ORIGINAL ARTICLE

Antifungal modes of action of tea tree oil and its two characteristic components against *Botrytis cinerea*

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Abstract

Aims: The essential oil of *Melaleuca alternifolia* (tea tree) has been evaluated as a potential eco-friendly antifungal agent against *Botrytis cinerea*. In this study, we investigated the antifungal activity and mode of action of tea tree oil (TTO) and its components against *B. cinerea*.

Methods and Results: Of the components we tested in contact phase, terpinen-4-ol had the highest antifungal activity, followed by TTO, α-terpineol, terpinolene, then 1,8-cineole. As one of characteristic components of TTO, terpinen-4-ol treatment led to pronounced alterations in mycelial morphology, cellular ultrastructure, membrane permeability under scanning electron microscope, transmission electron microscope and fluorescent microscope, and also reduced the ergosterol content of fungi. As another characteristic component, 1,8-cineole caused serious intracellular damage but only slightly affected *B. cinerea* otherwise. When terpinen-4-ol and 1,8-cineole were used together, the synergistic antifungal activity was significantly higher than either component by itself.

Conclusions: The results of our study confirmed that terpinen-4-ol and 1,8-cineole act mainly on the cell membranes and organelles of *B. cinerea*, respectively, and when combined are similar to TTO in antifungal activity due to their differences.

Significance and Impact of the Study: Understanding the mechanism of terpinen-4-ol and 1,8-cineole antifungal action to *B. cinerea* is helpful for investigation on their synergistic effect and explaining antifungal action modes of TTO.

Introduction

Due to growing health and environmental concerns regarding the use of synthetic fungicides to control postharvest spoilage, antifungal GRAS (generally recognized as safe) substances and sustainable nonchemical management techniques are increasingly being investigated (Walter *et al.* 2005; Combrinck *et al.* 2011). Essential oils (EO), which can be used to control biological spoilage and extend the storage life of perishables, have received particular attention for their broad-spectrum, biodegradable and eco-friendly properties (La Torre *et al.* 2014; Sivakumar and Bautista-Baños 2014; Guerreiro *et al.* 2015).

Many essential oils effectively inhibit postharvest fungi *in vitro* (Lopez-Reyes *et al.* 2010). Among them, tea tree oil (TTO), the volatile essential oil derived mainly from the Australian native plant *Melaleuca alternifolia*, has been used to treat a wide range of human and animal conditions and is considered as an effective alternative to the most commonly used antifungal drugs (Carson *et al.* 2006). In *in vitro* experiments, TTO vapour exhibited a high activity against spore germination and mycelial growth of the main postharvest pathogens *Botrytis cinerea* and *Rhizopus stolonifer* (Shao *et al.* 2013a). Mycelial growth in *Fusarium graminearum*, *F. culmorum* and *Pyrenophora graminea* was also significantly suppressed by contact with TTO concentrations ranging from 0·25 to 5% (Terzi *et al.* 2007). TTO has been compared with several fungicides and seaweed extracts in the control of fruit rot in strawberries, and results showed that only TTO significantly reduced the total incidence of rot (Washington *et al.* 1999). Compared with bergamot and

thyme, chitosan coatings containing TTO were the most effective in reducing microbial growth on infected oranges (Cháfer *et al.* 2012).

Many studies suggest that the overall antifungal activity of essential oils is due to the synergistic effects of their various components (Moon *et al.* 2011; Pinto *et al.* 2014). Some of the most abundant components of essential oils, such as α-terpineol in *Thymus caespititius* EO and menthone in *Mentha piperita L.* EO appear to be highly effective against fungus (Marcelo *et al.* 2012; Pinto *et al.* 2014). A study of the components of thyme oil revealed that the most active combinations against *Candida albicans* and *Candida krusei* were thymol/*p*-cymene or thymol/1,8-cineole (Pina-Vaz *et al.* 2004). Previous investigations demonstrated that oxygenated terpenoids are the main active components in TTO, among which terpinen-4-ol is the most effective (Carson *et al.* 2006). To optimize antimicrobial activity, the international standard ISO 4730 requires two characteristic components of commercial TTO, the terpinen-4-ol chemotype must have a minimum terpinen-4-ol content of 30% and a maximum 1,8-cineole content of 15%. Studies suggest that TTO and its main component, terpinen-4-ol, compromise the cytoplasmic membrane of both bacteria and fungi (Hammer *et al.* 2004). *Staphylococcus aureus* suspensions treated with TTO or terpinen-4-ol lost significant 260-nm-absorbing material, exhibited morphological alterations and were more susceptible to lysis (Carson *et al.* 2002). Levels of 1,8-cineole tend to be inversely proportional to the levels of terpinen-4-ol, and have lower antimicrobial activity. Mondello *et al.* (2006) showed that against clinical isolates of *C. albicans*, the minimum inhibitory concentration (MIC) of terpinen-4-ol ranged from 0·015 to 0·06% v/v and 1% to >4% v/v for 1,8-cineole.

Botrytis cinerea, the causal agent of the economically important grey mould disease, is a ubiquitous pathogen which causes considerable postharvest loss of fruits and vegetables (Jing *et al.* 2014; Jung *et al.* 2014). Our earlier study demonstrated that TTO can effectively inhibit the growth of *B. cinerea in vitro* and reduce grey mould on strawberry fruit *in vivo* (Shao *et al.* 2013a). To characterize TTO's antifungal mechanism more completely, it is necessary to investigate TTO and its constituents alone and in combination. Here, we first tested the inhibitory activities of TTO and its individual components against *B. cinerea*, and then examined the activity of terpinen-4-ol in combination with the other components. We more closely evaluated the effects of terpinen-4-ol and 1,8-cineole using a variety of methods to assess impacts on hyphal morphology (scanning electron microscopy), cell ultrastructure (transmission electron microscopy), the formation of lesions in the cell membrane (fluorescence microscopy) and membrane integrity (extracellular conductivity and ergosterol content).

Materials and methods

Pathogen, essential oil and single component

The highly virulent *B. cinerea* was isolated from strawberries and preserved at 25°C on potato dextrose agar (PDA) before use (Shao *et al.* 2013a). TTO was provided by Melalyn Bio-Technique Co. Ltd (Fujian, China). The TTO, analysed by gas chromatography–mass spectrometry, contained 43·4% terpinen-4-ol and 3·5% 1,8-cineole, adjusted to comply with the international standard. Terpinen-4-ol (95%), Terpinolene (85%) and γ-terpinene (95%) were purchased from TCI Shanghai (Shanghai, China). 1,8-cineole (99%) and α-pinene (98%), α-terpinene (90%) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). α-terpineol (98%) was purchased from Aladdin Reagent Co., Ltd (Shanghai, China). Limonene (96%) was purchased from J&K Chemical Reagent Co., Ltd. ρ-cymene (99%) was purchased from Shyuanye Reagent Co. Ltd (Shanghai, China).

Effect of TTO and its components on mycelial growth

Contact effects of TTO or its components on mycelial growth of *B. cinerea* were assessed *in vitro* using the agar dilution method (Zhou *et al.* 2014). TTO or a single constituent was dispersed as an emulsion in sterile PDA containing 1% (v/v) Tween-80 immediately before it was decanted into glass petri dishes (90 mm diameter) at a temperature of 50–65°C. The dilutions tested were 0·25, 0·5, 1·0 and 1·5 μl ml^{-1}. Control plates received the same quantity of Tween-80 mixed with PDA. A 5 mm diameter plug containing *B. cinerea* was taken from the edge of a 5 days old fungal culture and placed in the centre of each plate. After incubation at 25°C for 3 days, treatment efficacy was determined by measuring the average of two perpendicular diameters through each colony. The percentage inhibition of mycelial growth inhibition (MGI) was calculated according to the following formula:

$$\mathrm{MGI}(\%) = [(d_c - d_t)/d_c] \times 100,$$

where d_c (cm) is the mean colony diameter for the control sets and d_t (cm) is the mean colony diameter for the treatment sets. Each treatment was performed in quintuplicate.

Effect of component combinations on mycelial growth

Terpinen-4-ol, the principal active component of TTO, was combined with other TTO components (α-terpineol,

γ-terpinene, terpinolene, α-pinene, α-terpinene, ρ-cymene, 1,8-cineole and limonene) at a ratio of 1 : 1 (v/v). The concentration of terpinen-4-ol in PDA is 0·25 μl ml^{-1}, and the same volume of other components was added respectively. Stock solutions of all components were prepared in PDA medium at 0·25 μl ml^{-1}. Diluted terpinen-4-ol and PDA without added oil served as controls. Experimental manipulations and calculation of MGI (%) were as described above. Each treatment was performed in quintuplicate.

The MIC of TTO, terpinen-4-ol, 1,8-cineole and a 1 : 1 mixture of terpinen-4-ol and 1,8-cineole were also determined as described above. PDA (20 ml) was added to sterilized Petri dishes (90 mm diameter) and measured amounts of oils were added to give the following dilutions: TTO at 1·0, 1·5, 2·0, 2·5, 3·0, 3·5 and 4·0 μl ml^{-1}; terpinen-4-ol at 0·5, 0·6, 0·7, 0·8, 0·9 and 1·0 μl ml^{-1}; 1,8-cineole at 6·0, 6·5, 7·0, 7·5, 8·0, 8·5 and 9·0 μl ml^{-1}; and for paired components at 0·5, 0·6, 0·7, 0·8, 0·9 and 1·0 μl ml^{-1}. The MIC was expressed as the lowest dilution that completely inhibited the growth of the fungus. Each treatment was performed in quintuplicate.

Scanning electron microscopy (SEM)

A spore suspension of *B. cinerea* was obtained from 3-day-old cultures by adding 10 ml 0·9% NaCl solution to each petri dish and gently scraping the mycelial surface three times with a sterile L-shaped spreader to free the spores. A 1 ml spore suspension (10^7 spores ml^{-1}) of *B. cinerea* was added to 150 ml potato dextrose broth (PDB) medium and incubated at 25°C and 150 rev min^{-1} for 2 days. Mycelium was filtered by multi-layer gauze and washed three times with 0·9% NaCl solution. Identical amounts (wet weight) of mycelium were re-suspended in an equal volume of sterile water (w/v = 4%) containing 1% (v/v) Tween-80 after addition of TTO, terpinen-4-ol, 1,8-cineole or paired components to their MIC as described earlier. Samples without oil were used as controls. Mixtures were incubated for 2 h at 25°C and 150 rev min^{-1}. Each treatment was performed in triplicate.

The effects of TTO and its characteristic components on hyphal morphology were analysed using the modified method of Tao *et al.* (2014). For SEM observation, mycelium exposed to different treatments was fixed with 2·5% glutaraldehyde for 2 h at low temperature. Samples were washed three times for 15 min in 0·1 mol l^{-1} phosphate buffer (pH 7·2). After fixation, samples were dehydrated in a graded series of ethanol concentrations (30, 50, 70, 80, 90% and twice at 100%) for 15 min at each stage. They were then sequentially washed in 3 : 1, 1 : 1 and 1 : 3 mixtures of ethanol and tert butyl alcohol for

10 min each, and finally dipped into pure tert-butyl alcohol twice for 10 min. Samples were dried by sublimation in a freeze dryer (ES-2030; Hitachi, Tokyo, Japan), then gold-coated using a sputter coating machine (E1010, Hitachi) for 60 s. All samples were viewed in a SEM (Model S-3400N, Hitachi) operating at 25 kV at 2000× magnification.

Transmission electron microscopy (TEM)

The effects of TTO and its components on cell ultrastructure were analysed using the modified method of Liao *et al.* (2010). For TEM observation, mycelium samples obtained as described above were fixed using 2·5% glutaraldehyde then osmic acid and dehydrated in a graded ethanol (30, 50, 70, 90%) and acetone series (90%, three times at 100%) for 15 min at each stage. A solution of epoxy resin was added to specimens every 2 h, thereby gradually increasing the resin concentration. Samples were incubated in pure epoxy at 37°C for 12 h, then polymerized at 60°C for over 36 h. Ultrathin sections of about 70 nm were prepared for each sample using a diamond knife. Sections were treated with uranyl acetate followed by lead citrate (30 min each) and examined by TEM (Model JEM-1230, Hitachi).

Fluorescent microscope (FSM)

Membrane integrity was assayed following the method of Liu *et al.* (2010) with minor modifications. *Botrytis cinerea* was treated with TTO or its characteristic components as described previously. After 2 h, mycelium was sampled from each group and washed with cold 50 mmol l^{-1} sodium phosphate buffer (pH 7·0). Mycelium was then fixed by cold 70% ethanol at 4°C for 1 h. After removal of ethanol and two washes with PBS, the mycelium was stained with 50 μg ml^{-1} propidium iodide (PI) for 20 min at 4°C in the dark. Mycelium was collected by centrifugation and washed twice with buffer to remove residual dye. Samples were observed with a Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) equipped with an individual fluorescein rhodamine filter set (Zeiss no.15: excitation BP 546/12 nm, emission LP 590 nm). Fields of view from each cover slip were chosen randomly, and all experiments were repeated three times.

Determination of extracellular conductivity

As a proxy for changes in membrane permeabilization, the electrical conductivity of supernatants obtained from mycelium suspensions was determined according to the methods of Lee *et al.* (1998). After 2 days of growth,

mycelium was collected and treated with TTO or its components as described above. Suspensions were centrifuged at 2500 *g* for 10 min to obtain supernatants either immediately following the addition of oil or 2 h later. All experiments were repeated three times. Conductivity was measured using a conductivity meter (DDS-11A, Shanghai Precision Scientific Instrument Co., Ltd. Shanghai, China).

Determination of ergosterol content

The ergosterol content of the plasma membrane in *B. cinerea* was measured according to the method of Tian *et al.* (2012b) with minor modifications. Mycelium from *B. cinerea* was harvested and treated as described previously. Samples without oil treatment were used as controls. After treatment, mycelium was collected and washed three times with distilled water, followed by sublimation in a freeze dryer. The net dry weight of mycelium was determined. Three millilitre of 25% alcoholic potassium hydroxide solution was added to each sample and mixed by vortexing for 2 min (TS-1, Kylin-Bell Lab Instruments Co., Ltd., Shanghai, China). After ultrasonic extraction for 20 min, samples were incubated at 85°C for 2 h. Sterols were extracted from each sample by adding a mixture of 1 ml sterile distilled water and 3 ml *n*-heptane, then mixed by vortexing for 2 min and the layers allowed to separate for 1 h at room temperature. The *n*-heptane layer was analysed using scanning spectrophotometry (UV-1700, Shimadzu, Tokyo, Japan)

between 230 and 300 nm. The presence of ergosterol (at 282 nm) and the sterol intermediate 24(28) dehydroergosterol (at 230 and 282 nm) in the *n*-heptane layer produced a characteristic curve. Ergosterol was quantified as described by Tian *et al.* (2012b).

Statistical analyses

SAS Software (Ver. 8.2; SAS Institute, Cary, NC) was used to conduct statistical analyses. Data were analysed by one-way analysis of variance (ANOVA). Comparison of means was performed by Duncan's multiple range tests. A value of $P < 0.05$ was considered statistically significant.

Results

Antifungal activity of TTO and its components

The effects of different concentrations of TTO and its components on the MGI of *B. cinerea* are shown in Fig. 1. TTO, as well as its constituents terpinen-4-ol, α-terpineol, and terpinolene, had high antifungal activity against *B. cinerea* in a dose-dependent manner. At $0.25 \ \mu\text{l ml}^{-1}$, the MGI of terpinen-4-ol was higher than that for TTO ($P < 0.05$), and α-terpineol showed similar effects to TTO. When the concentration is $0.5 \ \mu\text{l ml}^{-1}$, terpinen-4-ol exhibited higher MGI than α-terpineol, and both of them inhibited mycelial growth more significantly than TTO. Terpinen-4-ol and α-terpineol completely
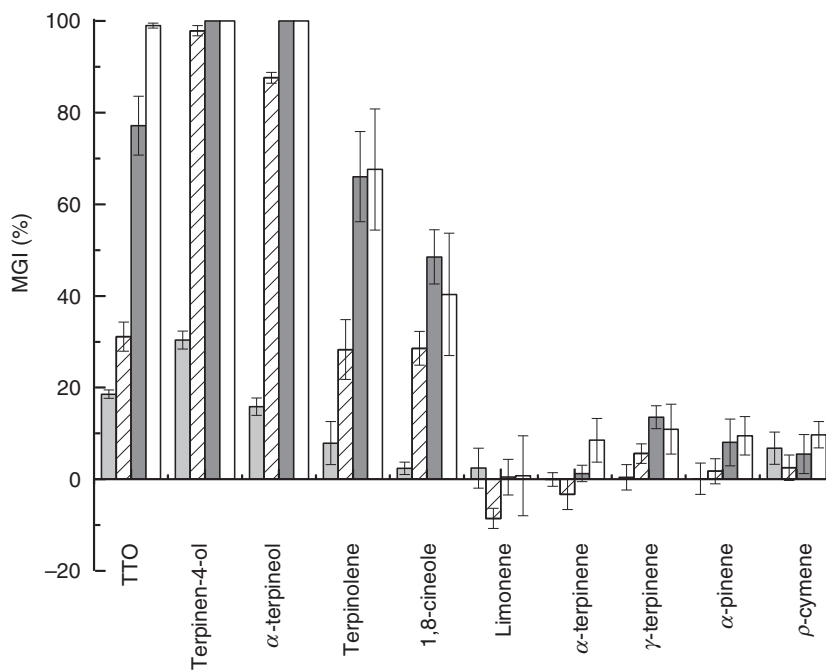

Figure 1 Antifungal effects of tea tree oil and its components on mycelium growth in *Botrytis cinerea*. ▨ $0.25 \ \mu\text{l ml}^{-1}$, ▧ $0.5 \ \mu\text{l ml}^{-1}$, ■ $1.0 \ \mu\text{l ml}^{-1}$, ☐ $1.5 \ \mu\text{l ml}^{-1}$.

inhibited mycelial growth of *B. cinerea* at concentrations of 1·0 and 1·5 μl ml^{-1}, whereas the MGI of 1,8-cineole reached only 48·52%. Meanwhile, MGI of ρ-cymene, limonene, γ-terpinene, α-pinene and α-terpinene did not exceed 13·55% at any concentration tested.

Effects of terpinen-4-ol in combination with other components

The antifungal activity of terpinen-4-ol was significantly enhanced when combined with other components at a ratio of 1 : 1 (v/v), demonstrating that each single component acts synergistically with terpinen-4-ol (Table 1). The combination of terpinen-4-ol and α-terpineol exhibited the highest inhibitory activity, followed by a combination of terpinen-4-ol and 1,8-cineole. Interestingly, 1,8-cineole, a less active component in TTO (Fig. 1), increased the inhibitory effect of terpinen-4-ol at 0·25 μl ml^{-1} from 37·67 to 87·10%. In tests with *B. cinerea*, the MIC of TTO, terpinen-4-ol, 1,8-cineole and a combination of terpinen-4-ol and 1,8-cineole (1 : 1) were 2·5, 0·8, 8·0 and 0·7 μl ml^{-1} respectively.

Effects of different treatments on hyphal morphology and ultrastructure

The hyphal morphology of *B. cinerea* was observed by SEM (Fig. 2). The control sample displayed normal morphology with uniform, robust and linearly shaped hyphae of constant diameter and with smooth surfaces (Fig. 2a-1). 1,8-cineole caused inconspicuous changes in hyphae morphology (Fig. 2c-1), while terpinen-4-ol, a combination of terpinen-4-ol and 1,8-cineole, and TTO, all severely altered the morphology of *B. cinerea* (Fig. 2b-1, d-1, e-1). Treating *B. cinerea* hyphae with terpinen-4-ol alone resulted in loss of linearity and the formation of warty surfaces on a majority of hyphae (Fig. 2b-1). Treatment with terpinen-4-ol plus 1,8-cineole (Fig. 2d-1) resulted in higher numbers of shrivelled, collapsed and flattened empty hyphae than in the terpinen-4-ol only group, and at levels similar to the those in the TTO treatment group (Fig. 2e-1).

TEM examination of sections from control *B. cinerea* revealed a typical fungal ultrastructure with normal cell wall thickness, intact plasma membranes, uniform cellular cytoplasm and regularly shaped mitochondria in the mycelia (Fig. 2a-2). After treatment with 1,8-cineole, most organelles were indistinct and many unidentifiable vesicular structures appeared, but no leakage of cyto-

Table 1 Antifungal effects of terpinen-4-ol combined with other components

Constituents	Mycelial growth inhibition (%)
Terpinen-4-ol	37·67 ± 4·68[f]
Terpinen-4-ol + α-terpineol	99·46 ± 0·76[a]
Terpinen-4-ol + 1,8-cineole	87·10 ± 4·76[b]
Terpinen-4-ol + Terpinolene	61·27 ± 4·84[c]
Terpinen-4-ol + α-pinene	55·47 ± 5·39[cd]
Terpinen-4-ol + ρ-cymene	49·02 ± 13·91[de]
Terpinen-4-ol + γ-terpinene	40·33 ± 4·91[ef]
Terpinen-4-ol + α-terpinene	43·23 ± 6·19[ef]
Terpinen-4-ol + Limonene	42·34 ± 4·18[ef]

The different letters between different treatments indicate significant differences ($P < 0.05$).
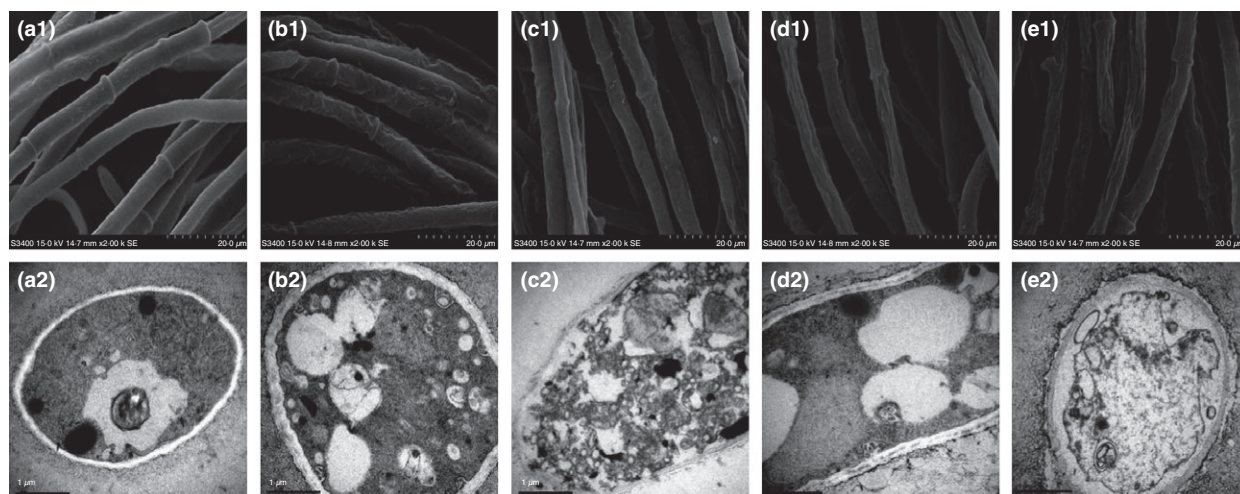

Figure 2 First row: scanning electron microscope images ($\times$ 2000) of morphology in hyphae. a-1, healthy hyphae control. b-1, c-1, d-1 and e-1, hyphae exposed to MICs of terpinen-4-ol, 1,8-cineole, the two components in combination and tea tree oil (TTO) respectively. Second row: transmission electron microscope images ($\times$ 30000) of hyphal ultrastructure. a-2, healthy hyphae control. b-2, c-2, d-2 and e-2, hyphae exposed to MICs of terpinen-4-ol, 1,8-cineole, the two components in combination and TTO respectively.

plasm was observed (Fig. 2c-2). In both terpinen-4-ol and the combined groups, TEM observation revealed thickened cell walls, numerous vacuoles and the extrusion of abundant material (Fig. 2b-2, d-2). Damage in the combined groups was more serious than terpinen-4-ol alone, with larger vacuoles and indistinct intracellular organelles. In the TTO-treated groups, plasmolysis occurred, plasmalemma ruptured and detached from the cell wall, and intracellular constituents were more seriously damaged (Fig. 2e-2) than those in the combined group (Fig. 2d-2).

Effects of different treatments on plasma membrane integrity

The results of staining *B. cinerea* hyphae with PI are presented in Fig. 3. PI penetrates hyphae treated with terpinen-4-ol (Fig. 3b-2), indicating that the integrity of the cell membrane has been compromised, while uptake after 1,8-cineole treatment is similar to that for untreated hyphae (Fig. 3a-2, c-2). Staining intensity in the combined group is markedly higher than in the terpinen-4-ol or 1,8-cineole only groups (Fig. 3d-2), and is like that in the TTO treatment group where PI penetrated almost all hyphae (Fig. 3e-2).

Exposure of *B. cinerea* to the MIC of TTO and its two characteristic components for 2 h resulted in changed levels of extracellular conductivity, another proxy for cellular integrity (Fig. 4). When the oils were initially added (0 h), extracellular conductivity in each group varied widely due to the effect of oil itself. Compared with the 0 h, larger changes in extracellular conductivity indicate more damage to the membrane after 2 h incubation with different components. Terpinen-4-ol alone and the combination of terpinen-4-ol plus 1,8-cineole increased the extracellular conductivity of treated *B. cinerea* suspensions similarly. In contrast, 1,8-cineole alone affected conductivity only slightly. The change in conductivity of suspensions treated with TTO was significantly higher ($P < 0.05$) than that in all other groups.

Ergosterol content

Figure 5 shows the effects of different treatments on the ergosterol content of the plasma membrane in *B. cinerea*. TTO produced the highest reduction in total ergosterol content (54·5%) compared with the control. Treatment with terpinen-4-ol alone reduced ergosterol content by 21·3% and treatment with 1,8-cineole alone caused no apparent decrease. When these components were combined, ergosterol content was reduced by 38·5%.

Discussion

Essential oils comprise a large number of components that are thought to impact a variety of targets in the microbial cell (Carson *et al.* 2006). Terpinen-4-ol clearly has the highest antifungal effect among all components tested (Fig. 1). Other components were considerably less active than TTO ($P < 0.05$). Our result is consistent with a previous report which showed that terpinen-4-ol is the principal active component responsible for TTO's antimicrobial efficacy (Cox *et al.* 2001). Numerous studies have demonstrated that whole essential oils have greater antifungal activity due to the synergistic effects between the constituent components, thus offering more promise in
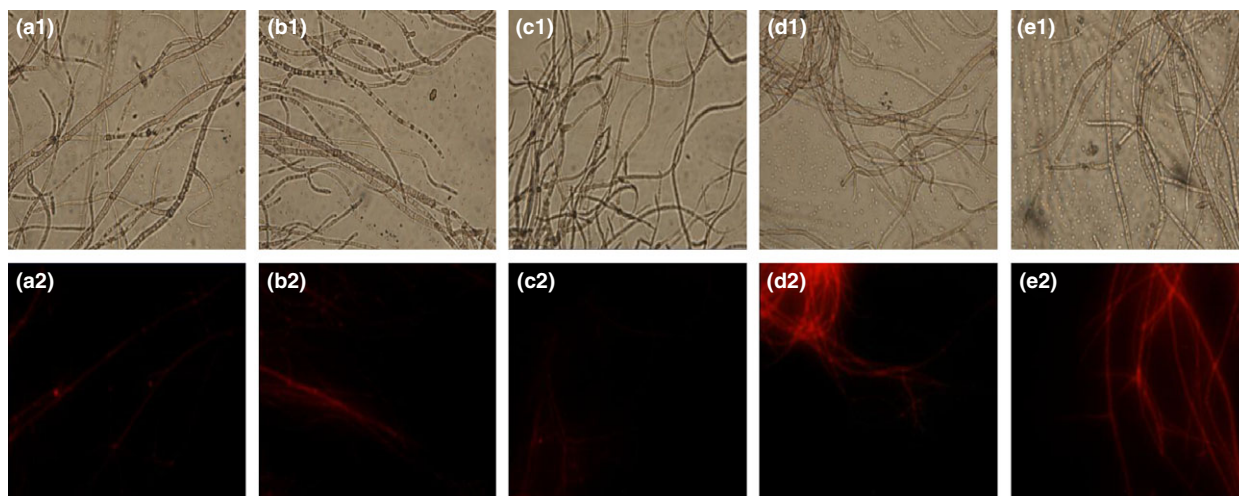

Figure 3 Images obtained by fluorescence microscopy (× 400). First row: bright field. Second row: propidium iodide (PI). (a) untreated; (b–e) treated with MICs of terpinen-4-ol, 1,8-cineole, the two components in combination and tea tree oil respectively.
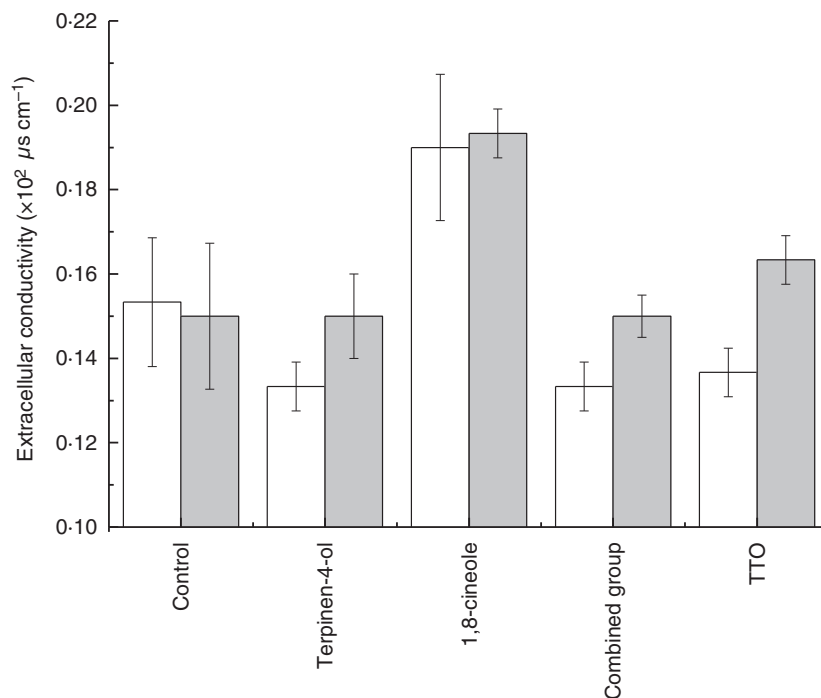
Figure 4 Effects of tea tree oil and its two characteristic components on extracellular conductivity in suspensions of *Botrytis cinerea*. ☐ 0 h; ▨ 2 h.
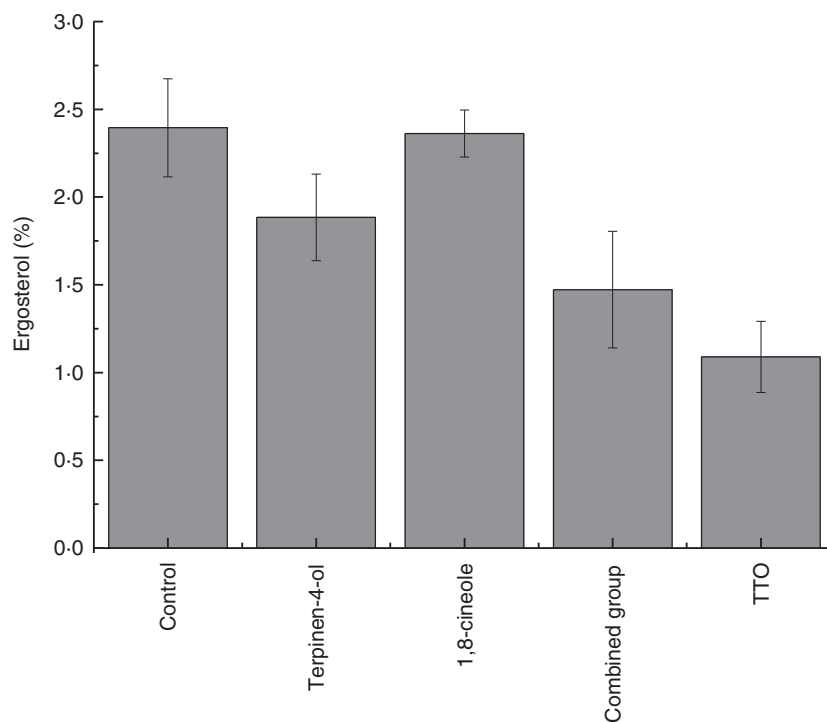

Figure 5 Effects of tea tree oil and its two characteristic components on ergosterol content in *Botrytis cinerea*.

commercial applications than single compounds (Hummelbrunner and Isman 2001; Bouchra *et al.* 2003; Deweer *et al.* 2012). The combination of eugenol and thymol, compared to either compound alone, induces a statistically significant increase in the number of damaged cells by interfering with the morphology of the envelope of *C. albicans* (Braga *et al.* 2007). A combination of γ-terpinene or ρ-cymene with an aqueous solution (0·1% w/v) of terpinen-4-ol is more active against *Escherichia coli* and *C. albicans* than either component individually,

although antagonistic effects are observed in *Pseudomonas aeruginosa* and *Staph. aureus*, reflecting that the combined effects of TTO constituents depend upon their modes of action and the susceptibility of the target micro-organism (Cox *et al.* 2001). In our study, 1,8-cineole, a less active component in TTO significantly enhanced antifungal activity of terpinen-4-ol (Table 1). To date there have been few studies of the interactions between the monoterpene components found in TTO. The MIC values also demonstrate that the antifungal activity of these two components together exceeds the activity of each alone as well as exceeding the antifungal activity of TTO. Since terpinen-4-ol and 1,8-cineole are two characteristic components in TTO and clearly act synergistically, we examined their ability to damage membranes or intracellular constituents more closely.

The suppression of fungal growth after treatment with many essential oils is associated with the degeneration of fungal hyphae and alterations in the endomembrane system (Romagnoli *et al.* 2005; Soylu *et al.* 2006). TTO can induce membrane or ultrastructure damage that can be visualized by electron microscopy in both bacterial and fungal cells (Carson *et al.* 2002; Shao *et al.* 2013b). Shao *et al.* (2013b) reported that TTO causes marked mycelial alterations, ruptured plasmalemma and the loss of cytoplasm in *B. cinerea*. Noteworthy, terpinen-4-ol combined with 1,8-cineole has a significant synergistic effect on hyphal morphology and ultrastructure compared with treatment using either component alone (Fig. 2d). Previous studies show that terpinen-4-ol interacts with plasma membranes and subsequently reorganizes membrane lipids in human melanoma cells (Calcabrini *et al.* 2004). It also causes formation of mesosomes and the loss of cytoplasmic contents in *Staph. aureus* (Carson *et al.* 2002). The deleterious effect of terpinen-4-ol on plasma membranes was confirmed in our SEM and TEM experiments (Fig. 2b). Few investigations, however, have been conducted on the effects of 1,8-cineole on cell ultrastructure. Our TEM observation demonstrates that organelles are mostly destructed in 1,8-cineole group (Fig. 2c-2). We speculate that 1,8-cineole and terpinen-4-ol act on different targets, and when combined have synergistic effects on both hyphal morphology and ultrastructure.

PI is a fluorescent probe often used to study membrane integrity. It is excluded from intact cells but can enter through membrane lesions and bind to nucleic acids, resulting in red fluorescence (Pinto *et al.* 2013). The results of TTO groups are similar to those obtained in yeast cells treated with the MIC of essential oil from *Ocimum sanctum* (Khan *et al.* 2010), which resulted in extensive lesions in the plasma membrane (Fig. 3). Moreover, our data show that plasma membrane damage in *B. cinerea* is markedly more severe after treatment using the paired components.

The antifungal modes of action of TTO are not fully understood, but mechanisms have been proposed based on the damage TTO causes to membranes (Cox *et al.* 1998; Carson *et al.* 2006). The plasma membrane plays a vital role in maintaining a homeostatic environment for the cell, exchanging materials and transferring energy and information (Cox *et al.* 2000; Shao *et al.* 2013b). Our extracellular conductivity experiments demonstrate that while terpinen-4-ol disrupts membranes and 1,8-cineole does not, the combination of these two components act synergistically to cause membrane damage (Fig. 4). These data reflects action modes of TTO and its two characteristic components on the membrane, which is consistent with our SEM and FSM results. In summary, terpinen-4-ol and 1,8-cineole act on the cell membranes and organelles of *B. cinerea* respectively. 1,8-cineole may penetrate the cell membrane and damage cellular organelles without causing lesions on the membrane.

Ergosterol is the major sterol component of the fungal cell membrane, helping to maintain cell function and integrity (Pinto *et al.* 2013). As mentioned earlier, natural and synthetic drugs can cause a considerable reduction in ergosterol quantity (Arthington-Skaggs *et al.* 2000). Tian *et al.* (2012a,b) suggested that the plasma membrane is the main target of dill essential oils against *Aspergillus flavus*, and that the oil causes a dose-dependent reduction in ergosterol quantity. The results shown in Fig. 5 support a model in which cellular membranes are the primary targets for TTO, terpinen-4-ol and the combination of terpinen-4-ol and 1,8-cineole. Treatment with 1,8-cineole alone does not disrupt the membrane or decrease ergosterol content.

In conclusion, the TTO component terpinen-4-ol is the most effective growth inhibitor of *B. cinerea in vitro*. It acts synergistically with 1,8-cineole, and the antifungal activity of the combination is second only to that of terpinen-4-ol combined with α-terpineol. 1,8-cineole, a limited constituent whose content is no higher than 15% in TTO, can penetrate the cell and damage cellular organelles without affecting membrane permeability. In contrast, terpinen-4-ol, the major component in TTO, destroys membrane integrity and increases permeability, resulting in ion leakage and membrane dysfunction. The synergistic effect of the combined treatment is probably due to these differences and has not been previously described. Overall, our results support the hypothesis that the components of TTO, including some not examined thus far, contribute to the antimicrobial activity of the oil by diverse modes of action beyond those involving the cytoplasmic membrane.

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Conflict of Interest

None to declare.

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