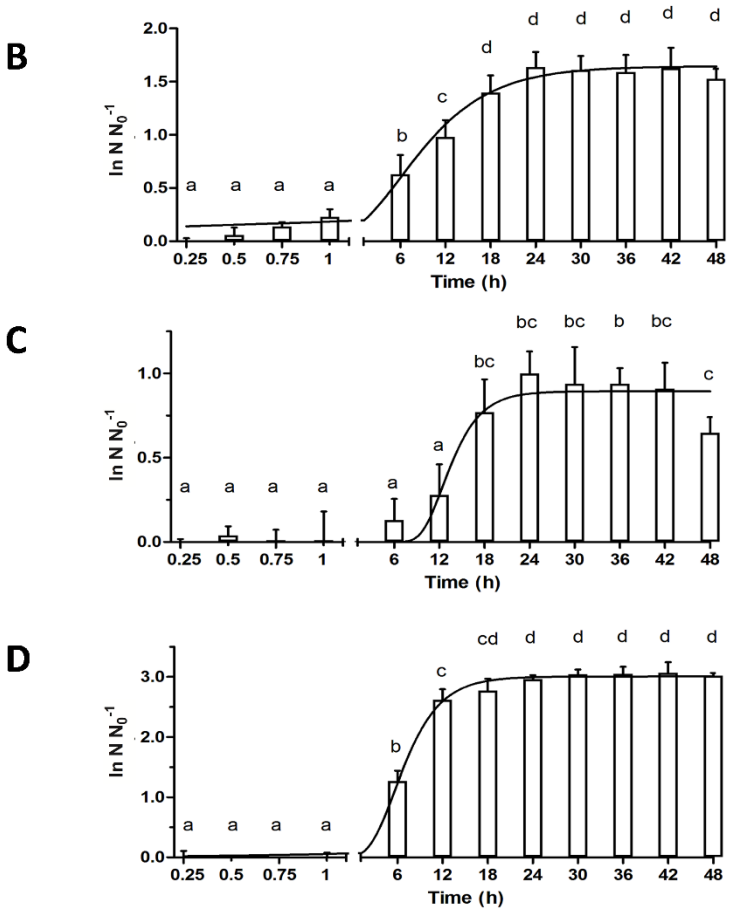


Fig. S5. Conidia kinetic adhesion. A: Number of adhered conidia at different times and adhesion kinetic parameters obtained by the Gompertz model. λ: lag phase duration, μ_m: maximum specific growth rate, A: maximum number of adhered conidia in the stationary growth phase, R^2: goodness of fit. B, C, D: Adhesion curve of *C. musae* (B), *F. oxysporum* (C) and *F. dimerum* (D) obtained by fitting the relative number of adhered conidia (log N N_0^{-1}) with the Gompertz model. Data represent the mean ± standard deviation of three independent measurements. Different superscript letters

indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between the time steps (lower letter) or fungal strains (capital letter).

References

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Supplementary S6: Conidia germination

Conc. (mg l^{-1})	Germinated conidia (%)					
	C. musae		*F. dimerum*		*F. oxysporum*	
	PK	PK-IK	PK	PK-IK	PK	PK-IK
0 (PBS)	100.0±0.0 ^{a} (1.0±0.0)	100.0±0.0 ^{a} (1.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	99.7±0.8 ^{a} (1.4±0.8)	99.7±0.8 ^{a} (1.4±0.8)
0 (3% MetOH)	99.0±1.7 ^{a} (0.0±1.7)	99.0±1.7 ^{ab} (0.0±1.7)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	98.3±2.7 ^{a} (0.0±2.7)	98.3±2.7 ^{ab} (0.0±2.7)
0.001	100.0±0.0 ^{a} (1.0±0.0)	95.3±1.2 ^{ab} (-3.7±1.2)	100.0±0.0 ^{a} (0.0±0.0)	98.0±2.0 ^{a} (-2.0±2.0)	100.0±0.0 ^{a} (1.7±0.0)	99.3±1.2 ^{ab} (1.0±1.2)
0.01	100.0±0.0 ^{a} (1.0±0.0)	100.0±0.0 ^{ab} (1.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (0.0±0.0)	100.0±0.0 ^{a} (1.7±0.0)	100.0±0.0 ^{ab} (1.7±0.0)
0.1	91.3±6.1 ^{a} (-7.7±6.1)	98.7±1.2 ^{ab} (-0.3±1.2)	96.7±3.1 ^{a} (-3.3±3.1)	96.7±3.1 ^{a} (-3.3±3.1)	100.0±0.0 ^{a} (1.7±0.0)	98.0±2.0 ^{ab} (-0.3±2.0)
1	90.0±5.3 ^{ab} (-9.1±5.3)	99.3±1.2 ^{ab} (0.3±1.2)	95.3±2.3 ^{a} (-4.7±2.3)	98.7±1.2 ^{a} (-1.3±1.2)	100.0±0.0 ^{a} (1.7±0.0)	100.0±0.0 ^{ab} (1.7±0.0)
10	91.3±3.1 ^{ab} (-7.7±3.1)	95.3±3.1 ^{b} (-3.7±3.1)	94.0±4.0 ^{a} (-6.0±4.0)	96.0±2.0 ^{a} (-4.0±2.0)	98.0±2.0 ^{a} (-0.3±2.0)	95.3±4.2 ^{ab} (-3.1±4.2)
100	90.7±5.0 ^{ab} (-8.4±5.0)	88.0±2.0 ^{c} (-11.1±2.0)	96.0±4.0 ^{a} (-4.0±4.0)	98.7±1.2 ^{a} (-1.3±1.2)	96.7±1.2 ^{a} (-1.7±1.2)	94.0±2.0 ^{b} (-4.4±2.0)
1000	88.0±2.0 ^{b} (-11.1±2.0)	15.3±3.1 ^{d} * (-84.5±3.1)	81.0±2.2 ^{b} (-19.0±2.2)	15.3±2.0 ^{b} * (-84.5±2.0)	81.0±1.4 ^{b} (-17.6±1.4)	0.7±1.2 ^{c} * (-99.3±1.2)
2000	0.0±0.0 ^{c} (-100.0±0.0)	0.0±0.0 ^{e} (-100.0±0.0)	0.0±0.0 ^{b} (-100.0±0.0)	0.0±0.0 ^{c} (-100.0±0.0)	10.0±4.0 ^{c} (-89.8±4.0)	0.0±0.0 ^{d} * (-100.0±0.0)

Fig. S6. Percentage of germinated conidia in the presence of *P. frutescens* essential oils. The percentage reduction, compared to the negative control prepared with the addition of 3 % MetOH, is reported in brackets. Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas an asterisk indicates a significative difference in the number of germinated conidia between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

Supplementary S7: Biofilm development

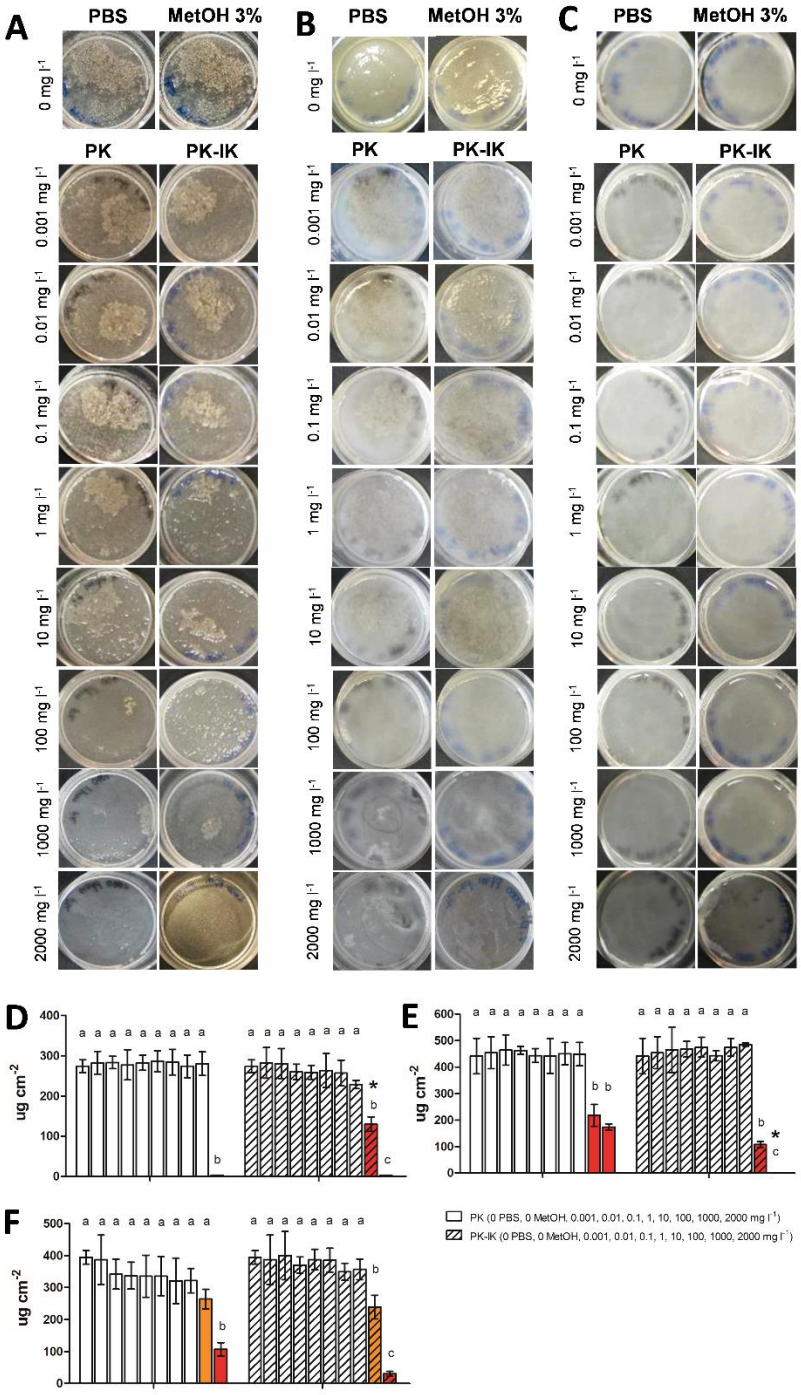

Fig. S7. Biofilm development in the presence of *P. frutescens* essential oils. Representative picture of *C. musae* (A), *F. dimerum* (B) and *F. oxysprum* (C) biofilms grown in semi-batch conditions in the presence of essential oils. Dry weight of *C. musae* (D), *F. dimerum* (E) and *F. oxysprum* (F) in the presence of the essential oils. Data represent the mean ± standard deviation of at least three independent measurements. Different superscript letters indicate statistically significant differences (Tukey's HSD, $p \leq 0.01$) between different concentrations, whereas an asterisk indicates a

significative difference in the dry biomass between PK and PK-IK at the same concentration. White: no effect; Yellow: low effect; Orange: moderate effect; Red: Excellent effect.

Supplementary S8: Planktonic growth of beneficial microorganisms in the presence of *P. frutescens* essential oils

Material and methods

Microorganism strains

Azotobacter vinelandii UW136, *Bacillus subtilis* Cu1065, *Escherichia coli* ATCC 25404, *Streptomyces lydicus* DEF49, *Streptomyces griseoviridis* DEF50, *Trichoderma asperellum* DEF51 (University of Milan collection) were maintained at −80°C in suspensions containing 20% glycerol. Working cultures were obtained by growing i) *A. vinelandii* in Burk's medium supplemented with 1% sucrose and 15 mmol l^{-1} ammonium acetate for 30 h at 30°C; ii) *B. subtilis* and *E. coli* in Tryptic Soy (TS) broth medium for 12 h at 30°C; iii) *S. lydicus* and *S. griseoviridis* on Czapek agar medium for 7 days at 21°C; and iv) *T. asperellum* on Potato Dextrose Agar (PDA) for 7 days at 21°C. Media were purchased from Sigma Aldrich, USA. Once sporulated, conidia of *S. lydicus*, *S. griseoviridis* and *T. asperellum* were collected in water and filtered through two layers of sterile gauze according to Kunova et al. (2016). Bacteria and conidia concentrations were determined by using a light microscope (Leica DM4000 M, Leica Microsystems, Germany) and a Thoma counting chamber.

Disk diffusion assay

The ability of PK and PK-IK *P. frutescens* oils to affect growth of beneficial microorganisms was assessed by disk diffusion assay (López-OviedoBaluoiri et al., 2006). Briefly, two hundred µL of 10^6 ml^{-1} bacteria or conidia were distributed uniformly on Petri plates containing i) Burk's agar medium supplemented with 1% sucrose and 15 mmol l^{-1} ammonium acetate for *A. vinelandii*; ii) TS agar medium for *B. subtilis* and *E. coli*, iii) Czapek agar medium for *S. lydicus* and *S. griseoviridis*; and iv) PDA for *T. asperellum*.

Filter-paper discs (6 mm diameter, Oxoid, United Kingdom) were imbibed with concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000, 2000 mg l^{-1} of PK or PK-IK *P. frutescens* oil with 3 % methanol (MetOH), and the discs were then placed at the center of the Petri plates. Filter paper discs were also prepared with Phosphate Buffer Saline (PBS, Sigma Aldrich, USA) or 3 % MetOH as negative controls. Positive controls were prepared with the streptomycin, erythromycin and chloramphenicol for *A. vinelandii*, *B. subtilis*, *E. coli*, *S. lydicus* and *S. griseoviridis* or the antifungal procloraz (0.01 %, Sportak, BASF, Germany) for *T. asperellum*. *A. vinelandii*, *B. subtilis*, *E. coli* were incubated at 30°C for 30 h whereas *S. lydicus*, *S. griseoviridis* and *T. asperellum* were incubated for 7 days at 21 °C. The diameter of inhibition growth around each disc was measured. Three biological replicates were performed for each treatment and three technical replicates for each experiment.

Results

Figures S8 and S9 show disk diffusion assays for each microbial strain. Experiments showed that both PK and PK-IK did not affect *A. vinelandii*, *B. subtilis*, *E. coli*, *S. lydicus*, *S. griseoviridis* and *T. asperellum* at all the tested concentrations as no growth inhibition was appreciable around each disk. The same result was obtained for the negative controls. On the contrary, a significant growth inhibition diameter was found around the disks imbibed with the positive controls.

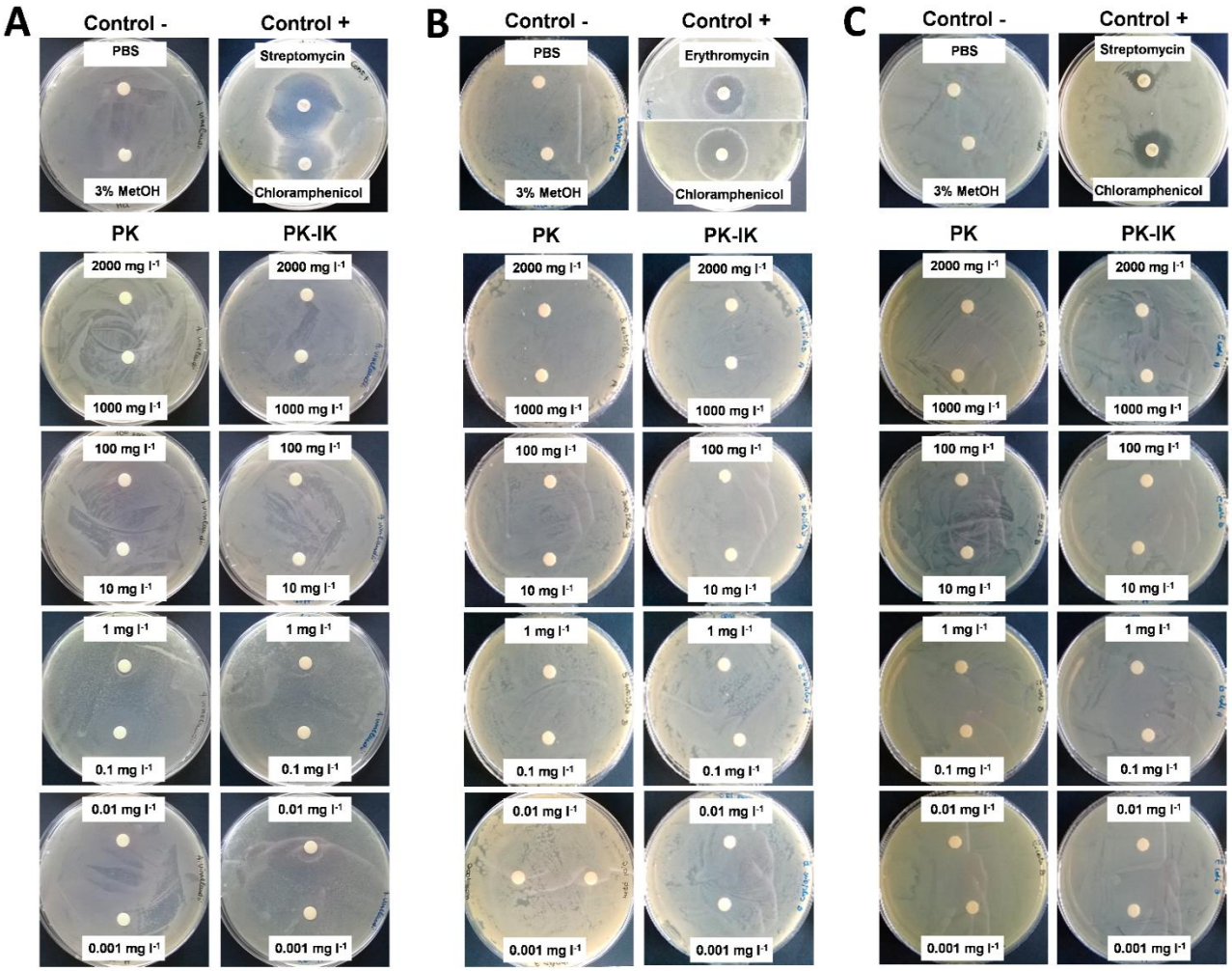

Fig. S8. Fungal growth in the presence of *P. frutescens* essential oils. Representative pictures of *A. vinelandii* (A), *B. subtilis* (B) and *E. coli* (C) grown in Petri plates with filter-paper discs at the center imbibed with PK or PK-IK oil. Filter paper discs were also prepared with PBS or 3 % MetOH as negative controls, and the antibiotics streptomycin, erythromycin and chloramphenicol as positive control.

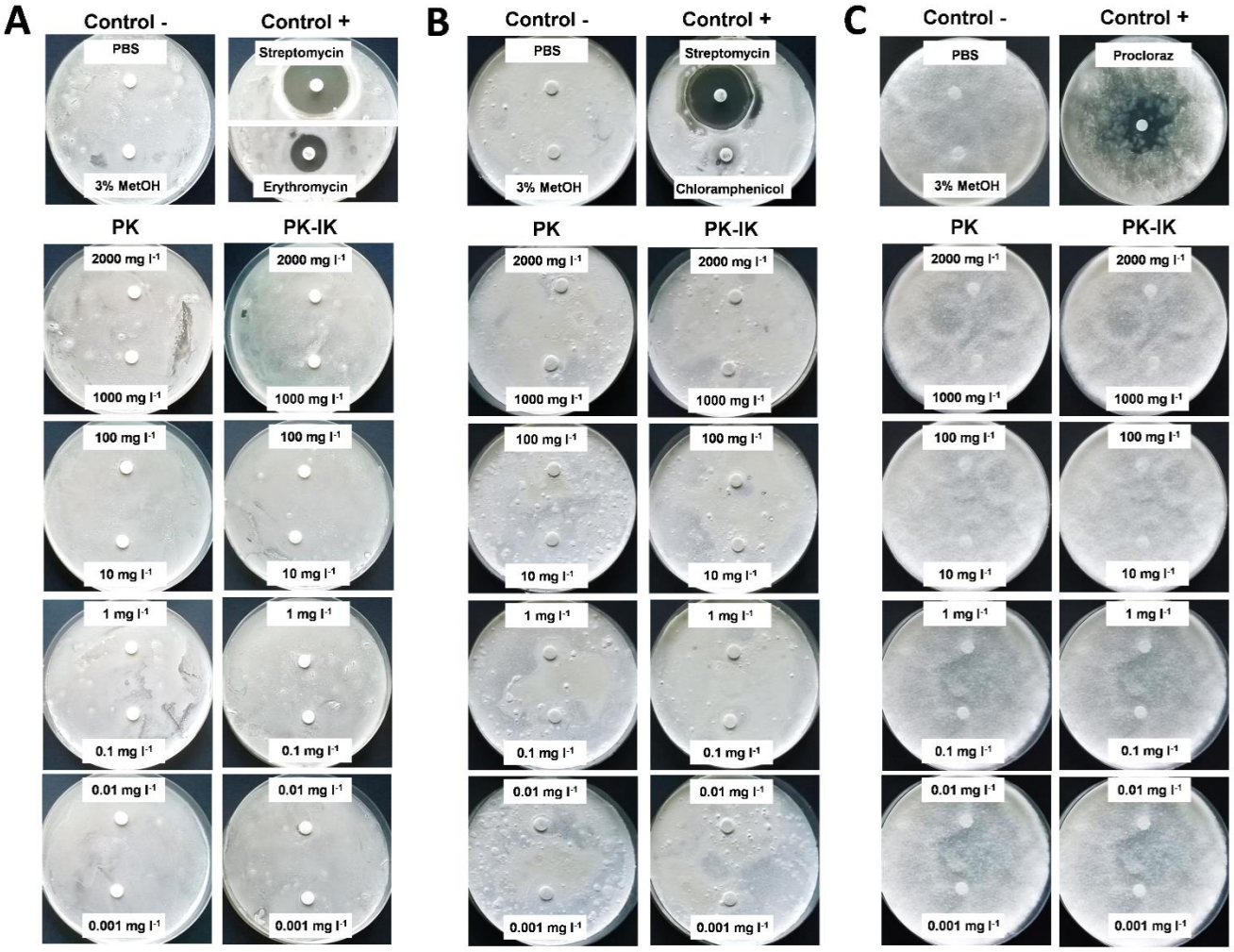

Fig. S9. Fungal growth in the presence of *P. frutescens* essential oils. Representative pictures of *S. lydicus* (A), *S. griseoviridis* (B) and *T. asperellum* (C) grown in a Petri plates with filter-paper discs at the center imbibed with PK or PK-IK oil. Filter paper discs were also prepared with PBS or 3 % MetOH as negative controls, and the antibiotics streptomycin, erythromycin and chloramphenicol or the antifungal procloraz as positive control.

References

López-Oviedo, E., Aller, A.I., Martín, C., Castro, C., Ramirez, M., Pemán, J.M., Cantón, E., Almeida, C., Martín-Mazuelos, E., 2006. Evaluation of disk diffusion method for determining posaconazole susceptibility of filamentous fungi: comparison with CLSI broth microdilution method. Antimicrob. Agents Chemother. 50, 1108−1111.