Activity of Tea Tree (*Melaleuca alternifolia,* Cheel) and thyme (*Thymus vulgaris,* Linnaeus.) Essential Oils against Some Pathogenic Seed Borne Fungi

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Abstract

Melaleuca alternifolia (tea tree) and *Thymus vulgaris* (thyme) essential oils were tested for their antifungal activity to investigate the possibility of their use for seed treatment. The ability of the two oils to inhibit mycelial growth was studied by *in vitro* assay on agar medium containing different concentrations of the essential oils (0%, 0.05%, 0.1%, 0.25%, 0.5%, 1% v/v). Seven seed-borne pathogen fungi of large interest *Ascochyta rabiei, Colletotrichum lindemuthianum, Fusarium graminearum, F. culmorum, Drechslera avenae, Alternaria radicina* and *A. dauci* were transferred on the modified medium in order to test the oils antifungal activity, by calculating the Percentage of Mycelial Growth Inhibition (%MGI) and the Minimum Inhibitory Concentration (MIC). Results showed that both the oils had a clear reducing effect on fungal growth, that was dose-dependent and it differed depending on the fungal species, confirming what is already reported in literature, with *T. vulgaris* oil to be one of the most potent agent against fungi. *M. alternifolia* and *T. vulgaris* oils can be considered potential alternative natural fungicides to the synthetic chemicals that are currently used to prevent and control seed-borne diseases, and could be used in agriculture for safe and eco-friendly seed-treatments.

Key Word Index

Ascochyta rabiei, Colletotrichum lindemuthianum, Fusarium spp, *Drechslera avenae, Alternaria* spp, mycelial growth, Minimum Inhibitory Concentration.

Introduction

In recent years, interest has grown in developing alternative measures to chemical treatments for crop and seed protection, including physical methods, biocontrol agents and plant extracts (1). Concerning plant extracts, many studies have been focused on the pharmacological actions of essential oils derived from aromatic and medicinal plants: these are metabolic products accumulating by plants and extracted from leaves, flowers, roots and barks, and their active principles (alkaloids, phenols, flavonods, monoterpenes and sesquiterpenes isoprenoids) have been broadly studied due to their many antimicrobial and antioxidant properties (2) (3). In particular, tea tree oil (*Melaleuca alternifolia* Cheel, TTO) and thyme oil (*Thymus vulgaris* Linnaeus, TO) have been reported to possess antifungal activity (4) (5) (6) and are considered to be potentially very important for agriculture, thanks to their effectiveness, low cost and easy availability.

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Most crops, mainly wheat and vegetable cultures, can be exposed to different seed borne diseases which results in considerable losses in yields: it is well known that seed can hide pathogen organisms which represent an important threat to crop yield and quality, causing germinability reduction, seedling damages, primary infection onset and species spreading. Therefore, seed borne diseases represent a critical problem for successful production at both qualitative and quantitative level, especially in organic farming systems, where less efficient plant protection agents are available for managing plant diseases. In this work, some seed-borne pathogen fungi of large interest on two legumes (chick-pea and bean), wheat and carrot are studied: *Ascochyta rabiei* (Pass.) Labrousse, *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cavara, *Fusarium graminearum* Schwabe, *F. Culmorum* (W.G. Smith) Saccardo, *Drechslera avenae* (Eidam) Scharif, *Alternaria radicina* (Meier) Drechsler & Eddy, *A. Dauci* (Kühn) Groves & Skolko.

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The aim of this study is to test the efficacy of the antifungal activity of TTO and TO against these pathogenic seed-borne fungi by *in vitro* assay, evaluating the potential use of the two oils for safe and eco-friendly seed-treatments.

Experimental

Fungi: A total of 7 fungi (*F. graminearum, F. culmorum, A. radicina, A. dauci, A. rabiei, C. lindemuthianum, D. avenae*), maintained on solid PDA medium (potato dextrose agar), were used for antifungal activity evaluation of TTO and TO. The strains of *F. graminearum, F. culmorum* and *D. avenae* were isolated from durum wheat seeds; the strains of *A. dauci, A. rabiei* and *C. lindemuthianum* were isolated from carrot, chick-pea and bean seeds, respectively; the strain of *A. radicina* was provided from Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre (Utrecht, The Netherlands).

Oils: Stocks of medicinal TTO and TO were purchased from Sovimpex (Marseille, France) and from Esperis s.p.a. (Milan, Italy) respectively. The main active components of the two oils used for *in vitro* trials are: terpinen-4-ol (40.7%) and γ-terpinene (20.3%) for TTO, thymol (41%) for TO, as reported by respective producer Companies.

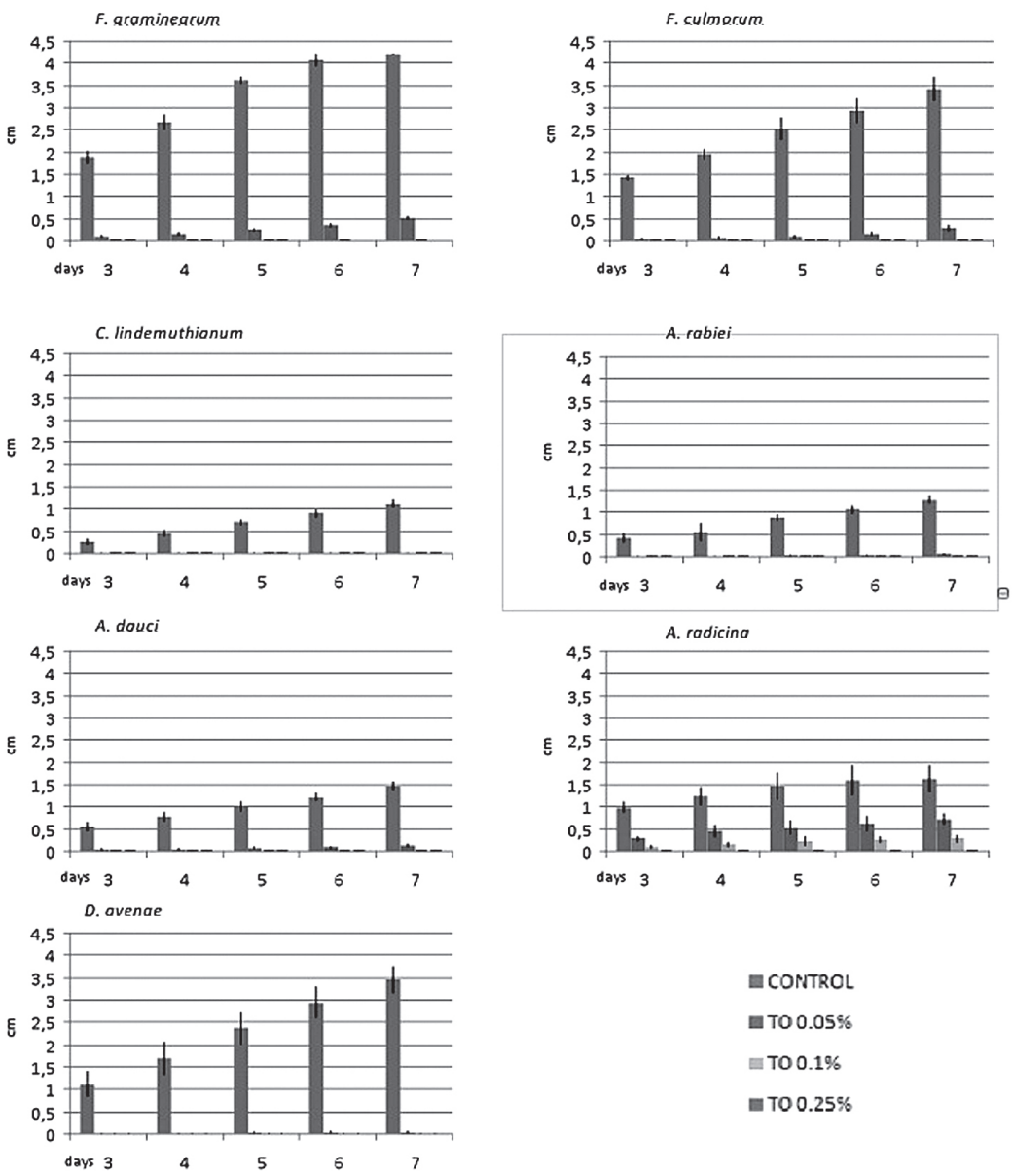

Figure 1. Micelial growth means of *F. graminearum, F. culmorum, A. radicina, A. dauci, A. rabiei, C. lindemuthianum, D. avenae* in presence of TO at different concentrations. Data of mycelial growth are expressed as radius length measured at the 3rd, 4th, 5th, 6th and 7th days after inoculation. The error bar show the Standard Deviation.

In vitro activity: Mycelial growth was determined on solid PDA medium amended with TTO and TO at the following concentrations: 0% (control), 0.05%, 0.1%, 0.25%, 0.5%, 1% (v/v) and in presence of Tween20 (0.5% v/v) as emulsifying agent. Experiments were conducted twice in duplicate in 90 mm Petri dishes containing the medium modified and inoculated with 6 mm plugs of PDA from actively growing cultures; after inoculations dishes were kept in the dark at 24°C.

Radial growth of colonies was measured every day (from the third to the seventh after inoculation) at two perpendicular points along the diameter of the plate, and the mean of these two readings was used to calculate the mean daily radius of the fungal colony. Percentage inhibition of mycelial growth (%MGI) by the oils was calculated using the formula: (C-T) x 100/C where C and T are the average of the colonies radius values in control and treatments, respectively, measured for each fungus at the seventh day after inoculation, obtained in both experiments. Minimum inhibitory concentration (MIC), the minimum dose that produced a 50% reduction of growth compared with the growth of the oil-free control, was computed for each combination of fungus/compound at the seventh day after inoculation using regression equation and FORCAST tool available with Microsoft Excel. In *F. graminearum* experiments, since mycelium covered the whole dish area on the sixth day after inoculation, the fifth day was taken as the last useable day to calculate MGI and MIC.

Analysis of variance (ANOVA) was performed on the data obtained using CoStat-Statistics Software version 6.4.

The significance of the differences among treated and control samples was evaluated using the Student-Newman-Keuls test for multiple comparison (significance level 0.05).

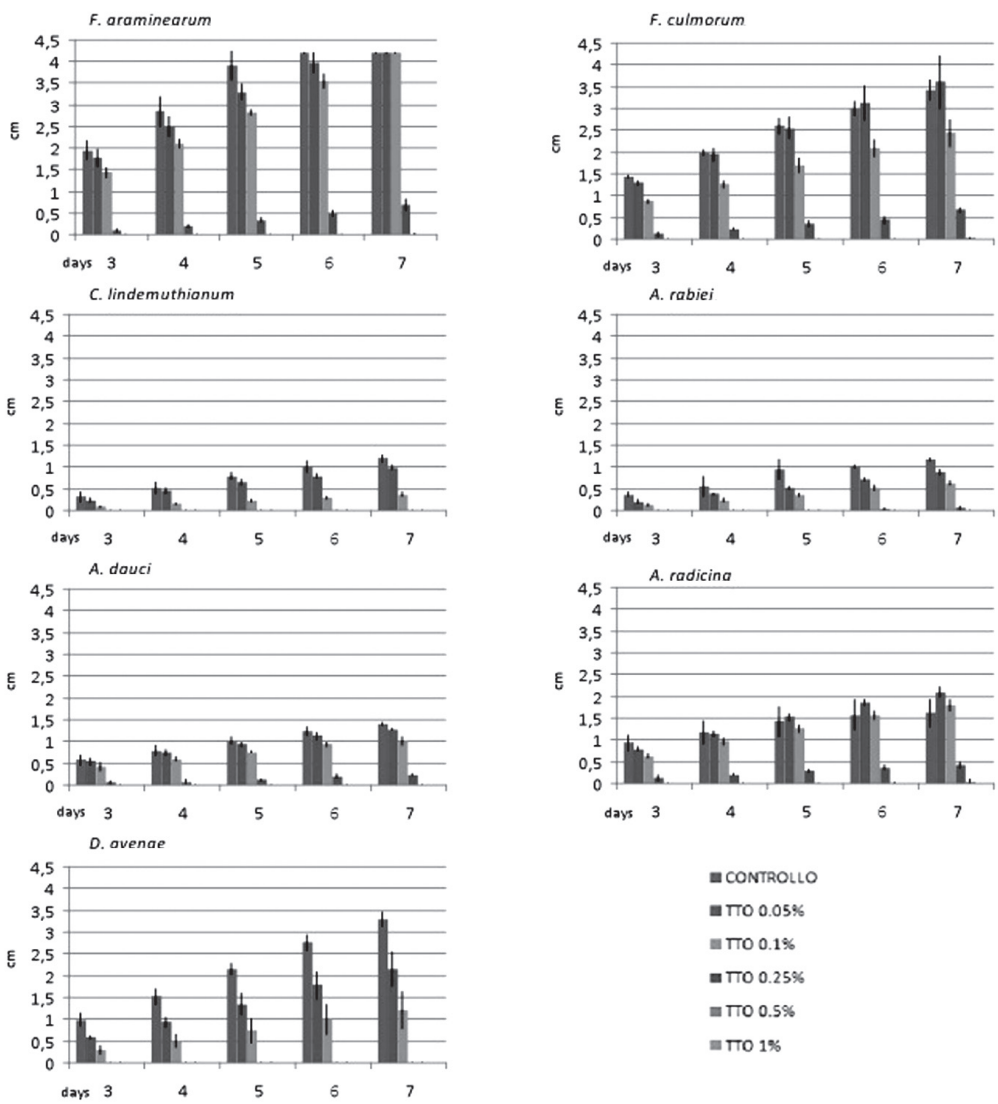

Figure 2. Micelial growth means of *F. graminearum, F. culmorum, A. radicina, A. dauci, A. rabiei, C. lindemuthianum, D. avenae* in presence of TTO at different concentrations. Data of mycelial growth are expressed as radius length measured at the 3rd, 4th, 5th, 6th and 7th days after inoculation. The error bar show the Standard Deviation.

Results and Discussion

Figures 1 and 2 show the mean radial growth of *F. graminearum, F. culmorum, A. radicina, A. dauci, A. rabiei, C. lindemuthianum* and *D. avenae* colony on solid medium containing TO and TTO, respectively. The standard deviations are also reported. The ANOVA analysis showed not significant differences between the two experiments ($p>0.05$). Data on the percentage inhibition of mycelial growth (%MGI) are reported in **Tables I and II**. In general, the fungi showed different mycelial growth depending on the strain and the oil concentration, proving a dose-dependent way of growth inhibition. The growth of the seven fungi on TO and TTO amended medium, compared to the growth of the oil-free control, was significantly reduced ($p=0.00$) for all the TO and TTO concentrations, excepting two cases, were the growth of *F. culmorum* and *A. radicina* was enhanced ($p=0.00$) at the lowest experimental concentration levels of TTO: 0.05% TTO for *F. culmorum* and 0.05% and 0.1% TTO for *A. radicina*. At these concentrations, they showed in both repetitions/tests mycelial growth stimulation, which results in minus sign in **Table II** data, supporting data previously reported by Angelini and colleagues (7) for *Pleurotus* species.

Although essential oils are known to be good antimicrobial agents, some microorganism are stimulated by them and use them as a carbon energy source (8); this may have been the case for the *A. radicina* and *F. culmorum* examined in this study.

The MICs of TTO were evaluated for all the tested fungi, and data are reported in **Table III**. The highest MIC value was 0.40% for *A. radicina*, while the lowest one was 0.08% TTO for *D. avenae*. About TO, all fungi showed no mycelial growth even at the lowest TO concentration tested, so the TO MIC value is surely <0.05% for each fungus. However, the comparison of MIC values of the two oils showed that TO is more effective than TTO in mycelial growth inhibition, and this occurred with all the fungi tested.

Results obtained from this work are consistent with previous studies (4) (5) (6), which demonstrate that TTO and TO posses antifungal activities, and these activities depend on oil concentration and on the fungal specie. Actually, first studies on essential oils were mainly focused on pharmacological and cosmetical potential of the oils against bacteria (9) (10), dermatophytes fungi and other filamentous fungal species like members of the genus *Candida* (11) (5) (12). Only in more recent years interest has grown in investigating essential oil potentialities as alternative measures to chemical treatments for crop and seed protection, and many authors have begun to study essential oil activities against plant pathogenic fungi of agricultural interest, and valuable results have been achieved. Zaker and Mosallanejad (13) studied the antifungal ability of five plant extracts against *Alternaria alternata*, the causal agent of Alternaria leaf spot of potato; Marin et al. (14) tested cinnamon, clove, oregano, palmarosa and lemongrass oils against *Fusarium graminearum*, one of the main causal agents of the root and foot rot and fusarium ear blight in wheat; Faria et al. (15) investigated the activity of *Ocimum gratissimum* essential

Table 1. Thyme oil (TO) in vitro activity: the percentage inhibition of mycelial growth (%MGI) is calculated for each fungus at different oil concentration as mean radius at the 7th (*) and the 5th (**) day after inoculation of both experiments. 100 value refers to complete inhibition with no mycelial growth. Significance differences ($p \leq 0.05$) found using the Student-Newman-Keuls are indicated as letters $^{a-e}$, where the letter a (not reported in the table) refers to the respective control range. Value followed by the same letter within the same row do not differ significantly.

	%MGI				
ORGANISM	**TO 0.05%**	**TO 0.1%**	**TO 0.25%**	**TO 0.5%**	**TO 1%**
F. graminearum **	93^{b}	100^{c}	100^{c}	100^{c}	100^{c}
F. culmorum *	91^{b}	100^{c}	100^{c}	100^{c}	100^{c}
*C. lindemuthianum**	100^{b}	100^{b}	100^{b}	100^{b}	100^{b}
A. rabiei *	96^{b}	100^{c}	100^{c}	100^{c}	100^{c}
A. dauci *	92^{b}	100^{c}	100^{c}	100^{c}	100^{c}
*A. radicina**	56^{b}	82^{c}	100^{d}	100^{d}	100^{d}
D. avenae *	99^{b}	100^{b}	100^{b}	100^{b}	100^{b}

Table II. Tea tree oil (TTO) in vitro activity : the percentage inhibition of mycelial growth (%MGI) is calculated for each fungus at different oil concentration as mean radius at the 7th (*) and the 5th (**) day after inoculation of both experiments. 100 value refers to complete inhibition with no mycelial growth. Significance differences ($p \leq 0.05$) found using the Student-Newman-Keuls are indicated as letters $^{a-e}$, where the letter a (not reported in the table) refers to the respective control range. Value followed by the same letter within the same row do not differ significantly.

	%MGI				
ORGANISM	**TO 0.05%**	**TO 0.1%**	**TO 0.25%**	**TO 0.5%**	**TO 1%**
F. graminearum **	16^{b}	28^{c}	91^{d}	100^{e}	100^{e}
F. culmorum *	-5^{b}	29^{c}	80^{d}	99^{e}	100^{e}
*C. lindemuthianum**	17^{b}	69^{c}	100^{d}	100^{d}	100^{d}
A. rabiei *	26^{b}	47^{c}	96^{d}	100^{d}	100^{d}
A. dauci *	9^{b}	28^{c}	83^{d}	100^{e}	100^{e}
*A. radicina**	-30^{b}	-10^{b}	74^{c}	98^{d}	100^{d}
D. avenae *	34^{b}	63^{c}	100^{d}	100^{d}	100^{d}

Table III. Minimum inhibitory concentration (MIC) of thyme oil (TO) and tea tree oil (TTO) at the 7th (*) and the 5th (**) day after inoculation

ORGANISM	**TO MIC**	**TTO MIC**
F. graminearum **	<0.05%	0.19%
F. culmorum *	<0.05%	0.31%
*C. lindemuthianum**	<0.05%	0.11%
A. rabiei *	<0.05%	0.14%
A. dauci *	<0.05%	0.20%
*A. radicina**	<0.05%	0.40%
D. avenae *	<0.05%	0.08%

oil against some phytopatogenic fungi like *Botryosphaeria rhodina, Rhizoctonia* sp. and *Alternaria* sp.; Feng and Zheng (16) studied the antifungal activity of essential oils of five plants (thyme, sage, nutmeg, eucaptus and cassia) against *A. alternata in vitro* and *in vivo*. Terzi and collegues (4) studied the *in vitro* antifungal activity of the tea tree essential oil and its major components against some cereals pathogen fungi as *Blumeria graminis f. sp. hordei*, *F. culmorum, F. graminearum, Pyrenophora graminea*. About the latter, the used TTO was proved to be effective against the fungi tested, in particular *F. culmorum* and *F. graminearum*, but with concentrations higher than those proved in our work. Being the experimental methods the same, this could be explained by the different TTOs available in the market. Nevertheless the oil sold as TTO is regulated by an international standard for "Oil of *Melaleuca*—terpinen-4-ol type," (17), which states the proportions of the oil components, like 30-48% for terpinen-4-ol and 10-28% for γ-terpinene. So the large naturally variation in oil chemical composition can affect the microbiological activities through the different concentrations of the active components and their interaction (18).

In conclusion, the examination of thyme and tea tree oils in this study showed promising prospects for the utilization of natural plant essential oils as a potential source of sustainable eco-friendly botanical fungicides, on the basis of their efficacy on different types of plant pathogens and their low cost and easy availability. Seed treatment against seed-born fungi could be one of this potentially use. However, because the *in vitro* effects did not always provide a good criterion for their *in vivo* performances, additional studies are necessary to verify the effectiveness in field conditions as seed treatment and their possible phytotoxicity on plant/seed material.

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