Majoritary compounds identified in essential oils of *Cymbopogon* species inhibits *Rhizoctonia solani*, causal agent of rice sheath blight

Compostos majoritários identificados em óleos essenciais de espécies de *Cymbopogon* inibem *Rhizoctonia solani*, agente causal da queima das bainhas do arroz

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ABSTRACT
The antifungal activity of plant essential oils (EOs) has been verified for several agricultural plant pathogens. The objective of this study was to evaluate the antifungal activity of EOs from *Melaleuca alternifolia*, *Eucalyptus citriodora*, *Cymbopogon martinii* and *C. citratus* against *Rhizoctonia solani*, causal agent of rice sheath blight. Essential oils were obtained by hydrodistillation, in a Clevenger type system, and characterized by GC and GC-MS analysis. Bioassays were conducted using EOs and commercial standards of majority compounds identified. The EOs from *C. martinii* and *C. citratus* and its majority compounds, geraniol and citral, respectively, inhibited the mycelial growth of *R. solani* in vitro. Biologic fungicides derived from these essential oils can became an alternative for the management of plant diseases.

Keywords: *Oryza sativa*, citral, geraniol, disease control.

1 INTRODUCTION
Sheath blight (ShB), caused by *Rhizoctonia solani* Kuhn [Teleomorph: *Thanatephorus cucumeris* (Frank) Donk] is a major soil borne disease in rice crop, causing crop losses corresponding to enough rice to feed 1.7 million people (Yellareddy gari et al., 2014; Tsiboe et al., 2017). ShB is associated with intensive and high-input production systems, including high-yielding susceptible varieties and excess of nitrogen fertilization (Slaton et al., 2003). In addition, other crops grown in rotation with rice, including soybeans, corn, grain sorghum and others, also serve as host plants for the fungus, which makes it difficult control of the disease (Bolkan & Ribeiro, 1985; Khandaker et al., 2008). Management of ShB could potentially be improved by the use of host genetic resistance, however no rice variety has been found presenting complete resistance to *R. solani* (Prasad & Eizenga, 2008; Srinivas chary et al., 2013; Ke et al., 2017). Nowadays, control of sheath blight is carried out mostly by use of fungicides, mainly Qols and DMIs (Groth, 2006; Pramesh et al., 2017; Naik et al., 2017). Despite the effectiveness in the disease management, chemical control increase the production costs, besides soil, water and grain contamination (Yan et al., 2015; Samarghandi et al., 2017, Teló et al., 2017). On the other hand, some samples collected in
2011 from a small number of fields in Louisiana, USA were found to contain less sensitive isolates of *R. solani* against QoIs fungicides (FRAC, 2018), which represents a concern about the effectiveness of chemical control of ShB over the time. Therefore, alternative control strategies are demanded in order to ensure efficient, long-lasting and environmentally safe management of ShB in the rice crop (Oard et al., 2004; Khaing et al., 2014; Yu et al., 2017).

Plant essential oils (EOs) exert biological activity naturally repelling insects and avoiding infection by plant pathogens (Sharifi-Rad et al., 2017). These products are generally assumed to be more acceptable and less hazardous for the ecosystems and could be used as alternative for treatment of plant diseases (Zaker, 2016). Several studies have demonstrated the effectiveness of EOs to inhibit bacterial, postharvest and soil borne pathogens, with potential to be used as biofungicides (Wilson et al., 1997; Silva et al., 2010; Ali et al., 2015; Hong et al., 2015; Amini et al., 2016). Among these, EOs of *Thymus vulgaris* and *Cymbopogon jwarancusa* are described suppressing the mycelial growth of *R. solani* (Lee et al., 2007; Bhuyan et al., 2010).

Despite the extensive number of works demonstrating the inhibitory effect of EOs against plant pathogens, few of them have focused on identify which compounds are effectively responsible for the inhibition (Numpaque et al., 2011; Shin et al., 2014). EOs are a complex mixture of molecules, which generally contains more than 20 different components of low molecular weight with variable concentrations (Sharifi-Rad, et al., 2017). Many of these molecules are found in low concentrations, while few of them are the main components that can represent up to 95% of total oil and will be the main responsible for the biological effects of the oil (Simon et al., 1986).

The general purpose of the current study was to investigate the efficacy of EO of four plant species in inhibit the mycelial growth of *R. solani* and to identify which of the compounds present in these oils are responsible for inhibition of the pathogen.

### 2 MATERIAL AND METHODS

#### 2.1 PLANT MATERIALS AND EXTRACTION OF ESSENTIAL OILS

The following plant species were used in this study: *Melaleuca alternifolia* Cheel (Myrtaceae); *Eucalyptus citriodora* Hook (Myrtaceae); *Cymbopogon martinii* (Roxb.) (Poaceae); *Cymbopogon citratus* Stapf (Poaceae). All plants were cultivated in the Itajaí Experimental Station of Santa Catarina State Agricultural Research and Rural Extension Agency – Epagri (26°57’09.1”S; 48°45’50.3” W). All plant material was dehydrated at 30°C. Essential oil was obtained using air-dried plant material by hidrodistillation for 4 hours. In order to remove the remaining water in the
oil, anhydrous sodium sulfate was used as drying agent. The oils were kept in amber flasks and stored at 4°C.

2.2 ESSENTIAL OIL ANALYSIS

The EOs were analyzed by gas chromatography coupled with a mass spectrometric detector (GC/MS) (Shimadzu, Model GCMS - QP2010) using a ZB-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The injection temperature was 250°C and the carrier (helium gas) flow was 1.0 mL min⁻¹. The chromatograph oven was optimized with an initial temperature of 60°C for 4 min up to 210°C, keeping for 6 min, in a 35 min run. The oil sample was diluted 200 times in hexane and subsequently injected into the GC/MS. The compounds were identified by comparison their mass spectra with a database (NIST-05) and also by comparing their retention indices (RI) with those reported in the literature (Adams, 2012). The retention indices were calculated according to Van den Dool & Kratz (1963) from n-alkane standards (C10-C21), under the same chromatographic conditions of the EOs samples. Quantification was performed by normalizing the area of each chemical constituent peak (%), and the total area obtained by the sum of all areas of the chromatogram.

2.3 IN VITRO ANTIFUNGAL ASSAY

*Rhyzoctonia solani* isolates from rice plants were gently provide by Dra. Valacia L. da Silva Lobo (Embrapa-CNPAF), and stocked in filter paper, -18°C. The EOs were emulsified with tween 80 (Biotec, Pinhais, BR) 1:1 (v/v) (< 45°C). Representing the majority compounds identified, the following commercial standards were used: geranyl acetate (CAS 105-87-3); citral (mixture of alpha and beta isomers) (CAS 5392-40-5); geraniol (CAS 106-24-1); and β-myrcene (CAS 123-35-3) (Sigma-Aldrich, St. Louis, MO). Both EOs and chemical standards were diluted in PDA medium and transferred 20 mL to each 90 mm Petri dishes. As a positive control it was used PDA medium containing only Tween 80 at the highest concentration of the dose curve, and as a negative control it was used tetracnazole (Arysta Life science, Salto do Pirapora, BR) 312,5 mg. L (i.a.) of medium. All treatments received antibiotic streptomycin sulphate 0.01% (Vetec, Duque de Caxias, BR). Five plates per treatment were inoculated with 2,5 mm PDA plugs from cultures on the same medium up to 5 days old and incubated at 28°C with 12 hof photoperiod. In order to verify the antifungal activity, radial growth of colonies was measured in two directions, 1,2,3,4,5,10 and 15 days after plate inoculations (DAI). Tests were performed in triplicate.
2.4 STATISTICAL ANALYSIS

Antifungal activity of essential oils and majoritary compounds were analyzed using the non-parametric Kaplan-Meyer estimators. Overall similarities among mycelial growth function curves and colony average diameter were tested by $\chi^2$ log-Rank test and the pairwise comparisons among curves.

3 RESULTS

3.1 ESSENTIAL OILS ANTIFUNGAL ACTIVITY

The inhibitory activity against *R. solani* *in vitro* was initially evaluated using EOs obtained from *M. alternifolia*, *E. citriodora*, *C. martinii* and *C. citratus* in four different concentrations. All oils tested inhibited the mycelial growth of *R. solani* in concentrations ≥ 0.3%, while the tetraconazole fungicide allowed a slightly growth (Table 1). However, in the lowest tested concentration of 0.075%, only *C. martinii* and *C. citratus* oils inhibited totally the mycelial growth until 15 days after inoculations of plates (Fig. 1). Due to the greater inhibitory capacity, EOs of *C. martinii* and *C. citratus* were selected to GC/MS analysis to identify compounds present in these oils responsible by inhibition of *R. solani*.

Table 1. Inhibition of mycelial growth of *Rhizoctonia solani* by plant essential oils

| Species                  | Concentration of essential oils (%) | Diameter of *R. solani* colonies (mm)* |
|--------------------------|-------------------------------------|----------------------------------------|
|                          | 0.075  | 0.15  | 0.3   | 0.6   |
| *Melaleuca alternifolia* | 91.0±0.0| 85.4±0.2| 0.0±0.0| 0.0±0.0|
| *Eucalyptus citriodora*  | 91.0±0.0| 0.0±0.0| 0.0±0.0| 0.0±0.0|
| *Cymbopogon martinii*    | 0.0±0.0| 0.0±0.0| 0.0±0.0| 0.0±0.0|
| *Cymbopogon citratus*    | 0.0±0.0| 0.0±0.0| 0.0±0.0| 0.0±0.0|
| **Controls**             |        |        |        |        |
| Tween 80                 | 91.0±0.0|        |        |        |
| Fungicide*               |         |        |        | 11.7±0.4|

*a* Values are represented as means ±SD of five replicates of two separate experiments, 15 days after plate inoculations.

*b* Tetraconazole (312.5 mg L⁻¹)

To find the minimum inhibitory concentration (MIC) of *C. martinii* and *C. citratus* EOs, a new set of experiments were performed with concentrations ranging from 0.15 to 0.009%. Results demonstrated that EO from *C. Martinii* significantly inhibited the *R. solani* mycelial growth until 0.075% up to 15 DAI (Fig. 2a). A partial inhibition occurred with 0.037%. No inhibition was observed in concentrations smaller than 0.037%. On the other hand, a complete inhibition was
observed using EO from *C. Citratus* in the concentration of 0.037% (Fig. 2b), suggesting more efficiency of *C. citratus* EO in the pathogen inhibition.

![Fig 1. Inhibition in vitro of mycelial growth of *Rhizoctonia solani* by plant essential oils. A- *Melaleuca alternifolia*, B- *Eucalyptus citriodora*, C- *Cymbopogon martinii* var. *motia*, D- *C. citratus*, E- Tween 80, F- Fungicide tetraconazole (312.5 mg L⁻¹). All EOs were tested at the concentration of 0.075%.

3.2 ESSENTIAL OILS COMPOSITION

Four majority compounds were identified in the *C. martinii* EO, representing 93.95% of the total registered compounds. Geraniol was the most abundant compound corresponding to 81.17% (Table 2). In the *C. citratus* EO were identified five different compounds representing 92.19% of the total, and β-citral, α-citral and β-myrcene were the most abundant compounds identified.

![Fig 2. Inhibition activities of *C. martinii* and *C. citratus* essential oils on the mycelial growth of *Rhizoctonia solani*. Vertical bars represent the standard errors. The box plots with the different lower-case letters are significantly different by pairwise χ² log-Rank test (P< 0.05). Control = tween 80; Fungicide = tetraconazole (312.5 mg L⁻¹).](image-url)
3.3 ANTIFUNGAL ACTIVITY OF MAJORITY CONSTITUENTS

Commercial standards representing the majority constituents were tested individually in a new set of experiments. Each compound was tested in a correspondent concentration identified in the EO (1.0 x), half dose (0.5 x) and twice the dose (2.0 x) (Fig 3a.). For β-mircene, no inhibition was observed.

In the highest dose (2.0x), geranyl acetate slightly inhibited mycelial growth in the beginning of the bioassays. However, a complete inhibition of R. solani mycelial growth was observed with citral and geraniol at doses 1.0 and 2.0x up to 15 DAI. Even at the lowest dose (0.5x), citral and geraniol inhibited the fungus development similarly to the tetraconazole fungicide (Fig. 3b).

Table 2. Chemical compounds identified in the essential oils of C. martinii and C. citratus

| Constituent       | CAS     | RT   | CRI | LRI       | C. martinii | C. citratus | Citral standard |
|-------------------|---------|------|-----|-----------|-------------|-------------|----------------|
| β-myrcene         | 123-35-3| 7.84 | 989 | 991       | -           | 11.03       | -              |
| Linalool          | 78-70-6 | 10.98| 1099| 1098      | 4.06        | 1.00        | -              |
| β-citral          | 106-26-3| 14.63| 1240| 1240      | -           | 48.07       | 47.09          |
| Geraniol          | 106-24-1| 14.90| 1251| 1255      | 81.17       | 2.20        | -              |
| α-citral          | 141-27-5| 15.34| 1268| 1270      | 1.18        | 29.89       | 52.91          |
| Geranyl acetate   | 16409-44-2| 17.89| 1377| 1381      | 7.54        | -           | -              |
| Unidentified*     | -       | -    | -   | -         | 6.05        | 7.81        | -              |

RT = retention time (min), CRI = calculated retention index, LRI = literature retention index (Adams, 2012).
*unmatched with mass spectral interpretation (NIST) or retention index divergent with any reference described.

Fig. 3. Inhibition activities in vitro of different majority compounds identified in EOs of C. martinii and C. citratus against Rhizoctonia solani. A - Diameter of colonies 15 DAI using three different concentrations of each compound. 1.0 x corresponding to: Geranyl acetate 58.2 ppm, citral 342.4 ppm, geraniol 678.1ppm, β-mircene 47.2 ppm. Fungicide tetraconazole 312.5 mg L⁻¹. B - Kinetics of the mycelial growth using half dose (0.5 x).
4 DISCUSSION

Plants throughout the evolutionary process developed different mechanisms of self-defense and some of them are present in EOs, which are a natural reservoir of antimicrobial compounds available to be explored for use in agriculture (Zaker, 2016).

In the present study, four plant species were selected to evaluate the effectiveness of their EOs against *R. solani*, based in a broad spectrum of antimicrobial activity found in these oils (Terzi et al., 2007; Wilson et al, 1997). All tested oils presented some inhibitory effect against the pathogen, however EOs of *C. martinii* and *C. citratus* were more effective. EOs from *Cymbopogon* species are known to inhibit pathogens belonging to different groups, including soil borne pathogens difficult to be controlled. Following Amini et al. (2010) *C. citratus* EOs sprayed directly in the soil significantly controlled three species of *Phytophthora* infecting melon, cucumber and pepper. Additionally, *Fusarium oxysporum* infecting different crops has been suppressed by EOs from *C. citratus* and *C. martinii* (Sharma et al., 2017). Paret et al. (2010) have demonstrated that EOs from both species significantly reduced the growth of *Ralstonia solancearum* Race 4. The effectiveness of EOs of *Cymbopogon* species has also been previously demonstrated against *R. solani* (Hajieghrari, et al., 2006; Bhuyan et a., 2010). Curiously, in some cases, the EOs from *C. citratus* did not inhibit the mycelial growth of *R. solani* (Lee et al., 2007). This discrepancy may be associated with the composition of the EOs that may be influenced by the cultivar employed, environmental conditions of plant growing, harvesting season and part of the plant used (Rao et al., 2009; Zouari et al., 2012; Gupta et al., 2016).

The chromatographic analyzes of EO from *C. martinii*, highlighted geraniol as the most abundant compound, representing 81% of the total oil composition. These results are in agreement with previous work descriptions in which the content of geraniol for *C. martinii* EO is around 80% (Siddiqui& Garg, 1990). For *C. Citratus* EO, the majority compounds identified were β-citral (48%), α-citral (30%) and β-myrcene (11%). Commonly, the *C. citratus* EO present higher variations in the chemical constituents present in the oil (Amini et al., 2016; Mourão et al., 2017). Vyshali et al. (2015) identified enormous variation in the content of geraniol, geranial (α-citral) and β-myrcene depending on the region in which the *C. citratus* were grown in India. In addition to variations in the constituent concentration within each species caused by environmental factors, there is significant variation in the constitution of the oils when comparing different species of the genus *Cymbopogon* (Bhuyan et al., 2010).

Analyzes of the majority compounds identified in the *C. martinii* and *C. citratus* EOs suggest that citral and geraniol are related with the inhibition of *R. solani*. The citral commercial
standard employed in this study is a mixture of α and β-citral, so is not possible to establish the contribution of each isomer in the inhibition observed. The mechanisms by which citral acts against microorganisms included down-regulating ergosterol biosynthesis, reduction in ATP biosynthesis and cell membrane hyperpolarization (OuYang et al., 2016; Shi et al., 2016; Wang et al., 2018).

Geraniol is an acyclic monoterpenic alcohol commonly found in several species of plants, reaching up to 95% of the EO composition of species as Monarda fistulosa (Simon et al., 1986). Geraniol is also associated with inhibition/ergosterol-binding, destabilization of fungal cell membranes and malformation of fungal hyphae (Miron et al., 2014; Pereira et al., 2015). Citral and geraniol have been individually effective in controlling several species of plant pathogens including Penicillium spp., Cladosporium spp., Aspergillus spp. and Fusarium spp. (Aoudou et al., 2010; Santos et al., 2017). The associated use of citral and geraniol may potentiate the inhibitory effect of the EO against pathogens as R. solani.

Field experiments should be performed to confirm the efficacy of these EOs on crops, developing microencapsulated formulations or nano-emulsions to ensure stability in field conditions.

5 CONCLUSIONS

The EOs from C. martinii and C. citratus and its majority compounds, geraniol and citral, respectively, inhibited the mycelial growth of R. solani in vitro. Our results suggest that geraniol and citral are promising tools for future use as a bio-fungicide to control of rice sheath blight.

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