Carvacrol: A Promising Environmentally Friendly Agent to Fight Seeds Damping-Off Diseases Induced by Fungal Species

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Abstract: Background: Gramineae damping-off disease is a growing problem worldwide, which affects a large range of seedlings in nurseries, glasshouses, gardens, crops, forests and untimely generates a heavy economic impact on the agriculture and related sectors. Objectives: The present study was conducted to evaluate the preventive potential of carvacrol on germination of Fusarium oxysporum, Neocosmospora solani, and Microdochium nivale spores as responsible agents for Lolium perenne seeds damping-off disease. Material and methods: Macrodilution method in agar medium, spore germination, spore destruction, and preventive treatment bioassays were used to achieve this goal. Results: The minimum inhibitory concentration (MIC) of carvacrol vs. tested strains existed in the range of 0.25–0.5 mg/mL. Carvacrol used in concentrations ranging from 0.2 to 0.4 mg/mL inhibited the germination of all fungal spores in a dose-dependent manner. Carvacrol showed a very strong sporidical effect against all studied fungal strains, and this effect was well confirmed by microscopic observations. The percentage of growth inhibition was found to be strictly correlated to carvacrol concentration. Conclusion: Based on the results obtained, carvacrol fulfills the requirement for being a natural alternative agent to fight Gramineae seedlings’ damping-off caused by fungal species without adverse effects on the plants.

Keywords: carvacrol; damping-off disease; fungal species; Lolium perenne

1. Introduction

Seeds damping-off disease is a disease complex that affects a wide range of seedlings in nurseries, glasshouses, gardens, crops, and forests, and can kill both germinating and young seedlings [1]. This disease is generally controlled by treating seeds with fungicides and pesticides, which have harmful effects on the environment and human health when used excessively [2]. As a result, many countries have limited the use of a variety of toxic
chemical products [3]. Thus, intensive research is needed to explore alternative and more sustainable management strategies for seed protection. In this sense, numerous studies have explored the potential use of natural substances for the control of soil-borne plant pathogens [4–6].

Several fungi including *Fusarium* spp., *Microdochium* spp., and *Neocosmospora* spp. can cause seeds damping-off [7,8]. Moreover, recent studies have shown that the *Fusarium* genus parasitises the grass seeds and can attack the whole plant [9,10]. *Microdochium nivale* (Fr.) Samuels and I.C. Hallet (*M. nivale*), *Fusarium oxysporum* described by Schltdl. (*F. oxysporum*), and *Neocosmospora solani* (Mart.) L. Lombard and Crous, three species owning to the “Fusarium complex” (*N. solani*) have devastating effects on species belonging to wild Gramineae, *Lolium perenne* L. (*L. perenne*) [11]. This disease is economically challenging since this fungus attacks several types of Gramineae including rice, maize, wheat, barley, oats, and rye and therefore induces significant losses especially when the environmental conditions are favourable [12].

Since the seeds damping-off is inevitably present in the majority of crops, most farmers often adopt a preventive strategy by treating the entire surface area using highly toxic products. These products can be used before planting and/or during cultivation, e.g., chloropicrin and benomyl [13,14]. It is reasonable that *Fusarium* wilt disease is one of the most fungicide consuming [10]. Due to excessive use, some fungicides can no longer be effective towards resistant strains among fungal species. Therefore, it is necessary to identify alternative weapons to control *Fusarium* wilt disease [15].

Over recent years, the effect of natural products in treating fungal diseases has been the subject of increasing interest, which is fuelled by a growing concern for better environmental protection [16–19]. Among them, there are plant crude extracts, essential oils, and their compounds that have proven efficacy in treating diseases caused by fungi without side effects on the environment [20,21]. Carvacrol is a natural agent derived from plants (*Thymus vulgaris* and *Origanum vulgare* described by Linnaeus). This monoterpene is considered nontoxic to humans and is commonly used as a flavouring substance. Carvacrol has been the subject of many investigations that place a higher priority on natural products with pharmacological activities to control such diseases. Previous investigations demonstrated that carvacrol is one of the potent monoterpenes that can be used to control fungal species [22–24].

The present work aimed to test Carvacrol as a promising environmentally friendly biofungicide to fight fungal species attacking seeds grasses without harming the environment.

2. Material and Methods
2.1. Fungal Strains

Strains of *F. oxysporum*, *N. solani*, and *M. nivale* used in the present study were isolated from diseased leaves, stems, and roots of *L. perenne* before being identified by Abdelilah Iraqi Housseini (Laboratory of Biotechnology, Environment, Agri-Food, and Health, Faculty of Sciences Dhar El Mahraz, Sidi Mohammed Ben Abdellah University, Fez, Morocco) [19].

2.2. Preparation of the Spore Suspension

Sporulation was obtained by culturing the fungi strains on potato dextrose agar (PDA) medium (purchased from Biokar, France) at 28 °C for 7 days [25]. Briefly, the spores were collected by flooding the plate with 5 mL of 0.05 % (v/v) tween 20, using a sterile spread rod. The number of spores in the mother suspension was counted using a hemocytometer calculator with light microscopy before being diluted to be about 10^6 spores/mL. Next, the number of cells per average square was counted (area: 0.0025 mm^2; depth: 0.2 mm) using microscopy before being diluted to 10^6 spores/mL [26].
2.3. Antifungal Activity Assay

2.3.1. Growth Inhibition Assay

Growth inhibition assay was performed as described in earlier work [27]. Briefly, a series of Petri plates (90 mm) were prepared with increasing concentrations of Carvacrol (99 %) 0.12, 0.25, 0.5, 1, and 2 mg/mL (the carvacrol (Figure 1) was kindly offered by FLAGRESSO, Austria). Then, each plate of PDA medium was inoculated by depositing 20 µL of a suspension of 10^6 spores/mL. Afterward, the plates were incubated at 28 °C for 7 days. Then, the diameter of the fungal colonies was measured daily during incubation. Growth inhibition percentage (GI %) of radial mycelium was calculated according to the following equation [28]:

\[
GI (%) = (\frac{dt - Dt}{dt}) \times 100
\]

where dt and Dt, respectively, represent the diameter of the control and the diameter in the presence of carvacrol concentrations. Quantitative data were expressed as means of triplicate experiments.

![Chemical structure of carvacrol](image_url)

**Figure 1.** Chemical structure of carvacrol [29].

2.3.2. Spores Germination Inhibition Assay

Using an Eppendorf tube of 1 mL, 100 microliters of freshly prepared spore suspension (10^6 spores/mL) were mixed with 900 µL of carvacrol solution at different concentrations (0.1, 0.2, 0.4, 0.8, and 1 mg/mL) using sterile malt extract broth (BEM) medium (was purchased from Biokar, France). The negative control tube contained 100 µL of freshly prepared spore suspension (10^6 spores/mL) with 900 µL of sterile BEM medium. The Eppendorf tubes were incubated under stirring for 24 h at 28 °C. Afterward, the evaluation of spore germination inhibition was determined by spreading a volume of 100 µL of each tube on the surface of sabouraud agar (SB) plates (90 mm) [15]. After 7 days of incubation at 28 °C, the emerged fungal colonies were manually counted. A spore is considered to have germinated when the length of the germ tube is longer than its smallest diameter, which will give a future monosporal fungal colony [30]. Quantitative data were expressed as means of triplicate experiments.

2.3.3. Spores Destruction Assay

A spore destruction test was used to evaluate the sporicidal effect of carvacrol. Briefly, 100 microliters of freshly prepared spore suspension (10^6 spores/mL) were mixed with 900 µL of carvacrol at various concentrations (0.1, 0.2, 0.4, 0.8 and 0.4 mg/mL) according to the method described by Remmal et al. [31,32]. The fungal spores destruction was evaluated after incubation at 28 °C for 24 h. Then, a 900 µL aqueous sterile solution of 0.9% NaCl containing 0.2% agar, mixed with 100 µL of the suspension, was used as a control tube (10^6 spores/mL). The number of conidia was determined using Malassez cell after 1, 3, 6, 12, and 24 h of incubation at 28 °C. After each time, 20 µL of spore suspension
was deposited between a slide and a coverslip before being observed using an optical microscope (×400). The percentage of spore destruction was calculated using the following equation [33]:

\[ I(\%) = \frac{N_c - N_t}{N_c} \times 100 \]  

where \( N_c \) and \( N_t \) represent the number of spores in the control and the test tubes, respectively. Quantitative data were expressed as means of triplicate experiments.

2.4. In Vitro Preventive Treatment Assay

2.4.1. In Vitro Preliminary Toxicity Testing for Carvacrol on Germination of \( L. \) perenne Seeds

The grass seeds of the English ryegrass variety, \( Lolium perenne \), used in this work were obtained from the technical team of the Golf Royal Dar Es-salaam of Rabat, Morocco. A total of 120 seeds were disinfected with 12° sodium hypochlorite solution for 20 min and then rinsed twice with sterile distilled water for 10 min before being transferred to Petri plates containing sterile layers of filter paper [34,35]. Afterward, the seeds were divided into 4 groups of 30 seeds, and each group (Table 1) was treated with a carvacrol solution according to the different modalities on the first day and 1 mL of sterile water in the following days of the experiment period. The experiment was carried out for 4 weeks. The percentage of seed germination was measured according to the method described elsewhere [11]. Quantitative data were expressed as means of triplicate experiments.

Table 1. Distribution groups for the carvacrol toxicity test.

| Distribution Groups | Description                              |
|---------------------|------------------------------------------|
| GI                  | 30 Intact seeds                          |
| GII                 | 30 Seeds + 0.05 mg/mL of carvacrol       |
| GIII                | 30 Seeds + 0.1 mg/mL of carvacrol        |
| GIV                 | 30 Seeds + 0.2 mg/mL of carvacrol        |

2.4.2. In Vitro Preliminary Test of Pathogenicity for Fungal Strains on Germination of \( L. \) perenne Seeds

In total, 150 seeds were disinfected with 12° sodium hypochlorite solution for 20 min and then rinsed twice with sterile distilled water for 10 min before being transferred to Petri plates containing sterile layers of filter paper [34,35]. For testing fungal strains’ pathogenicity on germination of \( L. \) perenne, the seeds were divided into 5 groups of 30 seeds, and each group (Table 2) was treated with 1 mL of the suspension adjusted to \( 10^8 \) spores/mL of each fungal strain separately and with a mixture of all strains (the ratio was 1/3, 1/3, 1/3) [27]. The experiment was carried out for 4 weeks. The percentage of seed germination was measured according to the previously reported method [11]. Quantitative data were expressed as means of triplicate experiments.

Table 2. Distribution groups for pathogenicity testing of fungal strain.

| Distribution Groups | Description                              |
|---------------------|------------------------------------------|
| GI                  | 30 Uninfested seeds                      |
| GII                 | 30 Seeds + \( F. \) oxysporum spores      |
| GIII                | 30 Seeds + \( M. \) nivale spores        |
| GIV                 | 30 Seeds + \( N. \) solani spores        |
| GV                  | 30 Seeds + mixture of all strain spores   |

2.4.3. In Vitro Preventive Treatment Assay on Germination of \( L. \) perenne Seeds

For this, 510 seeds were disinfected with 12° sodium hypochlorite solution for 20 min and then rinsed twice with sterile distilled water for 10 min before being transferred to
Petri plates containing sterile layers of filter paper [34,35]. In this assay, the seeds were divided into 17 groups of 30 seeds (Table 3) and were treated with 1 mL of carvacrol on the first day before being infested. The experiment was carried out for 4 weeks. The percentage of seed germination was measured as reported elsewhere [11]. Quantitative data were expressed as means of triplicate experiments.

Table 3. Distribution groups for the preventive treatment assay.

| Distribution Groups |   |
|---------------------|---|
| GI                  | 30 intact seeds |
| GII                 | 30 seeds + F. oxysporum spores |
| GIII                | 30 seeds + N. solani spores |
| GIV                 | 30 seeds + M. nivele spores |
| GV                  | 30 seeds + mixture of all strain spores |
| GVI                 | 30 seeds + F. oxysporum spores + 0.05 mg/mL of carvacrol |
| GVII                | 30 seeds + F. oxysporum spores + 0.1 mg/mL of carvacrol |
| GVIII               | 30 seeds + F. oxysporum spores + 0.2 mg/mL of carvacrol |
| GIX                 | 30 seeds + M. nivele spores + 0.05 mg/mL of carvacrol |
| GX                  | 30 seeds + M. nivele spores + 0.1 mg/mL of carvacrol |
| GXI                 | 30 seeds + M. nivele spores + 0.2 mg/mL of carvacrol |
| GXII                | 30 seeds + N. solani spores + 0.05 mg/mL of carvacrol |
| GXIII               | 30 seeds + N. solani spores + 0.1 mg/mL of carvacrol |
| GXIV                | 30 seeds + N. solani spores + 0.2 mg/mL of carvacrol |
| GXV                 | 30 seeds + mixture of all strain spores + 0.05 mg/mL of carvacrol |
| GXVI                | 30 seeds + mixture of all strain spores + 0.1 mg/mL of carvacrol |
| GXVII               | 30 seeds + mixture of all strain spores + 0.2 mg/mL of carvacrol |

2.5. Statistical Analysis

Quantitative data were expressed as means of triplicate experiments ± SD (standard deviation). The groups studied are greater than 2 (n > 2), follow a normal distribution, the variances of the groups are all equal, the samples are taken randomly and independently in the groups. In addition, there is a single quantitative variable: carvacrol. The significance of the difference between means was evaluated by ANOVA –IBM SPSS Statistics 21, United Kingdom. For performing multiple comparisons, Student’s t-test was used as a post hoc test. Statistically, a significant difference was considered at a threshold of α = 5% [36]. The variables that were considered as a factor in each assay are presented in Table 4.

Table 4. The distribution of the variables studied in all experiments.

| Assays | Variables |
|--------|-----------|
| Growth inhibition | Carvacrol concentrations |
| Spores germination inhibition assay | Carvacrol concentrations |
| Spores destruction assay | Carvacrol concentrations |
| In vitro preliminary toxicity testing for carvacrol on germination of L. perenne seeds | Carvacrol concentrations |
| In vitro preliminary test of pathogenicity for fungal strains on germination of L. perenne seeds | Species of fungal strains |
| In vitro preventive treatment assay on germination of L. perenne seeds | Carvacrol concentrations |
3. Results
3.1. Antifungal Activity Assay

3.1.1. Mycelial Growth Inhibition Assay

The antifungal activity results obtained showed that all fungal species were very sensitive to carvacrol since the MIC value of carvacrol vs. \textit{F. oxysporum} was 0.25 mg/mL and 0.5 mg/mL for both \textit{M. nivale} and \textit{N. solani}. Regarding growth inhibition percentage (GI\%) of radial mycelium, the results obtained seem to show that the percentage of growth inhibition was linearly correlated to carvacrol dose up, which reached 100\% at 0.5 mg/mL against all strains tested. With lower concentrations than MIC, carvacrol slightly controlled the growth of strains (Figure 2). The growth inhibition of \textit{F. oxysporum}, \textit{M. nivale}, and \textit{N. solani} differed statistically between the different doses of carvacrol used, and the concentrations of carvacrol were statistically more efficient on \textit{F. oxysporum} (See post hoc test in Table 7).

![Growth inhibition percentage](image)

**Figure 2.** Variation of the growth inhibition percentage of radial mycelium as a function of carvacrol dose. The dose of carvacrol had a significant effect on GI at a threshold \(\alpha = 5\%\) for the three studied fungal strains \((p\text{-value} = 7.71 \times 10^{-20}, p\text{-value} = 1.78 \times 10^{-23}\) and \(p\text{-value} = 5.45 \times 10^{-17}\). For \textit{F. oxysporum}, \textit{M. nivale}, \textit{N. solani}). Error bars show standard deviations.

3.1.2. Spore Germination Assay

Table 5 illustrates the effect of carvacrol on the germination of fungal strains. At 0.2 mg/mL, carvacrol completely inhibited the germination of \textit{F. oxysporum} spores, while for \textit{M. nivale} and \textit{N. solani}, the inhibition was observed at a concentration of 0.4 mg/mL.

| Strains       | Number of Colonies (CFU) |
|---------------|--------------------------|
|               | 0.1 mg/mL | 0.2 mg/mL | 0.4 mg/mL | 0.8 mg/mL | 1 mg/mL |
| \textit{F. oxysporum} | 17 ± 1.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| \textit{N. solani}    | ≥300 | 44 ± 3.50 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| \textit{M. nivale}    | 288 ± 2.40 | 23 ± 2.10 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| Negative control   | ≥300 | ≥300 | ≥300 | ≥300 | ≥300 |

Values are means \((n = 3)\) ± SD.
3.1.3. Spore Destruction Assay

The results of the sporicidal effects of carvacrol on fungal strains are presented in Figure 3. Carvacrol showed a very strong sporicidal effect vs. all tested Fusarium strains. At a concentration of 0.8 mg/mL, carvacrol destroyed F. oxysporum, M. niveale, and N. solani spores immediately after 6 h, 12 h, and 24 h, respectively. After increasing the concentration (1 mg/mL), all strains’ spores were immediately destroyed after 3 h only.

![Figure 3](image_url)

Figure 3. Time course of spore destruction after being treated with different concentrations of carvacrol. Concentrations of carvacrol: (●) 0.1 mg/mL, (■) 0.2 mg/mL, (▲) 0.4 mg/mL, (▼) 0.8 mg/mL and (♦) 1 mg/mL. Error bars show standard deviations.

Microscopic representations are presented in Figure 4. The observations showed a difference between the untreated spores (Figure 4C) and those treated with carvacrol (Figure 4A). The untreated spores have normal and intact morphology. Once treated, their envelope relaxes, resulting in swelling at the ends and increased in size. For the spores in the control tube (Figure 4B1,B2), the observations showed the appearance of germ tubes, which will later give hyphae and then mycelium.

![Figure 4](image_url)

Figure 4. F. oxysporum spores observed by light microscope (magnification × 400): (A) arrows showing the swelling of the spore tips after 1 h of treatment with 1 mg/mL carvacrol; (B1,B2) arrows showing the germination of spores by the emergence of the germ tube, which will give rise to hyphae; and (C) normal spores in the negative control.
3.2. In Vitro Preventive Treatment Assay

3.2.1. Preliminary In Vitro Tests

A first preliminary test was conducted to assess the effect of carvacrol on the germination performance of *L. perenne* seeds. Germination in the negative control was 85%, and 80% for seeds treated with the three doses of carvacrol (0.05, 0.1, and 0.2 mg/mL), as shown in Figure 5. The different concentrations of carvacrol had no effect on the germination performance of *L. perenne* seeds (p-value = 0.552); furthermore, the difference between the negative control and each carvacrol concentration were not significant (see post hoc test results in Table 7); therefore, carvacrol had no phytotoxic effect on seeds.

![Figure 5](image-url)

**Figure 5.** Germination percentage of *L. perenne* seeds treated with carvacrol. The results do not differ significantly at a threshold $\alpha = 5\%$ (Table 6). Error bars show standard deviations.

**Table 6.** The p-values of all results analysed by the ANOVA test.

| Assays | p-Value | Statistical Conclusion |
|--------|---------|------------------------|
| Growth inhibition | *F. oxysporum*: p-value $= 7.71 \times 10^{-20} << 0.05$  
*M. nivale*: p-value $= 1.78 \times 10^{-23} << 0.05$  
*N. solani*: p-value $= 5.45 \times 10^{-17} << 0.05$ | The dose of carvacrol had a significant effect on GI at a threshold $\alpha = 5\%$ for the three studied fungal strains. |
| In vitro preliminary toxicity testing for carvacrol on germination of *L. perenne* seeds | p-value $= 0.552 > 0.05$ | The different concentrations of carvacrol had no effect on the germination performance of *L. perenne* seeds. |
| In vitro preliminary test of pathogenicity for fungal strains on germination of *L. perenne* seeds | p-value $= 4.27 \times 10^{-7} << 0.05$ | The fungal strains (including no fungal strains and a mixture of fungal species) had an effect on seed germination. |
| In vitro preventive treatment assay on germination of *L. perenne* seeds | *F. oxysporum*: p-value $= 1.88 \times 10^{-5} << 0.05$  
*M. nivale*: p-value $= 1.0666 \times 10^{-7} << 0.05$  
*N. solani*: p-value $= 2.42 \times 10^{-4} << 0.05$  
Mixture of spores: p-value $= 1.168 \times 10^{-6} << 0.05$ | The concentrations of carvacrol had an effect on seeds germination in all the considered cases. |

The results presented in Figure 6 show that *M. nivale* was the most phytopathogenic strain since the percentage of germination obtained with this strain was only 5% when compared to 85% for the control. *F. oxysporum* and *N. solani* can be considered less phytopathogenic since the germination percentage obtained with these strains was 36% and 53%, respectively. In the presence of the three strains, the germination percentage obtained was about 36%. It was noticed that *M. nivale* damages seeds, root, and coleoptile development of *L. perenne* plantlets, unlike *F. oxysporum* and *N. solani*, which showed a less harmful effect on the studied plant. The ANOVA test at a threshold $\alpha = 5\%$ prove
that the fungal strains had an effect on seed germination (p-value = 4.27 × 10^{-7} < 0.05). The difference between the negative control and F. oxysporum, M. nivale, and mixture of spores, also the difference between F. oxysporum and M. nivale, between M. nivale and N. solani, and between M. nivale and mixture of spores are significant (see post hoc test results in Table 7). In contrast, the difference between the negative control and N. solani, as well as the difference between F. oxysporum and N. solani, between F. oxysporum and mixture of spores, between N. solani and mixture of spores are not significant (see post hoc test results in Table 7).

![Figure 6](image_url)

**Figure 6.** Germination percentage of L. perenne seeds infested by spores of the three fungal strains. Error bars show standard deviations.

| Figure 2. Mycelial growth inhibition of F. oxysporum | Difference between: | Post Hoc Tests Results | Statistical Conclusions |
|-----------------------------------------------------|----------------------|------------------------|-------------------------|
| 0.0625 and 0.125                                    | P(T ≤ t) two-tail = 6.78 × 10^{-6} < 0.0083 | The deference is significant |
| 0.0625 and 0.25                                    | P(T ≤ t) two-tail = 5.5 × 10^{-8} < 0.0083 | The deference is significant |
| 0.0625 and 0.5                                     | P(T ≤ t) two-tail = 5.5 × 10^{-8} < 0.0083 | The deference is significant |
| 0.0625 and 1                                       | P(T ≤ t) two-tail = 5.5 × 10^{-8} < 0.0083 | The deference is significant |
| 0.0625 and 2                                       | P(T ≤ t) two-tail = 5.5 × 10^{-8} < 0.0083 | The deference is significant |

| Figure 2. Mycelial growth inhibition of M. nivale | Difference between: | Post Hoc Tests Results | Statistical Conclusions |
|-------------------------------------------------|----------------------|------------------------|-------------------------|
| 0.0625 and 0.125                                 | P(T ≤ t) two-tail = 0.00042 < 0.0083 | The deference is significant |
| 0.0625 and 0.25                                  | P(T ≤ t) two-tail = 5.29 × 10^{-7} < 0.0083 | The deference is significant |
| 0.0625 and 0.5                                   | P(T ≤ t) two-tail = 3.2 × 10^{-8} < 0.0083 | The deference is significant |
| 0.0625 and 1                                     | P(T ≤ t) two-tail = 3.2 × 10^{-8} < 0.0083 | The deference is significant |
| 0.0625 and 2                                     | P(T ≤ t) two-tail = 3.2 × 10^{-8} < 0.0083 | The deference is significant |

| Figure 2. Mycelial growth inhibition of N. solani | Difference between: | Post Hoc Tests Results | Statistical Conclusions |
|-------------------------------------------------|----------------------|------------------------|-------------------------|
| 0.0625 and 0.125                                 | P(T ≤ t) two-tail = 4.43 × 10^{-5} < 0.0083 | The deference is significant |
| 0.0625 and 0.25                                  | P(T ≤ t) two-tail = 0.00075 < 0.0083 | The deference is significant |
| 0.0625 and 0.5                                   | P(T ≤ t) two-tail = 4.32 × 10^{-9} < 0.0083 | The deference is significant |
| 0.0625 and 1                                     | P(T ≤ t) two-tail = 4.32 × 10^{-9} < 0.0083 | The deference is significant |
| 0.0625 and 2                                     | P(T ≤ t) two-tail = 4.32 × 10^{-9} < 0.0083 | The deference is significant |

**Table 7.** Statistical conclusions of all results analysed by post hoc test.
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Table 7. Cont.

| Difference between: | Post Hoc Tests Results | Statistical Conclusions |
|---------------------|------------------------|-------------------------|
| Negative control and 0.05 | P(T ≤ t) two-tail = 0.32 > 0.01 | The deference is not significant |
| Negative control and 0.1 | P(T ≤ t) two-tail = 0.16 > 0.01 | The deference is not significant |
| Negative control and 0.2 | P(T ≤ t) two-tail = 0.32 > 0.01 | The deference is not significant |
| 0.05 and 0.1 | P(T ≤ t) two-tail = 0.93 > 0.01 | The deference is not significant |
| 0.05 and 0.2 | P(T ≤ t) two-tail = 1 > 0.01 | The deference is not significant |
| 0.1 and 0.2 | P(T ≤ t) two-tail = 0.93 > 0.01 | The deference is not significant |

3.2.2. In Vitro Preventive Treatment Assay

Figure 7 shows the preventive effect of carvacrol on the germination capacity of *L. perenne* seeds. The results obtained showed that most of the carvacrol doses tested reestablished the emergence of *L. perenne* seeds in comparison with the negative and positive controls (see the results of ANOVA tests and post hoc tests in Tables 6 and 7, respectively). The preventive treatment with carvacrol also showed satisfactory results even in the case of seeds infested by a mixture of spores. The results obtained showed also serious diseases occurred in nontreated plantlets when compared to treatments.
even in the case of seeds infested by a mixture of spores. The results obtained showed also serious diseases occurred in nontreated plantlets when compared to treatments.

Figure 7. Preventive effect of carvacrol treatment on germination of infested *L. perenne* seeds. The concentration of carvacrol had an effect on seeds germination in all the considered cases (\(p\)-value = \(1.88 \times 10^{-5}\); \(p\)-value = \(1.066 \times 10^{-7}\); \(p\)-value = \(2.42 \times 10^{-4}\) and \(p\)-value = \(1.168 \times 10^{-6}\) for *F. oxysporum*, *M. nivale*, *N. solani* and mixture of spores, respectively). Error bars show standard deviations.

4. Discussion

The present work aimed to find room for the intervention of natural products to mitigate fungal species attacking grasses. To achieve this goal, carvacrol, as an environmentally friendly agent, was tested in vitro to control fungal species responsible for seeds’ damping-off diseases.
The results obtained showed that carvacrol investigated in the present work can meet the requirement for being environmentally friendly argent to control strains spores due to its results in inhibiting this pathogenic strain. The findings obtained showed that carvacrol seriously controlled the tested genera even at low concentrations (MIC values ranged from 0.2 to 0.4 mg/mL). These results are consistent with those previously reported elsewhere [32,37], which showed that essential oil with natural carvacrol had an inhibitory effect on germination of *Fusarium oxysporum f. dianthi* spores. As regards the effect of carvacrol against *N. solani*, the MIC was 0.21 mg/mL. Moreover, earlier works reported closer MIC value for *M. nivale* treated with essential oils contained carvacrol [38–41]. The difference in MICs can be due to the use of different methods, in particular, the concentration of spore inoculum, effects of incubation temperature, time of reading on broth dilution, the use of detergents or solvents for carvacrol dispersion, which affect the antifungal activity [26,42]. Methods by which MIC is evaluated (liquid or agar medium), as well as the difference in culturing techniques, cannot be excluded [20,43].

The mechanism by which carvacrol kills fungal cells was investigated in earlier works [24,44], which showed that carvacrol exerts its antifungal activity by generating endoplasmic reticulum (ER) stress through the alteration of ER integrity. Therefore, carvacrol acts inside the fungal cells by modifying cell metabolism. In the present study, the sporidical effect of carvacrol on strains spores was assessed using a liquid medium. The results obtained show that carvacrol exhibited a serious sporidical effect on the strains tested in a dose deponent manner, which reached 100% inhibition at 0.4 mg/mL. These results agree with those reported in the earlier literature, which revealed that carvacrol possesses strong inhibitory effects on *Fusarium oxysporum f. dianthi* [32].

The microscopic observation showed a serious sporidical effect resulted in strains spores treated with carvacrol when compared to untreated spores. Microscopic observations revealed a serious decrease in the total number of spores in the group treated with high concentrations of carvacrol when compared to the negative control. This decrease in spore number is probably due to spore lyses. Similarly, Yamamoto-Ribeiro et al. [45] reported that spore cells of *Fusarium verticillioides* become deformed and devoid of cytoplasmic content after being treated with Ginger essential oil (EO) (*Zingiber officinale* Roscoe). These results could be explained by the fact that carvacrol acts at the level of the cell membrane. These results are corroborated by Bennis et al. and Chami et al. who elucidated the effect of clove and oregano EO on *Saccharomyces cerevisiae* cells [46,47]. Moreover, close results were obtained by Xing et al., who demonstrated the effect of cinnamaldehyde on *Fusarium verticillioides* using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) [48].

In the present study, the preventive potential effect of carvacrol was investigated in two different tests. The first was dedicated to studying the potential toxic effect of carvacrol on *L. perenne* seedling. Based on the results obtained, further processing was conducted to study the preventive potential. The results obtained showed that carvacrol had no toxic effects on *L. perenne* seedling when compared to untreated seeds since ANOVA 1 showed no significant difference (p-value > 0.05). Therefore, carvacrol did not adversely affect seedling germination at all tested doses. It is thus fitting that our results agree with those reported by Gonçalves et al., who confirmed the absence of phytotoxicity of carvacrol on tomato seeds [49]. Regarding the preventive effect of carvacrol investigated in the present work, the result obtained showed that in the absence of carvacrol, all fungal strains seriously inhibited seed germination whether individually or combined. These results were closely found to be similar to those reported by Boudoudou et al., who showed alterations in rice seed germination induced by fungal species [11]. *Fusarium graminearum* was the most pathogenic species to rice (Elio variety) since its inhibitory effect on seed germination reached 65%; meanwhile, *M. nivale* and *N. solani* have no serious adverse effect on germination as reported elsewhere [9,50].

The results obtained in our study showed that serious lesions occurred in the seed group inoculated with *M. nivale*. This notice is in accordance with the results reported in
earlier work [51], in which it was reported that seeds of Rice infected by *F. graminearum* got changed in terms of appearance by having white, yellow, reddish, or pink spots [51].

To the best of our knowledge, this is the first report concerning *L. perenne* seed treatment with carvacrol that resulted in inhibition of fungal diseases in seedlings without phytotoxic effects. The results obtained showed that pretreatment of seeds with carvacrol protects them against seeds damping-off disease induced by fungal species attack.

5. Conclusions

In summary, this study shed light on carvacrol as a potential alternative agent to fight fungal species that induce seeds damping-off. The results obtained showed that carvacrol seriously controlled fungal strains tested such as *F. oxysporum*, *N. solani*, and *M. nivale*. Therefore, carvacrol can be used as a natural weapon to protect plant seedlings from fungal disease without secondary effects on the environment. The current findings can serve as a valuable source for further research to exploit carvacrol as an environmentally friendly agent to fight seeds’ damping-off diseases.

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**References**

1. Agrios, N.G. *Plant Pathology*, 5th ed.; Academic Press: New York, NY, USA, 2005.
2. Guo, Q.; Dong, W.; Li, S.; Lu, X.; Wang, P.; Zhang, X.; Wang, Y.; Ma, P. Fengycin produced by Bacillus subtilis NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. *Microbiol. Res.* 2014, 169, 533–540. [CrossRef]
3. Gerhardson, B. Biological substitutes for pesticides. *Trends Biotechnol.* 2002, 20, 338–343. [CrossRef]
4. Ardakani, S.S.; Heydari, A.; Khorasani, N.; Arjmandi, R. Development of new bioformulations of *Pseudomonas fluorescens* and evaluation of these products against damping-off of cotton seedlings. *J. Plant Pathol.* 2010, 92, 83–88.
5. Lahlali, R.; Hiji, M. Screening, identification and evaluation of potential biocontrol fungal endophytes against *Rhizoctonia solani* AG3 on potato plants. *FEMS Microbiol. Lett.* 2010, 311, 152–159. [CrossRef]
6. Ascencion, L.C.; Liang, W.-J.; Yen, T.-B. Control of *Rhizoctonia solani* damping-off disease after soil amendment with dry tissues of Brassica results from increase in Actinomycetes population. *Plant Dis.* 1982, 66, 700–703. [CrossRef]
7. Stephens, C.T.; Herr, L.J.; Schmitthenner, A.F.; Powell, C.C. Characterization of *Rhizoctonia* isolates associated with damping-off of bedding plants. *Plant Dis.* 1982, 66, 700–703. [CrossRef]
8. Lamprecht, S.; Tewoldemedhin, Y. *Fusarium* species associated with damping-off of rooibos seedlings and the potential of compost as soil amendment for disease suppression. *S. Afr. J. Bot.* 2017, 110, 110–117. [CrossRef]
9. Ahmed, N.E.; Sugimoto, Y.; Babiker, A.G.T.; Mohamed, O.E.; Ma, Y.; Inanaga, S.; Nakajima, H. Effects of *Fusarium solani* isolates and metabolites on *Striga* germination. *Weed Sci.* 2017, 49, 354–358. [CrossRef]
10. Jaber, M.H.; Lahuf, A.A. Survey, pathogenicity and molecular identification of novel *Fusarium* species causing seed decay and damping-off disease of wheat crop in Kerbala Province, Iraq. *Plant Cell Biotechnol. Mol. Biol.* 2020, 21, 1–14.
11. Boudoudou, H.; Hassikou, R.; Touhami, A.O.; Badoc, A.; Douira, A. First demonstrations of fusaric acid on rice germination and plantlets. *Bull. Soc. Pharm. Bordeaux* 2009, 148, 45–54.
12. Summerell, B.A.; Laurence, M.H.; Liew, E.C.Y.; Leslie, J.F. Biogeography and phylogeography of *Fusarium*: A review. *Fungal Divers.* 2010, 44, 3–13. [CrossRef]
13. Gan, J.; Becker, J.O.; Ernst, F.F.; Hutchinson, C.; Knuteson, J.A.; Yates, S.R. Surface application of ammonium thiosulfate fertilizer to reduce volatilization of 1,3-dichloropropene from soil. *Pest Manag. Sci.* 2020, 56, 264–270. [CrossRef]
14. Amini, J.; Dzhahilov, F.S. The effects of fungicides on *Fusarium oxysporum f.* sp. lycopersici associated with fusarium wilt of tomato. *J. Plant Prot. Res.* 2010, 50, 172–178. [CrossRef]
15. Klieber, A.; Scott, E.; Wuryatmo, E. Effect of method of application on antifungal efficacy of citral against postharvest spoilage fungi of citrus in culture. *Australas. Plant Pathol.* **2002**, *31*, 329–332. [CrossRef]

16. Amrati, F.E.Z.; Bourhia, M.; Saghrourchi, H.; Slighoua, M.; Gafra, A.; Ullah, R.; Bousta, D. Carallumia europaea (Guss.) NE Br. Anti-Inflammatory, Antifungal, and Antibacterial Activities against Nosocomial Antibiotic—Resistant Microbes of Chemically Characterized Fractions. *Molecules* **2021**, *26*, 636. [CrossRef]

17. Jawhari, F.Z.; Moussauoi, A.E.; Bourhia, M.; Imtara, H.; Saghrourchi, H.; Ammor, K.; Bari, A. *Anacyclus pyrethrum* var. *pyrethrum* (L.) and *Anacyclus pyrethrurus* var. *depressus* (Ball) Maire: Correlation between Total Phenolic and Flavonoid Contents with Antioxidant and Antimicrobial Activities of Chemically Characterized Extracts. *Plants* **2021**, *10*, 149. [CrossRef] [PubMed]

18. Agour, A.; Missillou, I.; Saghrourchi, H.; Bari, A.; Lyoussi, B.; Derwih, E. Essential Oils of Haplophyllum tuberculatum (Forsskal) A. Juss from Morocco. *Trop. J. Nat. Prod. Res.* **2020**, *4*, 1108–1110.

19. Saghrourchi, H.; El Barnossi, A.; Chechchoua, H.; Mzabi, A.; Tanghort, M.; Remmal, A.; Fouzia, C. Study the effect of Carvacrol, Eugenol and Thymol on Fusarium sp. responsible for *Lolium perenne* fusariosis. *Ecol. Environ. Conserv.* **2020**, *26*, 1059–1067.

20. Abbaszadeh, S.; Sharifzadeh, A.; Saghrouchni, H.; Slighoua, M.; Grafov, A.; Ullah, R.; Bousta, D. *Caralluma europaea* (Guss.) NE Br: Effects of incubation temperature, inoculum size, and time of reading on broth microdilution susceptibility test results for amphotericin B against Fusarium. *Antimicrob. Agents Chemother.* **1997**, *41*, 808–811. [CrossRef]

21. Qiao, X.; Liu, Q.; Huang, Y.; Xia, Y.; Zhang, S. Management of bacterial spot of tomato caused by copper-resistant *Xanthomonas perforans* using a small molecule compound carvacrol. *Crop. Prot.* **2020**, *132*, 105114. [CrossRef]

22. Lahlol, R.A.; Bounechada, M.; Mohammedi, A.; Silva, L.R.; Alves, G. Dietary use of Rosmarinus officinalis and Thymus vulgaris as anticoagulants in poultry. *Anim. Feed Sci. Technol.* **2021**, *273*, 114826. [CrossRef]

23. Dinardo, F.R.; Deffiorio, M.; Casalino, E.; Crescenziani, G.; Centoducati, G. Effect of seed supplementation with *Origanum vulgare* L. essential oil on sea bass (*Dicentrarchus labrax*): A preliminary framework on metabolic status and growth performances. *Aquac. Rep.* **2020**, *18*, 100511. [CrossRef]

24. Chaillot, J.; Tebbji, F.; Remmal, A.; Boone, C.; Brown, G.W.; Bellaoui, M.; Sellam, A. The Monoterpene Carvacrol Generates Endoplastic Reticulum Stress in the Pathogenic Fungus *Candida albicans*. *Antimicrob. Agents Chemother.* **2015**, *59*, 4584–4592. [CrossRef] [PubMed]

25. Pujol, I.; Guarro, J.; Sala, J.; Riba, M.D. Effects of incubation temperature, inoculum size, and time of reading on broth microdilution susceptibility test results for amphotericin B against Fusarium. *Antimicrob. Agents Chemother.* **1997**, *41*, 808–811. [CrossRef]

26. Noman, E.A.; Rahman, N.N.N.A.; Shahadat, M.; Nagao, H.; Al-Karkhi, A.F.M.; Al-Gheethi, A.; Lah, T.N.T.; Omar, A.K.M. Supercritical Fluid CO$_2$ Technique for Destruction of Pathogenic Fungal Spores in Solid Clinical Wastes. *CLEAN Soil Air Water* **2016**, *44*, 1700–1708. [CrossRef]

27. Garg, S.; Naidu, J.; Singh, S.; Nawange, S.; Jharia, N.; Saxena, M. In vitro activity of terbinfine against Indian clinical isolates of *Candida albicans* and non-albicans sp. using a macrodilution method. *J. Mycol. Med.* **2006**, *16*, 119–125. [CrossRef]

28. Komaruzzaman, M.; Sani, M.A.; Ahmed, S.P.; Sultana, R. Effect of volatile organic compounds mediated fungal growth inhibition by Trichoderma asperellum HbGT6-07. *Res. Sq.* **2021**, *1*, 1–29.

29. Peixoto-Neves, D.; Silva-Alves, K.S.; Gomes, M.D.M.; Lima, F.C.; Lahlol, S.; Magalhães, P.C.; Leal-Cardoso, J.H. Vasorelaxant effects of the monoterpenic phenol isomers, carvacrol and thymol, on rat isolated aorta: Original article. *Fundam. Clin. Pharmacol.* **2017**, *31*, 8–10. [CrossRef] [PubMed]

30. Regnier, T.; Combrinck, S.; Veldman, W.; Du Plooy, W. Application of essential oils as multi-target fungicides for the control of Geotrichum citri-aurantii and other postharvest pathogens of citrus. *Ind. Crops Prod.* **2014**, *61*, 151–159. [CrossRef]

31. Remmal, A.; Bouchkiki, T.; Tantouai-Elaraki, A.; Ettaieb, M. Inhibition of antibacterial activity of essential oils by tween 80 and ethanol in liquid medium. *J. Pharm. Belg.* **2015**, *70*, 352–356. [PubMed]

32. Hamdani, H.; Remmal, A.; Oukhouia, M.; Sennouni, C.; Jabeur, I. In-vitro Study of Anti-Fusarium Effect of Thymol, Carvacrol, Eugenol and Menthol. *J. Plant Pathol. Microbiol.* **2017**, *8*, 253–258. [CrossRef]

33. Tzortzakis, N.G.; Economakis, C.D. Antifungal activity of lemongrass (*Cymbopogon citratus* L.) essential oil against key postharvest pathogens. *Innov. Food Sci. Emerg. Technol.* **2007**, *8*, 253–258. [CrossRef]

34. Kordali, S.; Cakir, A.; Ozar, H.; Cakmakci, R.; Kesdek, M.; Mete, E. Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish *Origanum acutidens* and its three components, carvacrol, thymol and p-cymene. *Bioreour. Technol.* **2008**, *99*, 8788–8795. [CrossRef] [PubMed]

35. Kordali, S.; Cakir, A.; Sutay, S. Inhibitory Effects of Monoterpenes on Seed Germination and Seedling Growth. *Z. Nat. C* **2007**, *62*, 207–214. [CrossRef] [PubMed]

36. El Barnossi, A.; Saghrourchi, H.; Moussaid, F.; Chahmi, N.; Houseini, A.J. Microbiological study of effects of solid organic waste (chicken droppings and sheep manure) decomposed in the soil used for *Pisum sativum* cultivation. *Int. J. Environ. Stud.* **2020**, *77*, 830–842. [CrossRef]

37. Mennti, A.; Gregori, R.; Nerl, F. Activity of natural compounds on *Fusarium verticillioides* and fumonisin production in stored maize kernels. *Int. J. Food Microbiol.* **2010**, *136*, 304–309. [CrossRef] [PubMed]

38. Kong, J.; Xie, Y.-F.; Guo, Y.-H.; Cheng, Y.-L.; Qian, H.; Yao, W.-R. Biocontrol of postharvest fungal decay of tomatoes with a combination of thymol and salicylic acid screening from 11 natural agents. *LWT* **2016**, *72*, 215–222. [CrossRef]

39. Shukla, R.; Singh, P.; Prakash, B.; Dubey, N. Antifungal, aflatoxin inhibition and antioxidant activity of *Callistemon lanceolatus* (Sm.) Sweet essential oil and its major component 1,8-cineole against fungal isolates from chickpea seeds. *Food Control* **2012**, *25*, 27–33. [CrossRef]
40. El Omari, N.; Guaouguaou, F.E.; Benali, T.; Aanniz, T.; Chamkhi, I.; Balahbib, A.; Taha, D.; Shariati, M.A.; Zengin, G.; et al. Phytochemical and biological activities of *Pinus halepensis* mill., and their ethnomedical use. *J. Ethnopharmacol.* **2021**, *268*, 113661. [CrossRef]

41. Bouyahya, A.; Chamkhi, I.; Guaouguaou, F.-E.; Benali, T.; Balahbib, A.; El Omari, N.; Taha, D.; El-Shazly, M.; El Menyiy, N.; Fatima-Ez-zahrae, G. Ethnomedicinal use, phytochemistry, pharmacology, and food benefits of *Thymus capitatus*. *J. Ethnopharmacol.* **2020**, *259*, 112925. [CrossRef] [PubMed]

42. Remmal, A.; Bouchikhi, T.; Rhayour, K.; Ettayebi, M.; Tantaoui-Elaraki, A. Improved Method for the Determination of Antimicrobial Activity of Essential Oils in Agar Medium. *J. Essent. Oil Res.* **1993**, *5*, 179–184. [CrossRef]

43. Kalemba, D.; Kunicka, A. Antibacterial and Antifungal Properties of Essential Oils. *Curr. Med. Chem.* **2003**, *10*, 813–829. [CrossRef] [PubMed]

44. Divband, K.; Shokri, H.; Khosravi, A.R. Down-regulatory effect of *Thymus vulgaris* L. on growth and *Tri4* gene expression in *Fusarium oxysporum* strains. *Microb. Pathog.* **2017**, *104*, 1–5. [CrossRef] [PubMed]

45. Yamamoto-Ribeiro, M.M.G.; Grespan, R.; Kohiyama, C.Y.; Ferreira, F.D.; Mossini, S.A.G.; Silva, E.L.; Junior, M.M. Effect of *Zingiber officinale* essential oil on *Fusarium verticillioides* and fumonisins production. *Food Chem.* **2013**, *141*, 3147–3152. [CrossRef]

46. Chami, F.; Chami, N.; Bennis, S.; Bouchikhi, T.; Remmal, A. Oregano and clove essential oils induce surface alteration of *Saccharomyces cerevisiae*. *Phytother. Res.* **2005**, *19*, 405–408. [CrossRef] [PubMed]

47. Bennis, S.; Chami, F.; Chami, N.; Bouchikhi, T.; Remmal, A. Surface alteration of *Saccharomyces cerevisiae* induced by thymol and eugenol. *Lett. Appl. Microbiol.* **2004**, *38*, 454–458. [CrossRef] [PubMed]

48. Xing, F.; Hua, H.; Selvaraj, J.N.; Zhao, Y.; Zhou, L.; Liu, X.; Liu, Y. Growth inhibition and morphological alterations of *Fusarium verticillioides* by cinnamon oil and cinnamaldehyde. *Food Control* **2014**, *46*, 343–350. [CrossRef]

49. Gonçalves, D.C.; de Queiroz, V.T.; Costa, A.V.; Lima, W.P.; Belan, L.L.; Moraes, W.B.; Iorio, N.L.P.P.; Póvoa, H.C.C. Reduction of Fusarium wilt symptoms in tomato seedlings following seed treatment with *Origanum vulgare* L. essential oil and carvacrol. *Crop Prot.* **2021**, *141*, 105487. [CrossRef]

50. Gray, L.E.; Achenbach, L.A.; Duff, R.J.; Lightfoot, D. Pathogenicity of *Fusarium solani* f. sp. *glycines* isolates on soybean and green bean plants. *J. Phytopathol.* **1999**, *147*, 281–284. [CrossRef]

51. Agarwal, P.C.; Mortensen, S.N.; Mathur, C. *Maladies du riz Transmises par les Semences et Tests Phytosanitaires*; Ede CTA/ADRAO: Wageningen, The Netherlands, 2015; p. 95.