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Research

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Effect of thyme, lemongrass and rosemary essential oils on *Aspergillus flavus* in cauliflower seeds

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ABSTRACT

Due to the action of essential oils of thyme, lemongrass and rosemary against microorganisms and few studies on them in seed treatment, this study is proposed with vegetable seed. Therefore, the aim was to evaluate the effect of treatments with essential oils of thyme, lemongrass and rosemary on cauliflower seeds, inoculated with *Aspergillus flavus*. Three essential oils were used, at concentrations 0.0; 0.4; 0.8; 1.6 and 2.0%, arranged in a 3x5 factorial scheme, in a completely randomized design, with four replications. The essential oils of thyme (1.8%) and lemongrass (1.9%) had a fungistatic effect against *A. flavus* in cauliflower seeds. Thyme essential oil at 2.0% and lemongrass at 1.6% didn't affect the physiological quality of seeds, being promising in the treatment of organic seeds. On the other hand, rosemary essential oil, despite not affecting germination and little affecting seed vigor, wasn't efficient in controlling the pathogen.

Keywords: *Brassica oleracea* var. *botrytis*, *Thymus vulgaris*, *Cymbopogon citratus*, *Rosmarinus officinalis*.

RESUMO

Efeito de óleos essenciais de tomilho, capim-limão e alecrim sobre *Aspergillus flavus* em sementes de couve-flor

Devido à ação dos óleos essenciais de tomilho, capim-limão e alecrim contra microrganismos e poucos estudos dos mesmos no tratamento de sementes, propõe-se este estudo com semente de hortaliça. Portanto, o objetivo deste trabalho foi avaliar o efeito do tratamento com óleos essenciais de tomilho, capim-limão e alecrim em sementes de couve-flor inoculadas com *Aspergillus flavus*. Foram utilizados os três óleos essenciais, nas concentrações 0,0; 0,4; 0,8; 1,6 e 2,0%, arranjados em esquema fatorial 3x5, no delineamento inteiramente casualizado, com quatro repetições. Os óleos essenciais de tomilho (1,8%) e capim-limão (1,9%) tiveram efeito fungistático contra *A. flavus* em sementes de couve-flor. Os óleos essenciais de tomilho a 2,0% e o de capim-limão a 1,6% não afetaram a qualidade fisiológica das sementes, sendo promissores no tratamento de sementes orgânicas. Por outro lado, o óleo essencial de alecrim, apesar de não afetar a germinação e afetar pouco o vigor das sementes, não foi eficiente no controle do patógeno.

Palavras-chave: *Brassica oleracea* var. *botrytis*, *Thymus vulgaris*, *Cymbopogon citratus*, *Rosmarinus officinalis*.

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Seeds are a relevant agricultural input, since from them begins the food production. But seeds can disseminate disease agents, such as fungus and bacteria. Thus, the use of seeds with sanitary quality is necessary to obtain a uniform stand and reach its maximum physiological potential, and less disease inoculum in the new fields. It is not always possible to obtain seed lots 100% guaranteed free of pathogens (Cardoso *et al.*, 2016). To protect seeds, it is necessary to treat the same with fungicides. In the conventional production system, there are already registered products (Agrofit, 2020), however, for the organic production system, there is still no product registration.

The presence of fungi affects the vigor of seeds, when they benefit from the reserve tissues or even from the embryo itself, which, according to Krzyzanowski & França Neto (2001), results in the slow growth of seedlings and production of abnormal seedlings. The fungi group with the highest contamination rate in stored seeds are of the *Aspergillus* species (Vecchia & Castilhos-Fortes, 2007).

The use of agrochemicals is not allowed in an organic production system, both in the production phase in the field and in seed treatment. In view of the increase of diseases in current agriculture, and the scarcity of organic products to mitigate the potential for environmental impact, it is necessary to develop alternative products for these problems. Therefore, the evaluation of the effect of essential oils has been promising due to its high antimicrobial activity (Romero *et al*., 2012).

According to Santos *et al.* (2010), studies have already proven the efficacy of essential oils acting as fungicides, inhibiting mycelial growth and the germination of conidia. Among them, the thyme essential oil (*Thymus vulgaris*) has already shown to be effective against stem and ear rot caused by *Stenocarpella maydi* (Teixeira *et al*., 2013), *Colletotrichum gloeosporioides* (Figueiredo *et al*., 2021), and *Aspergillus* sp. (Kohiyama *et al*., 2015).

In addition, there is the essential oil of lemongrass (*Cymbopogon citratus*) which, according to Shah *et al.* (2011), has antifungal action and in some studies it was shown to be efficient in the control of *A. flavus*, *A. niger*, *A. ochraceus* (Sonker *et al*., 2014) and *A. brasiliensis* (Oliveira *et al*., 2020). Oliveira & Sá (2014) detected an improvement in the physiological quality of rice, beans and soybean seeds, due to improving the sanitary quality. And finally, there is the rosemary essential oil (*Rosmarinus officinalis*) effective against *A. flavus*, *A. niger* (Sousa *et al*., 2013) and *A. flavus* (Prakash *et al*., 2015; Lee *et al*., 2020).

Most studies about the use of medicinal plants are against microorganisms in the food industry, but still scarce in the agronomic sector. Therefore, more detailed investigations should be made with emphasis on *in vivo* tests, in order to validate the *in vitro* results. Essential oils of thyme, lemongrass and rosemary act against microorganisms. Because of the lack of studies about the control of *Aspergillus flavus* on vegetable seeds, and their effect on seed quality, the present study is of considerable importance for the organic production system.

The aim of this study was to evaluate the effect of essential oils of thyme, lemongrass and rosemary on cauliflower seeds inoculated with *Aspergillus flavus*.

MATERIAL AND METHODS

The experiments were set out at the Seed Analysis Laboratory of the São Paulo State University (UNESP), College of Agricultural and Techonogical Sciences, Dracena.

Cauliflower seeds (Feltrin®) without fungicide treatment were inoculated with the fungus *Aspergillus flavus*, isolated from bean seeds. The inoculum was multiplied in PDA (potato-dextrose-agar) medium together with the water restrictor mannitol at a concentration of 33 g L^{-1}, to obtain -0.8 MPa. This technique has been used to prevent seed germination in sanitary tests (Machado *et al*., 2001). Then, the seeds remained on this medium in a single layer, for a period of one hour (Martins *et al*., 2016) and, after this time, the seeds remained under paper towels for a period of 48 hours.

Three essential oils were used (thyme, lemongrass and rosemary, from Company Laszlo®), at concentrations 0.0; 0.4; 0.8; 1.6 and 2.0%, arranged in a 3x5 factorial scheme, in a completely randomized design, with four replications. The composition of the main active principles of each essential oil are: rosemary: 1,8-cineole (41.2%), α-pinene (14.3%) and camphor (11.4%); thyme: thymol (46.6%), p-cymene (38.9%) and γ-terpinene (0.3%); lemongrass: geranial (44.4%) and neral (33.5%).

The water used to prepare the solutions was deionized and autoclaved. For the treatment of seeds with essential oils, an orbital shaker table (MA-model 376) was used for five minutes.

The evaluated characteristics were a) germination (11x11x3 cm plastic boxes were used with germination paper, moistened 2.5 times their weight and 50 seeds were sown in each repetition); b) percentage of normal seedlings [evaluated ten days after sowing (DAS) (Brasil, 2009a)]; c) first germination count [performed in the germination test, computing the percentage of normal seedlings on the fifth DAS (Brasil, 2009a)]; d) shoot, primary root and e) total seedling length (ten normal seedlings from the germination test were sampled and the average of each plot was obtained) f) seedling dry weight (the normal seedlings of the germination test were dried in an oven with forced air circulation, under 40^{0}C temperature, until reaching constant weight); g) emergency and emergency speed index [the emergency test was performed and the percentage was counted on the tenth DAS (Brasil, 2009a). Fifty seeds were sown per plot in polyethylene trays of 200 cells with substrate Carolina Soil® for vegetable seedlings production which remained in the greenhouse. The count of emerged seedlings was initiated when the first seedling emerged, with daily counting until stabilization to obtain the emergency speed index, calculated using the formula of Maguire (1962)]; h) sanitary test [the *blotter test* was used according to the Seed Sanitary Analysis Manual (Brasil, 2009b). In each plot 25 seeds were placed in petri dishes and kept in an incubation chamber for 24 hours at 20ºC. Then they were subjected to freezing for 24 hours at -20°C in order to suppress seed germination. Afterwards, they returned to the incubator at 20ºC for more 5 days, under alternating light and dark for 12 hours. After this period, fungi were evaluated, using a stereoscopic microscope, accounting for the presence or absence in each seed unit, and results were expressed in percentage].

Data were subjected to analysis of variance, and regression analysis was applied to the concentration factor, while for the essential oils factor the Tukey test was applied, both at 5% probability (Ferreira, 2011).

RESULTS AND DISCUSSION

A. flavus is one of the fungi which most affects seeds due to the consumption of their reserves, which are used in the germination process and directly affect seed vigor. Rosemary oil did not inhibit the development of *A. flavus* in cauliflower seeds. The essential oils of thyme and lemongrass had total control of *A. flavus* in its highest concentration (Figure 1).

It is worth highlighting the components and concentrations of each of them, even to compare with results in the literature, since each lot has its variation, it also depends on the place of cultivation, the time of harvest, among other factors that end up interfering. For example, the essential oil of thyme contains thymol (46.6%) and p-cymene (38.9%), which must have promoted a fungistatic action in controlling the fungus. Borugã *et al.* (2014) analyzed the components of the thyme essential oil and detected three major components, thymol (47.6%), p-cymene (8.4%) and γ-terpinene (30.9%) which have probably the greatest antimicrobial potentials, differing from the oil of the present study. Comparing oils (thyme), from the present study and from the study of Borugã *et al.* (2014), there is a similar amount of thymol and a high discrepancy between the other two components, however, having the same antimicrobial action.

The thyme essential oils from each mentioned study result in a difference in concentration as they are extracted from plants from different locations, as already mentioned and also can be observed in the work of Jakiemiu *et al.* (2010) who identified 33 compounds in thyme essential oil, the majority being thymol (50.4 to 55%), p-cymene (17 to 21%) and γ-terpinene (5 to 7%). There was a high variation of the three compounds compared to the one used in the present study. However, thymol is presented in greater quantity in all cited works being believed that this is the main antimicrobial component. Other researches had positive results with the use of thyme essential oil against fungi. Kohiyama *et al.* (2015) verified fungicidal effect against *A. flavus* at a concentration of 250 μg mL^{-1} which caused cell cytoplasm leakage. Tian *et al.* (2019) studied the use of thyme essential oil in the vapor and liquid phase to control *A. flavus*, and found positive effects at the concentration of 20 and 400 μg mL^{-1}, reducing aflatoxin production by 97.0 and 56.4%, respectively, concluding that the essential oil in the vapor phase can be a good strategy to control fungal contamination (Silva *et al*., 2012). Silva *et al.* (2012) reported also that the major antifungal component of thyme essential oil is thymol and that at a concentration of 50% (oil/dimethyl sulfoxide; v/v) it controlled *A. flavus*. Koch *et al*. (2010) observed the control of the *Alternaria dauci* and *A. radicina* in carrot seeds at a concentration of 1%.

Non treated seeds presented 100% infection from *A. flavus* (concentration 0%), showing the efficiency of inoculation. The essential thyme oil at 0.4% significantly reduced the contamination of cauliflower seeds contaminated with *A. flavus*, not differing from other essential oils, while in concentrations from 0.6% to 2.0% thyme and lemongrass essential oils significantly reduced the contamination, differing from rosemary essential oil (Table 1).

Shah *et al.* (2011) reported that lemongrass essential oil has antifungal action and components such as geranial and neral, among others that act to control fungi. Studies show the efficiency of lemongrass essential oil as occurred in the study by Sonker *et al.* (2014) who in their research verified the efficiency at a concentration of 0.33 μL mL^{-1} and inhibiting 100% mycelial growth of *A. flavus.* Martinazzo *et al.* (2019) also verified efficiency *in vitro* test to control 100% of *A. flavus,* using lemongrass essential oil at 1.0 μL mL^{-1} concentration.

Subramanian *et al.* (2015) concluded an antimicrobial effect and identified the chemical composition of the essential oil of lemongrass, as follows: geranial (32.4%), beta-citral (23.8%) and geraniol (12.0%), and the essential oil used in this research was composed of 44.4% geranial. Millezi *et al*. (2012) also found an antibacterial effect and again the concentration of geranial varied, resulting in 47.0%. This demonstrates that depending on the type of oil used, as they are from different locations, they may or may not be efficient in controlling phytopathogens.

Rosemary essential oil was not efficient in controlling *A. flavus* in cauliflower seeds, which continued

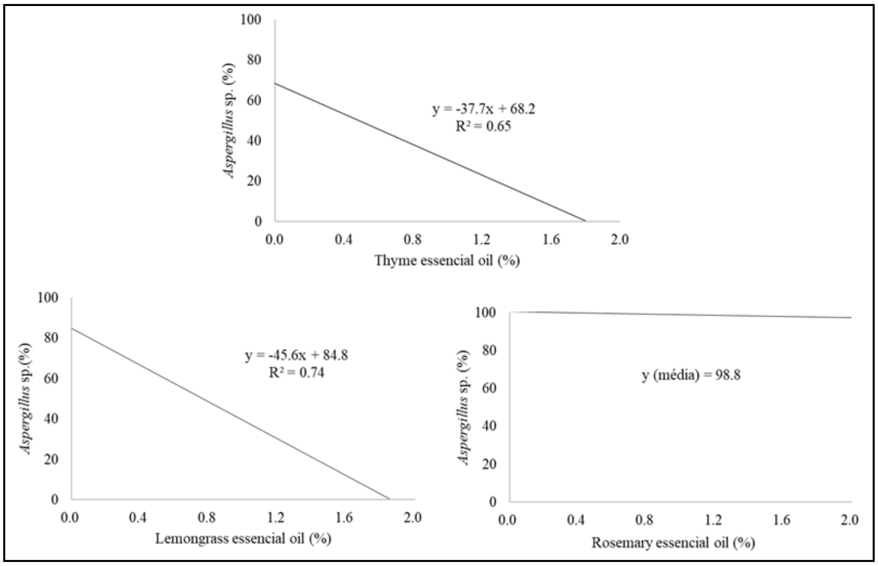

Figure 1. Percentage of cauliflower seeds with *Aspergillus flavus* in treated seeds with essential oils of thyme, lemongrass and rosemary in different concentrations. Dracena, UNESP, 2021.

Table 1. *Aspergillus flavus* in cauliflower seeds treated with essential oils of rosemary, lemongrass and thyme in different concentrations. Dracena, UNESP, 2021.

Essential oil	Concentration (%)					
	0.0	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**
Rosemary	100 a[1]	100 c	100 b	100 b	94 b	99 b
Lemongrass	100 a	77 b	26 a	20 a	17 a	13 a
Thyme	100 a	39 a	20 a	16 a	14 a	4 a
CV (%)			10,3			

[1]Means followed by the same letter in the column do not differ by the Tukey test at 5% probability.

to be highly contaminated (Table 1). Contrary results of the present study were found in the research of Iordache *et al*. (2006), studying rosemary essential oil; they related reduction of 22.2% in *A. niger* analized *in vitro*. Lee *et al.* (2020) concluded efficiency in the control of *A. flavus* using rosemary essential oil at 25 μL mL^{-1} concentration. Baghloul *et al*. (2017), reported that rosemary essential oil with 1,8-cineole major compound (63.6%), has a fungistatic activity on foods contaminated by *A. niger* at a minimum inhibitory concentration of 0.5%. Bomfim *et al*. (2015) detected three compounds that supposedly act in the control of microorganisms: 1,8-cineole (52.2%), camphor (15.2%) and α-pinene (12.4%). In the same way that it happened for thyme oil, it happened for rosemary, where there is a difference in the concentration of components, and 1,8-cineole is the component with the highest content in the oil of the present study, presenting 41.2%, however, it was not enough to control *A. flavus*. The essential oils of thyme and lemongrass have a fungistatic effect against *A. flavus* in cauliflower seeds, and especially as regards the essential oil of thyme. Rosemary essential oil, on the other hand, was not efficient in controlling this pathogen.

Germination and vigor of cauliflower seeds

Essential oils of thyme and rosemary, did not affect germination of cauliflower seeds and did not show any significant difference between them in any of the concentrations (Table 2). However, when the seeds were treated with lemongrass essential oil, germination was lower at 0.8 and 2.0%. In the research by Souza *et al*. (2007) there was an increase in corn seed germination when treated with 0.5% lemongrass essential oil. Oliveira & Sá (2014) also observed that this essential oil used in seed treatment at a concentration of 3 mL L^{-1} improved germination and vigor of rice, bean and soybean seeds.

For vigor characteristics, the first germination count and emergency speed index (ESI) had a significant effect only for essential oils (Table 3). For the first germination count, there was no significant difference between the rosemary and thyme oils, being superior to lemongrass essential oil. For the ESI, thyme essential oil was superior to the other oils which did not differ between itself.

For seedling dry weight (mean: 1.86 mg $seedling^{-1}$), emergence (mean: 83%), shoot length (mean: 2.8 cm), primary root (mean: 4.0 cm) and seedling length (mean: 6.8 cm) there was no significant difference among treatments. As for most physiological quality traits there was no significant effect for essential oils when treating the seeds; it is possible to assume a promising treatment via seeds for the organic production system, especially those that showed an effect in controlling *A. flavus*.

The essential oils of thyme (1.8%) and lemongrass (1.9%) had a fungistatic effect against *A. flavus* in cauliflower seeds. Thyme essential oil at 2.0% and lemongrass at 1.6% didn't affect the physiological quality of seeds, being promising in the treatment of organic seeds. On the other hand, rosemary essential oil, despite not affecting germination and little affecting seed vigor, wasn't efficient in controlling the pathogen.

Table 2. Germination of cauliflower seeds inoculated with *Aspergillus flavus* and treated with essential oil of rosemary, lemongrass and thyme in different concentrations. Dracena, UNESP, 2021.

Essential oil	Concentration (%)					
	0.0	0.4	0.8	1.2	1.6	2.0
Rosemary	86 a[1]	83 a	93 ab	96 a	91 a	88 a
Lemongrass	86 a	88 a	81 b	93 a	84 a	60 b
Thyme	86 a	87 a	97 a	94 a	87 a	93 a
CV (%)	8,4					

[1]Means followed by the same letter in the column do not differ by the Tukey test at 5% probability.

Table 3. First germination count and emergency speed index (ESI) of cauliflower seeds inoculated with *Aspergillus flavus* and treated with essential oil of rosemary, lemongrass and thyme in different concentrations. Dracena, UNESP, 2021.

Essential oil	First count (%)	ESI
Rosemary	85 a[1]	4.7 b
Lemongrass	67 b	4.8 b
Thyme	83 a	5.2 a
CV (%)	19,1	7,2

[1]Means followed by the same letter in the column do not differ by the Tukey test at 5% probability.

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