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ORIGINAL ARTICLE

In vitro antifungal activity of the tea tree (*Melaleuca alternifolia*) essential oil and its major components against plant pathogens

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Abstract

Aims: The aim of this study was to examine the effect of *Melaleuca alternifolia* essential oil (TTO) and its principal components on four cereal-pathogenic fungi.

Methods and Results: The antimycotic properties of TTO and of terpinen-4-ol, γ-terpinen and 1,8-cineole (eucalyptol) were evaluated *in vitro* on *Fusarium graminearum*, *Fusarium culmorum* and *Pyrenophora graminea*. Moreover, barley leaves infected with *Blumeria graminis* were treated with whole TTO. All the tested fungi were susceptible to TTO and its components.

Conclusions: TTO exerted a wide spectrum of antimycotic activity. Single TTO purified components were more active than the whole oil in reducing *in vitro* growth of fungal mycelium and, among the tested compounds, terpinen-4-ol was the most effective.

Significance and Impact of the Study: TTO and its components can be considered potential alternative natural fungicides.

Introduction

The use of synthetic fungicides in crops can result in problems like environmental pollution, phytotoxicity and selection of resistant pathogen populations. Therefore, alternative measures have been developed for crop protection, including biological agents, mineral salts and plant extracts. Among these, the complex mixtures of compounds, mainly monoterpenes and sesquiterpenes, that characterized the chemical composition of essential oils have been investigated for their wide spectrum of antimicrobial activity. In particular, *Melaleuca alternifolia* (tea tree) essential oil (TTO) has a long history of use as topical microbicide in human pharmacology (Markham 1999; Carson *et al.* 2006), and it has been re-evaluated in recent years as an alternative agent to antibiotics (Allen 2001). The mechanism of action of this oil, studied both in bacteria and fungi, involves both the loss of membrane integrity accompanied by the release of intracellular material and the inhibition of cellular respiration, with the consequent inability to maintain homeostasis associated to changes in cell morphology (Carson *et al.* 2006). Although there is a natural variation in the TTO essential oil content (Homer *et al.* 2000), whose genetic basis is not completely understood (Shelton *et al.* 2002), the composition of oil sold as TTO is standardized (ISO4730:2004) and characterised, as major components, by terpinen-4-ol (40% in a typical composition), γ-terpinen (23%), α-terpinen (10·4%) and 1,8-cineole (5·1%) (Brophy *et al.* 1989). Most components of TTO are active against a range of fungi that cause human diseases (Hammer *et al.* 2003) and, among them, terpinen-4-ol seems to be the most active agent (Oliva *et al.* 2003). Moreover, this monoterpene, present also in other plants like *Origanum majorana*, possesses contact and fumigant insecticidal action against several economically important pests (Isman 2000). The inhibitory activity of TTO has been demonstrated on sporulation and apical growth of

food-borne and phytopathogenic fungi, like *Aspergillus fumigatus, Fusarium solani, Penicillium espansum, Botritis cinerea* and *Rhizopus oryzea* (Bishop and Reagan 1998; Inouye *et al.* 1998; Bowers and Locke 2000; Inouye *et al.* 2000; Angelini *et al.* 2006); however little is known about its effect on fungi that infect small grain cereals. *Fusarium graminearum* and *Fusarium culmorum* are mycotoxigenic fungi responsible for FHB (Fusarium head blight) in wheat, barley and oat. *Fusarium* plant and grain contamination represents a major economic concern not only for yield loss but more for the quality and safety of the final products; in fact, deoxynivalenol (DON), the predominant toxin produced by these fungal species, is a trichothecene that causes both acute and chronic effects in humans and animals (Parent-Massin 2004). *Pyrenophora graminea* is a seed-borne pathogen responsible for barley leaf stripe, a common disease in barley districts with a cold sowing season. In susceptible cultivars, the fungus causes brown stripes on the leaves, stunted growth and severe yield reductions (Bulgarelli *et al.* 2004). *Blumeria graminis* f. sp. *hordei* is one of the grass powdery mildew pathogens and widespread biotrophic fungi that colonize plant epidermal tissues, causing large yield loss (Zhou *et al.* 2001).

In this study, we examined the effects of TTO and its major purified components (terpinen-4-ol, γ-terpinen and 1,8-cineole) on *F. graminearum, F. culmorum, P. graminea* and *B. graminis.* These four cereal-pathogenic fungi cause relevant yield loss in Mediterranean environments and are characterised by different life cycle, pathogenicity behaviour, transmission mode and effects on plants and derived products.

Materials and methods

Fungal material

Strains of *F. graminearum* and *F. culmorum* (provided by the Università Cattolica del Sacro Cuore, Piacenza, Italy, with repository codes MPVP74 and MPVP70, respectively) isolated from bread wheat plants in Italy (Emilia-Romagna) in 2002 as well as the highly virulent *P. graminea* isolate I2, collected from Northern Italy on barley (Gatti *et al.* 1992) and *Blumeria graminis* f. sp. *hordei* isolate K1 (kindly provided by Paul Schulze-Lefert, Max-Plank-Institut für Züchtungsforschung, Köln, Germany) were used.

Tea tree oil and its components

Stocks of medicinal tea tree oil extracted from the leaves and flowers of *M. alternifolia* grown in Australia were purchased from Campo Research (New York, USA). The stocks have been certified for their component content according to ISO4730. Terpinen-4-ol (≥99%, Fluka code 86477), γ-terpinen (≥98·5%, Fluka code 86476) and 1,8-cineole (≥99%, Fluka code 29210) were purchased from Sigma-Aldrich S.r.l. (Milan, Italy).

Evaluation of effects of Tea Tree oil on fungal growth

The mycelial growth of *F. graminearum*, *F. culmorum* and *P. graminea* was evaluated on solid PDA medium amended with 0·5% Tween 20 (Sigma, St Louis, MO, USA) and TTO ranging from 0% to 5%. The experiments were conducted twice in triplicate in 60 mm petri dishes inoculated with 8 mm PDA plugs from actively growing cultures. Mycelial growth was evaluated as mean diameter measured each day from fungal inoculation up to 6 days post-inoculation. The significance of the differences among treated and control samples were evaluated using the Student's *t*-test.

To evaluate the effect of seed dressing on fungal growth, stock solution of TTO was prepared using 0·05% Irol Plus® (Isagro Italia S.r.l., Milan, Italy) as emulsifying agent and by adding it to barley seeds at final concentrations of 1 g and 2 g essential oil kg^{-1} of barley. Eight-millimetre plugs of actively growing mycelium of *F. graminearum*, *F. culmorum* and *P. graminea* were inoculated on a single layer of barley seeds (cultivar Golden Promise) in 90 mm petri dishes. The experiments were conducted at 20°C in triplicate and the mycelium areas were evaluated 7 days after inoculation by Typhoon 9210 Imager scanner (Amersham Biosciences, Milan, Italy).

To evaluate the effect of TTO on *Blumeria graminis* f. sp. *hordei*, five 10-day-old leaves segments (40 mm length) of barley cultivar Golden Promise (susceptible to mildew) were laid out in a petri dish over a medium containing 0·5% agar. For each experimental condition, a set of four independent petri dishes was prepared. All the plates were placed on a rotating platform under an inoculation tower (diameter 50 cm, height 100 cm) and inoculated with K1 isolate. The leaf segments were then incubated for 72 h at 20°C in the dark and then sprayed with a control solution containing 0·05% Tween or a solution containing 0·05% Tween 20% and 0·5% TTO or 1% TTO. After 7 days of incubation at 20°C, the mildew colonies on the leaf segments were counted.

Effects of terpinen-4-ol, 1,8-cineole and γ-terpinen on fungal growth

The inhibition of mycelial growth of *F. graminearum*, *F. culmorum* and *P. graminea* was evaluated on solid PDA medium amended with 0·5% Tween 20 and the major TTO components (terpinen-4-ol, 1,8-cineole and γ-terpinen). The experiments were conducted twice in triplicate

in 60-mm petri dishes, inoculated with 8 mm PDA plugs obtained from actively growing cultures. Mycelial growth was evaluated by measuring the mean diameter daily (from fungal inoculation until 6 days). The significance of the differences was evaluated using the Student's *t*-test.

Effects of Tea Tree oil and of terpinen-4-ol, 1,8-cineole and γ-terpinen on barley seed germinability

Six hulled barley cultivars (Aiace, Aldebaran, Aliseo, Marjorie, Nure and Tidone) and six barley hulless cultivars (Alamo, Fibar, Fior 6328, Priora, Rattan and Zacinto), along with two barley near-isogenic lines (Fior 4503, hulless; Fior 4508, hulled), were used for germination tests. Stocks of 100 seeds/cultivar were treated for 2 h with solutions of 0·05% Irol Plus in sterile distilled water containing 0%, 0·5%, 1%, 2% and 5% TTO. After the treatments, the seeds were placed on a wetted filter paper (2 ml sterile distilled water) in a petri dish at room temperature and the percentage of germination was evaluated after 1 week. Two replicas of the experiment were done. Analysis of variance (AOV) was performed on the data obtained using the Systat package version 9.0 (SPSS Inc., Chicago, USA).

Stocks of 100 seeds of the above reported barley cultivar were treated for 2 h with solutions of 0·05% Irol Plus and sterile distilled water containing 0% essential oil or 0·25% terpinen-4-ol or 1% 1,8-cineole or 2% γ-terpinen. A chi-square analysis was performed to compare germination percentages of treated and non-treated seeds.

Results

Growth inhibition of fungal species by TTO and its components

Table 1 shows the mean radial growth of *F. graminearum*, *F. culmorum* and *P. graminea* mycelium on solid medium containing TTO. The growth of the three fungi on TTO amended medium was significantly reduced for all the TTO concentrations. The MIC50 for *F. culmorum*, *F. graminearum* and *P. graminea* was 2·3%, 1·2% and 2·12% TTO respectively.

The effects of three major TTO components were individually evaluated by measuring the radial growth of *Fusaria* and *P. graminea* on solid medium amended with several 0–2% concentrations of terpinen-4-ol, γ-terpinen and 1,8-cineole (Table 1). The growth inhibiting concentration was 0·5% for both γ-terpinen and 1,8-cineole. The MIC50 of γ-terpinen was 0·63% for *F. culmorum*, 0·53% for *F. graminearum* and 0·31% for *P. graminea*, whereas the MIC50 of 1,8-cineole was 0·33%, 0·17% and 0·25% for the three fungi, respectively. Terpinen-4-ol was more effective, causing a significant growth reduction at concentration as low as 0·05%, with MIC50 of 0·048% for *F. culmorum*, 0·039% for *F. graminearum* and 0·049% for *P. graminea*.

Table 1 Effects of various concentrations of TTO, terpinen-4-ol, 1,8-cineole and γ-terpinen on mycelial growth of *Fusarium culmorum*, *Pyrenophora graminea* and *Fusarium graminearum*. The radial mycelial growth was measured 6 days after inoculation (the data reported are the mean diameters expressed in cm, including the inoculation discs of 0·8 cm)

	0% TTO	0·25% TTO	0·5% TTO	1% TTO	2% TTO	5% TTO
Fusarium culmorum	6 ± 0·36	5·2 ± 0·11**	4·8 ± 0·1***	4·7 ± 0·08***	4·15 ± 0·05***	0·8 ± 0***
Pyrenophora graminea	5·15 ± 0·22	4·25 ± 0·19*	4·2 ± 0·09***	3·5 ± 0·15***	3·05 ± 0·1***	0·8 ± 0***
Fusarium graminearum	2·3 ± 0·2	2·08 ± 0·09***	1·8 ± 0·09***	1·5 ± 0·08***	1·23 ± 0·1***	0·8 ± 0***
	0% terpinen-4-ol	0·0125% terpinen-4-ol	0·025% terpinen-4-ol	0·05% terpinen-4-ol	0·1% terpinen-4-ol	0·25% terpinen-4-ol
Fusarium culmorum	5·8 ± 0·25	5·2 ± 0·81	4·4 ± 0·32***	2·7 ± 0***	1·1 ± 0·2***	0·8 ± 0***
Pyrenophora graminea	5·4 ± 0·28	5·3 ± 0·76	4·05 ± 0·4***	2·7 ± 0·2***	0·95 ± 0·05***	0·8 ± 0***
Fusarium graminearum	3·15 ± 0·57	3·2 ± 0·83	2·7 ± 0·44	1·4 ± 0·1***	0·8 ± 0***	0·8 ± 0***
	0% 1,8-cineole	0·1% 1,8-cineole	0·25% 1,8-cineole	0·5% 1,8-cineole	1% 1,8-cineole	2% 1,8-cineole
Fusarium culmorum	6 ± 0	6 ± 0	5·5 ± 0·15	0·8 ± 0***	0·8 ± 0***	0·8 ± 0***
Pyrenophora graminea	6 ± 0	5·06 ± 0·4	2·5 ± 0·05***	1·7 ± 0·05***	0·8 ± 0***	0·8 ± 0***
Fusarium graminearum	3·16 ± 0·25	3·3 ± 0	0·8 ± 0***	0·8 ± 0***	0·8 ± 0***	0·8 ± 0***
	0% γ-terpinen	0·1% γ-terpinen	0·25% γ-terpinen	0·5% γ-terpinen	1% γ-terpinen	2% γ-terpinen
Fusarium culmorum	6 ± 0	6 ± 0	6 ± 0	3·78 ± 0·97***	1·38 ± 0·07***	0·8 ± 0***
Pyrenophora graminea	6 ± 0	3·9 ± 0·58***	2·65 ± 0·25***	2·4 ± 1·4***	0·8 ± 0***	0·8 ± 0***
Fusarium graminearum	3·55 ± 0·1	3·25 ± 0·25	2·4 ± 0·14**	2·4 ± 0·19***	1·13 ± 0·18***	0·8 ± 0***

Significant differences from controls calculated using the Student's *t*-test are shown as $*P \leq 0{\cdot}05$, $**P \leq 0{\cdot}01$ and $***P \leq 0{\cdot}001$.

Effects of TTO and its components on barley seed germinability

Seven hulless and seven hulled barley cultivars, which differ for the absence/presence of seed teguments, were chosen to test the potential toxic effect on seed germination of TTO and its leading components. Table 2 reports the mean percentages of germination obtained after treatment of barley seeds with a set of TTO concentrations ranging from 0·5% to 5%. On these data, an AOV was made to test the effects of two factors (treatments, presence/absence of hull) as well as their interaction. The presence/absence of hull was not significant for germinability ($P = 0·609$); however a significant effect of the treatments ($P = 0·000$) as well as the interaction 'treatments × hull' ($P = 0·002$) was found. Table 3 reports the mean germination percentages obtained after treatment of seven hulless and seven hulled barley genotypes with minimal concentration of terpinen-4-ol, 1,8-cineole and γ-terpinen that completely inhibited the fungal growth. Significant differences in germinability, evaluated with Chi-square test, were found in hulless genotypes treated with 0·25% terpinen-4-ol and hulled genotypes treated with 1% 1,8-cineole in comparison with control seeds.

Fungal growth on TTO dressed seeds

To test the potential use of TTO as a dressing agent, the fungi were inoculated on a single layer of TTO barley dressed seeds. The growth of *F. graminearum* on barley seeds dressed with TTO final concentrations of 1 g seed kg^{-1} and 2 g seed kg^{-1} was reduced at 67·7% (±6·6) and 8·4% (±0·91) respectively of the control (seeds dressed with Irol Plus). The growth of *F. culmorum* was reduced to 72·37% (±4·7) and 10·14% (±1·04), whereas the growth of *P. graminea* was reduced to 44·13% (±5·3) and 10·33% (±0·58) of the controls (Fig. 1).

Table 2 Germination percentages of hulless and hulled barley seeds after treatment with different TTO percentages. The data represent the mean percentages of germination calculated on 100 seeds (two replicas) of each of seven hulless and seven hulled genotypes per treatments

	Control	0·5% TTO	1% TTO	2% TTO	5% TTO
Hulless genotypes	88%	88%	86%	76%	64%
Hulled genotypes	97%	93%	91%	79%	31%

Table 3 Germination percentages of hulless and hulled barley seeds after treatment with major TTO components. The data represent the mean percentages of germination calculated on 100 seeds (two replicas) of each of seven hulless and seven hulled genotypes per treatments

	Control	0·25% terpinen-4-ol	1% 1,8-cineole	2% γ-terpinen
Hulless genotypes	96%	87%*	92%	96%
Hulled genotypes	99%	92%	87%**	93%

Results of Chi-square test to compare the germination percentage of treated *vs* control seeds: *$P \leq 0·05$ and **$P \leq 0·01$.

Effects of TTO treatment on *Blumeria graminis*

To test the efficacy of TTO on an obligate leaf pathogen, barley leaf segments were inoculated with powdery mildew under controlled conditions: both 1% and 0·5% TTO solutions inhibited the formation of mildew colonies on leaf surface completely.

Discussion

All the tested fungi were susceptible to TTO at concentration equal to or higher than 0·25% (Table 1). Moreover, it appears that TTO single components are more active agents in reducing *in vitro* growth of *Fusaria* and *Pyrenophora*. Among the tested compounds, terpinen-4-ol is particularly effective in reducing fungal growth, causing complete growth inhibition of the three fungi at concentrations ranging from 0·1% to 0·25%. Eucalyptol and γ-terpinen determine complete growth inhibition at higher percentages, ranging from 0·25% to 1% and from 1% to 2% respectively. These data are consistent with previous studies, which demonstrate that terpinen-4-ol is the most active component of TTO on human pathogens (Carson and Riley 1995; Cox *et al.* 2001; Oliva *et al.* 2003). Antagonistic effects among different TTO constituents are responsible for lower level of fungicidal activity shown by the TTO complete formulation, most likely due to the reduction of terpinen-4-ol aqueous solubility by non-oxygenated terpenes (Cox *et al.* 2001).

Because *Fusaria* are plant pathogens that can contaminate the grain before and during storage and *P. graminea* is a seed-borne pathogen, the growth of these fungi was evaluated on TTO dressed seeds in comparison with non-dressed ones. The results obtained clearly show a marked inhibitory effect of TTO dressed seeds on fungal growth. To evaluate the potential toxic effect of TTO on barley, the germinability was tested on a panel of hulled and hulless seeds treated with essential oil. At low concentration, germinability was not affected by TTO; however statistical analysis showed that both TTO treatments and interaction between TTO and seed hull had an effect on germination. In particular, hulless genotypes were less affected by TTO treatments in comparison with hulled ones (Table 2). Germination tests on barley seeds treated with terpinen-4-ol, γ-terpinen and 1,8-cineole at concentrations that prevented fungal growth demonstrate very low

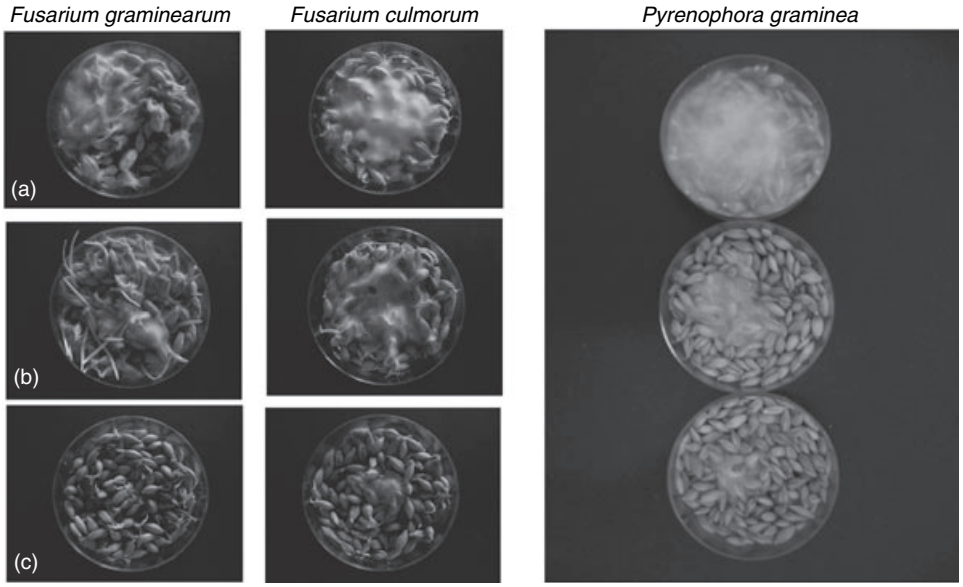

Figure 1 Growth inhibition of fungal inocula on TTO dressed seeds. Row a shows the fungal growth on control, not-treated seeds; row b shows the fungal growth on seeds treated with 1 g TTO kg^{-1} of barley; row c shows the fungal growth on seeds treated with 2 g TTO kg^{-1} of barley. Three replicates for each treatment have been done.

level of phytotoxicity of TTO leading components (Table 3). Given the worldwide diffusion and economic impact of the *B. graminis* infection, numerous studies have been focused on the development of crop protection strategies and use of synthetic fungicides (Letessier *et al.* 2001; Haugaard *et al.* 2002; Choi *et al.* 2004). Therefore, we tested TTO against this type of fungal pathogen characterized by a distinct way of transmission (wind-borne) and site of infection (epidermal tissue). The results obtained showed that even a single treatment with a spray solution containing TTO as low as 0·5% completely prevented the colonization of barley leaves.

In conclusion, TTO and its components can be considered potential alternative preharvesting natural fungicides, on the basis of their efficacy on different types of plant pathogens and their flexibility of use. However, because the *in vitro* effect of compounds did not always provide a good criterion for their *in vivo* performances, additional studies are necessary to verify the effectiveness and persistence of these antifungal treatments in field conditions.

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