

CONTROL OF *Colletotrichum gloeosporioides* (penz.) Sacc. IN YELLOW PASSION FRUIT USING *Cymbopogon citratus* ESSENTIAL OIL

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ABSTRACT

The use of antibiotics in agriculture is limited when compared to their applications in human and veterinary medicine. On the other hand, the use of antimicrobials in agriculture contributes to the drug resistance of human pathogens and has stimulated the search for new antibiotics from natural products. Essential oils have been shown to exert several biological activities including antibacterial and antifungal actions. The aim of this study was to determine the activity of 28 essential oils from medicinal plants cultivated at CPMA (Medicinal and Aromatic Plants Collection), CPQBA/UNICAMP, against *Colletotrichum gloeosporioides* (Penz.) Sacc., the anthracnose agent in yellow passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Deg), as well as evaluating their effect in the control of post-harvest decay. The oils were obtained by water-distillation using a Clevenger-type system and their minimal inhibitory concentrations (MIC) determined by the micro-dilution method. According to the results, 15 of the 28 essential oils presented activity against *Colletotrichum gloeosporioides*, and the following four oils presented MIC values between 0.25 and 0.3 mg/mL: *Coriandrum sativum*, *Cymbopogon citratus*, *Cymbopogon flexuosus* and *Lippia alba*. The evaluation of *Cymbopogon citratus* essential oil in the control of post-harvest decay in yellow passion fruit showed that the disease index of the samples treated with the essential oil did not differ ($P \leq 0.05$) from that of the samples treated with fungicide. The present study shows the potential of *Cymbopogon citratus* essential oil in the control of the anthracnose agent in yellow passion fruit.

Key words: yellow passion fruit; *Colletotrichum gloeosporioides*; anthracnose; antimicrobial activity; minimal inhibitory concentration.

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INTRODUCTION

After the introduction of antibiotics in the fight against bacterial diseases about six decades ago, phytopathologists quickly recognized the potential of using antibiotics against plant diseases, especially against those caused by bacteria (14). During the 1950s, about 40 drugs from bacterial and fungal origins were tested to control phytopathologies (10), the primary targets of the antibiotics being some bacterial diseases in vegetables and apple and pear rust.

Nowadays, the use of antibiotics in agriculture is limited when compared to their medicinal and veterinary applications (14). According to Iacobellis et al. (11), the control of plant diseases is difficult due to the limited availability of bactericides, and also, with the emergency of antibiotic-resistant bacterial strains, the control of phytopathologies has become further limited. The question of whether their use in agriculture contributes to the development of antibiotic resistant human pathogens has also been the subject of discussion in recent years in both Europe and North America.

This fact has stimulated the search for natural products with antimicrobial activity, and new strategies for this type of search, including the effective screening of natural products, should lead to the development of new antifungal drugs (12).

With respect to essential oils, many have shown good potential in the control of bacterial diseases, particularly in the eradication of those present in seeds (5), but studies on the use of essential oils as antimicrobial agents are limited (11). The pioneer research of Maruzzella et al. (13) was followed by a few studies on the action of essential oils in the control of phytopathogens (11).

Recently some studies have demonstrated the *in vitro* efficiency of essential oils and their extracts against important phytopathogenic microorganisms, such as *Allium sativum* extract, tested against 10 bacteria including *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* (6).

Yellow passion fruit (*Passiflora edulis* Sims f. *flavicarpa*) has achieved a space on the juice and juice products market,

due to its exotic taste, strong tropical aroma, high acidity and pulp yield (9). This plant is from tropical America and belongs to the Passifloriaceae family, which contains more than 200 native species in Brazil. Despite its commercial importance, yellow passion fruit presents significant losses due to withering and decay, and the main pathogens responsible are the fungi *Colletotrichum*, *Rhizopus*, *Cladosporium*, *Fusarium*, *Lasiodiplodia* & *Phomopsis*, *Alternaria alternata*, *A. passiflorae*, *Septoria passiflorae* and *Sclerotinia sclerotium* (3, 4, 19).

Colletotrichum gloeosporioides is responsible for anthracnose in yellow passion fruit and attacks the fruit before and post-harvest. Several chemical methods are used to control the disease, but increasing restrictions regarding the use of fungicides due to food and environmental safety, have stimulated the use of natural and biological methods. Thus, the aim of this study was to determine the *in vitro* activity of essential oils against *C. gloeosporioides*, and to evaluate its effects in the control of post-harvest diseases in yellow passion fruit.

MATERIAL AND METHODS

Microorganism

Colletotrichum gloeosporioides CBMAI 869 was isolated from infected fruits and identified by Dra. Eliane Aparecida Benato – Instituto de Tecnologia de Alimentos (ITAL) – Campinas, SP, Brazil. The culture was maintained on PDA (potato dextrose agar) and sub-cultured periodically with alternating light (12 h light/ 12 h dark) during 7 days. The culture was deposited at CBMAI – Brazilian Collection of Microorganisms from Environment and Industry.

Plant Material

A list of the plants studied, including the botanical name and voucher specimen, are listed in Table 1. The plants were cultured at CPMA – Medicinal and Aromatic Plants Collection of the Chemical, Biological and Agricultural Research Center (CPQBA), State University of Campinas - São Paulo – Brazil.

The plants were collected from November 2001 to February 2003, always in the morning after dewpoint. The maximum and minimal temperatures registered in the period were 32.2 °C and 17.3 °C, respectively with precipitation of about 1.777 mm. Voucher specimens were deposited at the State University of Campinas Herbarium (UEC) and identified by Dr. Washington Marcondes Ferreira Neto (curator) or at the CPQBA herbarium.

Table 1. Medicinal plants, voucher specimens, oil yields and antimicrobial activity (MIC) against *C. gloeosporioides*.

Medicinal species	Family	Voucher	Oil yield (%)	MIC mg mL ⁻¹
<i>Achillea colinal</i> L	Asteraceae	CPMA 373	0.27	*
<i>Allium tuberosum</i>	Liliaceae	CPMA 653	0.0011	2.0
<i>Aloysia gratissima</i> (Gill & Hook)	Verbenaceae	UEC 121.393	1.51	*
<i>Anthemis nobilis</i>	Asteraceae	UEC 121.411	0.05	2.0
<i>Artemisia annua</i>	Asteraceae	CPMA 1246	0.35	*
<i>Baccharis dracunculifolia</i>	Asteraceae	CPMA 622	0.30	2.0
<i>Baccharis trimera</i> (Less.) Dc.	Asteraceae	CPMA 1	0.30	*
<i>Cordia verbenaceae</i> (Jacq.) Roem. &	Boraginaceae	UEC 112.744	0.04	2.0
<i>Coriandrum sativum</i>	Apiaceae	CPMA 664	0.08	0.25
<i>Cymbopogon citratus</i>	Poaceae	UEC 127.112	0.40	0.25
<i>Cymbopogon flexuosus</i>	Poaceae	UEC 127.113	0.20	0.25
<i>Cymbopogum martinii</i> Motia.	Poaceae	UEC 127.115	1.36	0.70
<i>Cymbopogum winterianus</i> L.	Poaceae	UEC 121.414	1.88	*
<i>Cyperus articulatus</i>	Cyperaceae	UEC 121.396	6.70	*
<i>Cyperus rotundus</i>	Cyperaceae	CPMA 1252	0.03	*
<i>Lippia alba</i> (Mill) N.E. Br.	Verbenaceae	UEC 121.413	1.10	0.3
<i>Mentha piperita</i> L.	Lamiaceae	UEC 127.110	1.20	2.0
<i>Mikania glomerata</i> Sprengel.	Asteraceae	UEC 102.047	0.06	*
<i>Ocimum gratissimum</i> L.	Lamiaceae	UEC 121.407	0.74	2.0
<i>Ocimum selloii</i> Benth.	Lamiaceae	UEC 121.406	0.18	*
<i>Origanum applii</i> (Domin) Boros	Lamiaceae	UEC 121.410	0.20	1.0
<i>Origanum vulgare</i> L. Ssp Virens	Lamiaceae	UEC 121.409	0.12	1.0
<i>Piper aduncum</i> L.	Piperaceae	UEC 127.118	0.60	2.0
<i>Rosmarinus officinalis</i> L	Lamiaceae	CPMA 1792	1.85	*
<i>Santolina chamaecyparissus</i> L	Asteraceae	CPMA 1653	0.15	*
<i>Stachys byzantina</i> C.Koch.	Lamiaceae	UEC 121.404	0.44	*
<i>Stachytarphetta cayenensis</i> (L.C.	Verbenaceae	UEC 121.394	0.04	*
<i>Thymus vulgaris</i> L.	Lamiaceae	UEC 121.405	0.56	2.0

* = > 2.0 mg mL⁻¹

Essential oil extraction & GC and GC-MS analyses of the essential oils

The essential oils were obtained from 40 g of the aerial parts of fresh plants by hydrodistillation for 3 h using a Clevenger-type system. The aqueous phase was extracted three times with 50 mL dichloromethane. The pooled organic phases were dried with sodium sulfate, filtered and the solvent evaporated to dryness. The oil samples were stored at -70 °C in sealed glass vials.

Identification of the volatile constituents was performed using a Hewlett-Packard 5890 Series II gas chromatograph, equipped with a HP-5971 mass selective detector and HP-5 capillary column (25m x 0.2 mm x 0.33 µm diameter). GC and GC-MS were performed using split/splitless injection, with the injector set at 220 °C and the column initially at 60 °C, with a heating ramp of 3 °C.min⁻¹ to a final temperature of 240 °C, maintained for a further 7 min. The FID detector was set at 250 °C. Helium was used as the carrier gas at 1 mL min⁻¹. The GC-

MS electron ionization system was set at 70eV. A sample of the essential oil was dissolved in ethyl acetate for the analyses. The retention indices (RI) were determined by co-injection of hydrocarbon standards. The oil components were identified by comparison with data from the literature (1), the profiles from the Wiley 138 and Nis 98 libraries, by co-injection of authentic standards, when available.

Inoculum for the antimicrobial assay (16)

In order to induce conidia and spore formation, *C. gloeosporioides* was cultured in Petri dishes as indicated above. The culture surfaces were then washed with 1 mL 0.85% sterile saline solution and 0.01 mL Tween 20. The resulting mixture of conidia and mycelia fragments was transferred to a sterile tube and after 3-5 min the upper layer suspension was transferred to another sterile tube and submitted to vortex agitation for 15 sec. The density of the suspension was adjusted to between OD 0.09 and 0.11 in a spectrophotometer (Shimadzu UV mini1240) and then diluted 1:50 in RPMI-1640 medium.

Antimicrobial assay – minimal inhibitory concentration (MIC) Test

The assays were carried out according to NCCLS (16) using a tissue culture test plate (96 wells) and RPMI-1640 medium. The stock oil solutions were diluted and transferred into the first well, and serial dilutions made to obtain concentrations in the range from 2.0 to 0.0019 mg mL⁻¹. Amphotericin B (Merck) was used as the reference antimycotic control at 60.0-5.0 µg mL⁻¹. The spore solution was added to all the wells and the plates incubated at 35 °C for 48 h. The MIC was defined as the lowest oil concentration that inhibited visible growth, as indicated by a change in color of the medium (pink to yellow).

Effect of *Cymbopogon citratus* essential oil on the control of diseases in yellow passion fruit

The evaluation of disease control efficiency was carried out according to Benato et al. (3). The fruits cv. Afruvec with 1/3 of the peel yellow, were submitted to treatment with *C.*

citratus oil at the previously determined MIC concentration. Water and Sportak® (prochloraz), a control fungicide, were used as the standard treatment. A total of 7 treatments with 3 replicates of 10 fruits each were employed. The fruits were submerged for 2 min in 5 L of the following solutions: 1. water; 2. Sportak®, 0.5 mL L⁻¹(a.i.); 3. essential oil, 0.12 g L⁻¹; 4. essential oil, 0.25 g L⁻¹; 5. essential oil, 0.5 g L⁻¹; 6. essential oil, 1.0 g L⁻¹; 7. The concentration of the original essential oil was 2.0 g L⁻¹. For treatments 2-7, an adhesive emulsifier (Agral®) was added at 0.3 mL L⁻¹. All the fruits were numbered for identification during the subsequent evaluation. After the applications, the fruits were stored at 23-25 °C and 75-85 % relative humidity for 7 days.

Disease evaluation

The incidence and infection severity for each fruit on the second, fifth and seventh days after application were evaluated on a scale from 0 to 4, where: 0 = absence of symptoms; 1 = 25 % infected area; 2 = 26 ≤ 50 %; 3 = 51 ≤ 75 % and 4 = 76 ≤ 100 %. The results were expressed on a disease index scale according to the formula I.D. (%) = $\{[(1.n_1) + (2.n_2) + (3.n_3) + (4.n_4)].(R.4)^{-1}\}.100$, where I.D. = the disease index (%); n_{1...4} = number of fruits with respective score; R = number of fruits per treatment.

The means obtained for the different treatments were submitted to an analysis of variance and compared by Tukey at 5 % significance. For the statistical analyses the means were transformed by $\sqrt{x + 0.5}$.

Physicochemical analyses

The following analyses were realized at the beginning (0 – treatment zero) and on the eighth day, using 8 fruits from each treatment:

Peel color: The peel color was measured 4 times for each fruit using a Minolta (CR300) Colorimeter - System L*a*b*, subsequently calculating the mean value for each fruit.

Soluble solids: The soluble solids contents of the juice from each fruit were determined in a manual refractometer (Atago, 0-32°Brix).

Titrateable acidity and pH: The acidity and pH of the juice were measured using a pH- meter (Mettler Toledo 320). The results were expressed in g citric acid / 100 g juice. The acidity was determined using the formula: (g citric acid / 100 g juice) = (mL NaOH.N.64.100)/(g sample.1000).

Experimental design: The experimental design used was entirely randomized, with 3 repetitions and 8 or 10 fruits per treatment. The means were compared using the variance analysis and Tukey test at 5% probability, using the statistical program ESTAT.

RESULTS AND DISCUSSION

Essential oils *in vitro* activity against *Colletotrichum gloeosporioides*

Table 1 shows the medicinal species studied, the oil yields and the antifungal activities (MIC – mg mL⁻¹). The results showed great variation amongst the yields in essential oils, between 0.001 (*Allium tuberosum*) and 6.7 % (*Cyperus articulatus*). With respect to the antimicrobial activity, 15 of the 28 essential oils studied were able to inhibit *C. gloeosporioides* until 2.0 mg mL⁻¹. However, four medicinal species stood out, *Coriandrum sativum*, *Cymbopogon citratus*, *C. flexuosus* and *Lippia alba*, showing MIC values between 0.25 and 0.30 mg mL⁻¹. These species also presented good oil yields (0.08 – 1.35 %).

The chemical compositions of the oils from these four species were determined by GC-MS. The compounds identified are listed in Table 2, which shows that it was possible to identify 83.57 % (*C. sativum*) to 100 % (*C. citratus*) of the oil constituents. The results showed the presence of volatile substances with well known antimicrobial activities, such as decenol, decanol, dodecanal and dodecanol in *C. sativum*, linalool, geraniol and geranyl acetate in the majority of the species, and *trans*-cariophyllene in all of the species (18, 7, 17, 8, 15).

Based on the *in vitro* results, the essential oil from *C. citratus* was chosen for the tests with the yellow passion fruit, not only for its inhibitory effect but also because the cultivation

parameters of this specie are well established and the oil is easy to commercialize

The effect of *C. citratus* essential oil on the control of diseases in yellow passion fruit

Table 3 shows the results for the disease index for each treatment in relation to the time employed, and Figure 1 shows the appearance of the fruits on the seventh day after treatment with *C. citratus* essential oil. The data in Table 3 show that the disease indices of the fruits submitted to treatments 3 and 4 did not differ statistically from those treated with fungicide (Sportak® - prochloraz) – (treatment 2) at the 5 % level of probability. At some concentrations the use of the essential oil darkened the surface of the fruit peel, at about 33.3 % (treatment 3), 76.6 % (treatment 4) and 100 % (treatment 5), damaging their appearance and quality. Consequently, the higher essential oil concentrations resulted in higher disease indices, since the fruit peel became more susceptible to disease because of the darkening.

Table 4 shows the results of the physicochemical analyses for each treatment on the 7th day. With respect to the changes in peel color, the fruits from day 0 (zero) and treatment 7 were more opaque than the others (L*). In addition the fruits lost their green color and became yellow, except for those from treatments 6 and 7, which darkened due to a physiological disturbance. There were no statistical differences between the fruits with respect to soluble solids (°Brix). Finally, only the pH of treatment 6 was different on day 0 (zero), and only the total acidity of treatment 7 was similar to that found on day 0 (zero).

Prochloraz (imidazole fungicide) is widely used to control fungal diseases and acts by inhibiting the biosynthesis of ergosterol in the fungi. In addition in the presence of this fungicide, the mutation rate is low when compared to benzimidazoles (2). Its efficiency was previously compared with different compounds and alternative products (3) such as imazalil, thiabendazole, citric biomass, benzalconic chloride, organic and inorganic salts, *Bacillus subtilis* metabolites, chlorine dioxide and ozone. Prochloraz and imazalil were the

Table 2. Compounds identified in the essential oils. IR = Retention Index; Cs = *Coriandrum sativum*; Cm = *Cymbopogon martinii*; Cc = *Cymbopogon citratus*; Cf = *Cymbopogon flexuosus*; La = *Lippia alba*.

Compostos ^a	IR	Cs	Cm	Cc	Cf	La
Triciclono	923			0,12		
α -pineno	933			0.18		
Canfeno	948			0.94		
NI	961				0.25	
octen-4-ol	976					0.80
NI	985			0.54		
β -mirceno	990		0.12			
Octanal	1003			0.13		
dl-limoneno	1026			0.23	1.53	
1,8-cineol	1028					2.34
<i>cis</i> - β -ocimeno	1034		0.23	0.21	0.26	0.26
<i>Trans</i> - β -ocimeno	1044		0.98			0.15
M = 142	1071			0.42	0.30	
Linalool	1101	0.27		0.70	0.80	76.30
M = 152	1144			0.49		
Isopulegol	1144				0.47	
M = 152	1148			0.27	0.23	
Bergamal	1052				0.26	
Citronelal	1154			0.25	23.30	
<i>cis</i> -crisantemol	1164			2.30	0.21	
M = 152	1182			3.02	0.43	
α -terpineol	1187			0.18		0.49
Decanal	1205	5.15		0.37	0.52	
Nerol	1226			0.78	13.47	
Citronelol	1230					0.81
Neral	1240			35.54	9.64	0.62
<i>trans</i> -geraniol	1254		63.46	2.51	27.02	
-----	1261	2.61				
Geranial	1269			47.52	12.60	0.83
2-decen-1-ol	1270	26.02				
n-decanol	1273	26.03				
Undecanal	1307	0.49				
NI	1331				0.23	
delta-elemeno	1336	0.67				
acetato de citronelila	1352				0.89	
Eugenol	1355			0.50		
-----	1369	1.30				
Undecanol	1372	0.85				
acetato de geranila	1382		28.83	0.27	1.01	0.19
β -elemeno	1387					0.36
Dodecanal	1408	2.22				
<i>trans</i> -cariofileno	1414	0.43	2.15	1.46	1.85	1.91
α -humuleno	1447				0.25	0.26
-----	1465	3.02				
n-dodecanol	1471	7.56				
Dodecanol	1473	0.95				
M 204	1477					2.97
germacreno D	1478				0.27	
gama-cadineno	1512				1.58	
δ -cadineno	1520			0.28		
Delta-cadineno	1521				0.32	
Elemol	1546				0.27	
germacreno B	1522					1.91
n-butirato de citronelila	1527				0.34	
n-butirato de geranila	1560				0.34	
M 204	1562					0.53
germacreno D-4-ol	1569					0.19
germacreno D-4-ol	1573				0.41	
óxido de cariofileno	1579			0.81	0.53	
Tetradecanal	1612	0.36				
α -cadinol	1651				0.35	
-----	1670	4.40				
-----	1673	0.85				
(E,E) farnesol	1720		1.57			
-----	1776	0.39				
Total		83.57	97.34	100.00	99.93	90.92

most effective products in the control of yellow-passion fruit rots. The results from the present work show that the *C. citratus* essential oil was as efficient as Sportak® (prochloraz), with the advantage of being a natural product. In conclusion, at 0.12 mg L⁻¹ and 0.25 mg L⁻¹ this essential oil showed the potential for use in the control of post-harvest diseases, especially anthracnose (*C. gloeosporioides*), without causing physiological disturbances.

The emergence of antibiotic resistant microbial strains and

the fact that the use of drugs in agriculture could contribute to the development of resistance by human pathogens have stimulated the search for natural antimicrobials. The present *in vitro* and *in vivo* results indicated the potential of *C. citratus* essential oil against *C. gloeosporioides*, the anthracnose agent in yellow passion fruit and other fruits. The fact that *C. citratus* presents a good yield in essential oil and is easily commercialized favors its use in the control of post-harvest diseases in yellow passion fruit.

Table 3. Disease indices found for the different treatments on the 2nd, 5th and 7th days under environmental conditions. Results expressed in percentage (%). Means followed by the same letter do not differ significantly according to Tukey at 5%.

Treatments / days	2nd	5th	7th
Water (1)	0.83a	8.33ab	18.33b
Sportak® (2)	0.00a	3.33bc	5.83d
Essential oil (3)	0.83a	1.66c	8.33cd
Essential oil (4)	0.00a	6.66abc	8.33cd
Essential oil (5)	0.00a	8.33ab	15.83bc
Essential oil (6)	0.00a	10.83a	22.5b
Essential oil (7)	0.00a	14.16a	33.33a

Figure 1. Appearances of the passion fruits from the different treatments on the 7th day after treatment



Table 4. The color (L*a*b), soluble solids ($^{\circ}$ Brix), pH and total acidity of the fruits on the 7th day after treatment. Means followed by the same letter do not differ significantly according to Tukey at 5% in relation to the non-treated fruits

Treatment	color L*	color a*	color b*	$^{\circ}$ Brix	pH	acidity
0**	62.35bc	- 9.99b	33.42bc	12.22a	2.93b	4.59a
1	69.58 a	0.04a	40.56ab	10.47a	3.06ab	3.49b
2	70.31a	- 0.65a	41.85a	12.75a	2.97ab	3.78b
3	71.96a	0.24a	42.31a	11.22a	3.04ab	3.54b
4	70.60a	0.24a	41.16a	12.02a	3.05ab	3.53b
5	71.76a	0.68a	42.25a	12.17a	3.01ab	3.79b
6	66.49ab	- 1.15a	36.92ab	11.35a	3.11a	3.32b
7	57.74c	2.49a	26.28c	10.72a	3.06ab	3.85ab

**Day zero – non-treated

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