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Allium sativum produces terpenes with fungistatic properties in response to infection with *Sclerotium cepivorum*

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ABSTRACT

This study investigated terpene biosynthesis in different tissues (root, protobulb, leaf sheath and blade) of *in vitro*-grown garlic plants either infected or not (control) with *Sclerotium cepivorum*, the causative agent of Allium White Rot disease. The terpenes identified by gas chromatography–electron impact mass spectrometry (GC-EIMS) in infected plants were nerolidol, phytol, squalene, α-pinene, terpinolene, limonene, 1,8-cineole and γ-terpinene, whose levels significantly increased when exposed to the fungus. Consistent with this, an increase in terpene synthase (TPS) activity was measured in infected plants. Among the terpenes identified, nerolidol, α-pinene and terpinolene were the most abundant with antifungal activity against *S. cepivorum* being assessed *in vitro* by mycelium growth inhibition. Nerolidol and terpinolene significantly reduced sclerotia production, while α-pinene stimulated it in a concentration-dependent manner. Parallel to fungal growth inhibition, electron microscopy observations established morphological alterations in the hyphae exposed to terpinolene and nerolidol. Differences in hyphal EtBr uptake suggested that one of the antifungal mechanisms of nerolidol and terpinolene might be disruption of fungal membrane integrity.

1. Introduction

Garlic (*Allium sativum* L.) is an economically important horticultural crop grown in Argentina. One of the main fungal diseases affecting garlic crops worldwide is Allium White Rot (AWR) caused by the soil-borne fungus *Sclerotium cepivorum* Berk. This pathogen does not show a recognizable teleomorphic state, so it reproduces through copious production of small sclerotia that serve as both propagules and inoculum (Couch and Kohn, 2000). In the absence of a host, *S. cepivorum* persists in soil as sclerotia that can survive for more than 20 years, and which can germinate in response to sulfides of *Allium* spp. producing an infective mycelium (Coley-Smith et al., 1990). AWR is prevalent in many *Allium* cultivated regions worldwide and can reduce drastically yields of onion and garlic crops (Davis et al., 2007). While until now resistance to *S. cepivorum* has not been reported in the genus *Allium*, many approaches were used to control AWR including application of fungicides (Miñambres et al., 2010), soil fumigants and soil solarization (Entwistle, 1990), biological control agents (Kay and Stewart, 1994), sclerotia germination stimulants (Coley-Smith et al., 1987), and composted onion waste (Coventry et al., 2002). However, no single method efficiently controls AWR. Recently, transgenic garlic plants able to delay *S. cepivorum* infection have been genetically engineered by introducing tobacco chitinase and glucanase genes (Lagunes-Fortiz et al., 2013).

Plants have developed a diverse spectrum of defensive strategies against biotic stresses, including phytoalexins, low molecular weight defensive compounds produced by the plant's secondary metabolism (Croteau et al., 2000). Biosynthesis of phenylpropanoids and terpenes (phytoalexins) are mechanisms utilized by plants to fight pests and diseases (Degenhardt et al., 2009a; Jansen et al., 2011; Martin et al., 2003). Furthermore, production of terpenes in many plant species has been associated with fungal (Escoriaza et al., 2013; Neri et al., 2006) and herbivore attack (Kappers et al., 2005; Loughrin et al., 1994; Niu et al., 2012; Raffa and Smalley, 1995; Turlings et al., 1990). However, there is no information in garlic plants about biosynthesis of terpenes as a defense mechanism against fungal pathogens.

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In higher plants, the isoprene units of isopentenyl diphosphate and its isomer dimethylallyl diphosphate are the precursors of terpenes. These compounds can be synthesized either via the mevalonic acid (MVA) pathway in the cytosol/endoplasmic reticulum or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids (Croteau et al., 2000). A large family of enzymes, known as terpene synthases (TPS), catalyze the conversion of the prenyl diphosphate intermediates into various structural types of terpenes (Chappell, 1995; Degenhardt et al., 2009b; Keeling and Bohlmann, 2006). The MVA pathway provides the precursors for sesqui- and triterpenes, while the MEP pathway is responsible for the precursors for mono-, di-, and tetraterpenes (Chappell, 1995; Gershenzon and Kreis, 1999). Many of these compounds likely operate as defense agents, since they often exhibit toxic effects against plant pathogens (Geisler et al., 2013; Gershenzon and Kreis, 1999). In this regard, several studies have shown that levels of many monoterpenes with known antifungal activity increase in response to plant pathogens (Niu et al., 2012; Raffa and Smalley, 1995). However, the antifungal mechanism of monoterpenes at the molecular level is not fully understood. It has been suggested that inhibitory effects on *S. cepivorum* growth are due to an alteration in lipid composition (decrease in phospholipid/sterol ratio) associated with membrane lipids peroxidation (Lucini et al., 2006; Sikkema et al., 1995). In addition to monoterpene synthesis as the plant's defense response to pathogen attack, an increase in TPS activity with the subsequent accumulation of sesquiterpenes has been observed in cells suspension cultures (Escoriaza et al., 2013; Ma, 2008; Vögelli and Chappell, 1988).

The use of natural products for control of fungal diseases in plants is considered as an alternative to traditional use of synthetic fungicides (Hollis and Jones, 2009). Research on plant-derived fungicides is now being intensified, since plant secondary metabolites are often biodegradable to non-toxic products and possess no residual phytotoxic properties. These characteristics also make them attractive for their potential use as commercial fungicides and/or as adjuvants. The general antifungal activity of essential oils (mainly consisting of mono- and sesquiterpenes) and the antifungal effects of their pure components have been explored (Camele et al., 2012; Monsálvez et al., 2010; Sharma and Tripathi, 2006). Several authors have reported high inhibitory activity of cyclic terpenes (such as eugenol, carvacrol, thymol and citral) on growth and mycotoxin biosynthesis both in soil-borne plant disease-caused fungi (Dambolena et al., 2008; Mueller-Riebau et al., 1995), and fungi affecting post-harvest fruit (Camele et al., 2012).

Although there are studies *in vitro* showing antifungal effects of monoterpenes on growth and sclerotia development of *S. cepivorum* (Lucini et al., 2006), no information on terpene biosynthesis in response to *S. cepivorum* attack has been reported in garlic plants. Thus, the main objective of the present study was to analyze changes in terpene levels and TPS activities in different tissues of *in vitro* cultured garlic plants infected (or not) with *S. cepivorum*. Furthermore, to evaluate the antifungal activity of the most abundant terpenes biosynthesized by the infected garlic plants, *in vitro* inhibition of mycelial growth, sclerotial production, hyphal morphological alterations, and fungal membrane integrity were tested.

2. Results

2.1. S. cepivorum induces synthesis of monoterpenes and sesquiterpenes in garlic plants

Eight terpenes were identified by GC-EIMS in the different tissues (root, protobulb, leaf sheath and blade) of *in vitro* garlic plants infected with *S. cepivorum* (see Table 1, Fig. 1, and for GC-EIMS total ion chromatogram see Supplementary Fig. S1). These

Table 1
Terpene composition assessed by GC-EIMS (ng mg^{-1} fr. wt.) in different tissues of *in vitro* garlic plants un-inoculated (control) and inoculated with *Sclerotium cepivorum*. The data reported are means ± SE of $n = 5$. $P_{(I)}$: inoculated effect; $P_{(T)}$: tissue effect; $P_{(I \times T)}$: inoculated × tissue interaction effect. Different letters represent statistically significant differences between means of the different plant tissues (root, protobulb, sheath, blade) for each treatment (inoculated/control) and terpene ($P \leqslant 0.05$, LSD test). Nd, not detected.

Compound	Inoculated				Control				$P_{(I)}$	$P_{(T)}$	$P_{(I \times T)}$
	Root	Protobulb	Sheath	Blade	Root	Protobulb	Sheath	Blade			
α-Pinene (**4**)	185.1 ± 40.5a	168.4 ± 59.7ab	63.5 ± 12.9b	18.5 ± 2.2c	1.7 ± 0.4a	0.9 ± 0.4ab	0.4 ± 0.0b*	0.6 ± 0.1ab	0.0001	0.0004	0.0371
Limonene (**6**)	0.7 ± 0.4a	1.2 ± 0.7a	0.2 ± 0.0a*	Nd	Nd	Nd	Nd	Nd			
1,8-Cineole (**7**)	0.3 ± 0.1a	0.2 ± 0.1a	0.2 ± 0.0a*	Nd	Nd	Nd	Nd	Nd			
γ-Terpinene (**8**)	1.0 ± 0.5a	1.7 ± 0.6a	1.5 ± 0.5a	Nd	Nd	Nd	Nd	Nd			
Terpinolene (**5**)	29.4 ± 11.0a	29.8 ± 8.4a	20.4 ± 2.9a	3.0 ± 0.6b	0.9 ± 0.4a	0.4 ± 0.1a	0.4 ± 0.1a	0.6 ± 0.3a	0.0001	0.0513	0.1001
Nerolidol (**1**)	449.9 ± 118.6a	348.6 ± 133.3a	211.7 ± 47.3a	65.5 ± 10.6b	4.3 ± 0.7a	0.8 ± 0.3b	0.5 ± 0.4b	0.8 ± 0.3b	0.0001	0.0001	0.0347
Phytol (**2**)	7.3 ± 1.7c	34.3 ± 10.7b	47.7 ± 7.2b	123.8 ± 20.9a	20.3 ± 4.7c	18.0 ± 3.4c	71.8 ± 20.8b	184.1 ± 32.4a	0.2061	0.0001	0.0722
Squalene (**3**)	276.5 ± 52.6a	75.1 ± 14.5b	72.6 ± 3.7b	33.8 ± 1.5b	6.5 ± 3.3a	7.0 ± 0.2a	10.4 ± 3.8a	11.0 ± 3.1a	0.0001	0.7143	0.1719

* The value 0.0 means <0.05.

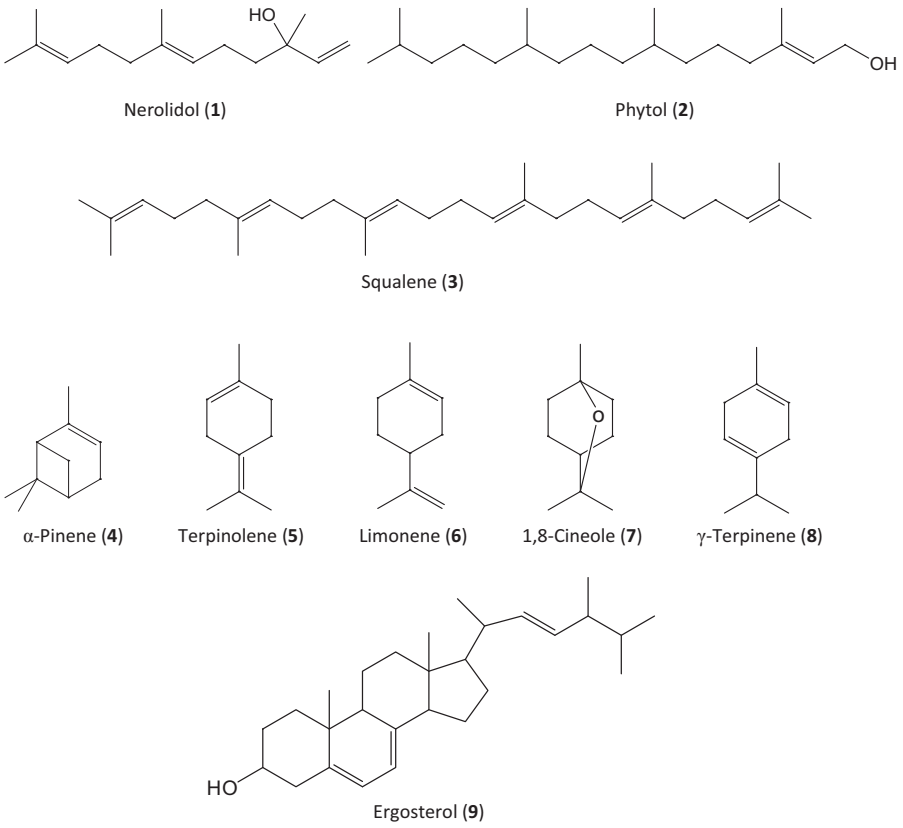

Fig. 1. Chemical structures of monoterpenes, a sesquiterpene, a diterpene, a triterpene and ergosterol identified by GC-EIMS in *in vitro* garlic plants un-inoculated (control) and inoculated with *Sclerotium cepivorum*.

compounds included the sesquiterpene nerolidol (**1**), the diterpene phytol (**2**), the triterpene squalene (**3**), and the monoterpenes, α-pinene (**4**), terpinolene (**5**), limonene (**6**), 1,8-cineole (**7**) and γ-terpinene (**8**) (see Fig. 1, Table 1, and for full mass spectra see Supplementary Figs. S2 and S3). As shown in Table 1, the concentrations of nerolidol (**1**) and α-pinene (**4**) were significantly higher in tissues of infected garlic plants, as compared with the control (Table 1). The major levels of these compounds were found in root and protobulb, as compared to values measured in the leaf blade (Table 1). In the case of terpinolene (**5**), *S. cepivorum* induced a significant increase in its level with respect to un-inoculated plants (Table 1). Terpinolene (**5**) content was similar in all infected tissues, except for the leaf blade where a lower concentration was observed (Table 1). Also limonene (**6**), 1,8-cineole (**7**) and γ-terpinene (**8**) were detected in infected plants, but in smaller amounts as compared to other terpenes (Table 1). The concentration of phytol (**2**) was also significantly affected by the type of tissue, but not by the effect of fungus infestation ($P_{(I)} \leqslant 0.2061$, Table 1). By contrast, the fungus induced a significant increase in the levels of squalene (**3**) in all the tissues analyzed, irrespective of tissue type ($P_{(T)} \leqslant 0.7143$, Table 1).

2.2. The increase of terpene levels induced by S. cepivorum is systemic

To verify whether *S. cepivorum* was responsible for the increase in terpene levels in the interaction with the plant (Table 1), the fungus was re-isolated from inoculated tissues. Only fungal development was obtained in root and protobulb (*ca.* 1.5 cm stem-and-leaves section above root disc, data not shown).

Ergosterol (**9**) is the major sterol component in cell membranes of filamentous fungi, while it is nearly absent in higher plants. Thus, it has been used to estimate fungal biomass in various environments (Mille-Lindblom et al., 2004; Pasanen et al., 1999). In order to calculate the fungal total mass of infected garlic tissues, ergosterol (**9**) content was measured by GC-EIMS either in plant or fungal mycelium (for full mass spectra see Supplementary Fig. S4). The ergosterol (**9**) content per milligram of fungal fresh weight (fr. wt.) was 556 ng, while in root and protobulb it was 0.01 and 0.02 ng mg^{-1} plant fr. wt., respectively. No ergosterol (**9**) was detected in leaf tissue. These results are in agreement with Arimura et al. (2004) suggesting that the fungus in root and protobulb tissue was able to induce increased terpene levels as a local and systemic response.

2.3. S. cepivorum increases TPS activity

The large diversity of carbon skeleta characteristic of terpenes is formed by the enzymes TPS, which convert prenyl diphosphate precursors into a wide variety of terpenoid products (Croteau et al., 2000). To reinforce the consideration that *S. cepivorum* induces terpene biosynthesis in the plant-fungus interaction, TPS activity with farnesyl diphosphate (FPP) (**10**) as substrate was measured (Gil et al., 2012). The potential destinies of FPP (**10**) are shown in Fig. 2. TPS activity, assessed in terms of radioactivity in the hexane-soluble fraction from tritiated FPP (**10**) increased 52-fold in root, 43-fold in protobulb and 8.3-fold in the leaf blade as compared to their respective controls, whereas among the controls they did not differ (Fig. 3).

2.4. Nerolidol (*1*) and terpinolene (*5*) inhibit fungal growth and sclerotial production

The antifungal effect of nerolidol (**1**), terpinolene (**5**) and α-pinene (**4**) on the growth of *S. cepivorum* is shown in Fig. 4a, with

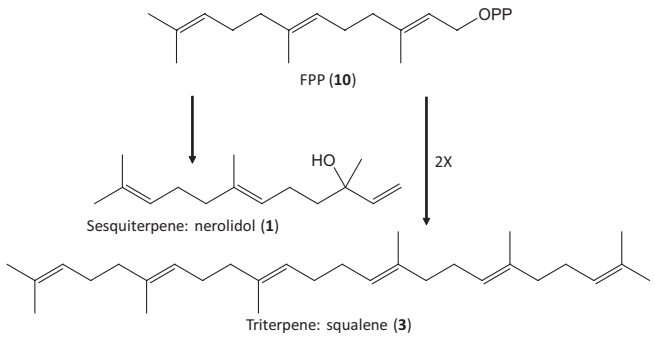

Fig. 2. Simplified scheme of the two potential fates of farnesyl diphosphate (FPP) (**10**).

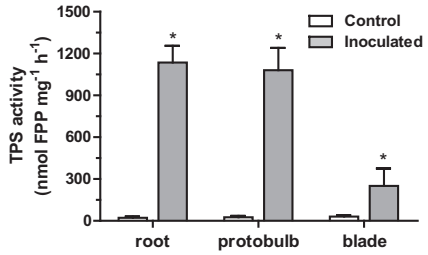

Fig. 3. Terpene synthase activity (TPS) expressed as nmol [^{3}H]-FPP (**10**) transformed mg of protein^{-1} h^{-1} (see Section 5.4 for details) in different tissues of *in vitro* garlic plants un-inoculated (control) and inoculated with *S. cepivorum*. Values are means ± SE, $n = 3$. Asterisks indicate significant differences comparing control and inoculated for the same tissue.

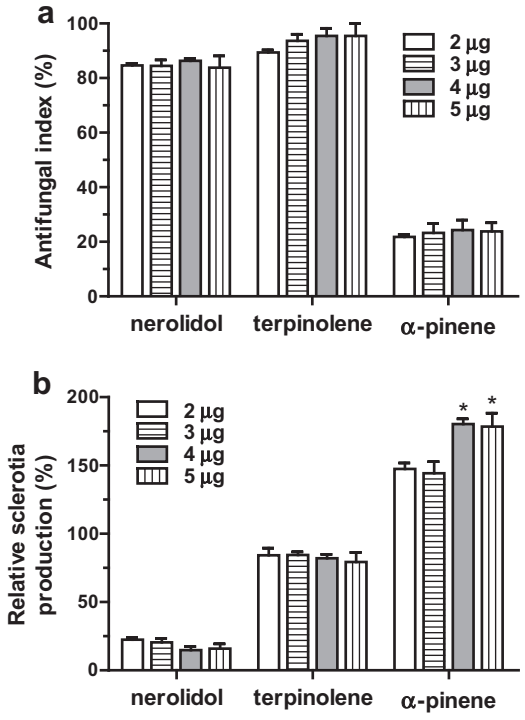

Fig. 4. Effect of nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**) on mycelial growth and sclerotial production of *S. cepivorum*. Plates treated with standards of nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**) in a range between 2.0 and 5.0 µg/disc, relative to the root terpinolene (**5**) content (18.0–29.0 µg root^{-1} fr. wt., see Section 5.5 for more details) were inoculated with the fungus and incubated at 20 ± 1 °C in darkness. (a) The antifungal index was calculated 5 dpi. Values are means ± SE, $n = 6$. (b) The relative production of sclerotia was calculated 45 dpi. Values are means ± SE, $n = 8$. Asterisks indicate significant differences among concentrations tested for each terpene.

the antifungal index calculated being in a range between 2.0 and 5.0 µg/disc according to terpinolene (**5**) content (18.0–29.0 µg root^{-1} fr. wt.) detected in the whole root of infected plants (see Section 5.5 for more details). Terpinolene (**5**) was the most effective compound inhibiting mycelial growth (around 93%), followed by nerolidol (**1**) (~85%), and α-pinene (**4**) (around 23%, Fig. 4a, and Supplementary Fig. S5a). The inhibitory effect on fungal growth was also permanent under treatment with nerolidol (**1**) since it was observed even 90 days post-inoculation (dpi; Supplementary Fig. S5b), while the effect of terpinolene (**5**) (Fig. S5b) and α-pinene (**4**) (data not shown) was transient, losing their inhibitory effects *ca.* 20 and 8 dpi, respectively. Nevertheless, in the cases, the fungus was able to grow when it was re-cultivated on fresh PDA (potato dextrose agar) plates showing that these terpenes had only fungistatic effect, at least at the concentrations tested (data not shown).

Treatment with nerolidol (**1**) and terpinolene (**5**) significantly reduced sclerotial production, where their effectiveness were *circa* 84%, and 18%, respectively, compared with the terpene-free control (Fig. 4b). An opposite effect was found for α-pinene (**4**), as it stimulated sclerotial production by 47% at a lower concentration, and by 80% at a higher one (Fig. 4b).

In the case of the less abundant monoterpenes 1,8-cineole (**7**) (0.3 µg root^{-1} fr. wt.), limonene (**6**) (1.2 µg protobulb^{-1} fr. wt.), and γ-terpinene (**8**) (1.7 µg protobulb^{-1} fr. wt. Table 1), the concentrations detected in root and protobulb of infected garlic plants evaluated *in vitro* were insufficient to inhibit *S. cepivorum* mycelial growth (data not shown).

*2.5. Nerolidol (**1**) and terpinolene (**5**) produce alterations in hyphal morphology and membrane permeability*

Observations with scanning electron microscopy (SEM) showed that untreated control *S. cepivorum* hyphae had a typical tubular shape with long branching (Fig. 5a and b). Seven days after being exposed to nerolidol (**1**) and terpinolene (**5**), the hyphae had shorter branching (Fig. 5c and e), as well as several morphological alterations such as hyphal shrinkage and partial distortion (Fig. 5d and f). In some cases, distorted hyphae appeared to be collapsed and squashed (Fig. 5d and f). No morphological changes in hyphae were observed in the α-pinene (**4**) treatment (data not shown). In order to test the potential antifungal mechanism of nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**), the ethidium-nucleic acid complex fluorescence generated by the influx of EtBr (ethidium bromide) into the hyphae was assessed. Fig. 6 shows the fluorescence differences of their respective EtBr uptake, as compared to the control. While nerolidol (**1**)-treated mycelia were markedly bright and stained at the periphery of the fungal colony, terpinolene (**5**)-treated mycelia were more uniformly stained, and α-pinene (**4**) as a control only showed weak fluorescence (Fig. 6).

3. Discussion

The results from this study provide evidence that *S. cepivorum* induces biosynthesis of terpenes in garlic plants grown *in vitro*. In this work, the sesquiterpene nerolidol (**1**) and the monoterpenes α-pinene (**4**), terpinolene (**5**), limonene (**6**), 1,8-cineole (**7**) and γ-terpinene (**8**) were identified. Nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**) were the most abundant in infected plants, with decreasing concentrations from root to leaf blade, whereas the structural phytol (**2**) and the precursor of sterols, squalene (**3**) were in un-infected plants. The accumulation of nerolidol (**1**) has been closely related with plant defense mechanisms against pathogens and herbivores. Moreover, several studies have shown that many plant species respond to herbivore attack by producing a mixture

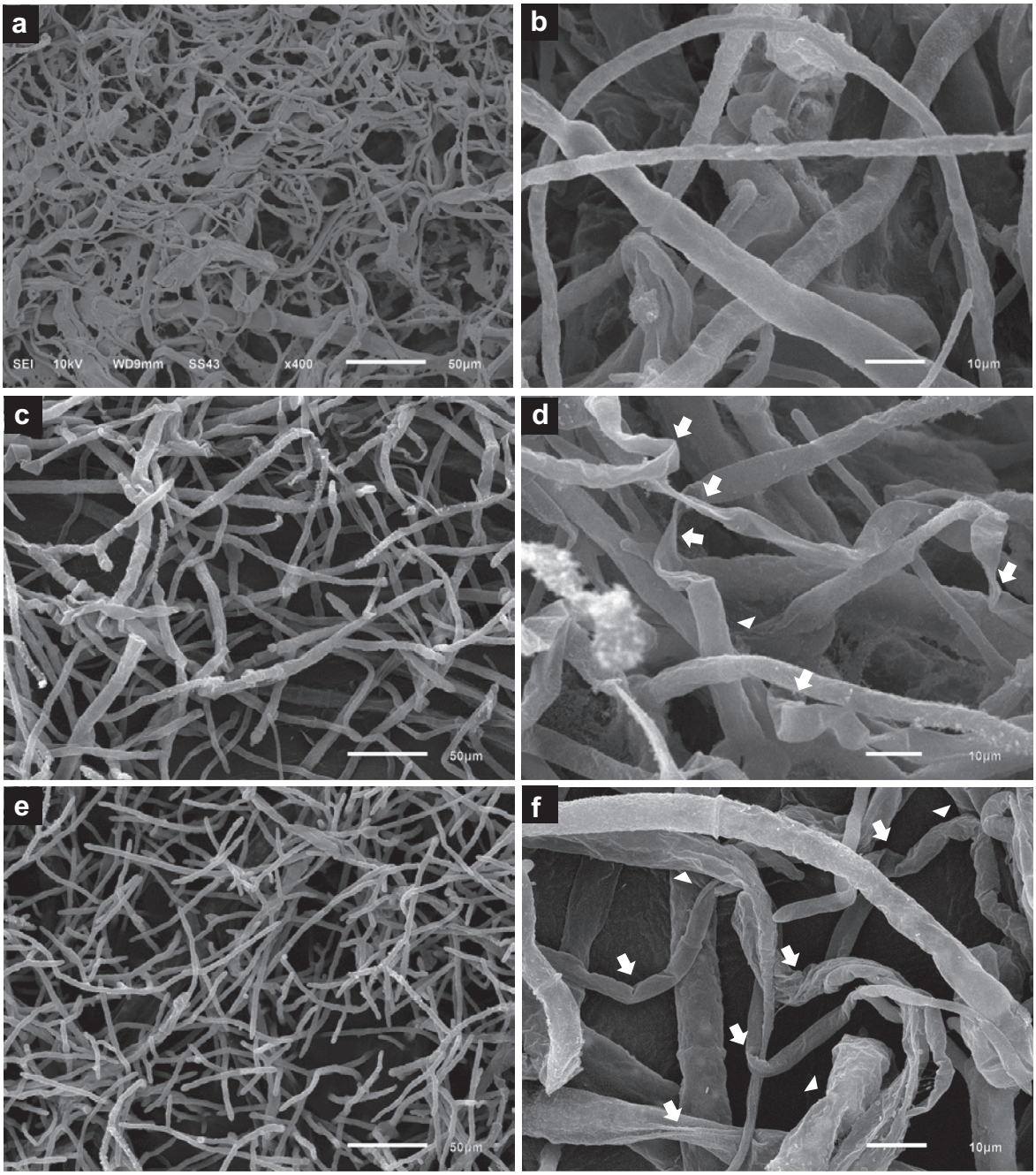

Fig. 5. Scanning electron micrographs showing the effects of nerolidol (**1**) and terpinolene (**5**) on *S. cepivorum*. The effect of each terpene was evaluated by using the disc diffusion method. (a and b) Control hyphae of 7 dpi at 20 ± 1 °C showing normal morphology. (c and d) Hyphae exposed to nerolidol (**1**) (4.0 µg/disc), and (e and f) terpinolene (**5**) (4 µg/disc) for 7 days at 20 ± 1 °C. Arrowheads and arrows indicate the shrinkage and partial distortion of hyphae, respectively. Images (a, c and e): scale bars = 50 µm; images (b, d and f): scale bar = 10 µm.

of volatiles, including 4,8-dimethyl-1,3(*E*),7-nonatriene derived from (3*S*)-(*E*)-nerolidol, that attract natural enemies of the herbivores (Bouwmeester et al., 1999; Kappers et al., 2005; Turlings et al., 1990). Also, an accumulation of nerolidol (**1**) and capsidiol has been observed in fungal elicitor-treated cell suspension cultures and plants (Chappell and Nable, 1987; Leitner et al., 2008). More recently, studies with grapevine showed an increment of nerolidol (**1**) and α-pinene (**4**) in response to infection with the fungus *Phaeoacremonium parasiticum*, a causal agent of 'hoja de malvon' disease that affects grapevines (Escoriaza et al., 2013).

In order to evaluate that fungus-induced terpene accumulation in garlic plants was due to *de novo* biosynthesis (Chappell and Nable, 1987; Chappell et al., 1987; Croteau et al., 1987; Ma, 2008), TPS activity was assessed. The infected plants showed high TPS activity, and this increment in levels was associated with increases in nerolidol (**1**) and squalene (**3**) contents, mainly in root and protobulb, suggesting they were synthesized *de novo* in response to fungal attack. These results are in agreement with Escoriaza et al. (2013), where an increase in TPS activity and nerolidol (**1**) accumulation in grapevine was correlated with an increment of *P. parasiticum*.

Monoterpenes are natural products that accumulate during plant development, and their abundance and composition vary in response to environmental conditions (Gershenzon et al., 2000;

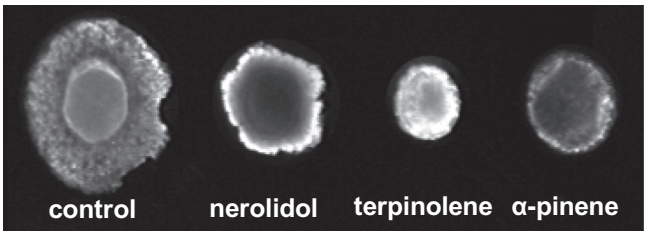

Fig. 6. Effect of nerolidol (**1**), terpinolene (**5**) and α-pinene (**4**) on EtBr uptake into hyphae of *S. cepivorum*. The effect of each terpene was evaluated by using the disc diffusion method. Plates treated with the terpenes at the same concentration (4.0 μg/disc) were inoculated with the fungus and incubated at 20 ± 1 °C in darkness for 4 days. The uptake of EtBr (15 μM) was detected using an ultraviolet transilluminator. A representative image from two independent experiments is presented.

Gil et al., 2012, 2013; Keeling and Bohlmann, 2006; Raffa and Smalley, 1995). Also, differences in terpene profiles have been observed in beneficial plant-bacteria interactions (Salomon et al., 2014). In addition to an increment in nerolidol (**1**) levels, the contents of α-pinene (**4**) and terpinolene (**5**) were significantly increased by fungal infection. It is known that terpinolene (**5**) has antifungal activity (Farooq et al., 2002), and also possesses good antioxidant capacity in lipophilic blood systems, protecting low-density lipoprotein from oxidation (Graßmann et al., 2001, 2003). Because of this, it is possible to infer that increased levels of terpinolene (**5**) protect membranes of plant cells against an oxidative burst generated by the plant in response to pathogen attack (Apel and Hirt, 2004). In the case of limonene (**6**), 1,8-cineole (**7**) and γ-terpinene (**8**), they were not detected in control garlic plants thus again suggesting *de novo* synthesis as result of fungal elicitation. Even though the antifungal properties of these monoterpenes have been reported (Dambolena et al., 2008; Espinosa-García and Langenheim, 1991; Hammer et al., 2003; Lucini et al., 2006; Sharma and Tripathi, 2006; Tao et al., 2014), the concentrations of 1,8-cineole (**7**), limonene (**6**) and γ-terpinene (**8**) measured in root and protobulb of infected plants were insufficient to inhibit *in vitro S. cepivorum* mycelial growth. This is in agreement with previous studies (Lucini et al., 2006; Sharma and Tripathi, 2006; Tao et al., 2014), which showed that higher concentrations of these terpenes might be required to inhibit mycelial growth of filamentous fungi, including *S. cepivorum*.

It was reported that *S. cepivorum* penetrates the root epidermis and that the hyphae invade cortical parenchyma thus reaching the stem base and affecting the whole plant (Abd-El-Razik et al., 1973; Metcalf and Wilson, 1999). Consistent with this, the fungus was re-isolated only in root and protobulb tissues, but not in the leaves (leaf sheath and blade). Previous reports in poplars showed that volatile compound emission in younger leaves induced by insect feeding older leaves is the result of acropetal systemic induction of sesquiterpene synthase genes (Arimura et al., 2004). The present results suggest that the plant-fungus interaction was responsible for high concentrations of terpenes localized in the root and protobulb, and for eliciting a systemic response in leaves.

When antifungal properties of nerolidol (**1**), terpinolene (**5**) and α-pinene (**4**) against *S. cepivorum* were evaluated *in vitro*, the first two ones significantly reduced mycelial growth (~85% and ~93%, respectively), and sclerotia production (~84% and ~18%, respectively), as compared to the terpene-free control. It is possible that the major effectiveness of nerolidol (**1**) in reducing sclerotia production might be a consequence of its long-term inhibitory effect on fungal growth, even when applied at levels 15 times less than the concentration detected in infected root. Although α-pinene (**4**) inhibited mycelial growth by ~23%, sclerotia production was highly stimulated in a concentration-dependent manner. Given the generally accepted association between high degree of lipid peroxidation and sclerotial biogenesis (Georgiou et al., 2000), then an α-pinene (**4**)-induced lipid peroxidation may explain the higher sclerotia production observed under this treatment, as compared with that of the control.

It was also proposed that antifungal activity of thymol and borneol on *S. cepivorum* could be correlated with alterations in membrane fluidity and functionality (Lucini et al., 2006). In the experiments herein, the inhibition of fungal growth by nerolidol (**1**) and terpinolene (**5**) was associated with changes in hyphae morphology, which appeared severely collapsed and squashed; meanwhile, with α-pinene (**4**) no modifications were observed. When terpene-induced hyphal damages were assessed by EtBr uptake, it was found that nerolidol (**1**)-treated mycelium was markedly bright and stained at the periphery of the fungal colony; it was more uniformly stained in terpinolene (**5**)-treated mycelium, while it was scarcely stained in α-pinene (**4**)-treated mycelium. The differences in EtBr uptake may be explained, on the one hand, by the hydrophobic nature of nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**), being responsible for their low diffusion through the agar media and, on the other hand, by the more volatile nature of α-pinene (**4**) and terpinolene (**5**). Regardless of the staining patterns observed in both treatments, the ability of EtBr to penetrate the cell compromising plasma membrane integrity suggests that one of the mechanisms of antifungal action of these terpenes might be disruption of the fungal membrane integrity.

4. Conclusions

This study provides the first evidence of terpene synthesis in response to garlic plants-*S. cepivorum* interaction. The terpenes identified in *in vitro* infected plants were nerolidol (**1**), squalene (**3**), α-pinene (**4**), terpinolene (**5**), limonene (**6**), 1,8-cineole (**7**) and γ-terpinene (**8**). Among them, nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**) were the most abundant with antifungal activity against *S. cepivorum* assessed in *in vitro* by mycelium growth inhibition. The higher levels of terpenes were measured in root and protobulb colonized by the fungus, and the terpenes identified in leaves far away from the fungus suggest a systemic response. The hyphal damages and enhancement of EtBr uptake observed in *S. cepivorum* treated with nerolidol (**1**) and terpinolene (**5**) suggest that the fungal membrane could be the target of these compounds. Considering the significant antifungal effect of nerolidol (**1**) and terpinolene (**5**), and the augmented sclerotia production by α-pinene (**4**), further experiments would be interesting to investigate in depth in terms of their modes of action, detoxification capacity by the fungi (Arakawa et al., 2013; Farooq et al., 2002) and their possible practical applications for the control of AWR.

5. Experimental

5.1. Plant material and in vitro growth conditions

Garlic plants cv. Sureño INTA belonging to the eco-physiological group IVa (Burba et al., 1989) from the INTA collection were grown in the experimental field of INTA La Consulta (33° 44′ S and 69° 04′ W, 940 m a.s.l.). Mature bulbs were harvested, and stored at 20 °C. When the cloves showed 70–80% visual index of dormancy (VID; Burba et al., 1989), they were selected by size and weight uniformity. Then, they were peeled and surface sterilized with EtOH:H_2O (70:30, v/v) for 4 min, with 5% commercial bleach (55 g l^{-1} active chlorine) for 15 min, this being followed by 20% bleach for 15 min with constant swirling and then finally rinsed several times with sterile distilled H_2O. The storage and sprouting

leaves were removed from the cloves and the explants were grown in glass tubes (one per tube, 2.4 cm diameter × 20 cm height) with 20 ml of MS medium (Murashige and Skoog, 1962) supplemented with 0.4 mg l^{-1} thiamine HCl, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 2 mg l^{-1} glycine, mg l^{-1} adenine sulfate, 30 mg l^{-1} sucrose, and 6 g l^{-1} agar (Conci et al., 1986), and capped with two layers of low-density polyethylene. Cultures were maintained in a growth chamber at 20 ± 1 °C under 12 h photoperiod provided by cool-white fluorescents tubes at a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1} for 16 days before inoculation.

5.2. Inoculation and fungal re-isolation from inoculated garlic plants

A pure culture of *S. cepivorum* Berk isolate Sc23 from the INTA La Consulta collection was used. The fungal stock cultures were made by placing a garlic clove of cv. Sureño INTA, disinfected as described above, in a plate containing potato dextrose agar (PDA, Difco) and incubated at 20 ± 1 °C in darkness until *ca.* 1 cm-root growth was observed. The clove was then inoculated by placing a mature sclerotium in contact with the root in darkness at 20 ± 1 °C until the plate surface was fully covered with mycelium. To prepare the inoculum, one mycelial disc (1 cm diameter) was taken at the periphery and sub-cultured on PDA medium in the same growth conditions for other 4 days.

For inoculation, two mycelial discs (1 cm diameter) from 4-day-old cultures were macerated in 600 μl 0.1% (w/v) H_2O agar. Then, 16-day-old explants were inoculated with macerated mycelium (600 μl) by slurring it over the roots. As un-inoculated control, 16-day-old plantlets were treated with a macerate of two discs of PDA in 0.1% (w/v) H_2O agar (600 μl). The plantlets were placed in darkness at 20 ± 1 °C for 48 h, and then in the same growth conditions described above (12:12 h light/dark cycle, 100 μmol m^{-2} s^{-1}, 20 ± 1 °C). Fourteen days after fungal inoculation, one set of inoculated and control plants were arbitrarily selected and fractionated into root, protobulb (*ca.* 2.5 cm stem-and-leaf sections above the root disc), leaf sheath (*ca.* 4 cm leaf sections above the end of the protobulb), and leaf blade. Also, fungal mycelium growing on MS (14 days) was collected. Mycelium and plant samples were immediately frozen and stored at −80 °C until they were used for metabolite extraction.

In parallel, and in order to evaluate the fungal presence along the garlic plant, another set of inoculated and control plants was surface disinfected with EtOH:H_2O (70:30, v/v) for 3 min and with 15% commercial bleach for 5 min with occasional swirling. Then, the plants were rinsed several times in sterile distilled H_2O and fractionated into root, protobulb (*ca.* 1.5 cm stem-and-leaf sections above the root disc), leaf sheath (*ca.* 1 cm leaf sections above the end of the protobulb), and leaf blade (*ca.* 1 cm leaf sections at 4 cm above the end of the protobulb). The different fractions of tissues were homogenized with 2 ml sterile phosphate buffer (50 mM potassium phosphate – pH 7 buffer, PBS), and 0.5 ml of each macerate was plated onto PDA medium and cultured at 20 ± 1 °C in darkness. Three independent biological replicates were used for fungal re-isolation from each tissue, and it was performed twice.

5.3. Terpene and ergosterol determinations by GC-EIMS

Terpene determinations were done according to Salomon et al. (2014) with modifications. Samples of different plant tissues (root, protobulb, leaf sheath and blade) were macerated with CH_2Cl_2:MeOH with 0.2% HCO_2H (1 ml, 2:1, v/v) per 400 mg fr. wt., and the same solvent mixture (3 ml) per 700 mg mycelia fr. wt. The macerates were transferred to glass tubes with screw caps, vortex-mixed vigorously, and kept overnight in darkness at 4 °C. As control, MS medium was extracted. Then, the macerates were centrifuged 10 min at 15,000*g*, the CH_2Cl_2 phases were collected, dried over anhydrous Na_2SO_4, and aliquots of each CH_2Cl_2 (100 μl) phase were injected with *n*-hexadecane (1 ng μl^{-1}) as internal standard (Supelco, Bellefonte, PA, USA) in a capillary gas chromatography–electron impact mass spectrometer (GC–EIMS; Clarus 500, PerkinElmer, Shelton, CT, USA) in split-splitless mode. The analyses of terpenes (except for ergosterol (**9**)) were carried out with the same GC column and program as described in Gil et al. (2012). The oven temperature program for ergosterol (**9**) analysis was: initial temperature at 60 °C for 1 min followed by an increase of 10 °C min^{-1} to 280 °C and held at 280 °C for 37 min. The identities of compounds were confirmed by comparison of their retention times and full scan mass spectra with those of authentic standards of terpinolene (**5**) (85%), nerolidol (**1**) (96%), α-pinene (**4**) (99%), limonene (**6**) (99%), and squalene (**3**) (98%, Sigma–Aldrich Steinheim, Switzerland), and with mass spectra of the National Institute of Standards and Technology (NIST) library. Quantification of each compound was performed on the basis of the peak area as compared to the peak area of a known amount of *n*-hexadecane co-injected with the sample. Five independent biological replicates were used for metabolic analysis in plant tissues and fungal mycelium. Each experiment was repeated twice.

5.4. Terpene synthase activity determinations

Samples of the different plant tissues (100 mg fr. wt. each of root, protobulb and leaf blade) collected at 8 dpi were homogenized with 1 M PBS (800 μl, pH 6.5–7), 20% (w/v) glycerol, 10 mM sodium metabisulfite, 10 mM ascorbic acid, 15 mM $MgCl_2$, 0.5% PVP (insoluble polyvinyl poly pirrolidone, Sigma Chem Co, St Louis, MO, USA, MW 40.000) and 1.47 mM 2-β mercapthoethanol, according to Gil et al. (2012). Each total protein homogenate was centrifuged 10 min at 9000*g* and the supernatant (10 μl) was incubated with 0.22 GBq of radioactive *trans*, *trans*-farnesyl pyro-phosphate triammonium salt ([1-^{3}H]-FPP, specific activity 740 GBq $mmol^{-1}$, Perkin Elmer, Boston, MA, USA), FPP (**10**) (0.14 μM, Sigma Chem. Co., St. Louis, MO, USA) as carrier, and reaction buffer containing 250 mM Tris:HCl (pH 6.5–7), 50 mM $MgCl_2$. Each mixture was incubated 20 min at 30 °C with the reaction stopped using H_3PO_4 (85%, 10 μl). Reaction products were partitioned with *n*-hexane (150 μl) and treated with 5 mg silica gel powder (240–300 Mesh, Sigma Chem Co, St Louis, MO, USA). An aliquot of *n*-hexane (50 μl) was placed in a scintillation vial with Fluka cocktail (4 ml, Sigma–Aldrich, St. Louis, MO, USA), with radioactivity measured using a Tricarb liquid scintillation analyzer (Perkin Elmer, Illinois, USA). TPS activities were expressed as nmol [^{3}H]-FPP transformed mg of $protein^{-1}$ h^{-1} according to Vögeli and Chapell (1988). The protein concentration of the extract was determined by the method of Bradford using bovine serum albumin (Bio-Rad Laboratories, Philadelphia, PA, USA) as standard. Results are reported as a mean of three independent replicated assays, and each experiment was repeated twice.

5.5. Evaluation of antifungal activity

To evaluate the antifungal activity of the terpenes identified in inoculated plants, the disc diffusion method was used. The concentrations of nerolidol (**1**), α-pinene (**4**) and terpinolene (**5**) were determined in relation to terpinolene (**5**) content calculated in the entire root of infected plants (18.0–29.0 μg $root^{-1}$ fr. wt.), considering that it was at the lowest concentration among these terpenes (see Table 1). Therefore, four sterile filter paper discs (Whatman No. 1,6 mm diameter) impregnated with a range between 2.0 and 5.0 μg of each pure authentic terpinolene (**5**), α-pinene (**4**) or nerolidol (**1**) were equidistantly distributed around the center of a PDA plate (see Supplementary Fig. S5). In the same

way, the concentrations of 1,8-cineole (**7**) (0.3 μg root^{-1} fr. wt.), limonene (**6**) (1.2 μg protobulb^{-1} fr. wt.), and γ-terpinene (**8**) (1.7 μg protobulb^{-1} fr. wt.) detected in the whole root and protobulb were evaluated with their pure standards, respectively. Four discs with an equivalent volume of *n*-hexane were tested in this experiment as a terpene-free control. Then, each plate was inoculated in the center with a disc (5 mm diameter) of 4-day-old mycelium, and incubated at 20 ± 1 °C in darkness until it reached the edges of the terpene-free control dishes (*ca.* 5 dpi). The accumulated radial growth was measured in the four axes, and the antifungal index was calculated as: AI (%) = (1 − Dt/Dc) × 100 (Bittner et al., 2009); where Dt is the average diameter of the fungal colony in the plate treated with pure terpenes, and Dc is that of the control. Each treatment had six replicates and was performed twice. At the concentrations of 1,8-cineole (**7**), limonene (**6**) and γ-terpinene (**8**) tested, no inhibition of *S. cepivorum* growth was observed. To evaluate the fungi toxicity (fungistatic/fungicidal nature) of nerolidol (**1**), terpinolene (**5**) and α-pinene (**4**), as well as a terpene-free control, discs of mycelium (7-day-old), were re-inoculated onto new PDA plates, incubated at 20 ± 1 °C in darkness to observe possible hyphal growth.

In order to evaluate the effect of terpenes on the number of sclerotia produced by *S. cepivorum* (45 dpi), four plates treated with each one of the pure authentic nerolidol (**1**), terpinolene (**5**), α-pinene (**4**) as described above, were divided into four quarters. Two quarters per plate were randomly selected and the number of sclerotia per quarter was counted at ×10 under a Zeiss Standard microscope. Relative sclerotia production was calculated as follows: relative sclerotia production (%) = [(1 − St/Sc) × 100]; where St is the average of sclerotia number per quarter in the plate treated with pure terpenes, and Sc is that of the control. The experiment was performed twice.

Since no differences in fungal growth inhibition were observed among the concentrations tested for nerolidol (**1**), terpinolene (**5**) and α-pinene (**4**) (see Fig. 4a), and considering that 4.0 μg/disc was the threshold of response for sclerotial production (see Fig. 4b), this concentration was selected to evaluate the ability of these terpenes to change the hyphal morphology and membrane permeability.

5.6. Scanning electron microscopy

Mycelial disc of *S. cepivorum* obtained after 7 days of exposure to pure nerolidol (**1**), terpinolene (**5**), α-pinene (**4**) (4.0 μg/disc) and *n*-hexane (control) were used for scanning electron microscopy observation. The discs were rapidly placed in vials containing 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C and fixed for 16 h. Fixed mycelia were washed with fresh cacodylate buffer five times for 10 min, dehydrated in a graded H_2O-acetone series of 30, 50, 70, 90, and 100% acetone for 10 min at room temperature, and finally stored in acetone. Dehydrated samples were then dried at critical point in liq. CO_2, and sputter-coated with gold. Observations were carried out with a scanning electron microscope (JSM-6610LV, JEOL Ltd, Tokyo, Japan) operated at an accelerating voltage of 20 kV.

5.7. Ethidium bromide uptake assay

The effect of nerolidol (**1**), terpinolene (**5**) and α-pinene (**4**) on fungal membrane permeability was evaluated by monitoring uptake of EtBr (a cell impermeant dye) within the hyphae. For that, discs from 4-day-old *S. cepivorum* cultures treated with the terpenes (4.0 μg/disc) and *n*-hexane as terpene free-control (see antifungal activity assay) were incubated in 15 μM EtBr (in PBS) for up to 30 min at 25 °C. After incubation, samples were washed with fresh PBS for 3.5 h and observed by using an ultraviolet transilluminator (BIO-RAD Gel Doc 1000). The assay was repeated three times and a representative image of the results is presented in the Fig. 6.

5.8. Statistical analysis

Statistical analyses of the data were performed using the software Statgraphics Centurion XVI version 15.0.10 (Statpoint Technologies Inc., Warrenton, VA, USA). Significance of differences was conducted with LSD of Fisher test at a probability level of $P \leqslant 0.05$. The effect of fungus inoculation, type of tissue and its interaction were determinate by multifactorial ANOVA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.02.003.

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