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Control of Seed-Borne Fungi by Selected Essential Oils

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Abstract: Seed-borne pathogens reduce the quality and cause infections at various growth stages of horticultural crops. Some of the best-known are fungi of genus *Alternaria,* that cause destructive vegetable and other crop diseases, resulting in significant yield losses. Over several years, much attention has been paid to environmentally-friendly solutions for horticultural disease management regarding the environmental damage caused by chemicals. For example, plant extracts and essential oils could be alternative sources for biopesticides and help to control vegetable seed-borne pathogens. This study aimed to evaluate essential oils' influence on the growth of seed-borne fungi *Alternaria* spp. The microbiological contamination of vegetable seeds (carrot, tomato, onion) was determined by the agar-plate method. The essential oils' impact on the growth of fungi was evaluated by mixing them with PDA medium at different amounts. The hydrodistillation was used for extraction of thyme and hyssop essential oils, and common juniper essential oil was purchased. The investigation revealed that the highest contamination of carrot and tomato seeds was by *Alternaria* spp. fungi. Furthermore, the highest antifungal effect on *Alternaria* spp. growth was achieved using 200–1000 μL L^{-1} of thyme essential oil. Meanwhile, the antifungal effect of other investigated essential oils differed from low to moderate. Overall, essential oils expressed a high potential for fungal pathogens biocontrol and application in biopesticides formulations.

Keywords: *Thymus vulgaris*; *Juniperus communis*; *Hyssopus officinalis*; *Alternaria* spp.; biocontrol

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1. Introduction

Vegetables are a crucial part of food production and are consumed worldwide. However, fungal diseases often lead to significant economic yield losses [1]. For example, horticultural production yield spoilage caused by fungal *Alternaria* species ranges from 20% to 80% [1,2]. This fungus can induce seedling death, petiole base blackening, leaf death or blight, leaf lesions, stem canker, black rot, and other symptoms depending on the host plant [3,4]. *Alternaria* spp. can also be considered a seed-borne pathogen, responsible for destructive diseases of various vegetables such as carrot, tomato, onion, etc. [1]. For example, *Alternaria radicina* Meier, Drechsler, and Eddy is known primarily as a carrot pathogen, responsible for root and crown disease and causing foliar blight under certain conditions. *Alternaria dauci* (Kühn) Groves and Skolko mainly cause carrot Alternaria leaf blight. However, *A. dauci* has also been documented to cause disease on parsnip, spinach, celery, and parsley [5]. *Alternaria solani* (Ellis and Martin) Sorauer causes early blight on foliage, collar rot on basal stems of seedlings, stem lesions on adult plants, and fruit rot of tomatoes [6]. Sources indicate that *Alternaria arborescens* Keissler also causes stem canker of tomato [7]. The purple blotch of onion is a disease caused by *Alternaria porri* (Ellis) Cif. [8]. *Alternaria alternata* (Fr.) Keissl. causes a black spot in many fruits and vegetables around the world. Some studies reported seed contamination with various *Alternaria* species, including saprotrophic *A. alternata*, *Alternaria tenuissima* Samuel Paul Wiltshire, *Alternaria longipes* (Ellis and Everh.) E.W. Mason [9–11]. In addition, due to its presence on the seeds' surface, *Alternaria* spp. can adversely affect seed germination [1,3,12]. Therefore, high-quality, fungi-free seeds are prioritised because vegetable consumption increases yearly [3]. Seed-borne diseases can be controlled by selecting resistant varieties, production technology, seed treatments and dressings, and soil disinfection [13]. Over the last several decades, seed and soil or foliar treatments with synthetic chemicals have been shown to prevent plant disease epidemics caused by seed-borne fungi [14–19].

Nevertheless, following regulation No. 1452/2003 developed by the European Commission, organic horticulture is limited to using only organic seeds [20]. Therefore, fungicides in organic production are not used for seeds to prevent the influence of micromycetes. Additionally, their non-target impact on pathogen resistance gain risks to human health and other organisms. Chemical nature and horticultural products pollution by pesticide residues has encouraged the investigation for alternative solutions to control and make horticulture more sustainable [21,22].

Essential oils, due to their broad applicability in various industries, like pharmacy or food industries, have received much attention [23,24]. Furthermore, more comprehensive studies of essential oils revealed their potential in environmental-friendly horticultural disease management as they have antiseptic, antiviral, antibacterial, and antifungal properties [25,26]. Additionally, essential oils, as secondary metabolites, exhibit high plant defence effects, are non-toxic, biodegradable, and limit pathogenic organisms [24,27]. Due to these features, they can be applied as biopesticides for alternative plant protection. For example, Karaca et al. [28] reported good inhibition of investigated fungal species growth under oregano, mint, and clove essential oils application. Muthukumar et al. [29] also stated significant results of geranium and palmarosa essential oils efficacy against rice micromycetes of genera *Cochliobolus* and *Fusarium.* According to other studies, thyme essential oil has a potent antifungal effect on the development of fungal plant pathogens [19,24,25,28,30–33].

The literature review showed that there is a lack of studies regarding environmentally friendly ways to prevent fungal infections of vegetable seeds. Hence, the aim of this study was to determine the predominant seed-born fungi in carrot, tomato, and onion seeds, then to evaluate the antifungal activity of essential oils of thyme, hyssop, and common juniper on the growth of *Alternaria* spp.

2. Materials and Methods

2.1. Seed Samples

For the research, three seed samples of carrot, onion, and tomato were obtained from the Department of Vegetable Breeding and Technology, Institute of Horticulture (IH), Lithuanian Research Centre for Agriculture and Forestry (LAMMC) (Table 1).

Table 1. Vegetable seeds used in the experiments.

Common Name	Botanical Name	Cultivar
Carrot	*Daucus carota sativus* L.	Svalia
Tomato	*Solanum lycopersicum* L.	Rutuliai
Onion	*Allium cepa* L.	Babtų didieji

Vegetable seeds were surface-sterilised by rinsing them in 70% ethanol for 3 min and then washing them three times with sterile distilled water for 5 min in total [34]. After this, seeds were left to dry for 5–10 min in laminar flow. The internal seeds infestation with fungi was determined when external microorganisms were removed during surface sterilisation.

2.2. Determination of Predominant Fungi

The microbiological contamination of seeds samples was evaluated using the agar-plate method [35]. The potato dextrose agar (PDA) medium (Sigma-Aldrich, St. Louis, MO, USA) composed of 15 g L^{-1} agar, 20 g L^{-1} dextrose, and 4 g L^{-1} potato extract was autoclaved and distributed to the Petri dishes [36]. Prepared surface-sterilised samples were arranged in a square shape (five rows and five columns) on each Petri dish (Figure 1) and kept at 22 ± 2 °C temperature in the dark [37,38].

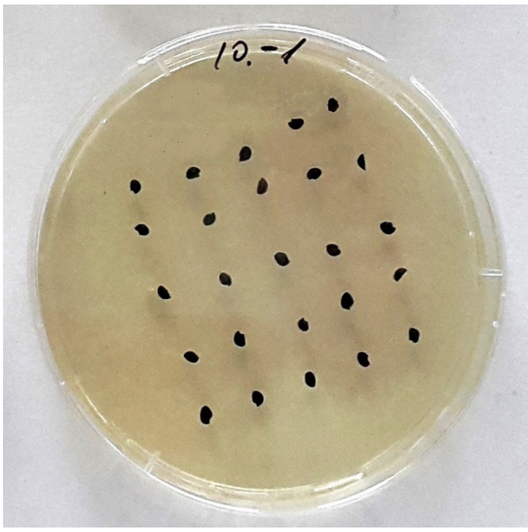

Figure 1. Arrangement of 25 seeds.

The experiment was repeated twice (four replications of each treatment). While inspecting the internal infestation of seeds, the settlements of fungi were counted to get the percentage of dominating fungi in the treatment after 2, 5, and 7 days of incubation (DOI). Visual and microscopical fungi identification was made based on morphological and cultural characteristics typical to the colonies [17,39]. Their detection frequency was determined using the detection rate of micromycetes: less than 30%—random species, more than 30%—typical species, more than 50%—dominant species [40].

2.3. Essential Oils Efficacy Assay

Three essential oils of thyme (*Thymus vulgaris* L.), hyssop *(Hyssopus officinalis* L.), and common juniper (*Juniperus communis* L.) were used in the experiment. The essential oils of thyme and hyssop were separately hydro-distilled for 2 h using Clevenger type of apparatus [41] from naturally dried herb material, harvested from the experimental fields of IH, LAMMC. The essential oil of the common juniper was purchased (Naujoji Barmune, Vilnius, Lithuania). The major compounds of thyme essential oil: thymol (41.35%), *p*-cymene (16.95%), and γ-terpinene (10.81%), were identified earlier by Morkeliūnė et al. [32]. The hyssop essential oil was characterised by cis-pinocamphone (40.16%), β-phellandrene (12.51%), and β-pinene (8.07%) and the process of chemical analysis was described previously by Šernaitė et al. [42]. The essential oil of the common juniper was mainly characterised by α-pinene (21.0–67.4%) and myrcene (7.8–18.7%) chemotypes [43].

Then, different amounts of each essential oil were added to one litre PDA medium after cooling to 45 °C, to get 200, 400, 600, 800, and 1000 µL L^{-1} concentrations, then mixed and poured into new Petri plates [44]. A control treatment was without essential oil in PDA, prepared as previously described. Treatments with thyme essential oil were coded T1, hyssop—T2, and common juniper—T3. Surface-sterilised samples of each seeds cultivars were placed in the same order as before (Figure 1) on PDA with different essential oil concentrations and incubated at 22 $\pm$ 2 °C in the dark for 7 days. There were four replicates for each vegetable seed cultivar, and the experiment was repeated twice.

The percentage of *Alternaria* spp. was calculated based on the number of grown fungal colonies in each plate after 2, 5, and 7 DOI. Fungi were identified according to cultural and morphological characteristics typical to the colonies [17,39]. Essential oils effect on *Alternaria* species was evaluated according to the disease incidence using the formula below (1) [12]:

$$\textit{Alternaria}\text{ spp. incidence (\%)} = \text{Number of seeds infected by } \textit{Alternaria}\text{ spp.} \times 100/\text{Total number of infected seeds} \quad (1)$$

Lower disease incidence showed effective essential oil mean activity for seed-borne fungi control.

2.4. Statistics

The experimental data were analysed using the analysis of variance (ANOVA) from the software SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test ($p < 0.05$) was used to determine differences among the treatments.

3. Results

The fungal contamination of vegetable seeds at the 7 DOI is summarised in Table 2. Carrot seeds were infected by 100%, and the predominant fungi were *Alternaria* spp. Fungi of genera *Penicillium* and *Fusarium* occurrence reached up to 4% and were considered random. However, the internal infection of tomato and onion seeds did not exceed 20%. The *Alternaria* spp. also dominated on tomato seeds. Fungi of the genera *Mucor* and *Penicillium* were typical for onion seeds and *Aspergillus* and *Mucor* for tomato seeds.

Table 2. Seeds contamination with fungi after seven days of incubation.

Seeds	Total Seeds Infected, %	Fungal Contamination, %					
		Alternaria spp.	*Fusarium* spp.	*Aspergillus* spp.	*Mucor* spp.	*Penicillium* spp.	*Mycelia sterilia*
Carrot	100	93.4	3.77	0	0	2.83	0
Tomato	20	50	0	15	25	0	10
Onion	15	20	0	0	40	40	0

As *Alternaria* species prevailed as the dominant fungi in vegetable seeds, it was decided to test the influence of three essential oils (T1, T2, and T3) on the growth of seed-borne fungi *Alternaria* spp. in vitro.

The incidence of *Alternaria* spp. on carrot seeds under the influence of T1, T2, and T3 treatments is presented in Figure 2.

The treatments applied to carrot seeds showed an intermittent effect. In the case of treatments with T1, all concentrations significantly suppressed ($p < 0.05$) the growth of seed-borne fungi. Furthermore, no colonies were detected at 2 DOI regardless of the amount of essential oil. Meanwhile, the emergence of *Alternaria* species reached 33% at 2 DOI, 36% at 5 DOI, and 64% at 7 DOI in the control treatment.

During the first assessment, seeds infection with *Alternaria* fungi was 1% at 400 and 1000 μL L^{-1} of T2 treatment. Later, the abundance of these fungi was higher: 63% (200 μL L^{-1}), 68% (400 μL L^{-1}), 74% (600 μL L^{-1}), 48% (800 μL L^{-1}), and 64% (1000 μL L^{-1}) at 5 DOI. Likewise, 400 μL L^{-1} of T2 cause significant decreation of *Alternaria* incidence at 7 DOI. However, the remaining T2 concentrations of 200, 600, 800, and 1000 μL L^{-1} did not affect fungal growth— *Alternaria* spp. incidence increased compared with the control at 5 and 7 DOI. Thus, the opposite effect of T2 was observed than expected.

The T3 treatment performed weaker on *Alternaria* spp. on the second incubation day. Nevertheless, the 600 μL L^{-1} had the best antifungal activity. The 200 μL L^{-1} and 600–1000 μL L^{-1} of T3 slightly controlled the prevalence of the fungi compared to controls at 5 DOI and did not differ significantly at 7 DOI. Still, the best fungal incidence suppression was exhibited by 400 μL L^{-1} of this treatment at the fifth and seventh DOI.

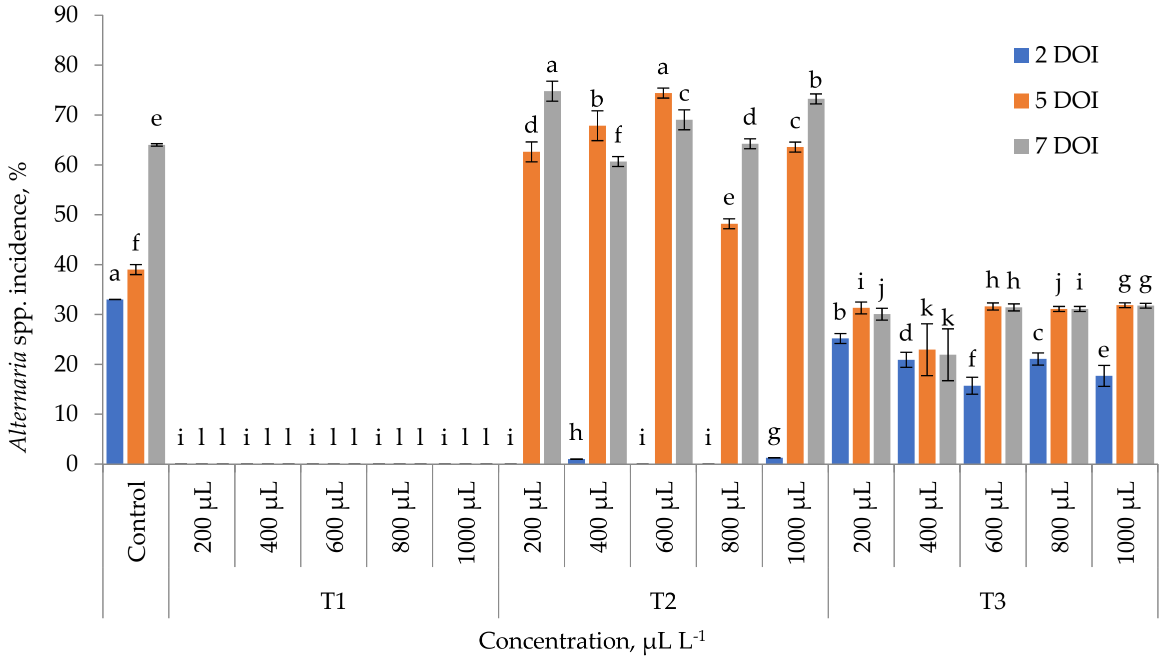

Figure 2. The incidence of *Alternaria* spp. on carrot seeds under the influence of thyme (T1), hyssop (T2), and common juniper (T3) essential oils treatments after 2, 5, and 7 days of incubation (DOI); according to Duncan's multiple range test ($p < 0.05$), the same letters demonstrate no significant differences between treatments at 2, 5, and 7 DOI.

The incidence of *Alternaria* spp. on tomato seeds under T1, T2, and T3 treatments is presented in Figure 3. Evaluating the effect of T1 concentrations from 200 to 1000 µL L^{-1}, no colonies of *Alternaria* spp. were observed at 2 and 5 DOI. However, 6% incidence was reached at 7 DOI under 200 µL L^{-1}.

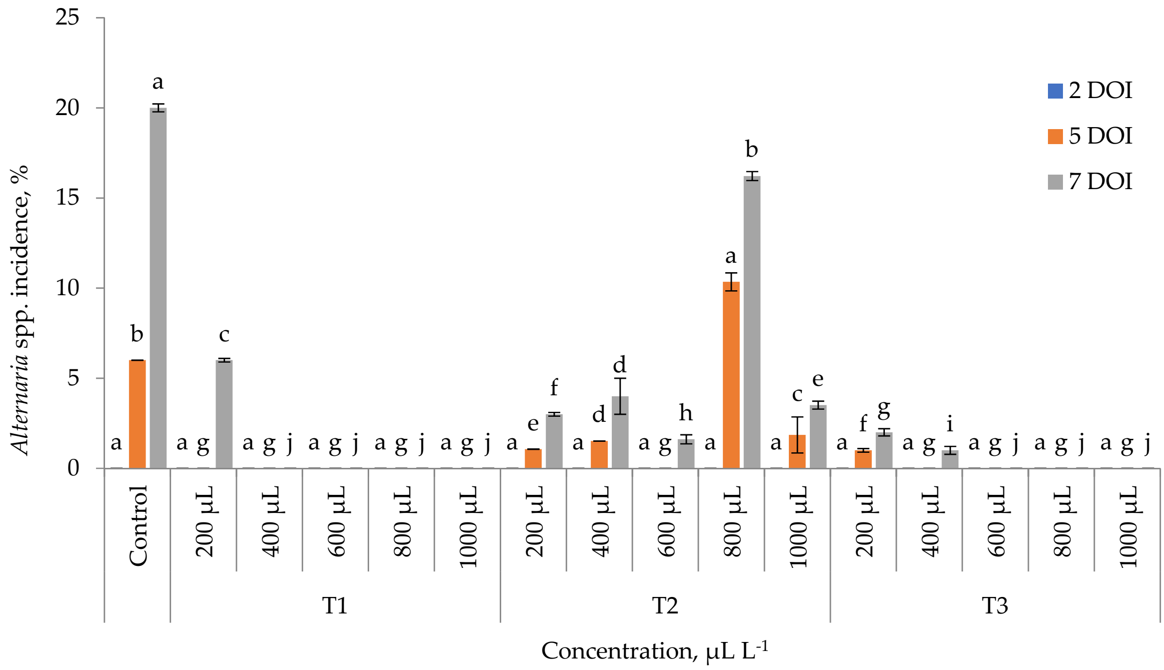

Figure 3. The incidence of *Alternaria* spp. on tomato seeds under the influence of thyme (T1), hyssop (T2), and common juniper (T3) essential oils treatments after 2, 5, and 7 days of incubation (DOI); according to Duncan's multiple range test ($p < 0.05$), the same letters demonstrate no significant differences between treatments at 2, 5, and 7 DOI.

Meanwhile, T2 performed a weaker impact on fungi occurrence, although no fungal colonies were noticed at 2 DOI. The development of micromycetes was observed: 1% under 200 μL L^{-1}, 2%—400 μL L^{-1}, 10%—800 μL L^{-1}, and 2%—1000 μL L^{-1} at 5 DOI. During the third estimation, the number of *Alternaria* spp. increased 2–6% from the previous evaluation. However, none of the T2 values exceeded those of the control incidence; the prevalence value of *Alternaria* spp. came up with 6% at 5 DOI and 20% at 7 DOI.

Estimation of tomato seeds incidence with *Alternaria* species at different concentrations of T3 revealed that fungi infected 1% of seeds under the lowest concentration used at 5 DOI. Later, the incidence increased to 2% under 200 μL L^{-1} and 1% under 400 μL L^{-1} of T3 at 7 DOI. The T3 treatment concentrations from 600 μL L^{-1} had significant antifungal activity at 2, 5, and 7 DOI.

The influence of T1, T2, and T3 treatments on the onion seeds infestation with *Alternaria* spp. is presented in Figure 4. At 5 DOI, the frequency in control was 18%, and at 7 DOI, 20%. The assay with T1 (200–1000 μL L^{-1}) revealed total development inhibition of the genus *Alternaria* fungi at all assessment days.

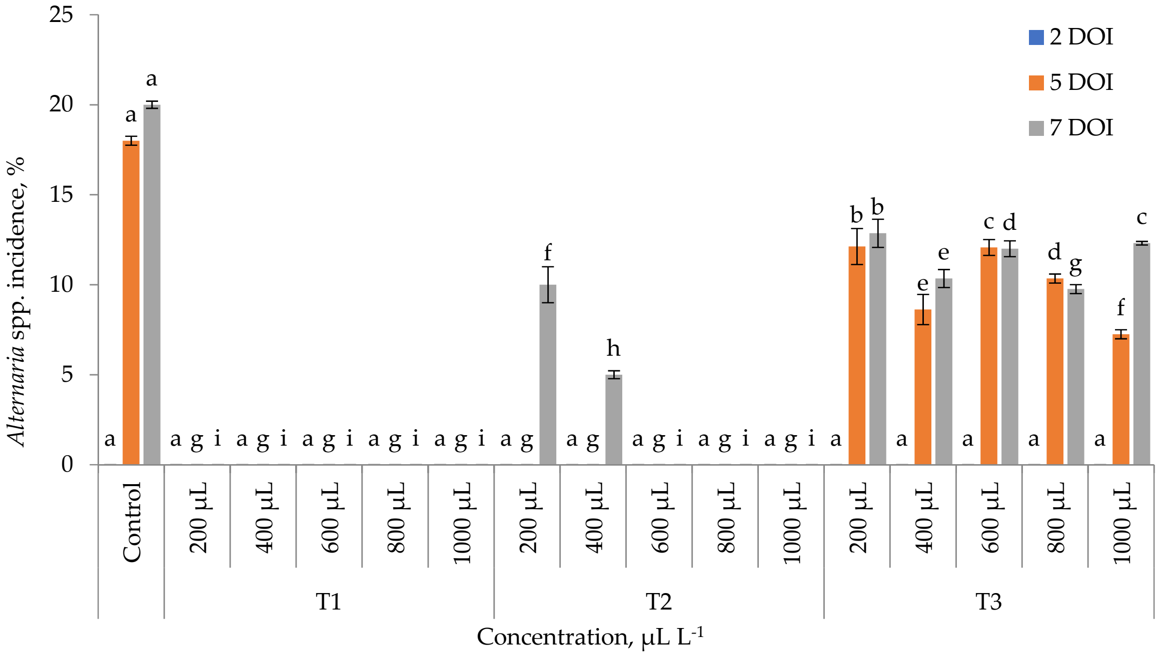

Figure 4. The incidence of *Alternaria* spp. on onion seeds under the influence of thyme (T1), hyssop (T2), and common juniper (T3) essential oils treatments after 2, 5, and 7 days of incubation (DOI); according to Duncan's multiple range test ($p < 0.05$), the same letters demonstrate no significant differences between treatments at 2, 5, and 7 DOI.

T2 treatment also gave excellent inhibition of *Alternaria* fungi growth recorded only with 200 and 400 μL L^{-1} at 7 DOI.

However, the overall antifungal effect of T3 treatment at concentrations from 200 to 1000 μL L^{-1} was average compared to the control evaluating onion seeds at 2, 5, and 7 DOI. In addition, the augmentation of *Alternaria* spp. was observed in all treatments (control, T1, T2, and T3) at 2 DOI. Therefore, the 400 and 1000 μL L^{-1} of all T3 treatment concentrations had the best antifungal activity on *Alternaria* spp. incidence.

From all T1, T2, and T3 treatments applied on the carrot, tomato, and onion seeds, the T1 at all rates most significantly inhibited the growth of *Alternaria* spp., and concentrations from 400 μL L^{-1} of T3 also showed modest antimycotic results. It is important to remember that tomato and onion seeds had less internal contamination with pathogenic fungi than carrot seeds.

4. Discussion

Innovative plant protection solutions are necessary due to the chemical fungicides' negative impact on ecology and seeds germination issues caused by infestation with *Alternaria* spp. [12,21,22]. Therefore, appropriate antifungal measures and their application strategy are crucial. The current study provides the latest findings regarding the antifungal effects of plant-based substances on the seed-borne fungi of the genus *Alternaria* isolated on tomato, carrot, and onion seeds.

Fungi incidence on vegetable seeds was significantly reduced by thyme (T1) treatment at all rates (200–1000 µL L^{-1}). There are numerous investigations about thyme essential oil antimicrobial impacts determined by the main chemotypes of thymol, γ-terpinene, *p*-cymene, β-caryophyllene, and carvacrol [19,24,25,27,28,30–33]. Some other experiments reported an effective control mechanism for *Alternaria* and other genera fungi on carrot and tomato seeds by this oil [30,31,33,45,46]. For example, Dorna and Szopińska [30] noticed that different applications of commercial oils involving thyme, possibly with 22–38% of thymol and 1–2% carvacrol, reduced the fungal contamination of the carrot seeds (cultivar 'Flakkese 2′), including *Alternaria alternata* (Fr.) Keissl. micromycetes. Contrary, the incidence of *Alternaria dauci* (J.G. Kühn), J.W. Groves and Skolko, and *Alternaria radicina* Meier, Drechsler, and E.D. Eddy increased under this oil influence in the case of seed cultivar 'Amsterdam 3′ [30]. The same good effects of the treatment were seen against *Alternaria* spp. when a 'Laguna' cultivar seed sample was stirred in 1% oil emulsion for four hours. Nevertheless, the authors emphasised that choosing the optimal concentration is critical due to inherent oil phytotoxicity, and they recommended pre-testing [31]. Our results with T1 (41.35% of thymol) treatment support Riccioni and Orzali research [46], where a thyme essential oil (41% of thymol) concentration range of 0.05–1% considerably reduced the development of *A. dauci* in vitro.

Our results revealed that the effect on vegetable seeds was unequal when applying hyssop (T2) essential oil. It had the most negligible impact on carrot seeds, then better than average on tomato seeds, and the best fungi growth inhibition on onion seeds. It did not inhibit fungal development on carrot seeds and even promoted it compared to the control. Nonetheless, Fraternale et al. [47] have described various phytopathogenic fungi, like *Rhizoctonia solani* Ell. et Mart, *Botrytis cinerea* Pers., *Fusarium graminearum* Schwabe (ATCC 15624) as significantly sensitive to two hyssop essential oils. These oils were mainly characterised by pinocamphone (34% and 18.5%), β-pinene (10.5% and 10.8%), and isopinocamphone (3.2% and 29%). Their experiment also revealed that concentrations of 1400 and 1600 µL mL^{-1} of both oils inhibited 13 different fungal plant pathogens by 100%, *Alternaria solani* Ell. et Mart either. The seeds utilised in our experiments were characterised by higher contamination of *Alternaria* species. Thus, according to previously discussed results, the tested concentrations of T2 treatment were not high enough to achieve expected efficacy in our experiment. Still, the similarity between chemical compositions of T2 (cis-pinocamphone, 40.16%; β-phellandrene, 12.51%; β-pinene, 8.07%) and earlier described hyssop oil [40] prompts a potentially optimistic effect of higher T2 concentrations. Moreover, many studies investigated hyssop antimicrobial properties on other microorganisms and substances of this herb exhibited undeniable prospects [48].

Our study found that common juniper T3 inhibited *Alternaria* spp. depending on seeds, and the concentration of 400 µL L^{-1} was most effective in all seed experiments. T3 active compounds were possibly α-pinene (21.0–67.4%) and myrcene (7.8–18.7%). Other authors found that common juniper essential oil was effective against some soil- and seed-borne pathogens. For example, in Zabka et al. [49] research, 1 µL mL^{-1} concentration of this oil moderately influenced pathogens, such as *Fusarium verticillioides* (Sacc.) Nirenberg, *Fusarium oxysporum* Schlechtendahl, *Aspergillus fumigates* Fresenius, *Aspergillus flavus* Link, *Penicillium expansum* Link, and *Penicillium brevicompactum* Dierckx; the effect on *Alternaria* species was not studied. Indeed, thyme oil was described as the most robust to reduce target fungi growth [49]. Additionally, good antimicrobial activity (*A. flavus*, *A. niger*, and

Candida albicans (C.P. Robin) Berkhout) of methanolic extract of *Juniperus communis* L. was highlighted, emphasising the leading activity against *A. niger* and A. flavus [50].

To conclude, essential oils of hyssop and common juniper resulted in a moderate capability to control the seed-borne fungi on tested samples of vegetable seeds. Besides, results demonstrated that thyme essential oil had a significant reducing impact on the carrot, tomato, and onion fungi, affirming what is already published in the literature. Despite this, as in vitro effects do not always positively affect in vivo performances, further studies are required to prove the effectiveness in field conditions as seeds treatments and their possible phytotoxicity on the plant or seed material. Furthermore, thyme essential oil is a promising agent for vegetable seed-borne fungi *Alternaria* spp. management.

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