

## Article

# Screening and Evaluation of Essential Oils from Mediterranean Aromatic Plants against the Mushroom Cobweb Disease, *Cladobotryum mycophilum*

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**Abstract:** The main aim of this study was to evaluate the use of essential oils (EOs) as an alternative to synthetic fungicides used in the control of cobweb disease of button mushroom (*Agaricus bisporus*) caused by *Cladobotryum mycophilum*. The EOs used were obtained by hydrodistillation from five Mediterranean aromatic species (*Lavandula × intermedia*, *Salvia lavandulifolia*, *Satureja montana*, *Thymus mastichina*, and *Thymus vulgaris*), analyzed by gas chromatography, and tested in vitro for their antifungal activity against *C. mycophilum*. In vitro bioassays showed that the EOs obtained from *T. vulgaris* and *S. montana* (ED<sub>50</sub> = 35.5 and 42.8 mg L<sup>-1</sup>, respectively) were the most effective EOs for inhibiting the mycelial growth of *C. mycophilum*, and were also the most selective EOs between *C. mycophilum* and *A. bisporus*. The in vivo efficacy of *T. vulgaris* and *S. montana* EOs at two different concentrations (0.5 and 1%) were evaluated in two mushroom growing trials with *C. mycophilum* inoculation. The treatments involving *T. vulgaris* and *S. montana* EOs at the higher dose (1% concentration) were as effective as fungicide treatment. The effect of these EOs on mushroom productivity was tested in a mushroom cropping trial without inoculation. They had a strong fungitoxic effect at the first flush. However, a compensatory effect was observed by the end of the crop cycle and no differences were observed in biological efficiency between treatments. The main compounds found were carvacrol and *p*-cymene for *S. montana*, and *p*-cymene and thymol for *T. vulgaris*. These results suggest that *T. vulgaris* and *S. montana* EOs may be useful products to manage cobweb disease if used as part of an integrated pest management (IPM) program.

**Keywords:** *Agaricus bisporus*; antifungal activity; biofungicides; mushroom cultivation; *Satureja montana*; *Thymus vulgaris*

## 1. Introduction

Cobweb disease, caused by the mycopathogenic fungus *Cladobotryum mycophilum* (Oudem.) W. Gams & Hooz., is found in all mushroom-growing countries worldwide. The disease is prevalent especially in white button mushroom (*Agaricus bisporus* (Lange) Imbach) [1–5], king oyster mushroom (*Pleurotus eryngii* (DC.:Fr.) Quél.) [6–8], and oyster mushroom (*Pleurotus ostreatus* (Jacq.) P. Kumm.) [9]. Cobweb disease is associated with final flushes and conditioned by the season. The

pathogen develops into a cottony growth of mycelium, colored grey to white, which spreads over the casing layer and mushrooms. Cobweb mycelium becomes dense and sometimes develops a rosy red or yellow hue. The pathogen may grow over primordia and mushrooms, affecting the salability of the product [10]. Methods to control cobweb include strict hygiene measures to prevent dispersion of the conidia and the application of fungicides, mainly prochloraz and metrafenone [11–13]. However, the use of conventional chemical fungicides is gradually being limited due to its negative effects on human health, environmental safety, pathogen resistance, etc. Therefore, improvements in the hygiene conditions of farming facilities, as well as a better understanding of the new, environment-friendly, and effective control methods are necessary to minimize the use of chemicals in the mushroom industry.

In order to develop alternatives to chemical agents within an integrated management strategy for cobweb disease, the possible use of essential oils (EOs) would present many advantages in terms of sustainability, low persistence in the environment, biological activity, mode of action, effectiveness, and toxicity. For many years, EOs have been studied as sources of natural antifungal products and they have been screened for their potential uses as alternative remedies for the treatment of many phytopathogenic fungi [14–16]. More specifically, some EOs have also been tested as control agents against the most important diseases of white button mushroom. The EOs from *Cinnamomum zeylanicum* Blume, *Origanum vulgare* L., *Syzygium aromaticum* (L.) Merr. & L.M. Perry, *Thymus vulgaris* L., *Zataria multiflora* Boiss, and *Satureja hortensis* L. showed strong in vitro activity against *Lecanicillium fungicola* var. *fungicola* (Preuss) Zare & W. Gams [17–19]; *Lippia citriodora* (Cav.) Kunth, *Cymbopogon citratus* (D.C) Stapf, and *T. vulgaris* EOs were found to substantially inhibit the growth of *Mycogone perniciosa* (Magnus) Delacroix [20]; and *S. aromaticum* EO also exhibited strong in vitro inhibition against *C. mycophilum* [21]. *Melaleuca alternifolia* (Maiden & Betchee) Cheel essential oil was less toxic in vitro than prochloraz-Mn against *Cladobotryum dendroides* (Bull.:Fr.) W. Gams & Hooz., although in in vivo trials it caused a significant reduction in cobweb disease levels compared with a control without fungicide [22]. Therefore, these studies suggest that EOs can be considered as suitable biocontrol agents for the control of mushroom diseases, particularly the cobweb disease.

Moreover, it must be borne in mind that the cultivation of aromatic plants is an important agricultural activity in many parts of Spain, especially in certain zones of Castilla–La Mancha, near the most important mushroom cultivation centres. Several preliminary studies focused on native Mediterranean aromatic species (*Lavandula* spp., *Thymus* spp., *Salvia lavandulifolia* Vahl, *Rosmarinus officinalis* L., *Santolina chamaecyparissus* L., *Hyssopus officinalis* L., and *Satureja montana* L.), adapted to local environmental conditions, have been carried out. In this respect, antifeedant, insecticidal, phytotoxic, nematocidal, antibacterial, and antifungal activities of EOs from the above-mentioned species have been reported [23,24]. However, many factors including the origin of the cultivar or variety, environment (climatic, seasonal, and geographical), type of plant material used, plant nutrition, application of fertilizers, and the method of extraction have influence not only in the identity of main compounds of an essential oil but also in their percentage, and thereby might affect its biocidal properties [25,26].

The aim of this study was to: (i) characterize the chemical composition of five EOs from Mediterranean aromatic plants belonging to the *Lamiaceae* family, such as *Lavandula × intermedia* Emeric ex Loiseleur, *Salvia lavandulifolia* Vahl, *Satureja montana* L., *Thymus mastichina* L., and *Thymus vulgaris* L.; (ii) determine the effectiveness of the above-mentioned EOs in cobweb control by in vitro antifungal activity bioassays and in vivo mushroom trials involving inoculation with *C. mycophilum*; and (iii) test the toxicity of these EOs to *A. bisporus* mycelium and/or mushroom yield.

## 2. Materials and Methods

### 2.1. Plant Material

The species used for EO extraction (*L. × intermedia* (lavandin), *S. lavandulifolia* (Spanish sage), *S. montana* (savory), *T. mastichina* (white thyme), and *T. vulgaris* (thyme)) were cultivated in the

experimental fields of CIAF Albaladejito-IRIAF (Cuenca, Spain). Harvests of aerial parts from plants (inflorescences, leaves, and stems) were made at the flowering phenological stage.

## 2.2. Essential Oil Extraction

The plant material was dried in an environmental chamber at 28 °C until a constant weight is obtained before extraction. Samples were hydrodistilled using a Clevenger-type apparatus for 4 hours according to the method recommended in the European Pharmacopoeia [27].

## 2.3. Components Identification and Quantification

The chemical components of the EO were identified by gas chromatography. GC-FID analyses were carried out using a Varian 400-GC gas chromatograph operated with a split/splitless injector (Varian Inc., Palo Alto, CA, USA). Column (non-polar): VF-5ms (cross-linked phenyl-methyl siloxane), 60 m × 0.25 mm × 0.25 µm film thickness (Varian Inc., Palo Alto, CA, USA). Temperature program: from 70–95 °C at 3 °C/min and from 95–240 °C (5 min) at 4 °C/min. Injection temperature: 250 °C. Injection volume: 0.5 µL. Carrier gas: He (1 mL/min). Injection mode: split (100:1). FID (300 °C): H<sub>2</sub> flow, 35 mL/min; air flow, 300 mL/min; and make up flow, 29 mL/min. Sampling rate: 50 ms. Relative retention times and Kovats Index of corresponding reference standards (Acros Organics BVBA/SPRL, Fisher Scientific S.A., and Sigma Aldrich Química A.) were used to identify the major components using the Adams library [28]. The relative amounts of individual components were calculated according to the area of the chromatographic peaks using Galaxie® Chromatography software (Varian Inc., Walnut Creek, CA, USA).

## 2.4. Fungal Isolates

Six *Cladobotryum mycophilum* isolates obtained from mushroom farms located in Castilla–La Mancha and La Rioja (Spain) were used in vitro to test for antifungal activity and selectivity index of five EOs. Isolates were collected between 2015 and 2017 from *Agaricus bisporus* crops with symptoms of cobweb disease, maintained on potato dextrose agar (PDA; Oxoid, Basingstoke, England) medium at 22 °C in darkness, and characterized morphologically and molecularly [10,29,30]. Two commercial mushroom strains (Mispaj 400 (Mispaj S.L., Pradejón, La Rioja, Spain) and Gurelan 90 (Gurelan S. Coop., Huarte, Pamplona, Spain)) were used to determine the toxic effect of the EOs on *A. bisporus* mycelium.

## 2.5. In Vitro Antifungal Activity and Selectivity Index of the Essential Oils on Mycelium Growth of *Cladobotryum Mycophilum* and *Agaricus bisporus*

The antifungal activities of the EOs against *C. mycophilum* and *A. bisporus* were calculated by in vitro sensitivity assays, with slight modifications [8,13]. The five EOs (containing 0.01% Tween 20) from each plant sample were added to molten sterile malt extract agar (MEA) medium at 45 °C prior to solidification. The EO concentrations selected for the *C. mycophilum* isolates were: *Lavandula × intermedia* (46, 92, 183, 366, and 732 mg L<sup>−1</sup>), *Thymus mastichina* (325, 650, 1300, 2600, and 5200 mg L<sup>−1</sup>), *Thymus vulgaris* (5, 9, 18, 36, and 72 mg L<sup>−1</sup>), *Salvia lavandulifolia* (83, 165, 330, 660, and 1320 mg L<sup>−1</sup>), and *Satureja montana* (10, 21, 41, 82, and 164 mg L<sup>−1</sup>). All EO-amended media were prepared a maximum of 24 h before use. Control consisted of unamended plates. Four replicates were used per combination of isolate and EO concentration. The experiment was repeated twice.

The percentage of relative inhibition (PI) of mycelial growth was calculated using the formula:  $PI = 100(C - T)/C$ , where *C* = rate of growth of the control and *T* = rate of growth in treated dishes). ED<sub>50</sub> values (mg L<sup>−1</sup> of essential oil inhibiting radial mycelial growth by 50%) were calculated using the data analysis software PoloPlus, Probit, and Logit Analysis (LeOra Software Company, Petaluma, CA, USA) [31].

The in vitro antifungal activity of these EOs on *A. bisporus* was evaluated using essential oil-amended agar-compost medium [32], following the methodology described for the pathogen. The studied EOs (containing 0.01% Tween 20) were added to produce final concentrations of 10, 100, 1000,

and 10,000 mgL<sup>-1</sup>. The unamended medium was used as a control. A mushroom spawn grain covered with actively growing mycelium of two commercial mushroom strains were placed on the medium and incubated for 10 days at 25 °C in the dark.

A selectivity index (SI) for each essential oil was calculated as the ratio between the mean ED<sub>50</sub> values for the *C. mycophilum* isolates tested and the corresponding mean ED<sub>50</sub> values were estimated for the two commercial *A. bisporus* strains. Selectivity indices lower than 0.1 express acceptable selective toxicity between the pathogen and the host [33].

ED<sub>50</sub> values were examined using analysis of variance (ANOVA). ED<sub>50</sub> values, where necessary, were transformed by log( $x + 1$ ) before analysis and were back-transformed for the tables. Mean comparisons were performed using the Fisher's LSD (least significant difference) test to examine if differences were significant at  $p < 0.05$ . Statistical analyses were made using Statgraphics Centurion XV (Statistical Graphics Corp., Princeton, NJ, USA).

## 2.6. Effectiveness of EOs Treatments in Mushroom Growing Trials with *Cladobotryum Mycophilum* Inoculation

The effectiveness of EOs to control cobweb disease was tested in two cropping trials (A and B) carried out in experimental mushroom growing rooms (20 m<sup>3</sup>), according to the standard practices used in mushroom farms in Spain. In each trial, experimental trays (0.15 m<sup>2</sup>) filled with 10 kg commercial phase III compost (Champinter SCL, Villamalea, Albacete, Spain) and spawned at 1% with the commercial mycelium Sylvan®A15 (Sylvan Inc., Kittanning, PA, USA) were used. The trays were arranged in shelves at three different levels in a completely randomized block design. Each trial was conducted using 42 trays, 36 of which were inoculated with *C. mycophilum*, while the remaining 6 trays were sprayed with water and used as uninoculated control. The 36 inoculated trays were divided into five treatments and an inoculated control, with six replicates per treatment.

On casing day (day 0) of the cropping cycle, the compost was cased with a peat-based casing as a 30–35 mm thick layer (5.5 L per experimental tray). The environmental conditions were: compost temperature of about 24–26 °C, 93–98% relative humidity (RH) with 8000–3200 ppm carbon dioxide (CO<sub>2</sub>). Inoculation was done eleven days after casing by watering 20 mL per tray of a conidial suspension of *C. mycophilum* (10<sup>6</sup> conidia m<sup>-2</sup>) onto the surface of the casing layer in the inoculated treatments. The non-inoculated control was sprayed with sterile distilled water. The *C. mycophilum* isolate CS1, collected in September 2016 in a mushroom farm from Casasimarro (Castilla-La Mancha, Spain), was used as an inoculum and prepared on the day of inoculation. The concentration of the conidial suspension was determined using a hemocytometer and diluted in sterile distilled water to 7.5 × 10<sup>3</sup> conidia mL<sup>-1</sup>.

Based on the in vitro antifungal activity results given in Table 3, the *Satureja montana* and *Thymus vulgaris* EOs were selected. Essentials oil formulations were diluted in tap water to reach 0.5% (trial A) and 1% (trial B) concentration (containing 0.01% Tween 20) and added as a single drench application, at a rate of 150 mL per tray. For each of the EOs selected, there were two treatments and six replicate trays per treatment. The first treatment (BI: before inoculation) was applied 1 day before inoculating with conidia of *C. mycophilum*, while the second treatment (AI: after inoculation) was applied 2 days after inoculation. There were three controls: first, a non-inoculated control (C), in which drench applications were 150 mL tap water per tray; second, an inoculated control (IC), each tray receiving a 20 mL conidial suspension of *C. mycophilum*, in which irrigation was by water alone; and third (IMFC), also inoculated, but including the fungicide Vivando® (metrafenone 50% SC, BASF Crop Protection Spain, Barcelona, Spain) at 1 mL L<sup>-1</sup> per m<sup>2</sup> on day 4, at a rate of 150 mL per tray (Table 1).

In each trial, three flushes of mushrooms were harvested. Disease incidence (DI) was calculated as a percentage value, based on the ratio of the crop area colonized by cobweb in each treatment to the total colonized and non-colonized crop area. The affected area by cobweb was left uncovered for 24 hours. After that, this area was covered with a piece of damp paper and salt to avoid pathogen dispersion [11,12]. The DI in the different trays was related to the uninoculated control disease incidence average using the Abbot's formula correction:

$$DI_{corr} = (DI_{exp} - DI_{cont}) / (1 - DI_{cont}),$$

where  $DI_{exp}$  = experimental treatment disease incidence, and  $DI_{cont}$  = mean uninoculated control disease incidence [34]. The effectiveness of EO treatments was calculated based on the DI using Abbott's formula [35]:

$$Effectiveness (\%) = [(DI_{in-cont} - DI_{corr}) / DI_{in-cont}] \times 100,$$

where  $DI_{in-cont}$  = disease incidence of the inoculated control and  $DI_{corr}$  = corrected disease incidence of the treatment.

The disease incidence and the effectiveness of treatments were examined using ANOVA. Mean comparisons were performed using the Fisher's LSD test to examine if differences were significant at  $p < 0.05$ . Statistical analyses were made using Statgraphics Centurion XV (Statistical Graphics Corp., Princeton, NJ, USA).

**Table 1.** Treatments applied in the mushroom crop trials.

| <b>Trials</b> | <b>Code</b> | <b>Inoculation<br/>(10<sup>6</sup> conidia m<sup>-2</sup>)<sup>a</sup></b> | <b>Treatment<sup>b</sup></b>                                                                                                    |
|---------------|-------------|----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------|
| A, B, and C   | C           | No                                                                         | Only tap water.                                                                                                                 |
| A and B       | IMFC        | Yes                                                                        | Vivando® at 1 ml L <sup>-1</sup> on day 4.                                                                                      |
| A and B       | IC          | Yes                                                                        | Only tap water.                                                                                                                 |
| A             | IEO1B       | Yes                                                                        | <i>Satureja montana</i> essential oil at 0.5% applied 1 day before inoculation with <i>Cladobotryum mycophilum</i> , on day 10. |
| A             | IEO1A       | Yes                                                                        | <i>Satureja montana</i> essential oil at 0.5% applied 2 days after inoculation with <i>Cladobotryum mycophilum</i> , on day 13. |
| A             | IEO3B       | Yes                                                                        | <i>Thymus vulgaris</i> essential oil at 0.5% applied 1 day before inoculation with <i>Cladobotryum mycophilum</i> , on day 10.  |
| A             | IEO3A       | Yes                                                                        | <i>Thymus vulgaris</i> essential oil at 0.5% applied 2 days after inoculation with <i>Cladobotryum mycophilum</i> , on day 13.  |
| B             | IEO1B       | Yes                                                                        | <i>Satureja montana</i> essential oil at 1% applied 1 day before inoculation with <i>Cladobotryum mycophilum</i> , on day 10.   |
| B             | IEO1A       | Yes                                                                        | <i>Satureja montana</i> essential oil at 1% applied 2 days after inoculation with <i>Cladobotryum mycophilum</i> , on day 13.   |
| B             | IEO3B       | Yes                                                                        | <i>Thymus vulgaris</i> essential oil at 1% applied 1 day before inoculation with <i>Cladobotryum mycophilum</i> , on day 10.    |
| B             | IEO3A       | Yes                                                                        | <i>Thymus vulgaris</i> essential oil at 1% applied 2 days after inoculation with <i>Cladobotryum mycophilum</i> , on day 13.    |
| C             | MFC         | No                                                                         | Vivando® at 1 mL L <sup>-1</sup> on day 4.                                                                                      |

|   |      |    |                                                                |
|---|------|----|----------------------------------------------------------------|
| C | EO1B | No | <i>Satureja montana</i> essential oil at 1% applied on day 10. |
| C | EO1A | No | <i>Satureja montana</i> essential oil at 1% applied on day 13. |
| C | EO3B | No | <i>Thymus vulgaris</i> essential oil at 1% applied on day 10.  |
| C | EO3A | No | <i>Thymus vulgaris</i> essential oil at 1% applied on day 13.  |

<sup>a</sup> Inoculation on day 11 after casing. <sup>b</sup> Dose per application: 1 L m<sup>-2</sup> for each drench, 150 mL per tray.

### 2.7. Effect of EOs Treatments on *Agaricus bisporus* Yield

The effect of essential oils on *A. bisporus* yield was determined in a third cropping trial (trial C). This trial was set up under conditions identical trial B. No conidial suspensions were applied in the treatments (Table 1). The experiment was a randomized complete block design with five replicates. The effect of essential oils and metrafenone treatments on *A. bisporus* yield was assessed by reference to daily recorded mushroom production (kg m<sup>-2</sup> per flush and total yield). The effect of treatments on mushroom productivity was evaluated by the biological efficiency of the crop and calculated as the percentage ratio of the fresh weight of total yield of harvested mushrooms over the weight of dry substrate at spawning and expressing the fraction as kg/100 kg compost. In addition, the earliness (days to first harvest for each treatment) was expressed as the number of days between casing and harvesting of the first flush.

An ANOVA was used to test for the effect of treatment and the different yield parameters. Mean comparisons were performed using the Fisher's LSD test to examine if differences were significant at  $p < 0.05$ . Data were analyzed separately for each flush. Statistical analyses were made using Statgraphics Centurion XV (Statistical Graphics Corp., Princeton, NJ, USA).

## 3. Results

### 3.1. Chemical Composition of the Essential Oils

The chemical composition of the main compounds of the five EOs is shown in Table 2. Linalyl acetate (34.20%) was the most abundant component, followed by linalool (28.75%), camphor (8.49%), and 1,8-cineole (6.90%), in *L. × intermedia* EO. The most abundant compound in *S. lavandulifolia* EO was 1,8-cineole (20.90%), followed by  $\beta$ -pinene +  $\beta$ -myrcene (13.99%), camphor (12.60%), and  $\alpha$ -pinene (11.39%). The main components of *S. montana* EO were carvacrol (17.22%), *p*-cymene (11.54%), and camphor (10.14%). In *T. mastichina* EO, 1,8-cineole (44.57%) and linalool (29.89%) were the major components, while the main components of *T. vulgaris* EO were *p*-cymene (29.67%) and thymol (25.78%).

**Table 2.** Percentages of the main compounds of the essential oils from *Lavandula × intermedia*, *Salvia lavandulifolia*, *Satureja montana*, *Thymus mastichina*, and *Thymus vulgaris*.

| Main compounds <sup>a</sup>        | KT <sup>b</sup> | RT <sup>c</sup> | <i>Lavandula × intermedia</i> | <i>Salvia lavandulifolia</i> | <i>Satureja montana</i> | <i>Thymus mastichina</i> | <i>Thymus vulgaris</i> |
|------------------------------------|-----------------|-----------------|-------------------------------|------------------------------|-------------------------|--------------------------|------------------------|
| $\alpha$ -Pinene                   | 943             | 11.40           | t <sup>d</sup>                | 11.39                        | 7.86                    | 2.33                     | 1.73                   |
| Camphene                           | 962             | 12.03           | t                             | 6.38                         | 3.63                    | t                        | t                      |
| $\beta$ -Pinene + $\beta$ -Myrcene | 991             | 12.97           | 1.22                          | 13.99                        | 8.40                    | 4.42                     | 2.68                   |

|                             |          |       |                |       |       |       |       |
|-----------------------------|----------|-------|----------------|-------|-------|-------|-------|
| <i>p</i> -Cymene            | 103<br>3 | 14.52 | t              | t     | 11.54 | t     | 29.67 |
| Limonene                    | 103<br>8 | 14.66 | 2.18           | 8.72  | 3.53  | 2.46  | t     |
| 1,8-Cineole                 | 104<br>4 | 14.95 | 6.90           | 20.90 | 4.91  | 44.57 | 1.97  |
| $\gamma$ -Terpinene         | 106<br>6 | 15.68 | - <sup>e</sup> | 1.4   | 6.79  | t     | 7.55  |
| Linalool                    | 110<br>2 | 17.12 | 28.75          | t     | 2.1   | 29.86 | 4.59  |
| Camphor                     | 116<br>5 | 19.32 | 8.49           | 12.60 | 10.14 | -     | 1.54  |
| $\alpha$ -Terpineol         | 120<br>6 | 20.84 | t              | t     | t     | 3.60  | t     |
| Linalyl acetate             | 125<br>4 | 22.45 | 34.20          | -     | -     | t     | t     |
| Thymol                      | 129<br>0 | 24.09 | -              | -     | 1.87  | -     | 25.78 |
| Carvacrol                   | 129<br>8 | 24.43 | -              | -     | 17.22 | -     | 2.41  |
| <i>trans</i> -caryophyllene | 144<br>1 | 28.71 | 1.86           | 4.45  | 3.36  | t     | 2.76  |

<sup>a</sup> Compounds listed in order of elution from an HP-5 column. <sup>b</sup> Kovats index. <sup>c</sup> Retention time (minutes). <sup>d</sup> Traces.

<sup>e</sup> Not detected.

### 3.2. In Vitro Antifungal Activity and Selectivity Index of the Essential Oils on Mycelium Growth of *Cladobotryum mycophilum* and *Agaricus bisporus*

The most effective essential oils for inhibiting the in vitro mycelial growth of *C. mycophilum* were *T. vulgaris* ( $ED_{50} = 35.5 \text{ mg L}^{-1}$ ) and *S. montana* ( $ED_{50} = 42.8 \text{ mg L}^{-1}$ ). In contrast, *L. × intermedia* ( $ED_{50} = 784.2 \text{ mg L}^{-1}$ ), *T. mastichina* ( $ED_{50} = 627.3 \text{ mg L}^{-1}$ ), and *S. lavandulifolia* ( $ED_{50} = 598.4 \text{ mg L}^{-1}$ ) EOs showed the lowest antifungal activity against *C. mycophilum* (Table 3). The activity of *S. montana* and *T. vulgaris* EOs differed significantly from the rest of the EOs ( $F_{4,59} = 54.87$ ;  $p = 0.0$ ).

The in vitro antifungal activity of the EOs toward *A. bisporus* was highest in *L. × intermedia* ( $ED_{50} = 146.6 \text{ mg L}^{-1}$ ) and *T. mastichina* ( $ED_{50} = 175.7 \text{ mg L}^{-1}$ ) and the least toxic EOs in this respect were *S. lavandulifolia* ( $ED_{50} = 587.1 \text{ mg L}^{-1}$ ) and *S. montana* ( $ED_{50} = 537.4 \text{ mg L}^{-1}$ ) (Table 3). *Lavandula × intermedia* and *T. mastichina* EOs had significant differences from the rest of the EOs ( $F_{4,19} = 8.98$ ;  $p = 0.0007$ ). According to values obtained for selectivity index, *S. montana* and *T. vulgaris* EOs showed the best selective toxicity (SI = 0.08 and 0.09, respectively) between pathogen and host.

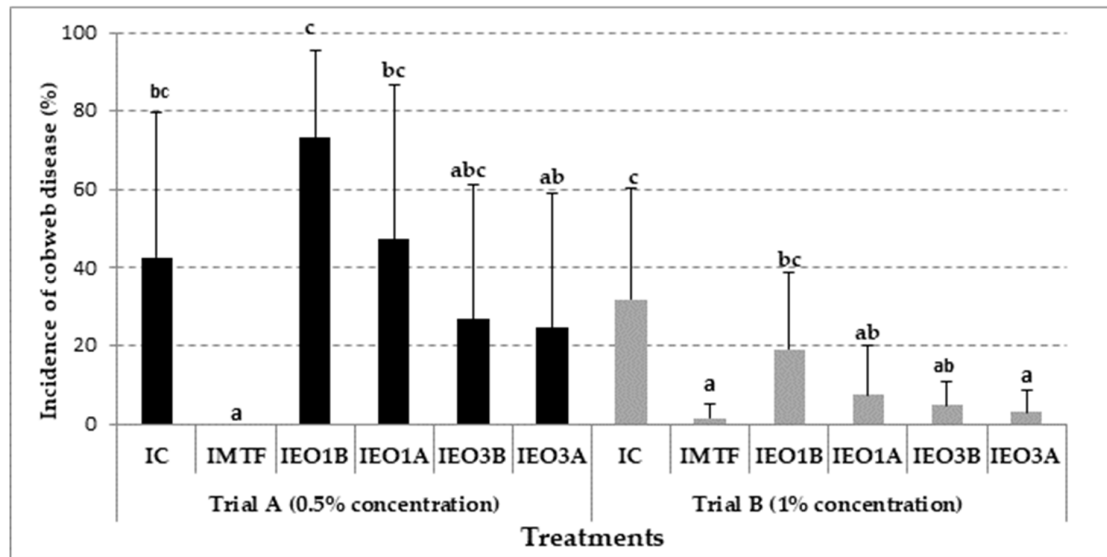
**Table 3.** Mean effective dose ( $ED_{50}$  values  $\pm$  SD (standard deviation)) of essential oils for isolates of *Cladobotryum mycophilum* and *Agaricus bisporus*. Selectivity index (SI) between pathogen and host.

| Essential Oil                 | <i>Cladobotryum</i>             | <i>Agaricus</i>                 | Selectivity Index <sup>a</sup> |
|-------------------------------|---------------------------------|---------------------------------|--------------------------------|
|                               | <i>mycophilum</i> (n = 12)      | <i>bisporus</i> (n = 4)         |                                |
|                               | $ED_{50}$ (mg L <sup>-1</sup> ) | $ED_{50}$ (mg L <sup>-1</sup> ) |                                |
| <i>Satureja montana</i>       | 42.8 $\pm$ 33.4 ab              | 537.4 $\pm$ 192.1 b             | 0.08                           |
| <i>Thymus vulgaris</i>        | 35.5 $\pm$ 23.0 a               | 389.7 $\pm$ 131.5 b             | 0.09                           |
| <i>Salvia lavandulifolia</i>  | 598.4 $\pm$ 430.9 b             | 587.1 $\pm$ 177.4 b             | 1.02                           |
| <i>Thymus mastichina</i>      | 627.3 $\pm$ 183.9 b             | 175.7 $\pm$ 60.2 a              | 3.57                           |
| <i>Lavandula × intermedia</i> | 784.2 $\pm$ 460.5 b             | 146.6 $\pm$ 39.2 a              | 5.35                           |

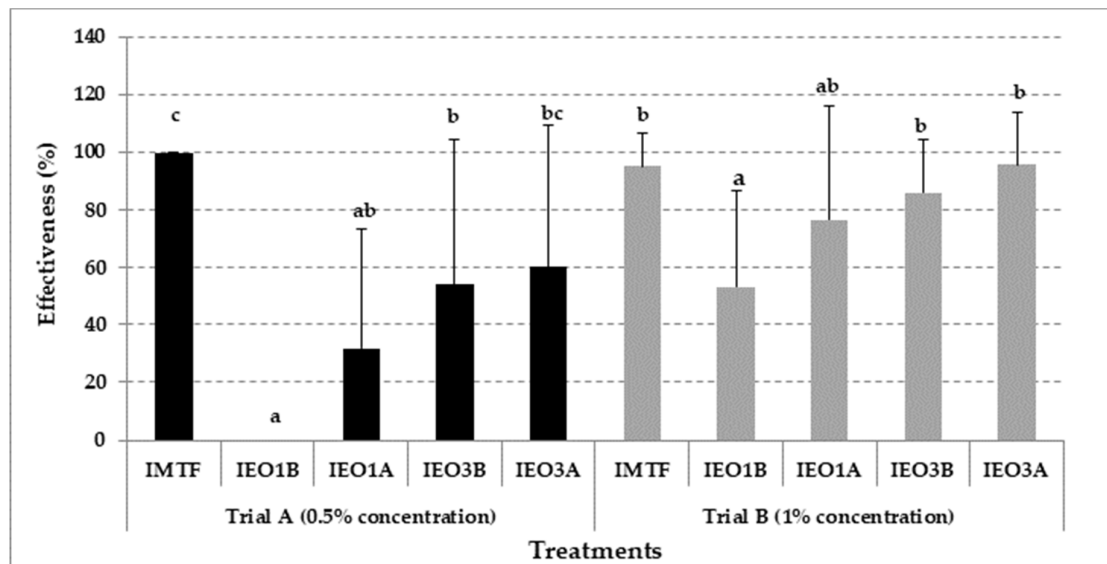
<sup>a</sup> Selectivity index expressed as the ratio between the mean  $ED_{50}$  value of *C. mycophilum* for each essential oil and the corresponding mean  $ED_{50}$  value of *A. bisporus*. <sup>b</sup> Values followed by different lowercase letters in the columns indicate statistically significant differences at  $p = 0.05$  according to Fisher's LSD (least significant difference) test.

### 3.3. Effectiveness of EOs Treatments in Mushroom Growing Trials with *Cladobotryum mycophilum* Inoculation

In trial A, the first symptoms of cobweb were detected at the end of the first flush (day 28 after casing) in the IEO1B treatment, and in the second flush in IEO3B (day 31) and IEO1A (day 33) treatments. Cobweb symptoms in C and IC appeared 39 days after casing. By the end of the crop cycle, the disease incidence ( $DI_{cont}$ ) in the non-inoculated control (C) was 28.3%. This  $DI_{cont}$  may be due to cross-contamination incidents. In the inoculated control (IC), the  $DI_{corr}$  was 42.6%. In the treatments IEO1B and IEO1A, the  $DI_{corr}$  was 73.2 and 47.3%, respectively, while in IEO3B and IEO3A it was 26.7 and 24.4%. By contrast, when metrafenone (IMTF) was used, the  $DI_{corr}$  was 0% at the end of the crop cycle. There were significant differences between IMTF and the rest of the treatments ( $F_{6,41} = 5.54$ ;  $p = 0.0004$ ) (Figure 1). At the end of the crop cycle, the most effective treatments were metrafenone (IMTF) and *T. vulgaris* (IEO3A and IEO3B) showing significant differences with *S. montana* EO treatment (IEO1B) ( $F_{4,29} = 6.52$ ;  $p = 0.0010$ ) (Figure 2).



**Figure 1.** Incidence of cobweb disease (*Dlcorr*) of different essential oils and fungicide treatments in two mushroom crop trials (A and B) with *Cladobotryum mycophilum* inoculation. Treatments are explained in Table 1. For each trial, different letters on columns indicate significant difference according to Fisher's LSD (least significant difference) test at  $p = 0.05$ .



**Figure 2.** Effectiveness (EF) of different essential oils and fungicide treatments at the end of the crop cycle in two mushroom crop trials (A and B) with *Cladobotryum mycophilum* inoculation. Treatments are explained in Table 1. For each trial, different letters on columns indicate significant difference according to Fisher's LSD (least significant difference) test at  $p = 0.05$ .

In trial B, the first symptom of disease was observed in treatment IC, 31 days after casing, at the end of the second flush, followed by treatments IEO1B (34 days) and IEO1A (35 days). Cobweb symptoms in C appeared 46 days after casing. At the end of the crop cycle, the *DI*cont in the non-inoculated control (C) was 6.7%. In the inoculated control (IC), the *DI*corr was 31.8% (Figure 1). In treatments IEO1B and IEO1A, the *DI*corr was 19.0 and 7.4%, respectively, while in IEO3B and IEO3A it was 4.7 and 3.0%. In the IMTF treatment it was 1.5%. There were significant differences between IC and C, IMTF, IEO1A, IEO3B, and IEO3A treatments ( $F_{6,41} = 3.2$ ;  $p = 0.0131$ ) at the end of the crop cycle. The most effective treatments at the end of the crop cycle were those involving metrafenone (IMTF), *S. montana* EO (IEO1A) and *T. vulgaris* EO (IEO3B and IEO3A) ( $F_{4,29} = 2.37$ ;  $p = 0.08$ ) (Figure 2).

### 3.4. Effect of EOs Treatments on *Agaricus bisporus* Yield

In trial C, metrafenone treatment (MTF) provided the highest yield at the first flush (Table 4), followed by the control (C), while treatments with essential oils applied after inoculation (EO1A and EO3A) had the lowest yield. The EO1A and EO3A treatments showed significant differences in yield from the rest of the treatments ( $F_{5,29} = 5.55$ ;  $p = 0.0016$ ). At the second flush, the opposite was the case, since the treatments with essential oils applied after inoculation (EO1A and EO3A) had the highest yield, while treatments C and MTF had the lowest yields, with significant differences between EO3A and C, MTF, EO1B, and EO3B ( $F_{5,29} = 3.55$ ;  $p = 0.0152$ ). At the end of the crop cycle, there were no significant differences between treatments in total yield or biological efficiency ( $F_{5,29} = 0.73$ ;  $p = 0.6089$ ). Regarding earliness, EO1B, EO3B, and EO3A showed a significant delay of 1 day compared with C and MTF ( $F_{5,29} = 16.32$ ;  $p = 0.0000$ ).

**Table 4.** Effect of the essential oils and fungicide treatments on *Agaricus bisporus* quantitative production parameters.

| Treatments <sup>a</sup> | Mushroom Yield (kg m <sup>-2</sup> ) |              |              | Biological Efficiency (kg dt <sup>-1</sup> compost) | Earliness (Days from Casing) |
|-------------------------|--------------------------------------|--------------|--------------|-----------------------------------------------------|------------------------------|
|                         | 1st Flush                            | 2nd Flush    | Total        |                                                     |                              |
| C                       | 11.0 ± 3.8                           | 5.8 ± 0.9 ab | 16.8 ± 3.4 a | 67.1 ± 13.8 a                                       | 24.0 ± 0.2 a                 |
|                         | bb                                   |              |              |                                                     |                              |
| MTF                     | 12.1 ± 2.6 b                         | 5.0 ± 0.6 a  | 17.1 ± 2.8 a | 68.4 ± 11.1 a                                       | 23.9 ± 0.3 a                 |
| EO1B                    | 9.4 ± 1.1 b                          | 6.6 ± 4.0 ab | 16.0 ± 3.9 a | 64.1 ± 15.4 a                                       | 25.4 ± 0.3 c                 |
| EO1A                    | 6.1 ± 2.2 a                          | 9.1 ± 2.6 bc | 15.1 ± 1.4 a | 60.4 ± 5.4 a                                        | 24.6 ± 0.5 b                 |
| EO3B                    | 10.7 ± 1.3 b                         | 7.2 ± 2.0 ab | 18.0 ± 1.8 a | 71.7 ± 7.2 a                                        | 25.2 ± 0.3 c                 |
| EO3A                    | 6.2 ± 2.6 a                          | 11.3 ± 4.1 c | 17.5 ± 2.0 a | 70.0 ± 8.0 a                                        | 25.0 ± 0.5 bc                |
| SED                     | 1.08                                 | 1.22         | 1.2          | 4.81                                                | 0.16                         |
| LSD                     | 4.72                                 | 5.32         | 5.26         | 21.05                                               | 0.69                         |
| d.f.                    | 29                                   | 29           | 29           | 29                                                  | 29                           |

<sup>a</sup> Treatments are explained in Table 1. <sup>b</sup> Values followed by different lowercase letters in the columns indicate statistically significant differences at  $p = 0.05$  according to Fisher's LSD (least significant difference) test. SED: Standard error of the difference between two means. LSD: Least significant difference between two means. d.f.: Degrees of freedom associated with the LSD and SED.

The treatment of EOs had a strong negative effect on *A. bisporus* yield at the first flush when applied on day 13 after casing, with reductions of 45% and 44% in EO1A and EO3A, respectively, compared with the control ©. These reductions were even greater compared with the yield obtained with MTF, the differences with EO1A and EO3A being statistically significant. However, in treatments applied 10 days after casing (EO1B and EO3B) the reduction in yield was less pronounced, 15% and 3%, respectively, compared with the control (C). Nevertheless, the yield obtained at the

second flush compensated for the drop in production at the first flush, and all EO treatments provided higher yields than the control and MTF treatment (14% increase in EO1B and 95% in EO3A, compared with the control). At the end of crop cycle, neither the total production nor biological efficiency showed significant differences among the different treatments applied. As regards earliness, there was a slight delay (0.6–1.4 days) in the harvest of the first flush in all treatments involving EOs, with significant differences between the control C and MTF and EO1B, EO3B, and EO3A. In other words, the EO treatments had a strong fungitoxic effect during the first flush.

#### 4. Discussion

The fungicidal effect of five EOs from Mediterranean aromatic plants on the mycopathogenic fungus *C. mycophilum* was assessed in this study. In vitro bioassays showed that *S. montana* and *T. vulgaris* EOs had the greatest inhibitory effect against *C. mycophilum*, while *S. lavandulifolia*, *L. × intermedia* and *T. mastichina* EOs were the least effective. The essential oils of *S. montana* and *T. vulgaris* were characterized by the phenolic compounds, carvacrol and thymol, which are consistent with the results of other studies [36,37]. *Satureja montana* contained a higher concentration of carvacrol (17.22%) than the other essential oils. Thymol was the main component of *T. vulgaris* oil (25.78%). These two EOs contained a significant proportion of biological precursors of the phenolic components,  $\gamma$ -terpinene and *p*-cymene. Carvacrol and thymol have been shown to have an important antifungal effect, causing damage in the cell membrane by interacting with sterols, particularly ergosterol [38,39]. However, the antifungal activity cannot be attributed to a single component, because there are many compounds acting synergistically [37,40,41]. For example,  $\gamma$ -terpinene and *p*-cymene, biological precursors of carvacrol, are not effective antibacterial agents when used alone, but, when combined with carvacrol, there is a synergism to facilitate the transport of carvacrol across the cytoplasmic membrane of fungi [41,42]. The essential oils of *C. zeylanicum* (cinnamon), *O. vulgare* (oregano), *S. aromaticum* (clove), *Pogostemon cablin* Benth. (patchouli), *T. vulgaris* (thyme), *M. alternifolia* (tea tree), *Mentha piperita* L. (peppermint), and *Pelargonium graveolens* L'Hér. (geranium) also showed antifungal activity against *Cladobotryum* sp. [22,43,44].

On the other hand, in vitro bioassays showed that *S. lavandulifolia*, *S. montana*, and *T. vulgaris* EOs were the least effective in inhibiting the mycelial growth of *A. bisporus*, while *L. × intermedia* and *T. mastichina* EOs were the most effective. In this case, the results were in contrast to those obtained in the bioassays with *C. mycophilum*. That is, the EOs of *S. montana* and *T. vulgaris* had greater antifungal activities against *C. mycophilum* than *A. bisporus*. These EOs, the only ones that contained thymol or carvacrol in their composition, showed no antifungal activity against *A. bisporus*. The reason for this low antifungal activity against *A. bisporus* may reflect the physiological trait of the mushroom to produce lignolytic enzymes, especially laccase and manganese peroxidase, which are able to decompose the phenolic compounds, while the materials produced by degradation are consumed by the mushroom [18,45,46].

The antifungal activity of *L. × intermedia* ( $ED_{50} = 146.6 \text{ mg L}^{-1}$ ) and *T. mastichina* EOs ( $ED_{50} = 175.7 \text{ mg L}^{-1}$ ) against *A. bisporus* could be attributed to the content of alcoholic monoterpenoids, which include linalool and linalyl acetate [15,42]. Other essential oils, like *M. alternifolia* and *P. graveolens* EOs, also showed in vitro toxicity against *A. bisporus* ( $ED_{50} = 98.0$  and  $128.36 \text{ mg L}^{-1}$ , respectively) [18,22].

The fungitoxic selectivity between the pathogen (*C. mycophilum*) and the host (*A. bisporus*) expressed by the selectivity indexes of the tested EOs indicated that *S. montana* and *T. vulgaris* EOs were the most selective essential oils ( $SI = 0.08$  and  $0.09$ , respectively). These values (lower than  $0.1$ ) are better than those obtained in previous studies with the fungicides chlorothalonil and thiophanate-methyl ( $SI = 0.38$  and  $0.21$ , respectively). However, the fungicides metrafenone, prochloraz-Mn, and thiabendazole showed better selectivity than the EOs tested ( $SI = 0.00001$ ,  $0.0031$ , and  $0.01$ , respectively) [13]. On the other hand, *L. × intermedia* and *T. mastichina* EOs had the greatest inhibitory effect against the *A. bisporus* mycelium, and their selectivity indexes were the highest (Table 3).

The results obtained using the two most selective essential oils, *S. montana* and *T. vulgaris*, in the cropping trials with inoculated treatments of *C. mycophilum* indicated that both EOs were effective

when used at the higher doses (1% concentration) (Figure 2). The *T. vulgaris* (IEO3B, IEO3A) and *S. montana* (IEO1A) EOs treatments, at 1% concentration, were as effective as those involving metrafenone. Until now, the most effective treatment for controlling cobweb disease has been with metrafenone [13]. These results agree with those of previous studies carried out in mushroom growing rooms, which showed that the preventative use of *T. vulgaris* oil was able to control the development of wet bubble disease caused by *Mycogone perniciosa* in mushrooms with no detrimental effect on the growth of *A. bisporus*, and provided an overall yield similar to that following the application of a commercial fungicide [20]. On the other hand, there were no significant differences between EO treatments applied before and after inoculation with conidia of *C. mycophilum*.

## 5. Conclusions

According to the obtained results, *S. montana* and *T. vulgaris* EOs showed potent in vitro antifungal activity against *C. mycophilum* compared with other EOs tested. These EOs were characterized by the presence of phenolic compounds such as carvacrol and thymol which were absent in the other EOs tested. In addition, the fungitoxic selectivity between *C. mycophilum* and *A. bisporus* indicated that *S. montana* and *T. vulgaris* EOs were the most selective of the essential oils tested. When used in mushroom growing trials and applied at a later time compared with standard fungicide products, the two EOs tested showed some efficacy at controlling cobweb disease when used at the 1% rate, especially *T. vulgaris*, but additional trials are needed to confirm this observation. However, there was a strong fungitoxic effect at the first flush, which increased with the time of application nearing the harvest period, although at the end of the crop cycle there were no differences between treatments in terms of biological efficiency. These results suggest that *T. vulgaris* and *S. montana* essential oils may be efficacious in managing cobweb disease if used as part of an integrated pest management (IPM) program.

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