Antibacterial activity of the essential oil of *Thymus vulgaris* and thymol in the control of *Curtobacterium flaccumfaciens*

Atividade antibacteriana do óleo essencial de *Thymus vulgaris* e timol no controle de *Curtobacterium flaccumfaciens*

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RESUMO

Curtobacterium flaccumfaciens é um dos principais patógenos que acometem sementes de feijão e soja, causando diversos prejuízos às culturas, já que não existe nenhum produto registrado para seu controle. Dentre os métodos de controle que possuem eficiência comprovada frente à diversos patógenos, destacam-se os óleos essenciais e seus derivados. Sendo assim, este trabalho buscou avaliar o efeito do óleo essencial (OE) de Thymus vulgaris (tomilho) e timol contra a bactéria Curtobacterium flaccumfaciens (cff). A cepa foi inoculada em meio BHI (Brain Heart Infusion) e incubada a 28ºC/96h. Os testes com OE e timol foram realizados sob duas perspectivas: prevenção e remediação. As microplacas foram incubadas por 24h e 48h em shaker orbital e posteriormente adicionou-se as soluções em contato com a cepa. As análises antibiofilme foram realizadas após 24h visando a prevenção da formação dessas comunidades. Para verificar o efeito de remediação sobre biofilmes maduros, as análises foram realizadas após 48h. Foram realizadas análises para quantificação de unidades formadoras de colônias e biomassa. Os resultados da análise antibiofilme demonstraram que ambos os antimicrobianos foram efetivos, sendo que, o timol foi mais eficaz, pois apresentou melhores resultados em todas as concentrações testadas. Dessa forma, pode-se concluir que a utilização de ambas as substâncias é uma alternativa viável para o controle da murcha vascular causada pelo patógeno.

Palavras-chave: antimicrobiano, biofilmes bacterianos, fitopatógenos.

ABSTRACT

Curtobacterium flaccumfaciens is one of the main pathogens that affects soybean and common bean seeds, causing several crop losses, since there is no product registered for its control. Among the control methods that have proven efficiency in relation to several pathogens, essential oils and their derivatives are highlighted. Thus, this study aimed to evaluate the effect of essential oil (EO) from Thymus vulgaris (thyme) and thymol against Curtobacterium flaccumfaciens (cff). The strain was inoculated in BHI medium (Brain Heart Infusion) and incubated at 28ºC/96h. The tests with EO and thymol were carried out from two perspectives: prevention and remediation. The microplates were incubated for 24 hours and 48h in an orbital shaker and solutions in contact with the strain were added. The antibiofilm analyzes were carried out after 24 hours, aiming at preventing the formation of these communities. To verify the effect of remediation on mature biofilms, the analyzes were performed after 48h. Analyzes were carried out to quantify colony forming units and biomass. The results of the antibiofilm analysis showed that both antimicrobials were effective, and that the thymol was more effective, because it presented better results in all the concentrations tested. Thus, it can be concluded that the
use of both substances is a viable alternative for the control of vascular wilt caused by the pathogen.

**Keywords:** antimicrobial, bacterial biofilms, phytopathogens.

**1 INTRODUCTION**

Seeds-transported pathogens can associate with them in different ways, contaminating them superficially, or colonizing their internal tissues. Various damages can be caused by these pathogens, such as pre-emergency death, root rot, tipping, deformations and wilting (Kobayasti & Pires, 2011). Among the pathogenic agents that cause disease in plants, the phytobacteria *Curtobacterium flaccumfaciens* (cfl) that causes vascular wilt in soybean and bean crops is noteworthy. This pathogen affects the vascular system of these species, promoting destabilization of sap transport. This destabilization generates initial damage to the stems, progressing to the leaf area and may culminate in plant death. (Valentini et al., 2010).

In addition, several studies, such as Harding et al., (2019) and Dees et al., (2016), show that this bacterium may have an important characteristic that gives it greater resistance to its control, which is the ability to produce biofilm. Biofilms are clusters of cells that are characterized by the ability to adhere to solid surfaces, with consequent production of extracellular polymeric substances, forming a gelatinous network that immobilizes and protects the cells. The formation of biofilms causes phenotypic changes of planktonic cells, which can be described as strategies for survival of microorganisms in adverse conditions (Oliveira; Brugnera; Piccoli, 2010). Studies show that cells organized in biofilms become more resistant to the effects of antimicrobials, when compared to planktonic cells of the same strain (Costa et al., 2014; Bellaver et al., 2022).

Due to these factors, the search for molecules that are capable of eliminating these pathogens is constant. Currently, several literatures confirm the antibacterial activity of essential oils (EO) and major compounds in the control of several bacterial strains (Lima et al., 2017; Santurio et al., 2011). The EO are secondary metabolites extracted from various parts of plants, having complex chemical composition and guaranteeing the plants
adaptive advantages in the environment in which they are inserted. The EO’s chemical composition varies between the species and stems from the same plant and the same botanical species may be affected by the place of cultivation, collection and storage conditions, as well as edaphoclimatic factors (Miranda et al., 2016). However, the major components of the EOs are those present in high quantities and, taking into account chromatographic results, they are the compounds that have the highest peaks, usually varying between two to three components (Miranda et al., 2016).

Thus, the objective of this study was to evaluate the antibacterial capacity of the \textit{Thymus vulgaris} EO and its major compound (thymol) in relation to the sessile and planktonic forms of the \textit{Curtobacterium flaccumfaciens} strain causing vascular wilt in soybean and bean crops.

2 MATERIAL AND METHODS

Microbiological analyzes were performed at the Laboratory of Biology and the EO chromatographies were performed at the Laboratory of Packaging Analysis of the Federal Catarinense Institute - Campus Concórdia. Thymol was commercially obtained from the company Dinâmica (Indaiatuba, SP), while the essential oil of \textit{Thymus vulgaris} was obtained from Ferquima (Vargem Grande, SP). The bacterial strain comes from the collection of cultures of Agricultural Microbiology (CCMA) - UFLA, courtesy of the Federal University of Lavras.

In order to measure the capacity of bacteria biofilm formation, the Violet Crystal methodology (VC) was used, according to Stepanovic et al., (2000). 96-well microplates were incubated in orbital shaker (Tecnal, Brazil) at 28ºC, using different times for biofilm formation, namely: 24h, 48h and 72h. In each of the wells corresponding to the times, 200 µL of inocular standardized at $10^9$ CFU/mL were inserted. To each period, the BHI (\textit{Brain Heart Infusion}) culture medium (Acumedia, Brazil) was disposed of with the plankton cells and the wells were washed with sterilized deionized water twice.

Then, 200 µL of methanol (Merck, Germany) was added for the fixation of the biofilm for 20 minutes, after the disposal of methanol and total drying of the plate at room temperature, 200 µL of violet crystal (NewProvv, Brazil) was added for 5 minutes. After
this time, the dye was discarded and the plate washed with deionized water to remove the violet crystal not adhered to the biofilm cells. For the violet crystal solubilization, 200 µL of 33% acetic acid (Synth, Brazil) were added in each well, followed by the optical density (OD) reading in a spectrophotometer (Thermoplate, Brazil) at 550 nm. The parameters followed for the classification of bacteria in each test time were: +, ODC < OD ≤ 2 doc, producer of weakly/weak biofilm; ++, 2xODc < OD ≤ 4xODc, producer of moderately adherent/moderate biofilm; ++++, 4xODc < OD, strongly adherent/strong producer of biofilm; where OD is the optical density of the negative control and ODC is the value of the cut-off defined as three standard deviation values above the mean of the negative control.

The EO-based solutions of *Thymus vulgaris* and thymol were adapted by Millezi et al., (2016) with modifications (used as diluent ethanol P.A). The concentrations used were: 0.05%; 0.1%; 0.2%; 0.4%; 0.6%; 0.8% and 1.0%, made from EO/thymol stock solution, which consists of 2.5% EO/thymol, 2.0% P.A ethanol and 0.9% saline water.

The chemical characterization of Thymus vulgaris essential oil was performed by Bellaver et al. (2022), containing its chemical composition identified as Table 1.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-mircene</td>
<td>1.54</td>
</tr>
<tr>
<td>p-cymene</td>
<td>29.92</td>
</tr>
<tr>
<td>(+)-sivilstreno</td>
<td>0.86</td>
</tr>
<tr>
<td>1.8-cineol</td>
<td>1.3</td>
</tr>
<tr>
<td>y-terpinene</td>
<td>6.81</td>
</tr>
<tr>
<td>Linalol</td>
<td>5.82</td>
</tr>
<tr>
<td>Isoborneol</td>
<td>0.73</td>
</tr>
<tr>
<td>Camphor</td>
<td>1.41</td>
</tr>
<tr>
<td>Borneol</td>
<td>1.33</td>
</tr>
<tr>
<td>4-terpineol</td>
<td>1.03</td>
</tr>
<tr>
<td>Thymol</td>
<td>39.94</td>
</tr>
<tr>
<td>Isothymol</td>
<td>6.38</td>
</tr>
<tr>
<td>β-cariophyllene</td>
<td>1.37</td>
</tr>
</tbody>
</table>

Source: Bellaver et al., (2022)
For the evaluation of the antibiotic effect of the EO of thyme and thymol, 100 µL of standardized inoculum and 100 µL of EO/thymol-based solution were added in sterile 96-well microplates. These were incubated in orbital shaker (Tecnal, Brazil) at 28°C/24h. A microplate was mounted for each analysis and for each test substance separately. After some time, the OD reading was performed (550nm) in a spectrophotometer (Thermoplate, Brazil), and microdrop plating was performed (Herigstad et al., 2001).

In parallel, analysis was performed to verify the reduction capacity of mature biofilm, 200 µL of standardized inoculum were added in microplate of 96 sterile wells. After 48h incubation, the planktonic cells were discarded and the wells were washed with sterile deionized water. The aliquots of each of the concentrations of the test solutions in the corresponding wells were added, allowing action for 30 minutes. After that, the substances were removed and the microplate was washed with sterile deionized water, counting colony forming units (CFU) and biomass quantification by violet crystal analysis (VC). To detach the cells in biofilm, 200 µL of sterile deionized water were added to the wells of interest, leading the microplate to the ultrasound bath equipment (Sanders, Brazil) for 5 minutes at room temperature. After that, microdrop plating was performed (Herigstad et al., 2001).

Data analysis was performed using GraphPad Prism 7.0 software, the statistical model used was a completely randomized design in a factorial scheme (7x2), containing three replicates and seven concentrations of the two different antimicrobials. Bonferroni's test was applied, with a significance level of 95%. Variance analysis (ANOVA) was performed for both 24 hours and 30 minutes of contact, without comparing these two times with each other. Tukey test was used to verify the biofilm formation, with 95% significance level.

3 RESULTS AND DISCUSSION

In this study, we used the EO of thyme previously characterized by Bellaver et al (2022), and the compounds in larger quantities were: thymol, p-cymene and y-terpinene, with 39.94%, 29.92% and 6.81%, respectively. In a study previously carried out by Jakiemiu et al., (2010) the compounds in larger amounts found were thymol (54 to 57%).
p-cymene (12 to 15%) and y-terpinene (6 to 7%). Romero et al., (2009) also found similar results, with thymol (50%), p-cymene (20%) and y-terpinene (18%) as the majority compounds. Although the studies cited show different results as to the percentage of oil constituents, it can be observed in all that the compound in larger quantities of the thyme EO was thymol.

The profile of biofilm formation by means of VC analysis showed that the highest adherence occurred in 48 hours of cultivation, statistically differing from 24 and 72 hours (Fig. 1a). In 72 hours, there has already been a reduction in OD, suggesting that cells and EPS matrix disaggregation may have occurred. This reduction also occurred for the CFU count (Fig 1b), thus corroborating these results. In UFC analysis (Fig. 1b), there was a significant difference between the time of 24 hours and 48 hours, whereas the training periods of 24 hours and 72 hours of biofilm did not differ, as well as between 48 and 72 hours.

Rech, et al., (2016), for colony forming units (CFU), when carrying out an experiment with Escherichia coli ATCC 25922, observed that after 32h of incubation, the strain obtained 16.38 log_{10}/cm^2, this demonstrates the presence of large amount of viable cells in the biofilm. Similarly, Boari, et al., (2009) found that after 96h incubation of Aeromonas hydrophila, the number of colony forming units was 9.5 log_{10}/cm^2, indicating the viability of the cells present in the biofilm matrix.

In the biofilm formation, the longer the incubation time, the more aggregated the cells are and, consequently, are more strongly adhered, which makes it more difficult to remove them, reason why the 24-hour time was chosen for the analysis of antibiotic activity of the EO of thyme and thymol. In the present study, and in all analyzed times, the cff strain was classified as a moderate biofilm formator, according to Stepanovic et al., (2000).
There was a progressive increase in the biofilm formation in the times from 24 hours to 48 hours. Harding et al. (2019) also observed the biofilm formation by \textit{cff} strain. In a study carried out with \textit{Curtobacterium flaccumfaciens} pv. \textit{flaccumfaciens}, \textit{Pseudomonas syringae} pv. \textit{phaseolicola}, \textit{P. syringae} pv. \textit{syringa} and \textit{Xanthomonas axonopodis} pv. \textit{phaseoli} it was possible to observe and confirm that all bacteria form the biofilm matrix at some stage of its cycle. By scanning electron microscopy, the authors observed the formation of this matrix in the external part of bean seed. Dees et al., (2016) also demonstrated the ability of the strain to form biofilm by sequencing the gene responsible for assigning this characteristic to the bacteria. Thus, the results of these studies reinforce that \textit{cff} is capable of adhering to the surfaces and forming biofilm, corroborating the results found.

Attempts to control biofilm can be made by the use of bactericidal substances – when they cause the strain to die – or bacteriostatic substances – when they inhibit growth, the latter being the most used control mode. Bacteriostatic substances inhibit bacterial growth through compounds that block cell adhesion, called antivirulence therapy, and do not result in bacterial death. The development of compounds that inhibit this adherence without affecting microbial growth has been investigated, since these, by keeping the cells in their plankton state, favor the external action of existing compounds, maintaining the efficacy of antimicrobials (Kauffmann et al., 2017).
In the treatments used in this study, it was observed that the EO of thyme and thymol decreased the bacterial adhesion significantly from the concentration of 0.05% (p<0.05), and all concentrations presented reduction potential (Figure 3a and 3c), proving the ability to prevent the biofilm formation. For CFU analysis, it was observed that the EO reduced the cells from the concentration of 0.2%, but at the concentration of 0.6% inhibited the growth of all the viable cells (Figure 3b). The thymol antibacterial activity as to the growth of CFU was higher than the thyme EO, because for thymol there was a significant reduction from the concentration of 0.05% and in the concentration of 0.2% there was no bacterial growth (Fig 3d).

Figure 3. Antibacterial effect of EO of Thymus vulgaris (thyme) and thymol before the strain of Curtobacterium flaccumfaciens: (a) OD thyme; (b) UFC thyme; (c) OD thymol; (d) UFC Thymol
*indicates statistically significant differences by ANOVA (Bonferroni) with P<0.05.
ns indicates statistically non-significant result.

Source: The authors
The results of the VC analyzes showed that thyme and thymol were not efficient in the control of mature biofilm formed by cff (p>0.05) (Figure 4). None of the seven concentrations of both antimicrobials tested showed significant results for the bacteria control regarding the effective biofilm matrix formation. In the treatment with thymol, there were satisfactory results for the analysis of CFU. From the concentration of 0.6%, the compound was capable of totally inhibiting the growth of the strain, (Figure 4d). For thyme, there were statistically significant results (p< 0.05) also from the concentration of 0.6%, however, it was only capable of zeroing bacterial growth at the concentration of 1.0% (Fig. 4b).

Figure 4. Antibacterial effect of EO of Thymus vulgaris (thyme) and thymol on mature biofilms of Curtobacterium flaccumfaciens; a) OD thyme; b) CFU thyme; c) OD thymol; d) UFC thymol. *Indicates statistically significant differences by ANOVA (Bonferroni) with p < 0.05 indicates statistically non-significant results.

Source: The authors
According to Costa et al., (2016) the biofilm formation occurs through a series of sequential events, which may be reversible or not. Initially, the adhesion of planktonic cells to the surface occurs, followed by the proliferation and accumulation of cell layers and, finally, by the microbial community formation. However, the adhesion and formation of biofilms are limited by the characteristics of the micro-organism itself, the adhesive material and the environment involving the micro-organism, such as pH, temperature, agitation time and a number of other factors.

There is a lack of studies in the literature reporting the activity of plant metabolites as antimicrobial against *c_ff* in the plankton and biofilm form, although many studies have been published reporting the antibacterial effect of these substances (Bellaver et al., 2022; Lima et al., 2017; Millezi et al., 2016; Nostro et al., 2007, Valeriano et al., 2012). In the present study, the results of the antibiofilm tests demonstrated that the EO of thyme and thymol are promising against *c_ff. biofilms*. The efficiency of the thyme EO was reported by Freire et al., (2014) in which antibacterial capacity was observed in relation to *Streptococcus mutans* and *Staphylococcus aureus* strains. The minimal inhibitory concentration (MIC) analysis showed that the EO was effective from 2.25 mg/mL and 0.5625 mg/mL, respectively. Santurio et al., (2007) also reported moderate antibiotic activity obtained by the thyme EO before several strains of *Salmonella enterica*. These authors performed the analysis of MIC, the OE presented means ranging from 400 to 1600 µg/mL. Whereas in order to present bactericidal effects, the minimum bactericidal concentration (CBM) analysis showed higher values, ranging from 800 to 1600 µg/mL.

As for thymol, works by Nostro et al., (2006) reported the antibiofilm effect against *Staphylococcus aureus* and *S. epidermidis strains*, being effective at an average concentration of 0.036% v/v for both bacteria. In the analysis of CBM, thymol was effective in the concentration of 0.073% v/v also for both strains. Ferreira et al., (2018) observed bacteriostatic and bactericidal effect of thymol against *Streptococcus mutans*, *S. oralis* and *S. salivarius cells*, namely 312.5 µg/mL for the first and 156.2 µg/mL for the second and third, respectively, thymol is the most effective compound of the study carried out.
Resistance mechanisms provide the sessile cells with favorable conditions of survival, which makes them less susceptible to eradication when compared to the same microorganisms in the plankton form (Oliveira, et al., 2010). Several factors have been suggested to explain the resistance of biofilms to antimicrobial agents: bacteria in biofilms, particularly those found in more internal layers, present reduced rates of metabolic and growth activities; the extracellular polymer matrix works as an adsorbent, reducing the amount of antimicrobial available to interact with the biofilm cells. In addition to limiting the diffusion of sanitizing agents, the exopolysaccharide matrix can react and cause their inactivation (Oliveira, et al., 2010).

In this study, the thyme and thymol EO were capable of decreasing and inhibiting bacterial growth of planktonic cells released from the biofilm. With the growth of the microbial population, the environment becomes toxic to the cells that form the biofilm, weakening the structure, causing the detachment of single cells or fragments. At the moment when the biofilm reaches critical mass, the dynamic balance is achieved and the outermost layer of its structure begins the production of planktonic bacterial cells (Oliveira, et al., 2010). However, even if the biofilm has not reached the critical state, viable microorganisms adhered to the surface will present the ability to detain even if the number of cells present is low within a certain area (Oliveira, et al., 2010).

Studies conducted by Mohsenipour (2015) demonstrated that the thyme EO is capable of reducing the biofilm formation of pathogenic strains when used in higher concentrations. Tests carried out with *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* showed that EO managed to reduce by 90%; 80%, 90%; 70%; 75% and 80%, respectively, the viability of the biofilm formed when 20mg/mL of the same was used. As well as Sper, et al., (2016) verified the efficacy of thyme extract before *Streptococcus mutans bacteria*. The 48h biofilm was exposed to thyme extract for 5 minutes, with a 64% reduction in the viability of the biofilm created after time. Sadekuzzaman et al., (2018) also found a reduction in the biofilm formed by *Salmonella spp.* and *Listeria monocytogenes* through the use of thyme essential oil. The reduction was 1.8 CFU/cm² and 2.3 CFU/cm² for the biofilm of *Salmonella spp.* and *L. monocytogenes*, respectively.
4 CONCLUSION

Based on the results obtained, we concluded that the EO of thyme and thymol have satisfactory action in relation to the strain, considering the in vitro tests performed. Based on the data presented, *in vivo tests* should be performed to verify the antibacterial potential of the compounds before the bacteria, taking into account the plants that are affected and the environment in which they are inserted into.
REFERENCES


BELLAVER FAV, JUNIOR AC, BELLO TCD, NEIS AJL, TRONCARELLI MZ, MILLEZI AF. Antibacterial potential of essential oils against planktonic and sessile cells of Escherichia coli isolated from diarrhea cases in swine. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas. v. 21, n.1, p. 81 – 93, 2022.


