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Study the effect of Carvacrol, Eugenol and Thymol on *Fusariums* sp responsible for *Lolium perenne* fusariosis

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ABSTRACT

Most fungi of the genus *Fusarium* can cause fusariosis in turf, especially in the species *Lolium perenne* (ray grass) in golf. The purpose of this study was to find biological control alternatives to control turfgrass blight. During our research, we studied *in vitro* the effect of Carvacrol, Thymol, and Eugenol against three *Fusarium* species responsible for ray grass fusarium head blight by the macrodilution method. The minimum inhibitory concentration (MIC) values obtained were confirmed that the three majority compounds tested, except Eugenol, have high activity against the three *Fusarium* species. This activity has been shown at very low doses (0.05 mg/mL of Thymol and Carvacrol against *F. nivale* and *F. solani*, the three major compounds continue to have a significant partial inhibitory action against the three isolates. A concentration that is four times lower than the MIC of Carvacrol has been induced more than 60% inhibition. With regard to soil disinfection, Carvacrol has been shown to have a dose-dependent anti-*Fusarium* action on both sterile and normal soils by the sporale technique. This action was detected from the 2nd week onwards and becomes significant at the 4th week for doses above 0.2 g/L. In the light of the results of the current study, we can suggest that Carvacrol could become a natural alternative that could play a protective role against ray grass fusariosis without any harmful effects on the environment and humans.

Key words : *Carvacrol, Thymol, Eugenol, Fusarium and Lolium perenne*

Introduction

On the borderline between saprophytism and parasitism, the ubiquitous phytopathogenic fungus *Fusarium oxysporum*, responsible for grass vascular *Fusarium* disease, is a fungus that causes enormous damage in the agricultural field (Xing *et al.,* 2014; Summerell *et al.,* 2010). The disease caused by this plant pathogen is economically frightening because this fungus attacks several types of grasses including turf (rye grass) and leads to significant losses on sports fields, particularly golf courses and green spaces (Leonard and Bushnell, 2003). Ryegrass maintenance requires a large investment in chemical fungicides and organic amendments (WHO, 2005).

Since the disease is inevitably present in most crops, professionals (agronomists and farmers) most often adopt a preventive strategy that consists in treating, before planting, the whole area to be cultivated with extremely toxic products such as methyl bromide, dichloropropene (Gan *et al.,* 2000), and, during cultivation, with other products with significant toxicity such as chloropicrin and benomyl

(Amini and Sidovich, 2010). It is what makes fusariosis one of the most pesticide-intensive diseases.

Soil disinfection using various chemicals is a traditional practice that has been widely used around the world (Cebolla *et al.,* 2000; Fravel *et al.,* 2005). As a result, eco-friendly alternatives to pesticides such as organic amendments (Céline *et al.,* 2007) and beneficial microorganisms are increasingly developing as conventional biological control methods against soil-borne diseases (Wang *et al.,* 2012).

In recent years, biological and environmental protection are imposed (Lahlou, 2004; Gurib-Fakim, 2006; Karamaouna *et al.,* 2013), as well as the increase in demand for high-quality products by consumers (Tinivella *et al.,* 2009).

The use of biological control agents became a necessity (El Barnossi *et al.,* 2020a). The essential oils represented a good alternative for the control of fungal diseases such as vascular fusariosis without resistance risk (Tinivella *et al.,* 2009).

In the present study, we evaluated the antifungal activity of the three major components of essential oils: Thymol, Carvacrol, and Eugenol on three isolates of the genus *Fusarium*, as well as the effect on the infested soil.

Materials and Methods

Culture media

The agar media used in this study for the isolation and identification of *Fusarium* isolates are Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Sabouraud (SB) (Leslie *et al.,* 2006). The liquid medium used for the determination of the MIC and the minimum fungicide concentration (MFC) is Malt Extract broth. The media were autoclaved at 121 °C for 20 min (Fothergill, 2012).

Antifungal agents

Thymol (99%) used in this work was provided by SEEMA International (India), Carvacrol (99%) by FLAGRESSO (Austria) and Eugenol (99%) by FLUKA (Switzerland) (Oukhouia *et al.,* 2017).

Sample collection

Isolates of the genus *Fusarium* were isolated from the soil, and diseased turf during a visit to three courses, within Golf Royal Dar-Es-Salaam in Rabat, Morocco in accordance with (INRA, 2014). After disinfection with sodium hypochlorite solution and rinsing with sterile distilled water (EDS), 5 fragments of the turf from each sample site were deposited on a Sabouraud agar (Bouakaz *et al.,* 2013). For soil sampling, decimal dilutions were prepared in sterile physiological water containing 0.9% NaCl, then a volume of 100 µL was sown by spreading on the agar media in Petri dishes (90 mm). The boxes were incubated at 28 °C for 5 days in the dark.

Fungal material

Fusarium isolates were purified in three different media PDA, MEA, and SB. Identification was based on the description given by Leslie *et al.,* (2006). This identification is essentially based on macro and microscopic characters. For each isolate, the mycelial growth rate (MG) was calculated after 7 days of culture, using Brewer, (1960) formula;

MG = Diameter of the thall - (Diameter of the explant/2)

To identify each fungal isolate. We based on macroscopic characteristics: growth, color, topography, and odor, and microscopic: the appearance of mycelial filaments, the presence or not of anastomosis loops and partitions, special structures (cystide, cuticular cells), and fructification, were photographed, using a small photonic microscope of a camera, from fresh and dried smears, then colored with lactophenol blue solution (Bovio *et al.,* 2017 and El Barnossi *et al.,* 2019). To determine the genus of each fungal colony obtained, we adopted the Saccardo classification system (Barnett and Hunter, 1972). Species identification was carried out by reference to different determination keys (Nelson *et al.,* 1983; Wang and Zabel, 1992).

Preparation of the sporadic suspension

The spore suspension was obtained from a 7 days culture of *Fusarium* on the SB medium at 28 °C. The spores are detached with a sterile loop and then transferred to a tube containing 0.9% sterile NaCl. The number of spores in the mother suspension was determined on a Malassez cell, then diluted to an inoculum of about 10^6 spores/mL. The number of cells per average square is counted according to the characteristics of the blade used (area: 0.0025 mm^2; depth: 0.2 mm).

Evaluation of antifungal activity by the macrodilution method

The macrodilution method was used to determine the minimum inhibitory concentration (MIC) and

the minimum fungicide concentration (MFC) of Carvacrol, Thymol, and Eugenol tested on the three *Fusarium* isolates (Oukhouia *et al.,* 2017). Determination of the MIC of the majority compounds tested.

The MICs of each compound tested separately were determined by the macrodilution method (Garg *et al.,* 2005). The concentrations tested for Carvacrol, Thymol and Eugenol are as follows: 1/250, 1/500, 1/1000, 1/2000 and 1/4000. All tubes were inoculated with 100 µL of the *Fusarium* spore mother suspension (10^6spores/mL). The final concentration in each tube was calculated for a final volume of 5 mL. The whole unit was incubated under agitation for 5 days at 28 °C. The MIC value was also measured by spectrophotometry at a wavelength of 600 nm (Oukhouia *et al.,* 2017).

The mycelial growth (MG) in liquid medium was calculated by measuring the optical density at 600 nm to determine the percentages of growth inhibition. The MG is calculated according to the formula of Dohou *et al.,* (2004);

MG (%) = (Dot-Doi)/Dot 100

Where Dot and Doi respectively represent the optical density of the control and the optical density in the presence of a majority compounds concentration.

Determination of the MFC of the majority compounds tested

The tubes where there has been no visible positive growth, a fraction of 100 µL was aseptically collected and transferred to tubes containing 5 mL of the ME broth. Thus, the risk of carrying the inhibitory effect of MCs was eliminated by dilution of 50 times. MFC is defined as the lowest concentration of MCs for which there was no fungal growth at all compared to the control (CLSI, 2002).

Effect of Carvacrol on soil disinfection

A sporal suspension of each isolate was incorporated into disinfected topsoil; previously screened using a 2 mm sieve and autoclaved at 121 °C for 30 min twice. The density of the inoculum was 10^6 spores/g of topsoil. Samples of 100 g of infested topsoil were placed in plastic pots.

The three experimental batches are as follows: a control containing disinfected and uninfected topsoil, a positive test containing disinfected topsoil infested with *F.oxysporum* spores, and batches containing topsoil infested and treated with 0.1; 0.2; 0.4 or 0.8 g/L. The same experiment was performed in normal topsoil in three replicates. The T^- and T^+ lots received 15 mL/day of well water, while the Tit lots received 15 mL of the Thymol dose 3 times per week (Oukhouia *et al.,* 2017). The load of the topsoil into spores was determined using the serial dilution technique. The fungal load was determined at 0 days (after infestation, before soil treatment began) then at 7 days, 14 days, 21 days and 28 days. The treatment started at 1 day. One gram of topsoil from each batch is placed in a sterile tube containing 9 mL of 0.9 % NaCl. From this stock solution, a series of dilutions were performed. Each dilution was then spread on Petri dishes containing the Sabouraud medium. These boxes were incubated at 28 °C for three days. Colonies of *F. oxysporum* are then counted by the agar enumeration technique (Coulibaly and Agathos, 2003).

Statistical analysis

All experiments were repeated three times. The results were designed and processed using Microsoft Excel 2016 software. The statistical analysis of the results obtained was carried out using the SPSS 20 software based on a mean analysis (Student t-test) and an analysis of variance (ANOVA I) at the threshold of $\alpha = 5\%$ (El Barnossi *et al.,* 2020b).

Results

Characterization of the three species of the genus *Fusarium*

After purification, the three species presented the characteristic aspect of the genus *Fusarium* on the three-agar media used. Figure (1) illustrated the macroscopic aspects of the three *Fusarium* species, and Figure (2) showed the fusiform spores of *Fusarium oxysporum*.

The texture of *Fusarium* isolates (Fig. 1) was fluffy white on the front side. On the reverse side, the pigmentation was white/salmon for *F. oxysporum*, white for *F. nivale*, and white/orange for *F. solani*. As for the microscopic characteristics of the species, hyphae are seven with the presence of macroconidia and microconidia.

The results showed that the mycelial growth rate after 7 days was rapid for *F. oxysporum* and *F. nivale*, while it was moderate for *F. solani*. The results showed that the growth rate did not change with the culture medium except for *F. solani*, where the

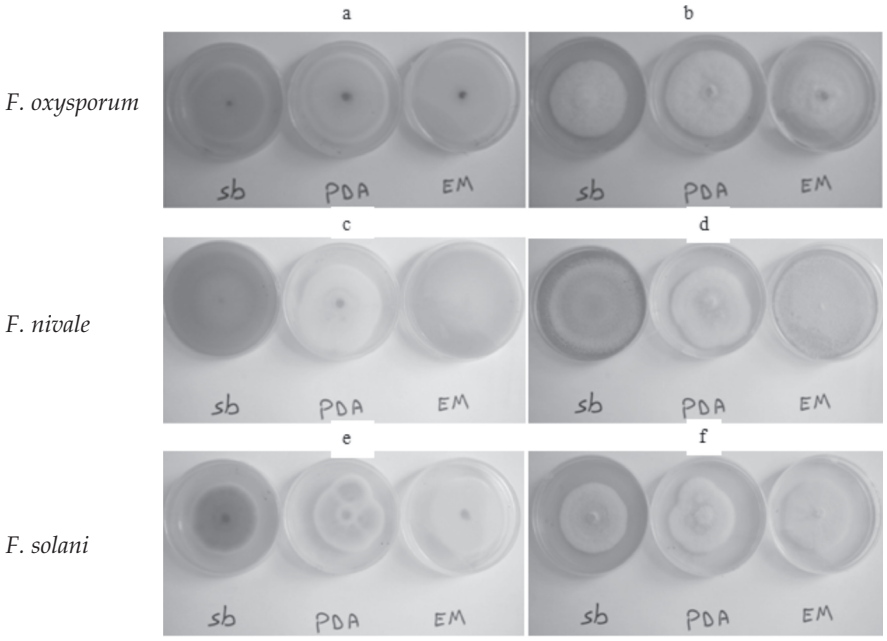

Fig. 1. Species of the genus *Fusarium* after transplanting into SB, PDA and MEA. a, c and e: bottom side. b, d and f: top side

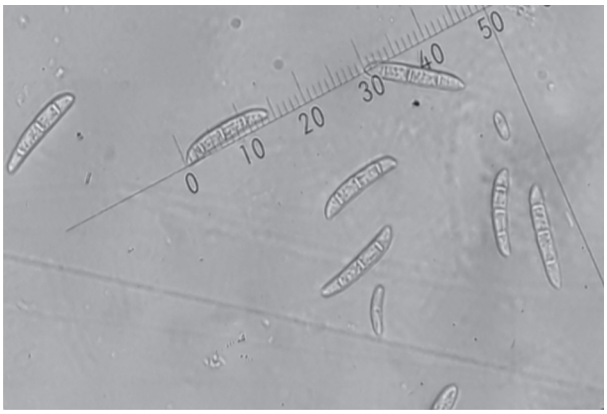

Fig. 2. *Fusiform* spores, curved, fairly pointed at the extremity of the *Fusarium oxysporum* isolate.

rate was varied between the MEA medium and the other media used.

Determination of MIC and MFC

The table below showed the MIC and MFC values of Thymol, Carvacrol and Eugenol tested against the three *Fusarium* species

The results Table 2 showed that Carvacrol had good antifungal activity against the three *Fusarium* species witha MIC of about 1/1000 against *F. oxysporum* and 1/2000 against *F. nivale* and *F. solani* and an MFC of about 1/1000 against *F. oxysporum* and *F. solani* and 1/2000 against *F. oxysporum*. Thymol came next with a MIC and MFC level of about 1/500 against *F. oxysporum* and 1/2000 against *F. nivale* and *F. solani*. While Eugenol was less effective than Thymol and Carvacrol with an MIC value between 1/250 and 1/1000 and an MFC of about 1/250 against *F. oxysporum* and 1/500 against *F. nivale* and *F. solani*.

The results Table 3 indicated that the percentage of inhibition of mycelial growth in liquid media (expressed as optical density at 600 nm) of Thymol, Carvacrol, and Eugenol against all three species.

Carvacrol was the most effective compound. It allowed a reduction of more than 60% with a value that represented half the value of the minimum in-

Table 1. Mycelial growth rate in cm of the *Fusarium* species studied in SB, PDA, and MEA

Isolate growth in cm	*F. oxysporum*	*F. nivale*	*F. solani*
SB	7.60 ± 0.60	8.71 ± 0.02	2.60 ± 1.41
PDA	7.46 ± 0.49	8.68 ± 0.07	2.06 ± 0.83
MEA	7.33 ± 0.58	8.64 ± 0.16	3.72 ± 0.37

Table 2. MIC and MFC of the different majority compounds (MC) tested against the species studied

Fusarium species	Majority compounds	MIC	MFC
F. oxysporum	Thymol	1/500	1/500
	Carvacrol	1/1000	1/1000
	Eugenol	1/250	1/250
F. nivale	Thymol	1/2000	1/2000
	Carvacrol	1/2000	1/2000
	Eugenol	1/500	1/500
F. solani	Thymol	1/2000	1/1000
	Carvacrol	1/2000	1/2000
	Eugenol	1/1000	1/500

Table 3. Inhibition percentages of essential oil compounds against *Fusarium* species by the liquid macrodilution technique

		Concentration tested					
Isolates	Majority compounds	1/250*	1/500*	1/1000*	1/2000*	1/4000*	Control +
F. oxysporum	Carvacrol	100 ± 0.00	100 ± 0.00	100 ± 0.00	62.81 ± 19.91	62.17 ± 8.94	0.00 ± 0.00
	Thymol	100 ± 0.00	100 ± 0.00	60.76 ± 8.35	57.55 ± 5.44	53.66 ± 2.66	0.00 ± 0.00
	Eugenol	100 ± 0.00	74.27 ± 16.16	62.65 ± 8.16	56.81 ± 8.31	54.50 ± 15.26	0.00 ± 0.00
F. nivale	Carvacrol	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	64.12 ± 8.82	0.00 ± 0.00
	Thymol	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	51.79 ± 15.94	0.00 ± 0.00
	Eugenol	100 ± 0.00	100 ± 0.00	51.32 ± 15.59	62.85 ± 16.28	46.79 ± 18.94	0.00 ± 0.00
F. solani	Carvacrol	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	38.66 ± 6.36	0.00 ± 0.00
	Thymol	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00	40.27 ± 28.73	0.00 ± 0.00
	Eugnol	100 ± 0.00	100 ± 0.00	100 ± 0.00	17.44 ± 11.71	41.18 ± 8.91	0.00 ± 0.00

The data displayed on the table is represented by the mean (n = 3) ± standard deviation. The results differ significantly ($p < 0.05$)

hibitory concentration against *F. oxysporum* and *F. nivale*. Thymol came in second place with a reduction of more than 50% for the same species. In addition, the results presented in Table 3 showed significant antifungal activity ($p<0.05$) between growth-positive controls and different MC concentrations for the three species studied.

Effect of Carvacrol on topsoil disinfection

Figure 3A showed the load of *F. oxysporum* in the previously disinfected soil, based on Carvacrol concentrations during 4 weeks of treatment. The charge of *F. oxysporum* in the batch containing only disinfected soil was zero throughout the experiment.

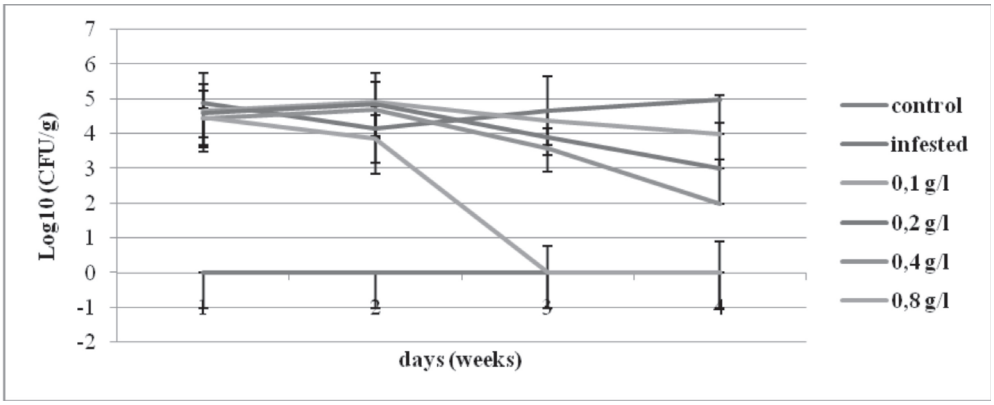

Fig. 3a. *Fusarium* loading during Thymol treatment in disinfected soil

However, the load of *F. oxysporum* in infested soil remained high and reached its maximum after the fourth week. Treatment with the four doses of Carvacrol resulted in a reduction in the dependent dose offungal load. The 0.8 g/L dose of Carvacrol was capable of reducing the *F. oxysporum* load to 0 after 3 weeks. Figure 3B showed the load of *F. oxysporum* in normal soil as a function of Carvacrol concentrations during 4 weeks of treatment. The *F. oxysporum* load of the uninfected control batch was high throughout the experiment, however, in infested soil, this load was higher. Treatment with all four doses of Carvacrol resulted in a load-dependent dose reduction. Again, the Carvacrol concentration of 0.8 g/L was capable to reduce the *F. oxysporum* load to 0 after three weeks of treatment.

Discussion

The method used for evaluating the action of MCs against the three species was the direct contact method (Bouddine *et al.,* 2012). The use of detergents such as tween 80, triton X100 or solvents such as ethanol and dimethylsulfoxide (DMSO) would inhibit antimicrobial activity. However, the advantage of this method is the use of a constant inoculum, as the size of the inoculum strongly influences the values of the MIC and MFC.

The three species have been tested for their sensitivity to the three CMs, Thymol, Carvacrol, and Eugenol. The choice of these three MCs is based on the fact that these compounds are not toxic at low doses (WHO, 2012) and have high antifungal activity (Manohar *et al.,* 2001; Pina-Vaz *et al.,* 2004; Tajkarimi, 2010).

The results (Table 3) showed that at doses lower than the MICs (0.5 mg/ml of Thymol and Carvacrol against *F. nivale* and *F. solani*, the 3 MCs had a significant partial inhibitory action on all three species. This means that in practice, it is not necessary to reach concentrations at the MIC in order to have an effect. A concentration that was four times lower than the MIC of Carvacrol caused more than 60% inhibition in liquid media. This inhibition also affects spore sporulation and germination of spores. This inhibition was carried out in a very rich culture medium and under very favorable temperature conditions.

Our results were in agreement with those reported by Divband *et al.,* (2017), which showed MIC values ranging from 5 to 20 mg/mL with Thymus vulgaris extract while CMF values ranged from 8 to 30 mg/ml. This activity is believed to be due to the hydroxyl group of Carvacrol (Pina-Vaz *et al.,* 2004). In a study by Kumar *et al.,* (2008), the essential oil of *T. vulgaris* had a broad fungitoxic spectrum against strains of *F. oxysporum*. Another report indicates that *T. vulgaris* oil has shown complete the inhibition of growth of *F. oxysporum* (Al-Rahmah *et al.,* 2013). Klaric *et al.,* (2007) showed in a study that the essential oil of *T. vulgaris* had a strong fungicidal activity against *Fusarium* spp. the MIC values obtained confirm that these 3 MCs except Eugenol, had a strong activity on the 3 species of *Fusarium.* It was noted that the MIC was very often equivalent to the MFC, which showed that the 3 MCs would have a fungicidal effect at very low doses. According to (Rasooli *et al.,* 2005; Sikkema *et al.,* 1994; Carson *et al.,* 2002), MCs caused irreversible damage to the cell wall, and to the cytoplasmic and nuclear membrane.

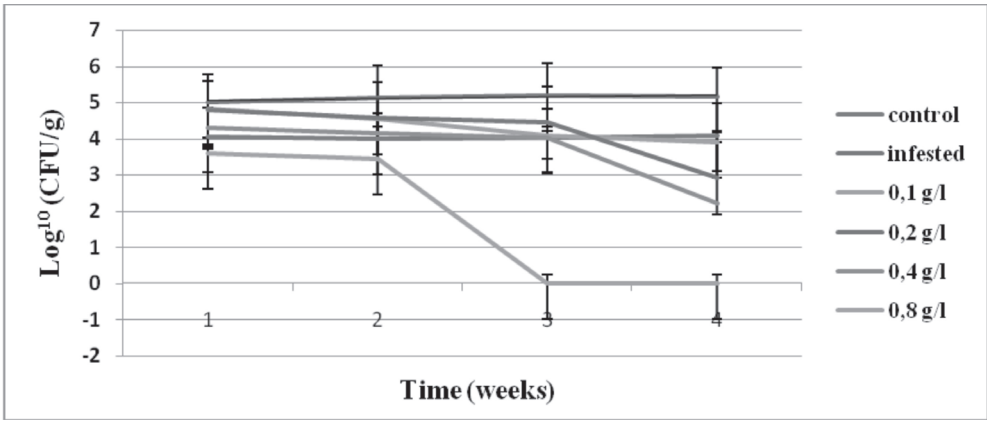

Fig. 3b. *Fusarium* loading during Thymol treatment in normal soil

Generally, soil treatment is carried out using systemic pesticides such as methyl bromide (Borrero *et al.,* 2009) or benomyl (Amini and Sidovich, 2010). These products are highly toxic either to humans or to the environment (WHO, 2005). After the results obtained, Carvacrol was chosen for soil treatment. For convenience, and it has shown very high antifungal activity. Other researchers have used plant extracts to treat soil infested with a *Fusarium* species (Bowers *et al.,* 2000) and some have chosen thymol over Ralstonia bacteria causing bacterial wilt in tomatoes (Ji *et al.,* 2005) and others have chosen thymol over *Fusarium*-infected soil (Oukhouia *et al.,* 2017). The action of Carvacrol has been tested on both types of soil. The first and one untreated topsoil, the second consisting of the same topsoil was disinfected by autoclave (30 min/121 °C) twice. Both were inoculated with a mixture of spores of all three species. Carvacrol doses ranged from 0.1 to 0.8 g/L. The results obtained show that Carvacrol has a dose-dependent anti-*Fusarium* action on both soils. This action was detectable from the 2nd week and became significant at the 4th week for doses above 0.2 g/L.

This experiment was carried out by (Oukhouia *et al.,* 2017) on soil not sterilized with Thymol, they used higher concentrations than our study, they managed to reduce the *Fusarium* soil load completely after 2 weeks with a high dose of 2 g/L. Thymol, on the other hand, we decreased the load with only 0.8 g/L Carvacrol. With the results of this study, Carvacrol could be considered as an alternative to the commonly used chemical fungicides; cycloheximide, and triazole triadimefon (Asami *et al.*, 2003) and the other products used for soil chloropicrin and benomyl (Amini and Sidovich, 2010). The species responsible for vascular fusariosis remain in the soil after cultivation and in crop residues (Edwards, 2004). For this purpose, it is necessary to apply soil disinfection before planting using biological antifungal agents to prevent the development of cryptogams (Isman, 2000; Yates *et al.,* 2002).

Conclusion

The majority of compounds are used as fungal growth inhibitors. In such a context, this research was carried out in order to study *in vitro* the antifungal activity of the majority compounds. The results obtained show a significant antifungal activity of the agents tested on *Fusarium* species responsible for ray grass fusariosis. The antifungal properties of the tested agents were observed at very low concentrations. Carvacrol has been shown to be most effective in inhibiting the growth of *Fusarium* species at concentrations below its own MIC. With regard to the preventive effect of Carvacrol. Soil treatment has shown efficacy in the disappearance of *Fusarium oxysporum* from the soil. These results suggest that Carvacrol could become a natural alternative that could play a very important role in combating fusariosiswithout any harmful effects on the environment or humans.

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