NOTAS CIENTÍFICAS

Detection of antifungal activity of plant extracts on *Alternaria citrus*

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ABSTRACT

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For approximately 15 years, Brazilian citriculture has suffered considerable losses in productivity and fruit quality as a result of the attack by the phytopathogen *Alternaria alternata* f.sp. *citri*, which causes Alternaria brown spot (ABS). The disease control is still performed with the massive use of agrochemicals, reaching up to twenty applications per reproductive period. The present study evaluated the efficiency of *in vitro* and *in vivo* ABS control by using fermented extracts of *Trifolium pratense* and *Daucus pusillus* as a means to control the disease. Fermented extracts autoclaved at 40% showed 100% inhibition, while the crude extract of both plants had no inhibitory effect. *In vivo,* the best results were found with the preventive test of non-autoclaved *Trifolium pratense*, which inhibited the onset of the disease by 93%.

Keywords: Alternative control, plant pathogens

RESUMO

Triaca, T.; Cavião, H,C.; Pansera, M.R.; Venturin, L.; Sartori, V.C. Determinação de atividade antifúngica de extratos vegetais em *Alternaria citrus*. ***Summa Phytopathologica,*** v.44, n.2, p.185-188, 2018.

Há aproximadamente 15 anos, a citricultura brasileira vem sofrendo consideráveis perdas em produtividade e qualidade dos frutos devido ao ataque do fitopatógeno *Alternaria alternata* f.sp. *citri*, causador da Mancha Marrom da Alternaria (MMA), o controle da doença ainda é feito com o uso massivo de agroquímicos, chegando a vinte aplicações por período reprodutivo. O presente trabalho avaliou a eficiência no controle do MMA *in vitro* e *in vivo* utilizando fermentados botânicos de *Trifolium pratense* e *Daucus pusillus* como meio de controle da doença. Nos fermentados autoclavados a 40%, a inibição foi de 100%, o extrato bruto de ambas plantas não apresentou poder inibitório. *In vivo,* os melhores resultados foram com o teste preventivo de *Trifolium pratense* não autoclavado, que inibiu em 93% o aparecimento da doença.

Palavras-chave: controle alternativo, fitopatógenos

Brazil is the third largest producer of mandarin fruits, presenting around 54,000 hectares of planted area and producing 1.1 million tons (5). The presence of Alternaria brown spot (ABS) in Brazil was confirmed in 2001, and it was rapidly disseminated to various states within one year (10). ABS is caused by the fungus *Alternaria alternata*, which has as characteristic the ease to establish in places of high humidity. This requires farmers to bear high costs of fungicide applications - between 12 and 15 applications per year (5). Other points that hinder the disease control include easy air dispersion and the possibility to establish in dry or dead material easily found inside crowns of old and neglected orchards (8). Prolonged use of synthetic agrochemicals has led to the selection of increasingly resistant pathogenic microorganisms, often with profile of multiresistance to chemical groups.

Therefore, the main research focus has been shifting towards adequate management of natural resources and reduced use of agrochemicals, aiming to stimulate the use of natural substances in agricultural systems. Thus, the present study aimed to evaluate the fungitoxic efficiency of fermented extracts of *Trifolium pratense* and *Daucus pusillus* on the phytopathogen *Alternaria alternata* f.sp. *Citri.* The plants *T. pratense* and *D. pusillus* were collected in 2014, from the town of Travessão Aquidabam, Flores da Cunha/RS (29° 01' 44" S 51° 10' 55"W). The microorganism *A. citri* (A67/11) was isolated from citrus at the town of São Sebastião do Caí, state of Rio Grande do Sul, Brazil. The fermented plant extracts were produced with 1.5 L untreated water and 500 g fresh plant. Spontaneous and aerobic fermentation occurred for 15 days. The mixture was maintained in a dark environment up to the end of fermentation (7). It was used in autoclaved and non-autoclaved forms. Crude extract was prepared with 1.5 L untreated water and 500 g fresh plant. This mixture was then blended and filtered.

***In vitro* evaluation of fungitoxic activity**

Fermented and crude extracts were filtered and added at the concentrations of 20 and 40% to PDA medium. They were placed in Petri dishes and the 3mm-diameter fungus was added to the medium (five replicates). As control, only PDA medium was used. All plates were incubated in a germination chamber (BOD) during 14 days, at 25°C. Mycelial diameter for colony growth was measured on the 3rd, 7th and 14th days after inoculation of the fungi of interest.

***In vivo* assessment of fungitoxic activity**

The tangerines (18 each treatment) were collected from an organic production system so that there was no interference of agrochemicals with the samples. Four lesions (4 mm) were made in each fruit, and they were colonized with a conidial suspension ($30x10^4$), totaling 72 lesions per treatment. Treatments were as follows: Control 1: only water; Control 2: only fungus; Treatment 1: curative tests with non-autoclaved fermented extract of *T. pratense*; Treatment 2: curative tests with autoclaved fermented extract of *T. pratense*; Treatment 3: curative tests with non-autoclaved fermented extract of *D. pusillus*; Treatment 4: curative tests with autoclaved fermented extract of *D. pusillus*; Treatment 5: preventive tests with non-autoclaved fermented extract of *T. pratense*; Treatment 6: preventive tests with autoclaved fermented extract of *T. pratense*; Treatment 7: preventive tests with non-autoclaved fermented extract of *D. pusillus*; Treatment 8: preventive tests with autoclaved fermented extract of *D. pusillus*. In the curative treatment, the pathogen was inoculated and the fermented extract was applied 6 hours later. In the preventive treatment, the fermented extract was applied and the pathogen was inoculated 6 hours later. The *in vivo* test was only performed with the best results of the *in vitro* tests. The methodology for analysis of phenolic compounds of crude extracts was high-performance liquid chromatography (HPLC). The means of total results were compared according to Tukey's test at 5% probability, using the software IBM SPSS 19 Statistics.

As shown in Figure 1, the fermented and crude extracts of *in vitro* tests had potential inhibitory effect. Fermented extract of *T. pratense,* pH 4.75*,* at 20%, inhibited fungal growth by 10% within 14 days of inoculation. When the dose of the fermented extract was increased to 40%, there was total inhibition of the phytopathogen. Fermented extracts of *D. pusillus*, pH 4.58, showed 100% inhibition at 40%. In contrast, the crude extract showed no inhibition at any of the tested concentrations. Fermented extracts from crushed plants may act on the development of microorganisms. Quite the contrary: endophytic microorganisms can play a relevant role in plant health, since they control phytopathogenic microorganisms and can also help control insects and even protect plants against herbivores. For this purpose, use of plant remains as a source of fermented raw materials can result in economy and high performance for the process (6). Spontaneous fermentation for plant extract production has low cost because it only uses raw material and non-treated water and offers the advantage of using microorganisms present in the native flora (7). In this sense, as different polyphenols with varying polarities can be found in plants, solvents with different polarities should be used to enable efficient extraction of these constituents; aqueous solutions of ethanol and methanol are commonly used and their effectiveness will depend on the polarity of polyphenols present in the sample. The fermentation process provides advantages compared with non-fermented plant extracts, increasing the quantity of "active substances" and "breaking down" certain molecules to a feasible size for deeper and more rapid penetration (4).

In the *in vivo* tests, fermented extracts of *D. pusillus* and *T. pratense* plants were only applied at the concentration of 40% (diluted in water) because they showed better *in vitro* results (Figure 2). The non-autoclaved fermented extract of *T. pratense*, applied preventively, showed favorable potential, inhibiting 90% fungal growth (Figure 3), followed by the autoclaved preventive treatment, with 46% lesion control. The non-autoclaved preventive test with *D. pusillus* showed 43% mycelial growth control. As for the curative treatment, the autoclaved extract of *D. pusillus* showed 40% phytopathogen control, compared with only 37% control by the autoclaved extract of *T. pratense*. Controls 1 and 2 (only water and only fungus, respectively) reacted as expected: with water, fruits had no infected lesion along 12 days; differently, with control 2, only fungus, fruits showed ABS symptoms.

As the non-autoclaved fermented extract offered the best result among all tests, the autoclaved fermented extract is assumed to have suppressed some important metabolites for fungicidal activity, as cited by Talamini (9): plant extracts may elicit defense reactions against phytopathogens; they may also have a direct action, since some of their substances may be toxic to the fungus, which means that they have

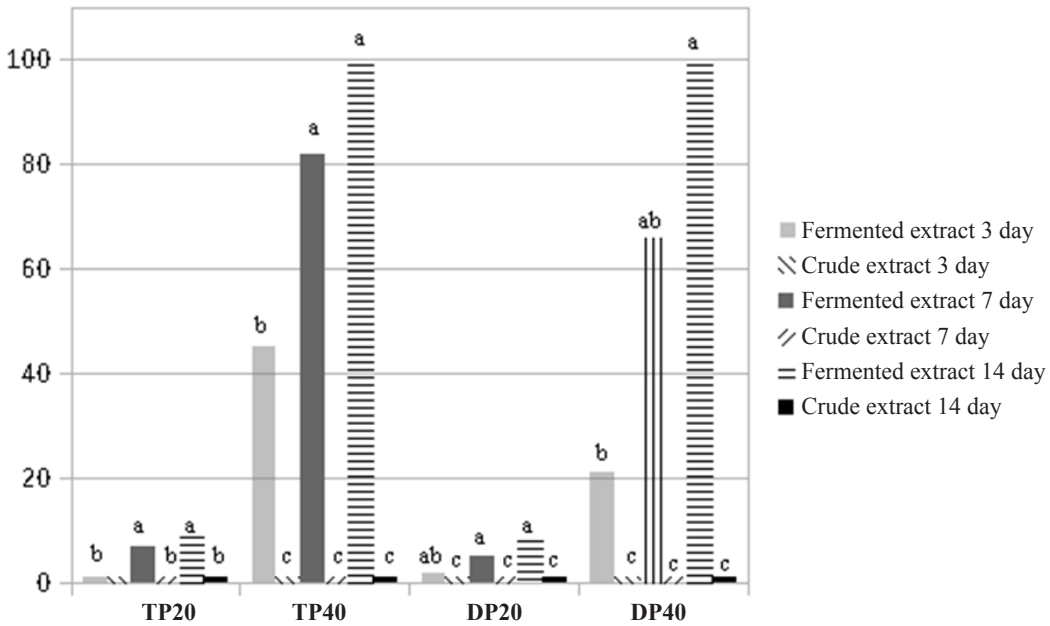

Figure 1. Percentage of inhibition of the phytopathogen *Alternaria alternata* f.sp. citri, using fermented and crude extracts of *T. pratense* (TP: 20 and 40%) and *D. pusillus* (DP: 20 and 40%) on the 3rd, 7th and 14th day of mycelial growth assessment.

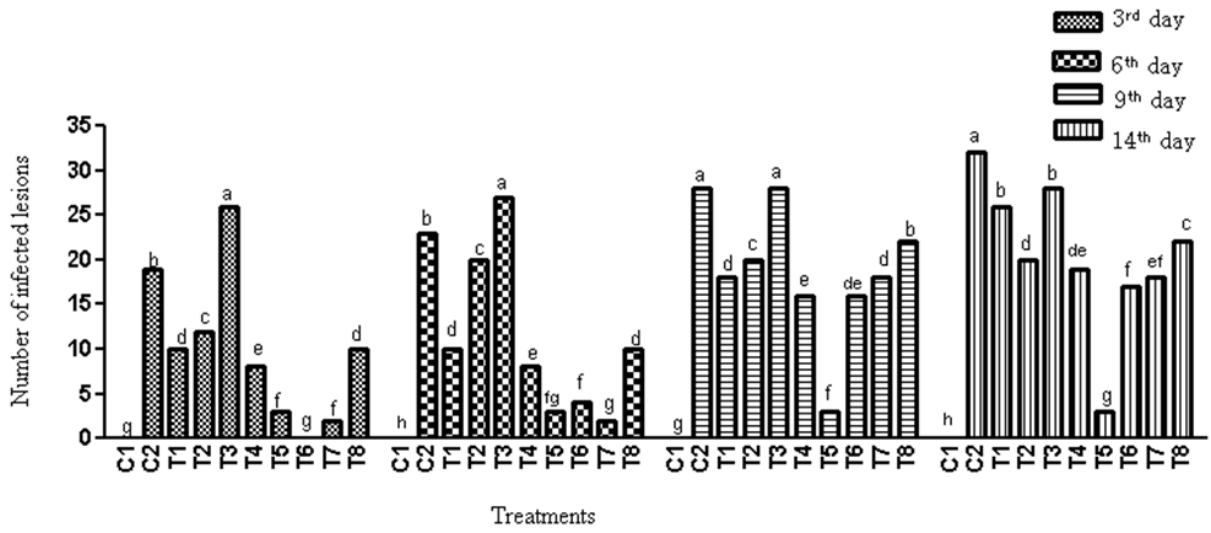

Figure 2. Number of ABS lesions at 3, 6, 9 and 12 days after inoculation of the fungus and application of treatments. C1: Only water; C2: Only fungus; T1: non-autoclaved dressing of *T. pratense*; T2: autoclaved dressing of *T. pratense*; T3: non-autoclaved dressing of *D. pusillus*; T4: autoclaved dressing of *D. pusillus*; T5: preventive application of non-autoclaved *T. pratense*; T6: preventive application of autoclaved *T. pratense*; T7: preventive application of non-autoclaved *D. pusillus*; T8: preventive application of autoclaved *D. pusillus*.

Figure 3. A) Only water; B: Only fungus; C: Preventive application of non-autoclaved *T. pratense*

potential for the development of bioproducts with fungicidal action (2, 4). The extract of *T. pratense* had 14 mg/mL ferulic acid and that of *D. pusillus* had rutin (9 mg/mL), epicatechin (5 mg/mL), apigenin (0.03 mg/mL), myricetin (1 mg/mL) and vitexin (1.6 mg/mL). The concentration of isoflavones in legumes is controlled by genetic and environmental factors and increases depending on responses to biotic and abiotic stress. Isoflavones play a role in the defense response of plants in various situations including attack to pathogens, UV rays, and chemical and physical damage; they are also involved in symbiotic plant-insect interactions (6). Studies involving the use of plant extracts as alternative defense agents, aiming to control phytopathogens, may contribute to the growing demand for either the development of products that control diseases in crops not covered by conventional agriculture or the replacement of synthetic fungicides for fungicides of low toxicity (1). Moreover, they can be used to develop viable options of fungal disease control in organic cultivation, as well as to develop effective methods for the integrated management of diseases (3). Fermented extract of *Trifolium pratense* showed potential to control *Alternaria alternata* f.sp. *citri*, in both *in vitro* and *in vivo* tests. Fermented extracts, used preventively, showed better results than those used curatively.

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