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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 sporicidal 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 dose up vs. all strains. Carvacrol increased the emergence of *L. perenne* seeds when compared to both uninfested and infested seeds. 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 biofongicide 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]:

$$GI\ (\%) = (dt - Dt/dt) \times 100$$

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.

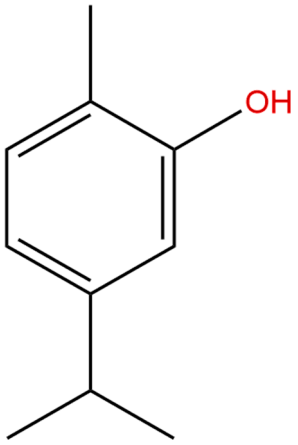


Figure 1. Chemical structure of carvacrol [29].

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]:

$$I\ (\%) = (Nc - Nt)/Nc \times 100 \quad (1)$$

where Nc and Nt 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 experiment.

Table 1. Distribution groups for the carvacrol toxicity test.

Distribution Groups	
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	
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. nivale* 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. nivale* spores + 0.05 mg/mL of carvacrol
GX	30 Seeds + *M. nivale* spores + 0.1 mg/mL of carvacrol
GXI	30 Seeds + *M. nivale* 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 $\alpha = 5\%$ [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 concentrationsTime
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. *F. oxysporum* was 0.25 mg/mL and 0.5 mg/mL for both *M. nivale* and *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 *F. oxysporum*, *M. nivale*, and *N. solani* differed statistically between the different doses of carvacrol used, and the concentrations of carvacrol were statistically more efficient on *F. oxysporum* (See post hoc test in Table 7).

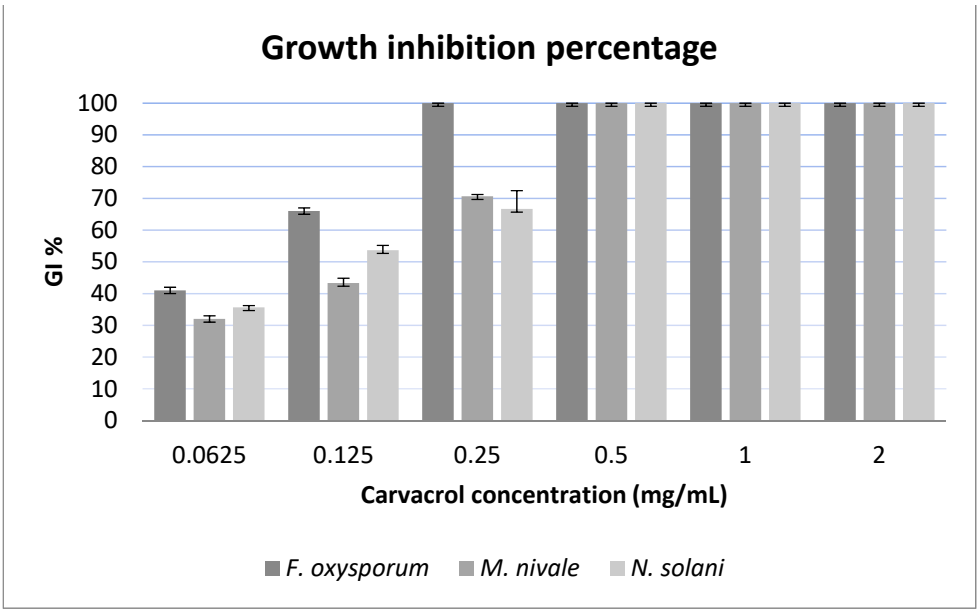


Figure 2. Variation of the growth inhibition percentage of radial mycelium as a function of carvacrol dose. The dose of carvacrol had a significant effect on GI at a threshold α = 5 % for the three studied fungal strains (p-value = 7.71 × 10^{-20}; p-value = 1.78 × 10^{-23} and p-value = 5.45 × 10^{-17}. For *F. oxysporum*, *M. nivale*, *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 *F. oxysporum* spores, while for *M. nivale* and *N. solani*, the inhibition was observed at a concentration of 0.4 mg/mL.

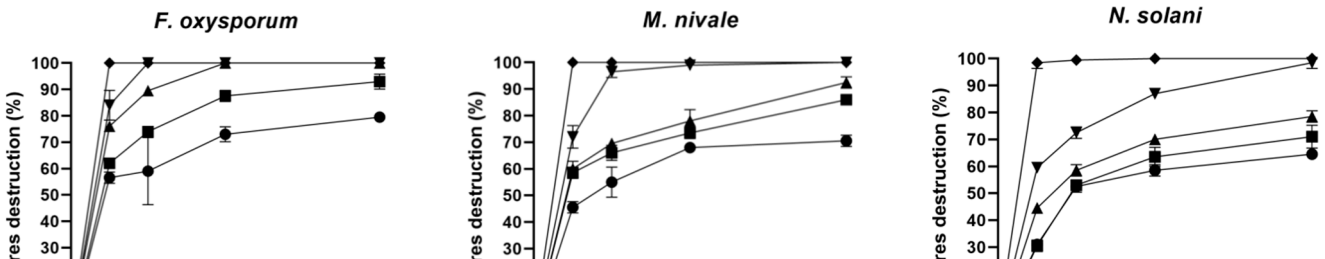
Table 5. Inhibitory effects of carvacrol at various concentrations on spore germination of fungal strains.

Strains	Number of Colonies (CFU)				
	0.1 mg/mL	**0.2 mg/mL**	**0.4 mg/mL**	**0.8 mg/mL**	**1 mg/mL**
F. oxysporum	17 ± 1.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
N. solani	≥300	44 ± 3.50	0 ± 0.00	0 ± 0.00	0 ± 0.00
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. nivale*, 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.



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.

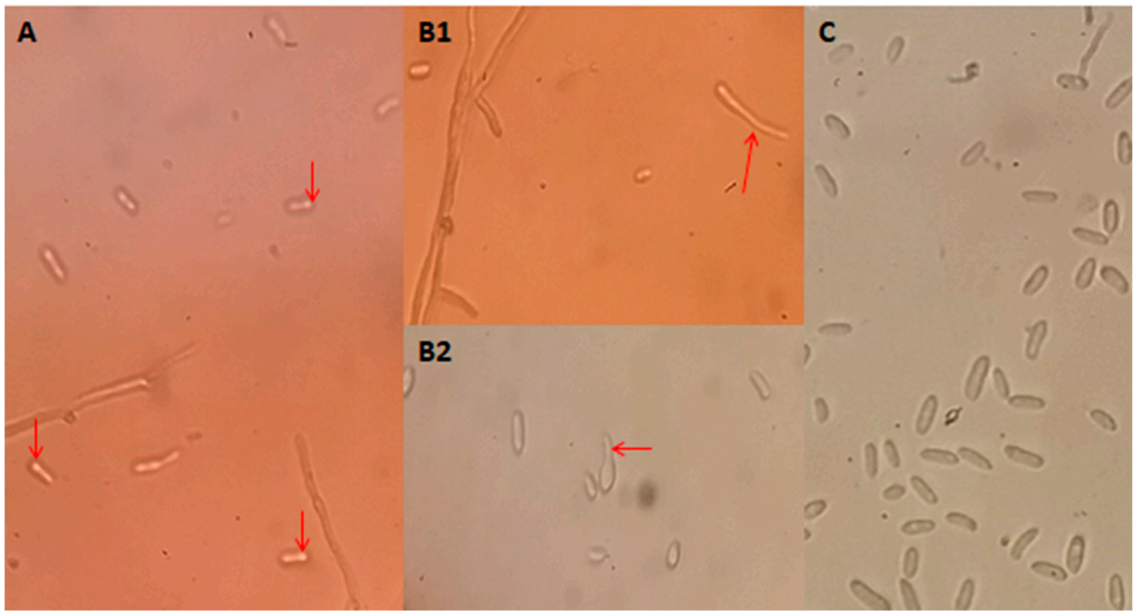


Figure 4. *F. oxysporum* spores observed by light microscope (magnification × 400): (**A**) arrows showing the swelling of the spore tips after 1 h of treatment with 1 mg/mL carvacrol; (**B1**,**B2**) arrows showing the germination of spores by the emergence of the germ tube, which will give rise to 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.

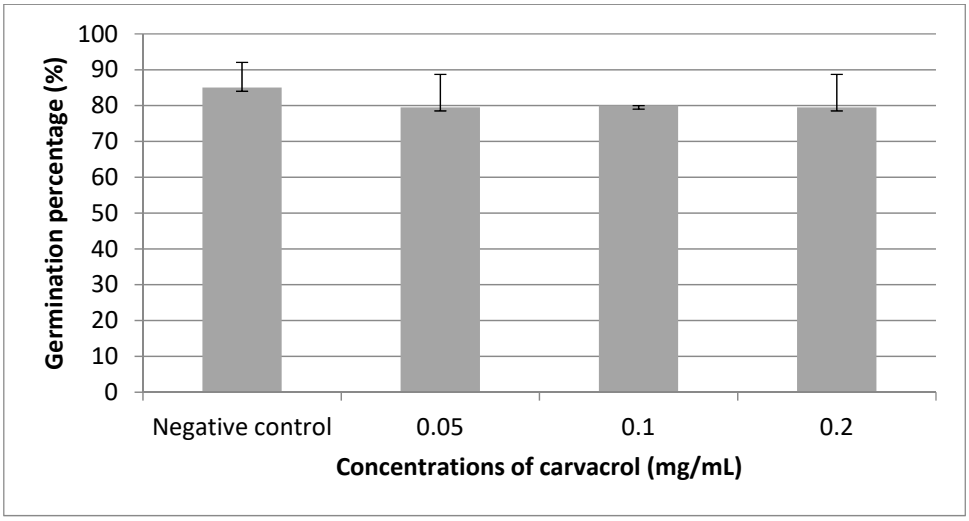


Figure 5. Germination percentage of *L. perenne* seeds treated with carvacrol. The results do not differ significantly at a threshold $\alpha = 5\%$ (Table 6). Error bars show standard deviations.

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).

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

	Difference between:	Post Hoc Tests Results	Statistical Conclusions
Figure 2. Mycelial growth inhibition of *F. oxysporum*	0.0625 and 0.125	$P(T \leq t)$ two-tail = $6.78 \times 10^{-6} < 0.0083$	The deference is significant
	0.0625 and 0.25	$P(T \leq t)$ two-tail = $5.5 \times 10^{-8} < 0.0083$	The deference is significant
	0.0625 and 0.5	$P(T \leq t)$ two-tail = $5.5 \times 10^{-8} < 0.0083$	The deference is significant
	0.0625 and 1	$P(T \leq t)$ two-tail = $5.5 \times 10^{-8} < 0.0083$	The deference is significant
	0.0625 and 2	$P(T \leq t)$ two-tail = $5.5 \times 10^{-8} < 0.0083$	The deference is significant
Figure 2. Mycelial growth inhibition of *M. nivale*	0.0625 and 0.125	$P(T \leq t)$ two-tail = $0.00042 < 0.0083$	The deference is significant
	0.0625 and 0.25	$P(T \leq t)$ two-tail = $5.29 \times 10^{-7} < 0.0083$	The deference is significant
	0.0625 and 0.5	$P(T \leq t)$ two-tail = $3.2 \times 10^{-8} < 0.0083$	The deference is significant
	0.0625 and 1	$P(T \leq t)$ two-tail = $3.2 \times 10^{-8} < 0.0083$	The deference is significant
	0.0625 and 2	$P(T \leq t)$ two-tail = $3.2 \times 10^{-8} < 0.0083$	The deference is significant
Figure 2. Mycelial growth inhibition of *N. solani*	0.0625 and 0.125	$P(T \leq t)$ two-tail = $4.43 \times 10^{-5} < 0.0083$	The deference is significant
	0.0625 and 0.25	$P(T \leq t)$ two-tail = $0.00075 < 0.0083$	The deference is significant
	0.0625 and 0.5	$P(T \leq t)$ two-tail = $4.32 \times 10^{-9} < 0.0083$	The deference is significant
	0.0625 and 1	$P(T \leq t)$ two-tail = $4.32 \times 10^{-9} < 0.0083$	The deference is significant
	0.0625 and 2	$P(T \leq t)$ two-tail = $4.32 \times 10^{-9} < 0.0083$	The deference is significant

Table 7. *Cont.*

	Difference between:	Post Hoc Tests Results	Statistical Conclusions
Figure 5. Germination percentage of *L. perenne* seeds treated with carvacrol	Negative control and 0.05	$P(T \leq t)$ two-tail = 0.32 > 0.0125	The deference is not significant
	Negative control and 0.1	$P(T \leq t)$ two-tail = 0.16 > 0.0125	The deference is not significant
	Negative control and 0.2	$P(T \leq t)$ two-tail = 0.32 > 0.0125	The deference is not significant
	0.05 and 0.1	$P(T \leq t)$ two-tail = 0.93 > 0.0125	The deference is not significant
	0.05 and 0.2	$P(T \leq t)$ two-tail = 1 > 0.0125	The deference is not significant
	0.1 and 0.2	$P(T \leq t)$ two-tail = 0.93 > 0.0125	The deference is not significant
Figure 6. Germination percentage of *L. perenne* seeds infested by spores of the three fungal strains	Negative control and *F. oxysporum*	$P(T \leq t)$ two-tail = 0.00016 < 0.01	The deference is significant
	Negative control and *M. nivale*	$P(T \leq t)$ two-tail = 1.1×10^{-5} < 0.01	The deference is significant
	Negative control and *N. solani*	$P(T \leq t)$ two-tail = 0.013 > 0.01	The deference is not significant
	Negative control and Mixture of spores	$P(T \leq t)$ two-tail = 0.00016 < 0.01	The deference is significant
	F. oxysporum and *M. nivale*	$P(T \leq t)$ two-tail = 0.00011 < 0.01	The deference is significant
	F. oxysporum and *N. solani*	$P(T \leq t)$ two-tail = 0.088 > 0.01	The deference is not significant
	F. oxysporum and Mixture of spores	$P(T \leq t)$ two-tail = 1 > 0.01	The deference is not significant
	M. nivale and *N. solani*	$P(T \leq t)$ two-tail = 0.0024 < 0.01	The deference is significant
	M. nivale and Mixture of spores	$P(T \leq t)$ two-tail = 0.00011 < 0.01	The deference is significant
	N. solani and Mixture of spores	$P(T \leq t)$ two-tail = 0.088 > 0.01	The deference is not significant
Figure 7. Preventive effect of carvacrol treatment on germination of infested *L. perenne* seeds by *F. oxysporum*	Negative contol and 0.05	$P(T \leq t)$ two-tail = 0.75 > 0.01	The deference is not significant
	Negative contol and 0.1	$P(T \leq t)$ two-tail = 0.014 > 0.01	The deference is not significant
	Negative contol and 0.2	$P(T \leq t)$ two-tail = 0.56 > 0.01	The deference is not significant
	Positive control and 0.05	$P(T \leq t)$ two-tail = 1.69×10^{-5} < 0.01	The deference is significant
	Positive control and 0.1	$P(T \leq t)$ two-tail = 5.7×10^{-5} < 0.01	The deference is significant
	Positive control and 0.2	$P(T \leq t)$ two-tail = 0.0059 < 0.01	The deference is significant
Figure 7. Preventive effect of carvacrol treatment on germination of infested *L. perenne* seeds by *M. nivale*	Negative contol and 0.05	$P(T \leq t)$ two-tail = 0.012 > 0.01	The deference is not significant
	Negative contol and 0.1	$P(T \leq t)$ two-tail = 0.058 > 0.01	The deference is not significant
	Negative contol and 0.2	$P(T \leq t)$ two-tail = 0.081 > 0.01	The deference is not significant
	Positive control and 0.05	$P(T \leq t)$ two-tail = 6.73×10^{-6} < 0.01	The deference is significant
	Positive control and 0.1	$P(T \leq t)$ two-tail = 4.42×10^{-5} < 0.01	The deference is significant
	Positive control and 0.2	$P(T \leq t)$ two-tail = 0.00036 < 0.01	The deference is significant
Figure 7. Preventive effect of carvacrol treatment on germination of infested *L. perenne* seeds by *N. solani*	Negative contol and 0.05	$P(T \leq t)$ two-tail = 0.26 > 0.01	The deference is not significant
	Negative contol and 0.1	$P(T \leq t)$ two-tail = 0.32 > 0.01	The deference is not significant
	Negative contol and 0.2	$P(T \leq t)$ two-tail = 0.0025 < 0.01	The deference is significant
	Positive control and 0.05	$P(T \leq t)$ two-tail = 0.0067 < 0.01	The deference is significant
	Positive control and 0.1	$P(T \leq t)$ two-tail = 0.026 > 0.01	The deference is not significant
	Positive control and 0.2	$P(T \leq t)$ two-tail = 0.23 > 0.01	The deference is not significant
Figure 7. Preventive effect of carvacrol treatment on germination of infested *L. perenne* seeds by mixture of spores	Negative contol and 0.05	$P(T \leq t)$ two-tail = 0.0028 < 0.01	The deference is significant
	Negative contol and 0.1	$P(T \leq t)$ two-tail = 0.058 > 0.01	The deference is not significant
	Negative contol and 0.2	$P(T \leq t)$ two-tail = 0.080 > 0.01	The deference is not significant
	Positive control and 0.05	$P(T \leq t)$ two-tail = 0.00059 < 0.01	The deference is significant
	Positive control and 0.1	$P(T \leq t)$ two-tail = 0.00086 < 0.01	The deference is significant
	Positive control and 0.2	$P(T \leq t)$ two-tail = 0.00021 < 0.01	The deference is 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.

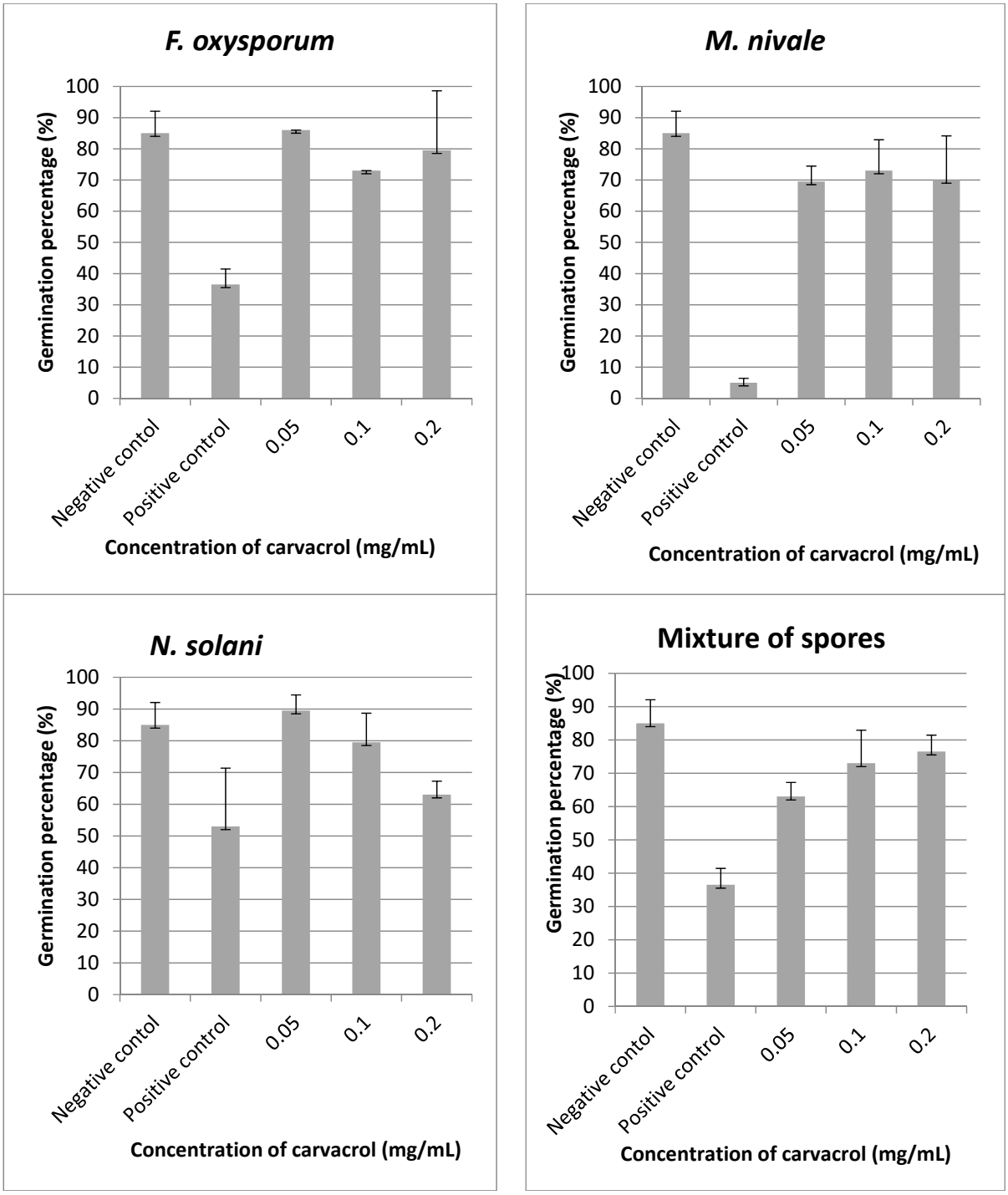


Figure 7. Preventive effect of carvacrol treatment on germination of infested *L. perenne* seeds. The concentration of carvacrol had an effect on seeds germination in all the considered cases (p-value = 1.88×10^{-5}; p-value = 1.066×10^{-7}; p-value = 2.42×10^{-4} and p-value = 1.168×10^{-6} for *F. 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 sporicidal effect of carvacrol on strains spores was assessed using a liquid medium. The results obtained show that carvacrol exhibited a serious sporicidal 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 sporicidal 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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