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Full Length Research Paper

Preventive control of cotton ramulosis using clove oil at low concentration

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The present study aimed to evaluate the toxicity and preventive action of essential oils in growth of cotton ramulosis, *Colletotrichum gossypii* var. *cephalosporioides*, *in vitro* and *in vivo* conditions. Six fungus-isolates obtained from cotton belts, in Southern and Mid-West Brazilian regions were submitted to different oil concentrations in bioassays and inhibition of mycelia growth were evaluated during 7 days. Then, the selected oil was used in a preventive bioassay carried out in greenhouse using a susceptible cotton cultivar infected with fungus-conidia at 1.0 × 10^6 mL-1. Plants in greenhouse were daily evaluated during 30 days. Among all samples evaluated, clove oil at low concentration (500 ppm) provided best results as to fungus-growth inhibition. The disease severity was reduced to 60% in relation to control (no-infected plants). These results mean an interesting alternative in the preventive control of cotton ramulosis, especially to small farmers which often have adopted an agro-ecological management for disease control of crops.

Key words: *Colletotrichum*, control, disease, fungus, growth, inhibition.

INTRODUCTION

Cotton is an important agricultural commodity traded worldwide and their products are raw materials for several textile and oil products. Despite its broad economic importance, the cost of production is often high, especially due to the use of pesticides for insect, weed and pathogen control. Cotton management uses more pesticides than any other single crop, nearly $2.6 billion annually (Greenberg et al., 2012).

Brazil is an important cotton producer at world level (Schnef, 2010). Given the tropical, humid environment of Brazil, several pests, especially fungus, threaten cotton production. Control is often achieved by the use of synthetic pesticides, which increases the production cost. However, with the emergence of pathogen-resistant strains and also residues in the final product and environmental contamination, it is necessary to develop researches involving alternative methods of control in order to minimize such risks.

Ramulosis, caused by *Colletotrichum gossypii var. cephalosporioides*, is a highly prevalent and virulent disease that occurs in all cotton belts, mainly in savanna region, leading to severe losses in fiber yield depending on environment conditions (Monteiro et al., 2009). The losses caused by the disease can get 80% or more, depending on the cultivar susceptibility (Freire et al., 1997). As genetic resistant varieties are limited in Brazilian commercial cultivars, the control is performed with synthetic fungicides by big farmers, whose fields exceed 500 ha. However, due to high cost, this practice is often out of reach to small ones, whose management activities rely on family support. As an alternative control, they often use empirical formulations, based on plants oil and extracts, since commercial biofungicides are not available in the national market.

An increased use of biopesticides in the agricultural market, with high consumption of organic products in

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several countries, is driven by the advantages of low toxicity, safety, efficacy in pest controls and benefits to the ecological chain. The use of biopesticides alleviates the hazards caused to the environment and human health derived from the chemical pesticide residues (Isman, 2000). In terms of active ingredients, microbial, botanical extracts and semiochemical biopesticides dominated the global biopesticides market in 2010, with over 63% share, creating an expressive market of alternative products for plant defense (Leng et al., 2011; Isman, 2000).

Although biopesticides can be used to control a wide variety of pests, each active ingredient often shows relative specificity for its target. Among natural pesticides reported in the literature as alternative crop protectants, those derived from essential oils have traditionally been used to protect plants against insects, nematodes and fungus, demonstrating contact and fumigant toxicity to several economically important pests (Leng et al., 2011). According to Muller-Riebau et al. (1995), biopesticide activity in oils is strongly associated with monoterpenic phenols, especially thymol, carvacrol and eugenol.

In the literature, several vegetal species have been reported with bioactivity against fungus, such as *Curcuma longa L.* (saffron), *Lippia alba Mill.* (lemon balm), *Rosmarinus officinalis L.* (rosemary), *Corymbia citriodora Hook.* (lemon eucalyptus), *Cymbopogon nardus L.* (citronella), *Azadirachta indica A. Juss* (neem), *Thymus vulgaris (L.)* (thyme), *Piper*, among others (Tagami et al., 2009; Lima et al., 2008; Balb-Peña et al., 2006; Dubey et al., 1987). The efficacy of the control depends on the action on inhibition of physiological events, such as germination, mycelia growth and formation of appressoria. With *Colletotrichum* genus, some essential oils have demonstrated fungitoxicity in *in vivo* and *in vitro* bioassays. Ribeiro and Bedendo (1999) showed inhibitory effect on papaya-*C. gloeosporioides* with garlic extract (*Allium sativum* L.) at 10,000 ppm. According to these authors, extract reduced radial growth of the colony up to 68%; Lima et al. (2008) found growth inhibition of cotton-*C. gossypii* with citronella oil (*Cymbopogon nardus* L.) *in vitro* and reduction in final disease index, in field trials, both assays at 2000 ppm. This index is often used to assess disease severity. Duamkhanmanee (2008) controlled anthracnose of mango (*C. gloeosporioides*) with lemon grass (*Cymbopogon citratus* Stapf.) at 4000 ppm. The results showed minimal disease scores (1.85), that is, with lower values of severity, when mango was dipped into lemon grass oil in hot water after inoculation.

Despite the results above, it is worth investigating the activity of these oils at lower concentrations, below to 1000 ppm, because it allows a reduction in cost of product, taking into account the price of essential oils in Brazilian market (average: U$ 41.00/100ml), as well as it could provide fungus control at longer period. The present study aimed to evaluate the toxicity and preventive action of several essential oils at low concentration, against cotton ramulosis in *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Origin of *C. gossypii* var. cephalosporioides isolates and experimental assay

Six isolates of *C. gossypii* var. *cephalosporioides* (*Cgc*) were obtained from different plants in cotton-belts in Primavera do Leste (15° 31' 40" S, 54° 20' 45" W, 636 m) and Campo Verde (15° 33' 60" S, 55° 10' 08" W, 736 m) both in Savanna-Mato Grosso and in Piracicaba (22° 43' 30" S, 47° 38' 56" W, 547 m), in Southeast-São Paulo regions, in Brazil.

All isolates were further isolated in Potato-Dextrose-Agar (PDA) medium using bioassays.

Eleven essential oils were evaluated, all gently provided by the Chemistry Department, at Rural-Federal University, in Pernambuco, Brazil (UFRPE). They were as follows: *Citrus reticulata sinensis* Tangor (shells), *Xylopia* sp. (leaves), *Schinus terebinthifolius* Raddi. (immature fruits), *Syzygium aromaticum* L. (leaves), *Croton sonderianus Mull.* Arg. (leaves), *Citrus limon L.* (leaves), *Croton regenelianus L.* (leaves), *Cinnamomum zeylanicum* Blume. (stem), *Lantana canescens* Kunth. (leaves), *Cymbopogon citratus* (DC) Stapf.) and *Croton heliotropiifolius* Kunth. (leaves).

At first, all oils were previously bioassayed for their ability to inhibit mycelia growth of fungus at 3000 ppm. Among them, just two, *Syzygium aromaticum* and *C. citratus*, were selected and again bioassayed in five lower concentrations (500, 1000, 1500, 2000 and 2500 ppm), following the methodology described in Lima et al. (2008).

Oils were added separately to PDA culture medium (Gibco) (45 - 50°C) and poured into Petri dishes. A 0.5 cm diameter disk of PDA medium with a 7 days-mycelium (*Cgc*-SP5 isolated from Piracicaba, SP) was deposited in the center of each plate. Then, plates were randomized and incubated in Biochemical Oxygen Demand (BOD)-growth chamber, at 25°C and 12:12 h photoperiod. The bioassay was completely randomized with seven replications for each concentration. Thereafter, diameter of colonies was measured every 24 h during 7 days.

Inhibition assays with different *Colletotrichum gossypii* var. cephalosporioides (*Cgc*) isolates

This bioassay was carried out with other five *Cgc* isolates obtained from cotton fields, in Mato Grosso and São Paulo States. Experiment was carried out following the same methodology described above. Four new concentrations were chosen based on the lowest (500 ppm) disclosed in the results of mycelial growth bioassay.

Table 1. Grade scale adopted to estimate disease incidence and severity of ramulosis.

Grade	Symptom descriptions visualized in plants
1	No symptoms
2	Necrotic spots on younger leaves
3	Necrotic spots on leaves, shortened internodes and early super-budding
4	Necrotic spots on leaves, shortened internodes, super- budding, no reduction in height
5	Necrotic spots on leaves, shortened internodes, expressive super-budding, reduction in height

In vivo validation assay

The effectiveness of selected oil was evaluated by *in vivo* assay carried out under field conditions, at an experimental area of the Agronomy Department (UFRPE), in Recife, PE (8º03'14" S, 34º52'51" W). Seeds of a susceptible cotton cultivar (BRS 187), developed by Brazilian Company of Agricultural Research (EMBRAPA), were sown in 15 L pots containing fertilized soil. A 2 plants/pot density was further maintained for the following treatments: 1 - Positive control (plants sprayed with fungus suspension); 2 - Preventive 1, plants spayed with selected oil at 750 ppm + 0.05% Tween-20; 3 - Preventive 2, plants spayed with selected oil at 1000 ppm + 0.05% Tween-20; 4 - Preventive control, plants spayed with commercial fungicide (Tebuconazole 200 g/l, Trifloxystrobin 100 g/l) at 3 ml/L; and 5 - Negative control, plants sprayed with water + 0.05% Tween-20. Both oil concentrations (750 and 1000 ppm) were chosen taking into account the safety margins disclosed in previous *in vivo* assays. The assay was completely randomized with seven replicates.

Cotton plants (30 days old) were sprayed with a fungus suspension at 10^{10} conidia/mL and incubated in moist chamber for 60 h. *Cgc*-MT121 isolated from Primavera do Leste, MT, was chosen for this trial due to the high severity shown in previous bioassays, based on precepts of Koch's postulates (data not shown). Disease incidence and severity were monitored during 30 days, every 3 days after inoculation. A grade scale was adopted, recommended by Araújo et al. (2003), as shown in Table 1.

The infection rate was estimated based on the McKinney (1923) formula:

$$\text{Infection rate (\%)} = (\text{Grade} - 1) \times 25$$

Finally, the effectiveness of disease control (DCE) was estimated according to Abbott (1925), as described hereunder:

$$\text{DCE (\%)} = \frac{(\text{\% Disease of control} - \text{\% Disease of treatment})}{\text{\% Disease of control}} \times 100$$

STATISTICAL ANALYSIS

An analysis of variance (ANOVA) was used to detect differences between the control and concentration groups. Data were subjected to analysis of variance, using SISVAR program, software 5.1 (Ferreira, 2011). A Scott-Knott test ($p < 0.05$) was used for multiple comparisons.

RESULTS AND DISCUSSION

Prospecting vegetal oils to ramulosis control

Among eleven essential oils evaluated for a period of seven days, just two, the essential oil from *Syzygium aromaticum*n (clove) and the essential oil from *Cymbopogon citrates* (citronella), inhibited *Cgc*-mycelia growth. Clove oil was the most effective and inhibited fungus growth at a lower concentration (Table 2).

Based on the results with clove oil, the same bioassay was performed using pure eugenol, a major component of clove oil, at lower concentrations. Commercial Eugenol (Vetec) was evaluated at 400, 300, 200, 150 and 50 ppm. The trial lasted seven days and was evaluated according to the same methodology mentioned above.

In this study, we verified a 43% reduction of fungus growth at the lower concentration of eugenol (50 ppm) and complete inhibition at 300 ppm (Table 3). This result is relevant because it suggests an opportunity of disease control by using pure oil in low concentration, with further possibility of minimizing costs since eugenol is available in the essential oil market.

In the literature, eugenol has been reported as an antimicrobial metabolite present in the essential oils or extracts of many other plants, including cinnamon, basil, peppermint, sweet basil and nutmeg. The oil is active against a variety of vegetal pathogenic fungi (Iranpour et al., 2012; Wang et al., 2010; Cheng et al., 2008).

As shown by Gill and Holley (2006) and Braga et al. (2007), eugenol leads to the disruption of fungal and bacterial membranes. Depending on its concentration, it can enter between the fatty acid chains that make up the membrane lipid bilayers, altering the fluidity and permeability of cell membranes. At low concentration, up

Table 2. *Cgc* SP5-mycelia growth (cm) in citronella and clove oils at different concentrations.

	Concentration (ppm)					
Treatment	**Control**	**500**	**1000**	**1500**	**2000**	**2500**
Citronella	8.6[a]	6.7[a]	5.5[a]	0.2[b]	0.1[b]	0.1[b]
Clove	8.5[a]	0.2[b]	0.2[b]	0.2[b]	0.1[b]	0.1[b]
Coefficient of Variation (%): 0.61						
General mean: 2.6						
Square mean (treatment): 60.40; degree of freedom: 1						
F test (isolates): 241602.67**						
Square mean (treatment × concentration): 24.14; degree of freedom: 5						
F test (isolates × concentration): 1.99**						

Means with same letters do not differ statistically by Scott-Knott test ($p < 0.05$), when compared to control.

Table 3. *Cgc* SP5-mycelia growth (cm) in different concentrations of eugenol.

	Concentration (ppm)					
Treatment	**Control**	**50**	**150**	**200**	**300**	**400**
Eugenol™	8.6[a]	4.9[b]	3.9[c]	3.2[c]	0.2[d]	0.2[d]
Coefficient of Variation (%): 2.03						
General mean: 3.53						
Square mean (treatment): 50.65; degree of freedom: 5						

Means with same letters in line do not differ statistically based on Scott-Knott test ($p < 0.05$).

to 5 to 200 µg/ml, Iranpour et al. (2012) found inhibition effects of eugenol against *Colletotrichum gloeosporioides* and *Agaricodochium camellia* with the growth rate method, with EC_{50} of 30.37 µg/ml and 51.55 µg/ml, respectively. Faria et al. (2006) found antifungal activity of eugenol against *Alternaria* isolated from tomato and against *Penicillium chrysogenum*. According to these authors, the minimal inhibitory concentrations of eugenol were 0.16 and 0.31 mg/disc for *Alternaria* sp. and *P. chrysogenum*, respectively. Other reports are available in the literature demonstrating the efficiency of clove-essential oil or active component against several plant diseases.

Growth of *Cgc* isolates in PDA-clove oil

Six isolates were grown on PDA-clove oil at 100 to 500 ppm concentrations during seven days (Table 4). SP5-Piracicaba and MT36- Primavera do Leste isolates showed 100% inhibition at 400 ppm, but MT37 and MT121 still grew up at maximum concentration, although with significantly lower radial growth (around 1 cm). This result suggests the existence of natural variability among isolates collected from Savanna-Mato Grosso, even within the same location. Although most of the isolates evaluated in this work were controlled at 500 ppm, for further control of ramulosis in cotton field, we recommend increasing the safety margin to 750 ppm, in order to provide more protection for the plants longer.

In vivo validation assay

In this assay, clove oil was used in field cotton-plots aiming to certify the preventive control of ramulosis. The first symptoms of the disease were verified at 5 days after inoculation, characterized by necrotic spots on young leaves and petioles, in control plants (sprayed with fungus) and mild symptoms in 750 ppm-preventive 1 and 1000 ppm-preventive 2 treatments. Symptoms increased during development of control plants exhibiting super-budding and reduced height. The infection rate grew up progressively reaching 78% at 30 days after inoculation. In these experiments, the infection rate in both preventive 1 and 2 treatments was less than 23%, during all assays (Table 5). Moreover, no statistical differences among them were found and infection rate was statistically similar in all period, indicating that in a lower concentration (750 ppm), clove oil could be used to

Table 4. Mycelia growth (cm) of *Cgc* isolates in PDA-clove oil.

Isolate	Origin	Concentration (ppm)					
		Control	100	200	300	400	500
SP5	Piracicaba, SP	7.6^{a}	4.9^{b}	3.5^{c}	2.7^{c}	0.2^{d}	0.2^{d}
MT36	Primavera do Leste, MT	8.4^{a}	5.8^{b}	4.0b^{c}	2.5^{d}	0.2^{e}	0.2^{e}
MT37	Campo Verde, MT	8.6^{a}	6.7^{b}	5.3^{c}	3.9^{c}	1.4^{d}	1.1$_{d}$
MT112	Primavera do Leste, MT	8.3^{a}	5.4^{b}	3.3^{c}	2.7^{c}	2.7^{c}	0.2^{d}
MT121	Primavera do Leste, MT	8.3^{a}	6.0^{b}	5.4^{b}	3.8^{c}	3.8^{c}	1.1^{d}
SP1147	Piracicaba, SP	8.6^{a}	5.6^{b}	4.8^{b}	3.9^{b}	2.2^{c}	0.3^{d}
Coefficient of Variation (%): 2.78							
General mean: 4.11							
Square mean (treatment): 16.75; degree of freedom: 5							
F test (isolates): 1286.38**							
Square mean (treatment x infection rate): 1.83; degree of freedom: 25							
F test (isolates x concentration): 1.00**							

Control: Plates sprayed just with water. Means with same letters in line do not differ statistically based on Scott-Knott test (p <0.05), when compared to control.

Table 5. Infection rate in cotton plants inoculated with *Cgc* and treated with clove oil.

Treatments	Infection rate (%)		
	5 dai	15 dai	30 dai
Positive control	25^{aA}	43^{aB}	78^{aC}
Preventive 1	15^{bA}	19^{bA}	22^{bA}
Preventive 2	15^{bA}	18^{bA}	21^{bA}
Preventive control	0.1^{cA}	0.1^{cA}	0.1^{cA}
Negative control	0.1^{cA}	0.1^{cA}	0.1^{cA}
Coefficient of variation (%): 4.27			
General mean: 17.20			
Standard error: 11.52			
Square mean (treatment): 5866.99; degree of freedom: 4			
F test (treatment): 10849.07**			
Square mean ((treatment x infection rate): 663.97; degree of freedom: 8			
F test (treatment x infection rate): 1.00**			

Dai: Days after inoculation. Positive control (plants sprayed with fungus suspension, at 10^{10} conidia/mL); Preventive 1, plants spayed with clove oil at 750 ppm + 0.05% Tween-20; Preventive 2, plants spayed with clove oil at 1000 ppm + 0.05% Tween-20; Preventive control, plants spayed with commercial fungicide (Tebuconazole 200 g/l, Trifloxystrobin 100 g/l) at 3 ml/L; and Negative control, plants sprayed with water + 0.05% Tween-20. Means with same letters do not differ statistically by Scott-Knott test (p <0.05), when compared to control. Letters in columns (minuscule) show comparison among treatments; letters in line (capital) show comparison among infection rates.

control ramulosis, reducing costs in acquisition and application of the product. As seen in Table 6, disease rate was controlled to about 67% at 30 days after inoculation, in both 750 and 1000 ppm.

This result strengthens the recommendation of clove oil to reduce the incidence and severity of cotton ramulosis. The use of clove oil is an attractive alternative in crop protection systems based on natural resources, with the

Table 6. Disease control rate in cotton plants after inoculation.

Treatments	Disease control rate (%)		
	5 dai	15 dai	30 dai
Positive control	0.6aA	0.6aA	0.8aA
Preventive 1	40bA	55bB	66bC
Preventive 2	40bA	58bB	68bC
Preventive control	99cA	99cA	99cA
Coefficient of Variation (%): 13.01			
General mean: 53.15			
Standard error: 6.98			
Square mean (treatment): 22799.98; degree of freedom: 3			
F test (treatment): 476.93**			
Square mean (treatment x infection rate): 243.65; degree of freedom: 6			
F test (treatment x infection rate): 1.00**			

Dai: Days after inoculation. Positive control (plants sprayed with fungus suspension, at 10^{10} conidia/mL); Preventive 1, plants spayed with clove oil at 750 ppm + 0.05% Tween-20; Preventive 2, plants spayed with clove oil at 1000 ppm + 0.05% Tween-20; Preventive control, plants spayed with commercial fungicide (Tebuconazole 200 g/l, Trifloxystrobin 100 g/l) at 3 ml/L; and Negative control, plants sprayed with water + 0.05% Tween-20. Means with same letters do not differ statistically by Scott-Knott test ($p < 0.05$), when compared to control. Letters in columns (minuscule) show comparison among treatments; letters in line (capital) show comparison among infection rates.

advantage of presenting low mammalian toxicity and being non-persistent in the environment. Moreover, this method provides lower selective pressure on pathogen population due to reduced risks in emergence and proliferation of resistant genotypes with favorable vertical mutability, as often takes place in Colletotrichum isolates (Zambolim and Souza, 1985; Santos et al., 2006; Pereira et al., 2011).

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