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# Antifungal potential of thyme essential oil as a preservative for storage of wheat seeds

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**Abstract –** Plant essential oils are potential food preservatives due to their inhibitory effects on bacterial and fungal growth. Antifungal activities of common thyme (*Thymus vulgaris*) essential oil were tested against endophytic fungi grown from wheat (*Triticum aestivum*) grain, molecularly identified as *Alternaria alternata, Alternaria infectoria, Aspergillus flavus, Epicoccum nigrum* and *Fusarium poae*. Their susceptibility to thyme essential oil was tested *in vitro*, and ranged from fungicidal to fungistatic. Treatment combinations of prior grain surface sterilization with hypochlorite and direct/indirect treatment with the essential oil were used, which showed strong effects on infection incidence and germination. Direct soaking of the wheat grain in the essential oil was particularly effective, but inhibited both fungal growth and seed germination. In contrast, indirect treatment of the grain with the essential oil (i.e., fumigation) inhibited fungal growth without negative effects on seed germination. In combination with grain surface sterilization with hypochlorite, indirect treatment with thyme essential oil reduced these fungal infections even more. Since thyme essential oil is safe for plants and consumers, in the form of fumigation it could be used as a protectant of storage containers for wheat grain intended for sowing and for food production.

**Keywords:** antifungal activity, essential oil, seed spoilage, *Thymus vulgaris*, wheat

## Introduction

Wheat is an important cereal crop that can be attacked by a number of fungi. From seed germination to harvest, soil-borne and seed-borne diseases can reduce the vigour and yield of wheat plants (Soliman and Badeaa 2002). Several wheat-associated fungi are carried from the field with the harvested grain, and can spread further during post-harvest storage, and can reduce their later germination (Özer 2005, Perelló and Larrán 2013, Rajput et al. 2005). Among these fungi, *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. are known to have negative impacts on grain during storage, which can result in serious risk for animal and human health through fungal production of a range of mycotoxins (D'Mello et al. 1998). Furthermore, infected grain and grain transmission represent a primary source of infection in the field, from which these fungi can spread to broader areas, which can potentially lead to fungal epidemics (Perelló and Larrán 2013).

Treatment of cereal plants with synthetic fungicides in the field before harvest has resulted in fungal resistance to antifungal agents, and it can often be problematic due to the high toxicity of fungicide residues to mammals (Chen et al. 2008). Post-harvest synthetic fungicide treatment of stored grain can influence the quality of cereals and can involve serious health hazards for consumers (Osman and Abdulrahman 2003). Therefore considerable interest has developed in recent years in the preservation of grain using more consumer- and nature-friendly protectants.

Plants contain a broad spectrum of antimicrobially active compounds that fit into this category and show effective inhibition of fungal growth. Essential oils are posed to become an important seed-decontamination alternative to synthetic seed preservatives, because in addition to strong fungicidal effects (Krisch et al. 2011) they are biodegradable and show low toxicity to humans and animals (Krisch et al. 2011, Sivakumar and Bautista-Baños 2014) In recent years, essential oils from different plants have been used to prevent fungal growth and the consequent mycotoxin accumulation in cereals (Batish et al. 2006, Sumalan et al. 2013). In particular, oils from aromatic and spice plants have been applied, because of their safety and their common use in the food industry for centuries.

Many studies have documented the antimicrobial activities of *Thymus* spp. essential oils and extracts (Bouzidi et al. 2013, Gonçalves et al. 2010, Soković et al. 2009, Solomakos et al. 2008). In a previous study, we reported that the

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essential oil from common thyme (*Thymus vulgaris*) shows promising antibacterial and antifungal activities against foodborne isolates of a *Fusarium* sp. and *Armillaria mellea*, as model fungi from Basidiomycetes and Ascomycetes, respectively (Anžlovar et al. 2014).

The main aim of the present study was to determine the antifungal activities and potential use of common thyme essential oil as a disinfectant against fungi grown on wheat grain to be used for sowing or germination. We hypothesised that treatment with thyme essential oil will decrease the infection rate of the stored wheat grain, although high concentrations might also have negative impact on germination of the treated grain. To test our hypothesis, we: (i) isolated fungal endophytes from wheat grain and tested the effectiveness of thyme essential oil on their radial growth *in vitro*; (ii) evaluated the effectiveness of the thyme essential oil on fungal infection rates of wheat grain; and (iii) tested for potential negative impact of the essential oil treatments on germination of the treated wheat grain. Different concentrations of thyme essential oil were tested, as well as in combination with initial surface sterilization of the grain with hypochlorite, to define the best possible antifungal treatments for the wheat grain, for effective real-life treatments.

## Materials and methods

### Preparation of thyme essential oil

Seedlings of common thyme (*Thymus vulgaris* L. cv. 'Deutcher winter') were grown from seeds under greenhouse conditions and transplanted to the experimental field of the Biotechnical Faculty (University of Ljubljana, Slovenia: 46°2'53.7''N, 14°28'30.47''E; altitude, 292 m a.s.l.) in March 2010. The plantation was regularly cultivated and was grown in the experimental field until October 2010, when the non-flowering shoots were harvested and processed to extract the essential oil. The thyme essential oil was isolated by water-steam distillation using a Clevenger apparatus (Anžlovar et al. 2014), following the procedures of the European Pharmacopoeia monograph on *Thymi herba*.

### Isolation and molecular identification of fungi from wheat grain

Wheat grain with developed fungal colonies on their surface were left in the dark at 20 °C until a single colony was large enough for transfer to fresh potato dextrose agar (PDA) medium (Biolife). The cultures are deposited in the fungal bank of the Plant Physiology Laboratory at the Department of Biology (Biotechnical Faculty, University in Ljubljana, Slovenia), under accession numbers KP271953-KP271958.

The fungal mycelia were ground in liquid nitrogen and the DNA was extracted using GeneElute Plant Genomic DNA miniprep kits (Sigma), following the manufacturer instructions. All of the PCR reactions were carried out in a thermal cycler (MJ Research) using Taq DNA polymerase (Promega). The 25-µl reaction mixture contained: 2.5 µl 10× PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each nucleotide, 500 nM of each primer, 0.75 U DNA polymerase, and 12.5 µl 100-fold–diluted template. The PCR conditions for the ITS1F-ITS4 primer pairs (Gardes and Bruns, 1993, White et al., 1990) were: 1 min at 94 °C, followed by 35 cycles of 45 s denaturation at 94 °C, followed by 53 s annealing at 55 °C, and 30 s elongation at 72 °C. The elongation step was at 72 °C for 10 min. The PCR amplicons were cleaned and sequenced by Macrogen (The Netherlands), using the ITS1F and ITS4 primers. The nucleotide sequences obtained were subjected to BLAST analysis, to determine their homology with other sequences available in the GenBank database.

### Fungal growth inhibition assay

The inhibitory effect of the thyme essential oil on radial growth of fungal mycelia was tested using the agar dilution method, as described by Zabka et al. (2009). The concentrated essential oil was added to the autoclaved PDA medium (medium temperature, ~50 °C) to prepare final essential oil concentrations of 0.05%, 0.01%, 0.005% and 0.0025% ($V_{essential\ oil}/V_{medium}$).

Fungal mycelia disks (diameter, 5 mm) were cut from the margin of the 7-day-old original cultures and aseptically inoculated by placing them in the centre of the medium with the essential oil in a Petri dish (diameter, 90 mm). The control samples were prepared at the same time, but without the essential oil. The fungal colonies were incubated at 23 °C in the dark for 7 days, each experiment being carried out twice.

Inhibition of fungal radial growth was calculated according to the following formula:

$$\text{Inhibition (\%)} = (DC - DT) / DC \times 100$$

where DC is the diameter of the control colonies, and DT is the diameter of the treated colonies. To evaluate the responses of the fungal isolates to the thyme essential oil and to calculate the 50% effective concentration ($EC_{50}$) and 90% effective concentration ($EC_{90}$), the DRC software package, version 2.3, was used (Ritz and Streibig 2005).

To determine the nature of the essential oil toxicity, the inhibited fungal disks grown on the medium with 0.05% thyme essential oil were re-inoculated onto fresh medium without essential oil, and the revival of the fungal growth was assessed after 7 days. The essential oil toxicity was designated as fungistatic when the fungal colonies grew again, and fungicidal when there was no growth of the fungal colonies (Kumar et al. 2008).

### Essential oil treatment of wheat grain

In the present study, two types of wheat (*Triticum aestivum* L.) grain were used: conventionally grown grain obtained from the Agricultural Institute of Slovenia (*T. aestivum* cv. 'Savinja'; designated as 'conventional grain' hereafter), and grain from the ecological farm Vila Natura (Slovenia, Prlekija, Vučja vas; designated as 'eco-grain' hereafter). Each of the grain types was divided in two groups: one group was left without any disinfection of the grain (non-sterile grain), while the other group was sterilized by soak-

ing in 3% hypochlorite for 3 min, to eliminate saprophytic microorganisms on the grain surface, followed by washing four times in sterilized distilled water (sterile grain).

The non-sterile and sterile wheat grains (10 g) were treated with the thyme essential oil, with each treatment repeated twice. Three concentrations of the thyme essential oil were prepared, as 2%, 0.2% and 0.05% by dilution of the essential oil in 10% dimethylsulphoxide (DMSO) in distilled water. The grain was treated with the thyme essential oil according to two protocols: (i) direct treatment, where the grain was submerged and incubated in the essential oil; and (ii) indirect treatment, where sterile Whatman filter paper was placed into the inner side of the top of the Petri dishes and impregnated with 4 ml of essential oil suspension, according to concentration (i.e., exposure to the essential oil vapour only). The grains were distributed on the bottom of each Petri dish, which were then sealed with parafilm. For these essential oil treatments, the grains were incubated under shaking (50 rpm) for 24 h at 25 °C. The control grains were soaked in the vehicle, as 10% DMSO, or exposed to the 10% DMSO vapours, for direct and indirect exposure controls, respectively.

### Germination and fungal contamination of wheat grain

The assessments of the fungal infection and seed germination were performed by the direct plating method (Sumalan et al. 2013). Ten subsamples (where each subsample contained 10 wheat grains) from each previously described treatment were placed on 2% PDA in Petri dishes (diameter, 90 mm) and incubated at 25±2 °C in the dark. After 72 h, the development of fungal colonies on the surface of the wheat grain was determined, with the seed contamination index calculated according to Doolotkeldieva (2010). At the same time, the germination of the wheat grain was determined by counting the number of germinated grains, expressed as a percentage of all grains used under each condition.

### Statistical analysis

All of the statistical analyses were performed with the software package R 3.1.2 (http://cran.r-project.org). The concentration/response analysis for growth of fungal isolates was performed using the drc package (v2.5-12) and a four-parameter log-logistic function. The effects of the seed type, prior sterilization, essential oil treatments, and essential oil concentrations on the fungal infection rates and the germination rates of the wheat grain were tested using multiway-ANOVA, with the level of significance set at $p<0.05$. All post-hoc comparisons were performed using Holm-Sidak post-hoc tests, with the level of significance set at $p<0.05$.

## Results

### Fungal isolation and growth inhibition tests

Five fungal species were isolated from the wheat grain: *Alternaria alternata* (2 strains)*, Alternaria infectoria* (*=Lewia infectoria*)*, Aspergillus flavus, Epicoccum nigrum*, and *Fusarium poae* (Tab. 1). The majority of these fungal endophytes were saprophytes, although *F. poae* is considered a plant pathogen. All of these fungal species were isolated from conventional grain as well as eco-grain, although the level of fungal infection was higher for eco-grain.

The potential of the thyme essential oil as an antifungal preservative was tested against all of the isolated fungal endophytes (Fig. 1). The thyme essential oil showed *in vivo* fungitoxicity against all of the tested fungi, with *A. infectoria, E. nigrum* and *F. poae* showing the lowest $EC_{50}$ and $EC_{90}$ values. The highest tolerance to the thyme essential oil was for *A. flavus*, with an $EC_{50}$ of 0.010% and an $EC_{90}$ of 0.018% ($V_{essential\ oil}/V_{medium}$).

After re-inoculation, the nature of the essential oil toxicity was determined as fungicidal for *A. alternaria, E. nigrum* and *F. poae*, and fungistatic for *A. infectoria* and *A. flavus* (Tab. 1).

### Infection and germination of wheat grain after essential oil treatments

The conventional grain and eco-grain were subjected to four treatment combinations, without and with surface sterilization with 3% hypochlorite, and direct and indirect exposure to the thyme essential oil, which yielded similar inhibitory effects on the fungal infection rates and different effects on the germination of the wheat grain (Fig. 2, Tab. 2).

The thyme essential oil significantly reduced the fungal infection of the wheat grain under all of the treatment combinations (Fig. 2A). Several factors affected the infection rates of the wheat grain (Tab. 3), including the source of the grain (conventional grain vs. eco-grain: $F=59288$, $p<0.0001$).

**Tab. 1.** Details of the fungal endophytes isolated from the wheat grain. E-value – expectation value. End – endophyte, Sapro – saprophyte, Path – pathogen.

| Isolate No. | Accession No. | Nearest match | | E-value | Maximum identity (%) | Putative ecological niche | Essential oil toxicity |
|---|---|---|---|---|---|---|---|
| 9C | KP271953 | KJ739880 | *Alternaria alternata* | 0.0 | 99 | End/Sapro | Fungicidal |
| 11C | KP271955 | JF440581 | *Alternaria alternata* | 0.0 | 100 | End/Sapro | Fungicidal |
| 13C | KP271957 | GU584953 | *Alternaria infectoria* | 0.0 | 99 | End/Sapro | Fungistatic |
| 12C | KP271956 | JX164075 | *Aspergillus flavus* | 0.0 | 99 | Sapro/Path | Fungistatic |
| 10C | KP271954 | JQ781728 | *Epicoccum nigrum* | 0.0 | 100 | Sapro | Fungicidal |
| 14C | KP271958 | JQ912669 | *Fusarium poae* | 0.0 | 99 | Path | Fungicidal |

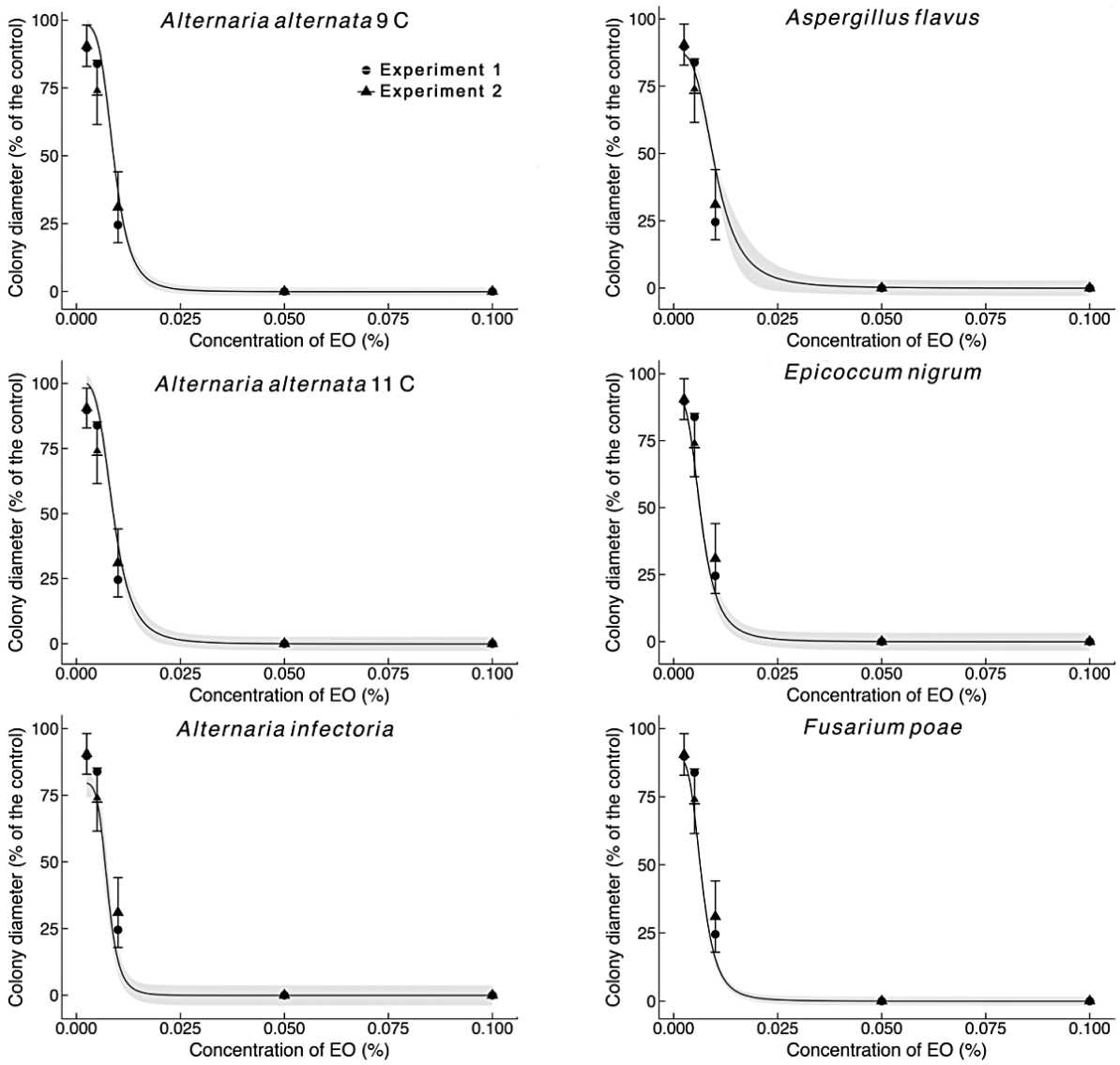


**Fig. 1.** Fungal growth–response curves in the presence of thyme essential oil (EO) in the growth media. Data are means±standard errors of two independent experiments (● experiment 1, ▲ experiment 2) with three individual measurements (n=6). A 95% confidence interval is shown in grey.

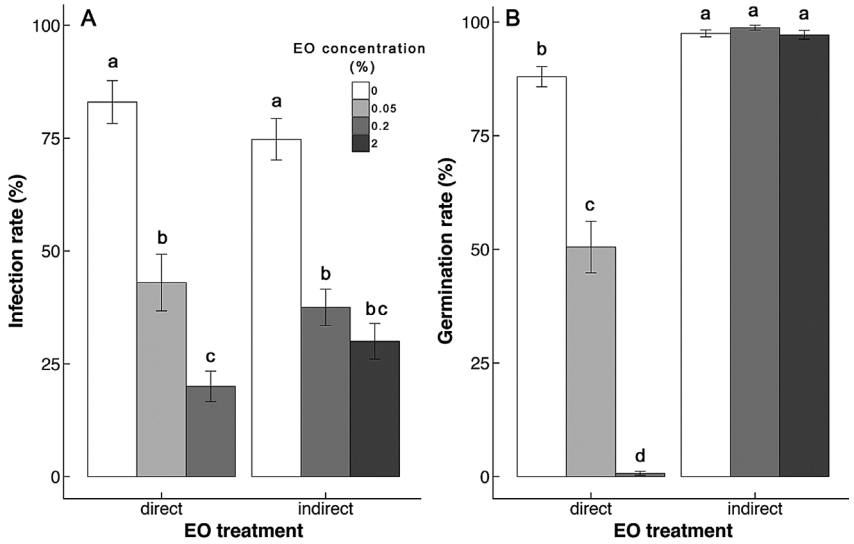


**Fig. 2.** Infection rates (A) and germination rates (B) of the wheat grain according to the concentrations and treatments with thyme essential oil (EO). Data are means±standard errors (n=40). Data with different letters are significantly different between treatments (Holm-Sidak post hoc tests, $p<0.05$).

**Tab 2.** Infection and germination rates of wheat grain after combinations of seed sources, surface sterilization and essential oil (EO) treatments. Data are means±standard errors (n=10).

| Grain source | Prior grain sterilization | EO treatment | EO concentration (%) | Infection rate (%) | Germination rate (%) |
|---|---|---|---|---|---|
| Conventional | Non-sterile | Direct | 0 | 100±0.00 | 97±1.53 |
| | | | 0.05 | 27±4.73 | 52±6.63 |
| | | | 0.2 | 15±4.53 | 0±0.00 |
| | | Indirect | 0 | 99±1.00 | 100±0.00 |
| | | | 0.2 | 38±7.57 | 99±1.00 |
| | | | 2 | 31±5.26 | 98±1.33 |
| | Sterile | Direct | 0 | 60±13.74 | 99±1.00 |
| | | | 0.05 | 3±1.53 | 2±1.33 |
| | | | 0.2 | 7±2.13 | 0±0.00 |
| | | Indirect | 0 | 37±4.23 | 97±1.53 |
| | | | 0.2 | 11±3.15 | 99±1.00 |
| | | | 2 | 2±1.47 | 97±1.47 |
| Ecological | Non-sterile | Direct | 0 | 100±0.00 | 70±3.33 |
| | | | 0.05 | 85±6.37 | 83±3.67 |
| | | | 0.2 | 47±5.78 | 3±1.53 |
| | | Indirect | 0 | 100±0.00 | 96±2.21 |
| | | | 0.2 | 69±4.07 | 98±1.33 |
| | | | 2 | 61±4.82 | 98±1.33 |
| | Sterile | Direct | 0 | 72±8.00 | 86±3.71 |
| | | | 0.05 | 57±13.75 | 65±9.69 |
| | | | 0.2 | 11±5.04 | 0±0.00 |
| | | Indirect | 0 | 63±6.33 | 97±1.53 |
| | | | 0.2 | 32±2.91 | 99±1.00 |
| | | | 2 | 23±3.67 | 95±3.07 |

In addition, the surface sterilization of the seeds prior to essential oil exposure also reduced the infection rates of the seeds (non-sterile vs. sterile: F=111096, p<0.0001). The inhibition of fungal growth depended on the essential oil concentrations (0.05% vs. 0.2% vs. 2%; F=53974, p<0.0001) and the concentrations of the essential oil showed interactions with the treatment methods (direct vs. indirect: F= 102801, p<0.0001), with the direct essential oil treatment showing greater reduction of the infection rate than the indirect essential oil treatment (Tab. 2).

On the other hand, the germination rates of the grain showed different susceptibility to these essential oil treatments. Direct treatment with the essential oil (i.e., submergence of grain in the essential oil) significantly reduced the germination rate of the grain, while the indirect treatment did not affect the germination rate, which remained at the same level as for the control treatment (Fig. 2B). When compared to the above-described fungal infection rates, more of the factors affected the germination rates of the seeds (Tab. 4). In addition to the essential oil treatments (i.e., method and concentrations), the source of seeds was also an important factor (conventional grain vs. eco-grain; F=4212, p=0.041).

As previously indicated, prior surface sterilization significantly contributed to the reduction of the fungal infection rates (Tab. 2). Both the conventional grain and the eco-grain were similarly contaminated with fungi when no sterilization was applied (99%, 100%, respectively) (Tab. 2). The surface sterilization without the essential oil treatments significantly reduced the fungal contamination (non-sterile vs. sterile; F=111096, p<0.0001), which on average dropped to 58% (Tab. 2). The additional direct and indirect treatments with thyme essential oil further reduced the fungal contamination of the wheat grain (Tab. 2). The direct treatment was generally more effective than the indirect

**Tab. 3.** ANOVA for the differences in the infection rates among the grain sources, sterilization, and essential oil treatments and concentrations. Df – degrees of freedom, SS – sum of squares, MS – mean square.

| Factor | Df | SS | MS | F-ratio | p |
|---|---|---|---|---|---|
| **Source** | **1.00** | **34031** | **34031** | **59288** | **<0.0001** |
| **Sterilization** | **1.00** | **63769** | **63769** | **111096** | **<0.0001** |
| Treatment (essential oil) | 1.00 | 108 | 108 | 0.19 | 0.666 |
| **Concentration** (essential oil) | **1.00** | **30981** | **30981** | **53974** | **<0.0001** |
| Source: sterilization | 1.00 | 77 | 77 | 0.14 | 0.714 |
| Source: treatment | 1.00 | 368 | 368 | 0.64 | 0.424 |
| Sterilization: treatment | 1.00 | 1835 | 1835 | 3197 | 0.075 |
| Source: concentration | 1.00 | 307 | 307 | 0.53 | 0.466 |
| Sterilization: concentration | 1.00 | 559 | 559 | 0.97 | 0.325 |
| **Treatment: concentration** | **1.00** | **59008** | **59008** | **102801** | **<0.0001** |
| Source: sterilization: treatment | 1.00 | 285 | 285 | 0.50 | 0.482 |
| Source: sterilization: concentration | 1.00 | 830 | 830 | 1445 | 0.231 |
| Source: treatment: concentration | 1.00 | 53 | 53 | 0.09 | 0.761 |
| Sterilization: treatment: concentration | 1.00 | 490 | 490 | 0.85 | 0.357 |
| Source: sterilization: treatment: concentration | 1.00 | 1752 | 1752 | 3053 | 0.082 |
| Residuals | 223 | 128001 | 574 | | |

**Tab. 4.** ANOVA for the evaluation of the differences in the germination rates according to the grain sources, sterilisation, and essential oil treatments and concentrations. Df – degrees of freedom, SS – sum of squares, MS – mean square.

| Factor | Df | SS | MS | F-ratio | p |
|---|---|---|---|---|---|
| **Source** | **1** | **1123** | **1123** | **4212** | **0.041** |
| **Sterilization** | **1** | **1507** | **1507** | **5655** | **0.018** |
| **Treatment** (essential oil) | **1** | **157609** | **157609** | **591348** | **<0.0001** |
| **Concentration** (essential oil) | **1** | **1703** | **1703** | **6390** | **0.012** |
| Source: sterilization | 1 | 894 | 894 | 3353 | 0.068 |
| **Source: treatment** | **1** | **1692** | **1692** | **6349** | **0.012** |
| Sterilization: treatment | 1 | 959 | 959 | 3597 | 0.059 |
| Source: concentration | 1 | 2 | 2 | 0.01 | 0.931 |
| Sterilization: concentration | 1 | 14 | 14 | 0.05 | 0.820 |
| **Treatment: concentration** | **1** | **145536** | **145536** | **546050** | **<0.0001** |
| Source: sterilization: treatment | 1 | 687 | 687 | 2576 | 0.110 |
| Source: sterilization: concentration | 1 | 93 | 93 | 0.35 | 0.555 |
| Source: treatment: concentration | 1 | 35 | 35 | 0.13 | 0.719 |
| Sterilization: treatment: concentration | 1 | 23 | 23 | 0.09 | 0.771 |
| Source: sterilization: treatment: concentration | 1 | 727 | 727 | 2726 | 0.100 |
| Residuals | 223 | 59435 | 267 | | |

treatment, and the highest fungal inhibition was seen for 0.2% thyme essential oil directly applied to the wheat grain. Further comparisons of these treatments showed that the indirect essential oil treatment had similar antifungal effects to the surface sterilization with hypochlorite (Tab. 2). With the conventional grain, the fungal infection rate was 31% when the grain were fumigated (i.e., indirect treatment) with 2% essential oil, and 37% when only surface sterilization was used. For the eco-seeds, the corresponding values were 61% and 63% (Tab. 2). Also, direct essential oil treatments resulted in range of fungal inhibition comparable to indirect essential oil treatments but at ~one-tenth of the concentrations used (e.g., 7% infection with direct treatment of the grain with 0.2% essential oil, and 2% infection with indirect treatment of the grain with 2% essential oil).

The combined effects of the surface sterilization with hypochlorite and the indirect treatment with the thyme essential oil had no effects on seed germination, but provided greatly decreased fungal infection, thus retaining the benefits of both of these treatments (Fig. 3).

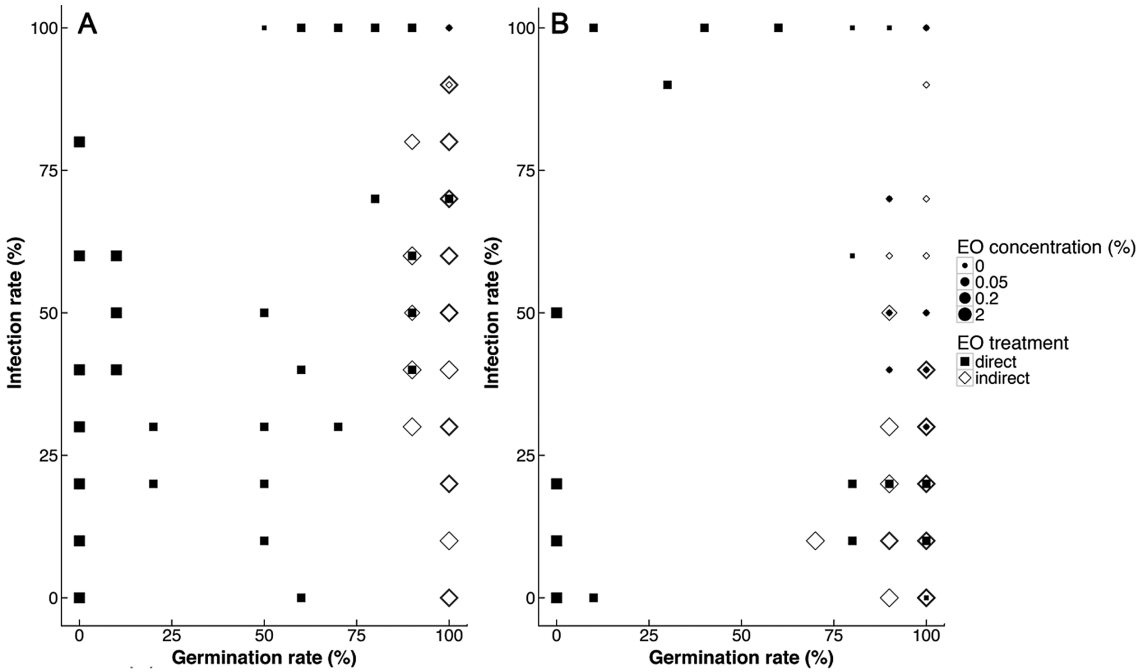


**Fig. 3.** Correlations between infection and germination rates for the non-sterile (A) and sterile (B) wheat grain according to the concentrations and treatments with thyme essential oil (EO). The sizes of the symbols depict the essential oil concentrations. Black squares denote direct essential oil treatment; white diamonds denote indirect essential oil treatment.

## Discussion

During plant growth as well as after harvest and during storage, wheat and other cereal grains are exposed to fungal colonisation. The use of such colonised seed for future sowing only exacerbates the problems of fungal pathogens and requires the application of fungicides. As essential oils from spice herbs are known to be safe for animal and human health, they represent a possible source for use in the decontamination and storage of grain that is intended not only for sowing, but also for food production (e.g., flour and its products, sprouts, dietary supplements).

In the present study, five indigenous fungal species were isolated from wheat grain and identified using molecular methods: *A. alternata*, *A. infectoria, A. flavus, E. nigrum* and *F. poae* were isolated from the grain from wheat grown in conventionally managed fields and in fields with sustainable eco-management. With the exception of *A. flavus*, these species appear to be common colonisers of wheat grain (Nicolaisen et al. 2014). However, in contrast to Nicolaisen et al. (2014), who identified the genera *Phaeosphearia* and *Microdochium* as the core operational taxonomic units in 99% of wheat grain samples, we did not isolate any fungi from these genera. In addition to *Fusarium* spp. and *Alternaria* spp., which are both known as soil fungi, the so-called storage fungi of *Penicillium* spp., *Aspergillus* spp. and *Rhizopus* spp. have also been reported for wheat grain (Bensassi et al. 2011, Bottalico and Perrone 2002, Gohari et al. 2007). In the present study, only *A. flavus* was isolated, which suggests the potential contamination of the grain with aflatoxins, which would negatively impact on the usability of the grain for animal and human consumption. Accordingly, the fungitoxic effects of thyme essential oil on the growth of *A. flavus* might prove to be extremely beneficial, as it would reduce the spoilage of food due to contamination with fungal toxins.

As post-harvest synthetic fungicide treatments of stored products can influence the quality of the grain (Osman and Abdulrahman 2003), natural essential oils have advantages over synthetic agents due to their biodegradability and low toxicity. In the present study, *in vitro* thyme essential oil showed a broad spectrum of fungitoxicity against all of the fungi isolated from the wheat grain. The $EC_{90}$ of the thyme essential oil was < 0.02% for all of these tested fungi, with a mainly fungicidal nature of the toxicity. The $EC_{90}$ for thyme essential oil against the five fungi in this study fit well within ranges of values reported in other studies, where the fungitoxicity of thyme essential oil has been given as between 0.025% (Soliman et al. 2002) and 0.07% (Kumar et al. 2008), although Zabka et al. (2009) reported a higher MIC for *A. flavus* (0.23%) and a *Fusarium* sp. (~0.15%). The antifungal effects of thyme essential oil can be explained by modifications of fungal morphogenesis and growth through interference of the essential oil components with the fungal enzymes that are responsible for cell wall synthesis, which will lead to changes in hyphae integrity and to plasma membrane disruption and mitochondrial destruction (Rassoli et al. 2006). The antifungal activities of essential oils are generally strongly associated with monoterpenic phenols, and especially thymol, carvacrol and eugenol (Bluma et al. 2008). Both thymol and carvacrol are also present in thyme essential oil and they are characterised by their high antimicrobial activity (Bouzidi et al. 2013, and references therein, Gonçalves et al. 2010, Sokovič et al. 2009, Šegvić Klarić et al. 2007). Chemical analysis of the thyme essential oil used in the present study has shown that the major constituent is indeed thymol (68.9%), whereas carvacrol constitutes only 1.6% (Anžlovar et al. 2014).

Due to the possible negative effects of essential oils on seed germination, we examined the effects of this thyme essential oil treatment on seed germination. Our results show that treatment with thyme essential oil is effective for the reduction of fungal contamination of wheat grain, although it can also affect the germination of the treated grain, which is an important aspect for the potential use of these treated grains. Direct treatment with thyme essential oil significantly inhibited seed germination, and so despite the good reduction in infection seen, this treatment is less appropriate as a prevention method for stored grain. In contrast, the indirect essential oil treatment had no effects on the germination of these wheat grains, but did successfully inhibit fungal growth, which was even more pronounced in combination with the prior surface hypochlorite sterilization that was also used here. Our results are in agreement with observations from several studies that have reported negative effects of direct essential oil treatments on seed germination (Kordali et al. 2008, Kotan et al. 2013, Paudel and Gupta 2008). In contrast, Kedia et al. (2014) demonstrated that wheat grain that has been indirectly fumigated with cumin seed essential oil retain viability even 12 months after storage. Accordingly, in combination with other studies, our results indicate that indirect treatment (i.e., fumigation) with thyme essential oil has great potential as a bioprotection technique. We also show that the combination of surface sterilization and indirect thyme essential oil treatment further diminishes fungal infection without negative effects on the germination of the treated wheat grain.

To conclude, fumigation with thyme essential oil shows a good *in vivo* efficacy for the protection of wheat grain from fungal colonisers. Indirect treatment with thyme essential oil in combination with prior surface sterilization of wheat grain provided an even more efficient treatment that significantly reduced fungal infection and spread on these grain and was accompanied by retention of high germination rates of the treated seeds. For the sake of safety for plants and consumers, thyme essential oil can be applied as a protection agent in storage containers for grain intended for sowing and food production.

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