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In Vivo Antifungal Control of Citrus Black Spot (CBS) Using Thyme Oil as an Alternative to Synthetic Fungicides

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Abstract: The spread of Citrus black spot (CBS) is a major concern in citrus industry as it threatens fruit marketability and citrus tree health. Additionally, there are public concerns about the safety and side effects of synthetic fungicides used to control CBS. Synthetic fungicides are reported to be carcinogenic to humans and toxic to the environment. microorganisms often develop resistance to synthetic fungicides. This has prompted research into the identification of new ways with broad activity in treatment of microbial disease in plants such as the use of essential oils (EOs). Previous study by Magunga (2023) has proven the effectiveness thyme oil in vitro in the treatment of CBS using the agar diffusion bioassay, minimum inhibitory concentration (MIC) and scanning electron microscopy (SEM), furthermore the study indicated that the life cycle of CBS revolves around the citrus leaves and the fruits, however no study has been done in vivo to treat CBS using Thyme oil. The study current study aimed to investigate the use of thyme oil in vivo as an alternative antifungal treatment for CBS on lemon leaves and fruits. Thyme oil effectiveness together with that of its hydrosol, was tested in vivo on healthy lemon leaves and fruits infected with *Phyllosticta citricarpa* (CBS causative agent) as these are parts of the life cycle of the CBS infection. The results showed that Thyme oil demonstrated the highest severity of reduction because it almost completely inhibited fungal growth on the leaf and on the fruits surface. This indicates that Thyme oil can be used as potential alternative antifungal agents against CBS.

Keywords: *Citrus Black Spot*; *Phyllosticta citricarpa*; Thyme oil; essential oils; Hydrosol

Introduction

South Africa's citrus industry has received much media and policy attention due to concerns over increased fungal infection, namely CBS interceptions on the country's EU-bound shipments, leading to fears of a possible ban on South African citrus exports (Olivier, 2017). It is evident that CBS negatively affects the agricultural industry in terms of food production, security, and trade. As such, some trading partners were reluctant to import fresh fruit from production areas where CBS occurs; this has resulted in South Africa losing more than 45% of its export revenue due to postharvest infection of citrus fruit (80-90% losses), which was intended for the EU and Asian markets (Christie, 2016; Lesar, 2013; Montesinos-Herrero *et al.,* 2009). One of the main difficulties related to fungal pathogens is their progressive nature because they sometimes do not show during harvest or fruit grading in the pack house; symptoms can develop during storage or weeks after harvesting (Erasmus *et al.,* 2015). In exporting fruit, this window period matches the commercial shipping period and the fruit's arrival at the point of sale. The result is customer complaints at best and huge financial losses at worst (Erasmus *et al.,* 2015).

These losses affect profit margins, initial input costs, and lower retailer and consumer confidence in the marketplace (Erasmus *et al.,* 2015). The application of synthetic fungicides usually minimizes symptoms and yield losses caused by CBS infection during the fruit's susceptibility period; however, their use raises health and environmental pollution concerns, including resistance to pathogens (Arraiza *et al.,* 2018). Moreover, the treatment used currently against this pathogen has raised consumer concerns on issues such as food safety (Du Plooy *et al.,* 2009). The food safety concerns are due to the chemical residues left on food when synthetic fungicides are applied. Hence, research into developing alternative agents for replacing synthetic fungicides for fungal disease control in the agricultural sector continues

(Christie, 2016; Du Plooy *et al.,* 2009). An alternative antifungal needs to (a) overcome resistance problems observed against established commercial products, (b) have acceptable levels of persistence in the environment, and (c) have market and technical advantages over synthetic fungicides (Arraiza *et al.,* 2018). Essential oils (EOs) and their hydrosol can represent a new class of CBS antifungals due to their antimicrobial effects (Arraiza *et al.,* 2018). In addition, the probabilities of creating new resistant strains by using EOs as fungicidal agents are less likely since they have different chemical constituents that work in synergy simultaneously (Arraiza *et al.,* 2018). Previous study by Magunga (2023) has proven the effectiveness of thyme oil in vitro in the treatment of CBS using the agar diffusion bioassay, minimum inhibitory concentration (MIC) and scanning electron microscopy (SEM), furthermore the study indicated that the life cycle of CBS revolves around the citrus leaves and the fruits, however no study has been done in vivo to treat CBS using Thyme oil. The Thyme oil used in Magunga (2023) was characterised using GC-MS, were the chemical composition consisted mostly of terpenes as major compounds. Terpenes and their derivatives play a major role in the antimicrobial activities of EOs. This included thymol (32.1%) and ρ-cymene (20.4%) as major compounds, while other minor compounds detected were ranging between 0.5 and 5.1%, which included α-Pinene (5.1%), terpinene (3.0%).

Study aim

The overall aim of the study was to investigate the *in vivo* effect of thyme oil and thyme oil hydrosol against citrus fungal pathogen *Phyllosticta citricarpa*.

Objectives

To achieve the overall aim, the objectives of this study was to evaluate the inhibitory effect of both thyme oils and thyme oil hydrosol over *P. citricarpa in vivo*:

The evaluation of the inhibitory effect of thyme oils and thyme oil hydrosol on *P. citricarpa* pathogen was done on citrus leaves and citrus fruits infected with the fungal pathogen.

Materials and methods

Strain to be used

Phyllosticta citricarpa that Kiely preserved at the national collection of fungi (ARC-PPRI) in Pretoria, South Africa, was used in the study. It was cultivated using the yeast malt (YM) agar at 25 °C in Petri dishes until spore-releasing structures were observed (Ncango *et al.,* 2010). Spore suspensions (10^6 spores/mL) were prepared seven days after the incubation period by adding sterile ¼-strength Ringer's solution to colonized Petri dishes.

Inhibition of pathogen growth on leaves

Fragments (2.0 cm x 1.5 cm) of healthy leaves of the lemon tree (*Citrus sinensis*) were washed and autoclaved at a temperature of 120 °C and 1 Pa pressure for 20 minutes. Each leaf fragment was placed on a water-agar plate and inoculated with a mycelial disc of *P. citricarpa* (1 mm diameter). Then, 10 µL of treatment (thyme oil and thyme oil hydrosol) or controls were applied over the inoculated leaf fragment, and the plates were incubated for 21 days at 25 °C. Ethanol and Thiabendazole were used as the negative and positive controls, respectively. The results were reported by determining the highest to the smallest growth of pathogen development on the leaf.

Inhibition of pathogen growth on citrus fruits

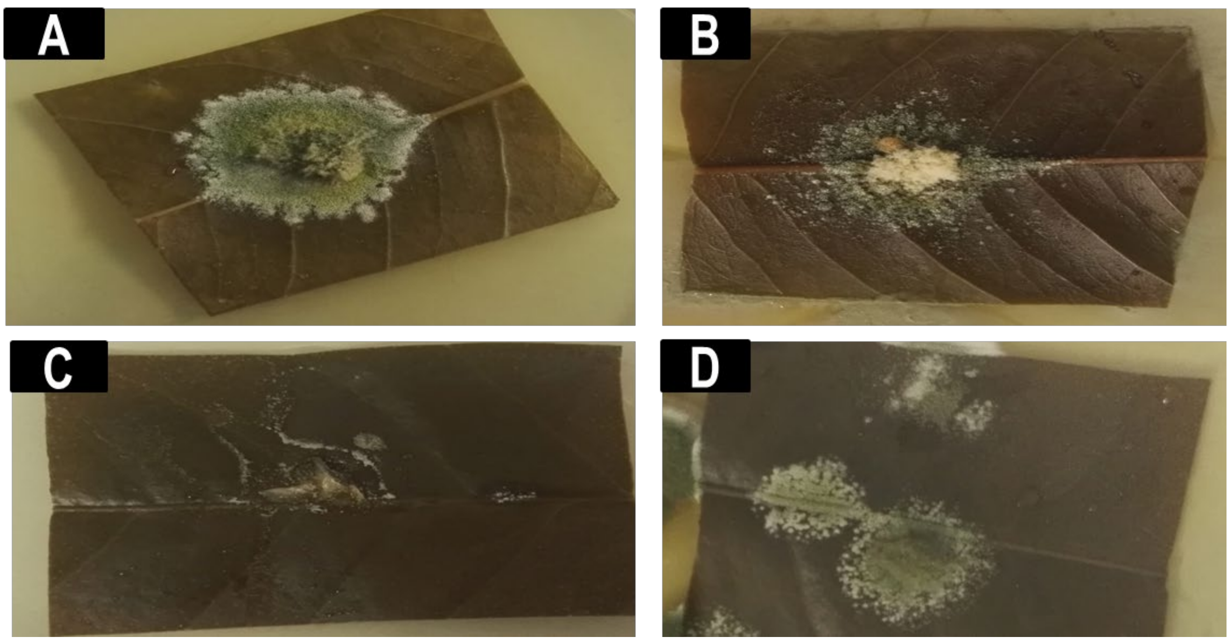
The experiment was done according to the method of Rodriguez *et al.* (2018) with slight modification. A total of 15 lemons were used in the study. Spore suspensions were prepared from 21-day-old cultures of *P. citricarpa* on yeast malt agar incubated in the dark at 25 °C. Spores were suspended in 2 mL of yeast malt broth and removed from sporulating colonies with a sterile pipette. Suspensions were adjusted to 10^6 conidia/mL. Lemons were wounded with a 1-mm-wide and 2-mm-deep sterile tip and were inoculated by injecting 5 µL of the spore suspension. Then, 10 µL of treatment (thyme oil and thyme oil hydrosol) was applied over the inoculated wounds. Ethanol and Thiabendazole were used as the negative and positive controls, respectively. Inoculated fruit was kept in sealed plastic trays in a security chamber at 25 °C and 80% relative humidity. Non-inoculated lemons wounded with a 1-mm-wide and 2-mm-deep sterile tip were used. Fruits were evaluated for the severity of symptoms by observing the lesion area without considering the point of inoculation (1 mm). The results were photographed weekly until the end of the experiment. All the experiments were done in triplicate.

Results and Discussion

Inhibition of pathogen growth on leaves

In this study, the antifungal activity of thyme oil was assessed *in vivo*, and the activity of Thiabendazole (positive control), hydrosol and ethanol (negative control) were also assessed on lemon leaves infected with *Phyllosticta citricarpa,* as shown in Figure 1. The activity of the abovementioned compounds was evaluated to determine their potential ability to inhibit or limit the development of *P. citricarpa* on the surface of sterile (20 min; 120 °C; 1 atm) lemon leaves. After a 21-day incubation period at 28 °C, the growth of *P. citricarpa* on the leaves of all the tested compounds was studied. The results of antifungal activity of the Thiabendazole (positive control), thyme oil, and hydrosol against *Phyllosticta citricarpa in vivo* on citrus leaves indicated a reduction of the severity incidence of *Phyllosticta citricarpa* compared with the untreated control.

Figure 1: Growth inhibition of *Phyllosticta citricarpa in vivo* on leaves. (A) Ethanol (negative control), (B) Thyme oil hydrosol, (C) Thyme oil (*Thymus vulgaris)* and (D) Thiabendazole.



Among all compounds tested, thyme oil (*Thymus vulgaris)* demonstrated the highest severity of reduction because it almost completely inhibited fungal growth on the leaf surface (Figure 9 C). Savi *et al.* (2019) conducted a similar study where secondary metabolites 7-O-β-D-glucosyl-genistein and 7-O-β-D-glucosyldaidzein produced by the strain *Microbacterium* spp. were used to inhibit the formation of *P. citricarpa* in citrus leaves. Their results indicated that both compounds could considerably inhibit the development of CBS of *P. citricarpa* on leaves. The current study also showed that Thiabendazole and hydrosol reduced the growth of the fungi, but the reduction observed was not as effective as thyme oil. There was no reduction difference between Thiabendazole and hydrosol (Figure 1 B and D) in terms of size. The ethanol used as negative control indicated no reduction in the fungal growth on the leaves; therefore, it was concluded that ethanol alone had no effect against *P. citricarpa,* the causative agent of CBS *in vivo,* Figure 1 A. The data from the current study is of great importance since both thyme oil and thyme oil hydrosol inhibited the development of *P. citricarpa*. These inhibitions are fundamental for the interruption of the CBS disease life cycle. Furthermore, the results suggest that certain secondary metabolites produced by plants, such as EOs and their hydrosol, may represent an alternative to decreasing the use of synthetic fungicides in treating CBS and their negative impacts. These compounds (thyme oil and their hydrosol) repressed the development of CBS necrosis, suggesting that they can be an ideal eco-friendly alternative to reduce fungicide use in citrus orchards to control CBS disease.

Inhibition of pathogen growth on the citrus fruits

Phyllosticta citricarpa survives and continues sporulation during harvest, storage, and transportation. Therefore, antifungal applications are paramount to preventing pathogen infection that occurs postharvest. As a result, the inhibition of *P. citricarpa* development postharvest would have a great advantage for suppression of the infection due to this fungal pathogen. Mycelial growth of *Phyllosticta citricarpa* on citrus fruits (lemon) was monitored for 28 days (four weeks) when treated with ethanol (negative control), thyme oil hydrosol, thyme oil (*Thymus vulgaris*) and Thiabendazole (positive control), as well as a non-inoculated fruit (Figure not shown), respectively. The results were monitored and recorded weekly (seven days) interval for four weeks (28 days). The results of *in vivo P. citricarpa* treatment with essential oil, hydrosol extract and Thiabendazole (positive control) on citrus fruits (lemon), respectively, are presented in Figures 2.1.-4.4. Throughout the four weeks, the most promising results were obtained from thyme oil (*Thymus vulgaris*) (Figure 2.4).

Figure 2.1: Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with ethanol (negative control) monitored over four weeks.

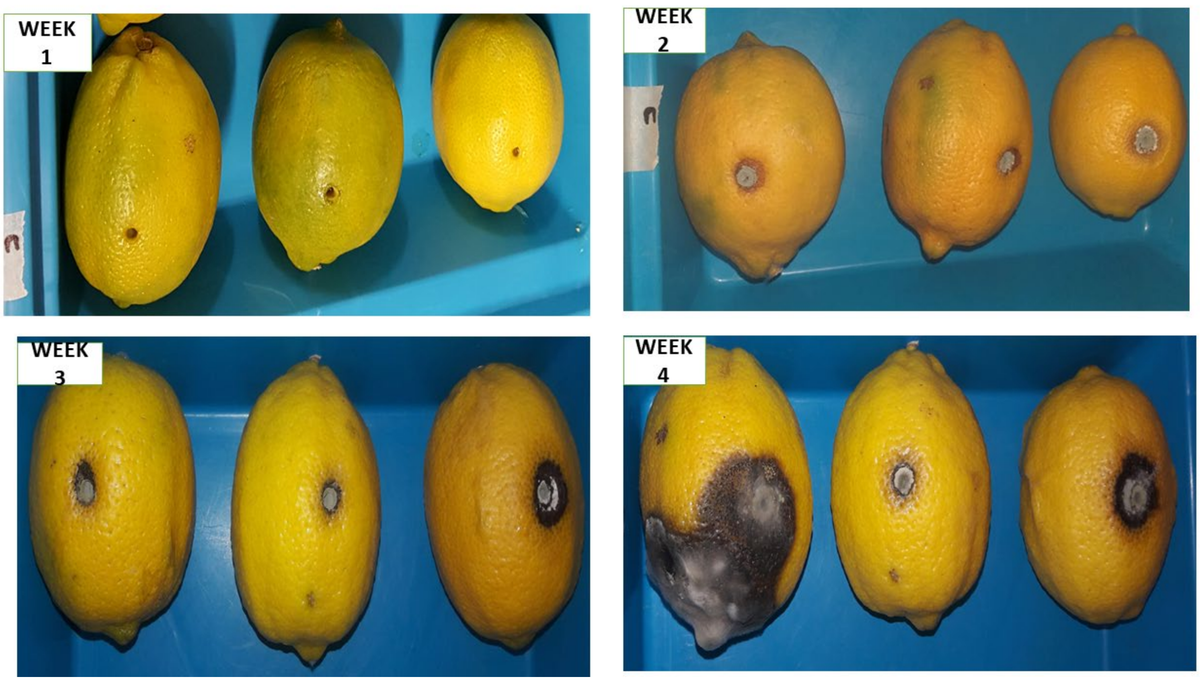


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Figure 2.2: Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with hydrosol monitored over four weeks.

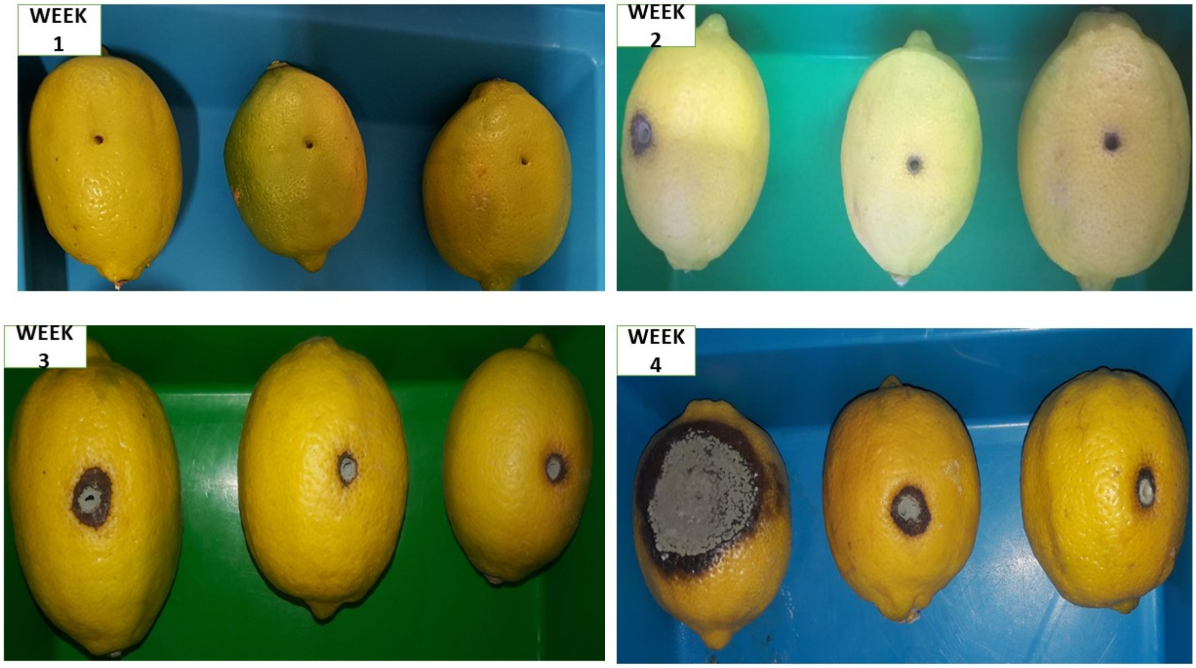


Figure 2.3: Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with Thiabendazole and monitored over four weeks.

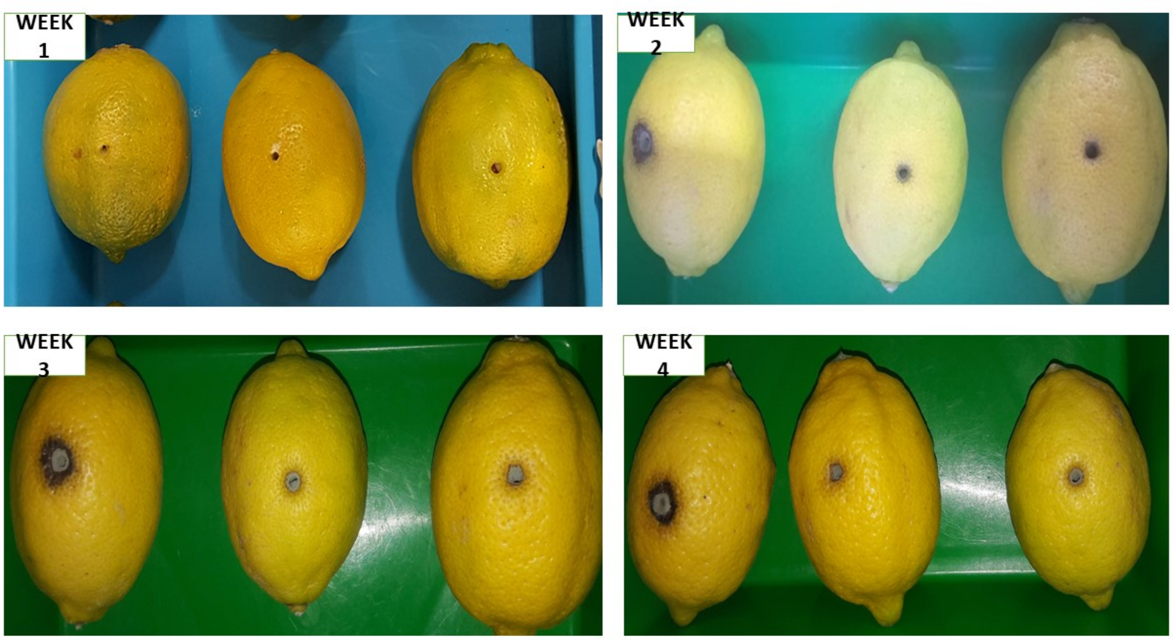
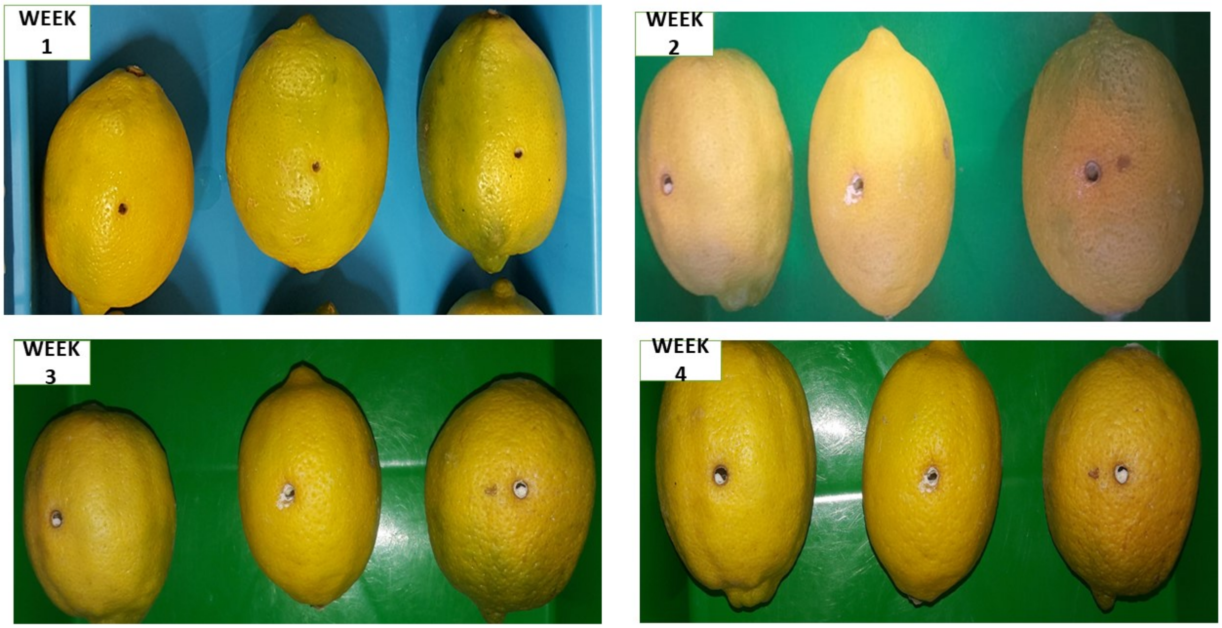


Figure 2.4: Growth inhibition of *Phyllosticta citricarpa in vivo* on citrus fruits treated with thyme oil and monitored over four weeks.



According to the results, thyme oil had substantially prevented the growth of the fungi from week one until week four, as there was no growth of the fungal pathogen throughout. This indicates that thyme oil has high antimicrobial activity against *P. citricarpa,* the causative agent of CBS, even on the citrus fruits themselves. The results align with a study by Tabti *et al.* (2014) on *Citrus sinensis* (orange) fruits infected with *Penicillium italicum* and treated *in vivo* with thyme oil; their results indicated no infection on the oranges as there was no mycelial growth on the fruits.

However, the synthetic fungicide Thiabendazole (positive control) prevented the growth during week one (this was not surprising, as the organism normally takes days to start growing), but as the weeks progressed (weeks two, three and four), the growth was observed and increased as the weeks progressed (Figure 2.3). These results mean that the compound used as a positive control managed to control fungal growth. However, it does not completely limit the development of fungal growth postharvest. These results support the findings by Agostini (2006), where Thiabendazole indicated inhibitory effects against conidial germination of *P. citricarpa* in a postharvest fungicide application study after the citrus fruits (lemon) were infected with *P. citricarpa.* Furthermore, another *in vitro* study conducted by Korf *et al*. (1998) showed that CBS cultured on Thiabendazole-amended plates reduced conidial germination incident of *P. citricarpa*, leading to the conclusion that Thiabendazole is effective in controlling CBS *in vitro*. On the other hand, the hydrosol prevented the fungal growth during weeks one and two, while in week three, some growth was observed (Figure 2.2). However, the growth started to develop extensively during week four. These hydrosol results were not surprising as the hydrosol contains traces of thyme oil, which is why it was not as effective as thyme oil. However, these results differ from the study by Tabti *et al.* (2014), where the thyme hydrosol extracts were tested *in vivo* on orange citrus fruits infected with *Penicillium italicum,* the causative agent of citrus rot. In their study, the hydrosol showed a complete absence of infection on the oranges and no disease incidence of citrus rot throughout the experiment. These results might indicate that thyme oil hydrosol is more effective against *Penicillium italicum* than on *P. citricarpa*; however, these results will have to be investigated further in future studies. The ethanol used as the negative control did not prevent the fungal growth; the growth was visible from week two and continued to grow extensively until week four (Figure 2.1). The results indicate that ethanol alone does not affect this fungal pathogen. Furthermore, uninoculated fruits did not develop CBS symptoms during the experiment (Figure not shown). This experiment also noted that all the compounds tested did not have any infection during week one, including the ethanol used as the negative control. These results suggest that at this point, the *P. citricarpa* conidia were not fully matured to cause severe infection, but from week two onwards, the fungal pathogen started to show infection growth in some of the tested compounds.

Concluding remarks

Worldwide, postharvest fungal disease control relies on the use of synthetic fungicides such as imazalil (IZ), Thiabendazole (TBZ), sodium orthophenyl phenate (SOPP), fludioxonil (FLU), Bordeaux mixture, and pyrimethanil (Ritenour *et al.,* 2004). These synthetic fungicides play a huge role in preventing and controlling many fungal diseases in the agricultural industry. If these fungicides are not used, the damages and the loss could increase drastically (Talibi *et al.,* 2014; Du Plooy *et al.,* 2009). These fungal reduction methods minimize the loss in the agricultural industry, ease pressure on the production resources, and generate much-needed revenue. However, recently there has been public concern about human health and the environmental over-use of synthetic fungicides, coupled with the development of resistance by the fungus (Abd-Elsalam *et al.,* 2015). In this respect, natural bio-control agents have shown great potential as an alternative to synthetic fungicides and offer an environmentally friendly alternative to synthetic fungicides (Setlhare, 2017; Tonial *et al.,* 2017; Chutia *et al.,* 2009). An attempt to reduce crop losses caused by pathogenic fungi and, simultaneously, the increasingly evident defects of synthetic fungicides lead to a constant search for natural substances limiting the development of fungi. The risk of spreading dangerous pathogens (including *Phyllosticta citricarpa*) is increased because they can gradually become resistant to synthetic fungicides, and most are very ecotoxic. In addition, fungi often have a cross-resistance phenomenon with synthetic fungicides (Hu *et al.,* 2019; Sivakumar and Bautista, 2014). If a given phytopathogen species becomes resistant to one preparation, at the same time, it becomes resistant to the whole group of chemical substances to which this preparation belongs (Sivakumar and Bautista, 2014).

The current study has demonstrated that *Thymus vulgaris* EOs has fungal activity against *Phyllosticta citricarpa*. This citrus fungal pathogen indicated an appreciable sensitivity towards the EOs used in the experiment. Furthermore, thyme oil hydrosol used in the study has indicated a moderate antifungal activity against the fungal pathogen used in the study in both the citrus fruits and the leaves, where the life cycle of *P. citricarpa* normally occurs in the field. The results imply that hydrosols can also be used as natural anti-microbials in agriculture. In fact, as aqueous solutions, the hydrosols could be easily rinsed off surfaces and do not have a strong, persistent smell, unlike EOs. From the results, thyme oil and its hydrosol have shown considerable damage to the citrus leaves and fruits, respectively. It is also important to note that both the citrus leaves and the citrus fruits are needed for the completion of the life cycle of this fungal pathogen. In addition, both the thyme oil and its hydrosol could be used to combat and prevent antifungal resistance. In fact, EOs and their hydrosols are unlikely to induce antimicrobial resistance, having multiple different targets in the microbial cells, unlike synthetic fungicides, which often only have a single target.

Future research

Due to the less effectiveness of hydrosol, future studies should explore the possibility of hydrosols being used synergistically with other molecules to enhance their antifungal properties in the citrus industry against CBS. Furthermore, more research needs to be done on thyme oil and its hydrosol to identify their sensory characteristics, and their persistence in the environment also needs to be investigated.

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