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Effect of essential oils from citrus varieties on *in vitro* growth and sporulation of *Phaeoramularia angolensis* causing citrus leaf and fruit spot disease

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Abstract

Citrus leaf and fruit spot disease caused by *Phaeoramularia angolensis* is a serious production constraint in tropical Africa. In previous studies, essential oils extracted from fruit peels of two tolerant varieties exhibited a strong antifungal activity *in vitro* against *P. angolensis* as compared to oils from susceptible ones. In order to investigate if the susceptibility of citrus varieties is associated with the antifungal activity of their essential oils, some 22 varieties of different susceptibility levels (tolerant, susceptible and highly susceptible) and belonging to different botanical groups were studied. Oils extracted from fruit peels were evaluated for their activity against radial growth and sporulation using the poisoned food technique. The optimal doses for growth inhibition and conidial reduction were 2500 and 1000 ppm, respectively. At these doses, radial growth and sporulation exceeded the untreated control respectively for four and nine varieties suggesting that oils from these varieties promote fungal development. In general, oils from the tolerant group were most effective in reducing radial growth irrespective of dose. The highly susceptible group ranked first in reducing sporulation at dose 1000 ppm (45.93%) while at higher doses of about 2000–2500 ppm, oils from the tolerant varieties could reduce sporulation up to 100%. The marked dose effect in reducing sporulation suggests that there may be different compounds acting with changing dose. Botanically, oils from pummelo (*Citrus maxima*, tolerant group), were best in reducing radial growth (>87% inhibition) while those from grapefruits (*C. paradisi*, highly susceptible group) were most effective in reducing sporulation (>64% reduction).

Introduction

Phaeoramularia angolensis, the fungus causing citrus leaf and fruit spot disease had been reported some decades ago in about 20 countries in tropical Africa and in the Republic of Yemen (Kuate, 1998). It is known to be a serious production constraint causing 20–100% yield loss to growers especially in highlands (of above 200 m elevation) where the disease incidence and severity are very high (Seif and Hillocks, 1993; Diarri-Diallo, 1995; Kuate, 1997; Kuate et al., 2002). It also affects the yield and quality of essential oils (Kuate et al., 2003). The disease attacks leaves, fruits (Figure 1) and young twigs causing many spots resulting in premature abscission and dieback (Kuate et al.,1994a, b). Citrus leaves, fruit peels and flower petals, organs attacked by this disease, contain valuable essential oils that can be extracted using various techniques (Huet, 1991).

Figure 1. Symptoms of citrus leaf and fruit spot disease on (a) grapefruit leaf (Marsh variety); (b) oranges (Hamlin variety).

The antibacterial and antifungal properties of such oils have long been reported (Misra et al., 1988; Knobloch et al., 1989; Kishore et al., 1992; Berahia, 1993; Violon, 1993; Mishra and Dubey, 1994; Siddiqui et al., 1996; Baratta et al., 1998; Guynot et al., 2003; Suhr and Nielsen, 2003). This is why antifungal activity of essential oils was suspected when an inhibition of *P. angolensis* colonies was observed on a culture medium made up of citrus fruit peel extracts (Kuate, 1997).

A subsequent study was conducted to assess the effect of essential oils extracted from six different citrus varieties on the growth of this fungus *in vitro* (Dongmo et al., 2002). It was observed that oils from *Citrus limon* and *C. aurantifolia* (both varieties tolerant to leaf and fruit spot disease in the field) strongly inhibited fungal growth as compared to oils from very susceptible varieties (*C. paradisi* and *C. sinensis*). Nevertheless, oils from some tolerant varieties did not show such a strong antifungal activity. It is interesting to know if essential oils do actually contribute to partial resistance against this disease. To better understand this phenomenon, we assessed the effect of oils from many citrus varieties with different susceptibility levels and belonging to different botanical groups on the development of the fungus *in vitro* with respect to this disease. Essential oils extracted from some citrus varieties cultivated in Yaounde (Cameroon) were studied for their effect on mycelial growth and sporulation of *P. angolensis*.

Materials and methods

Citrus varieties and the extraction of essential oils

A total of 22 citrus varieties (V) planted in 1984 in the Institute of Agricultural Research for Development (IRAD) experimental orchard in Yaounde was studied (Table 1). These varieties and many others had been evaluated for their field susceptibility against *P. angolensis* (Rey, 1982; Rey et al., 1986, 1988; Bella-Manga et al., 1999). Based on disease incidence in the field (percentage of diseased fruits), three levels of susceptibility were identified: (i) tolerant (<30% disease incidence), (ii) susceptible (30–70% disease incidence) and (iii) highly susceptible (>70% disease incidence). Fruits from these varieties were harvested at usual maturity and oils extracted from fruit peels using the hydrodistillation technique as previously described (Dongmo et al., 2002; Kuate et al., 2003).

Culture media and fungal isolate

Potato dextrose agar (PDA) was used for radial growth studies. Sporulation studies were conducted on amended V8 medium known to be conducive to conidial production (Mourichon et al., 1987). The isolate CMR4 of *P. angolensis* was chosen for its known growth and sporulation ability (Kuate, 1997; Kuate et al., 1997; Dongmo et al., 2002; Kuate et al., unpublished).

Essential oils and P. angolensis *radial growth*

Mycelial plugs of *P. angolensis* measuring 3 mm diam were taken from a 15-day-old culture on PDA plates. Essential oils were mixed with dimethyl sulfoxide (DMSO) so as to ease its incorporation to the agar medium in the proportion 1 volume oil to 9 volumes DMSO. Five different concentrations of

Table 1. Citrus varieties and their susceptibility against leaf and fruit spot disease

Susceptibility level	Variety code	Variety name
Tolerant	V1	Lime Tahiti (*Citrus latifolia*)
	V4	Pamplemoussier Reinking (*C. maxima*)
	V5	Satsuma Saint Jean (*C. unshiu*)
	V8	Mandarinier Obala (*C. reticulata*)
	V9	Citronnier Lisbonne (*C. limon*)
	V14	Citronnier Eurêka (*C. lemon*)
	V16	Tangelo Orlando (*C. reticulata*×*C. paradis*i)
	V21	Mandarinier King of Siam (*C. reticulata*)
	V19	Mandarinier Beauty (*C. reticulata*)
	V12	Lime Mexicaine (*C. aurantifolia*)
Susceptible	V3	Oranger Valencia Late (*C. sinensis*)
	V11	Oranger Pineapple (*C. sinensis*)
	V20	Oranger Don Joao (*C. sinensis*)
	V22	Satsuma Kowano (*C. unshiu*)
	V17	Mandarinier Kara (*C. reticulata*)
	V7	Mandarinier Fremont (*C. reticulata*)
	V18	Tangor Ortanique (*C. reticulata*×*C. sinensis*)
	V2	Pomelo Reed (*C. paradisi*)
	V6	Pomelo Marsh (*C. paradisi*)
Highly susceptible	V10	Mandarinier Fairchild (*C. reticulata*)
	V13	Pomelo Shambar (*C. paradisi*)
	V15	Tangelo Minneola (*C. reticulata*×*C. paradisi*)

oils were studied: 500, 1000, 1500, 2000 and 2500 ppm (that is μl l^{-1}). Essential oils were incorporated to the culture medium through a 0.45 μm Millipore filter (MILEX-HA, 67 Molsheim, France). The medium amended with oil was then poured into 55 mm Petri dishes and inoculated with mycelial plugs when solidified. Some plates prepared as controls received no oil but only DMSO. A total of 5 plates were prepared for each treatment. Plates were then sealed with parafilm and incubated 30 days in total darkness at 20–22 °C. Diameter of fungal colony (mm) was measured every 10 days and the percentage inhibition of radial growth (PIG) calculated: $PIG = 100(D_c - D_t)/D_c$ where D_c and D_t are the diameter (mm) in the control and treated plates, respectively.

Essential oils and sporulation of P. angolensis

Petri dishes of 90 mm diam containing V8 medium amended with the above concentrations of essential oils were prepared. Different sets of control (DMSO + 0 ppm of essential oil) were used for different sets of experiments involving respectively V1 and V2; V3, V4 and V5; V6, V7 and V8; V9, V10 and V11; V12 and V13; V14 and V15; V16 and V17, V18 and V19; V20 and V21; and V22. This was because all experimentation involving conidia counting could not be carried out on the same day. A 15 day-old *P. angolensis* culture was ground in a sterile mortar containing 3 ml distilled water. The mycelial suspension obtained was then inoculated on V8 plates and incubated 10 days under continuous light at 22 °C. After incubation, 3 ml distilled water was added to each plate and conidia were brushed. The conidial suspension was then filtered and counted with a Malassez haemocytometer (2 plates per treatment and 4 counts per plate). The percentage reduction of conidia (PRC) was then calculated: $PRC = 100\ (N_c - N_t)/N_c$ where N_c and N_t are the number of conidia ml^{-1} in the control and treated plates respectively.

Statistical analysis

All experiments were replicated twice. Data presented are the average of the two replicates. The data were entered into Microsoft excel and the Statistical Analysis System (SAS) version 8 where most of the analyses were done. The generalised linear model was used to investigate if essential oils from different varieties of citrus, different doses of oils, number of plates, counts made and the incubation period had different effects on the diameter

of the colony and on sporulation. A plot of mean PIG and mean PRC against dose were used to determine the optimum doses respectively. Using these doses, plots of PIG and PRC against variety were used to determine the best varieties that significantly reduce respectively the diameter of colony and sporulation. Based on the activities of their essential oils, cluster analysis was used to group these varieties with the hope of matching these groups with the susceptibility groups to *P. angolensis* and botanical groups of citrus that already existed (Rey et al., 1986, 1988; Kuate, 1998; Bella-Manga et al., 1999). Non-parametric tests were used to compare the PIG and PRC of essential oils from varieties of different susceptibility and botanical groups. Analyses were done at the 5% level of significance.

Results

Effect of essential oils on P. angolensis *radial growth*

The generalised linear model used to investigate the effect of variety, dose and number of plates on the diameter of colonies fitted well for the three intervals (10, 20 and 30 days after inoculation) ($P=0.0001$). The mean diameter of colonies increased from day 10 (7.77 mm) through day 20 (15.16 mm) to day 30 (23.64 mm). There was a difference observed amongst the varieties ($P=0.001$) and levels of dose ($P=0.0001$) on the three day intervals. But there was no difference due to the number of plates used (5 df, $F=0.47$, $P=0.8$). The optimum dose that reduced radial growth of *P. angolensis* colonies was 2500 ppm (Figure 2a) whereas dose 500 ppm induced a small increase in diameter compared to the control. The PIG of the fungus due to essential oils was estimated and compared to the control plates and a ranking made (Table 2). Furthermore, based on the PIG of colonies after 30 days of incubation, five clusters were generated. The varieties were grouped into clusters (Table 2) which had a mean cluster PIG ranging from 76.62% to −8.43%.

Effect of essential oils on the sporulation of P. angolensis

The generalised linear model was used to observe the effect of variety, dose, number of plates and

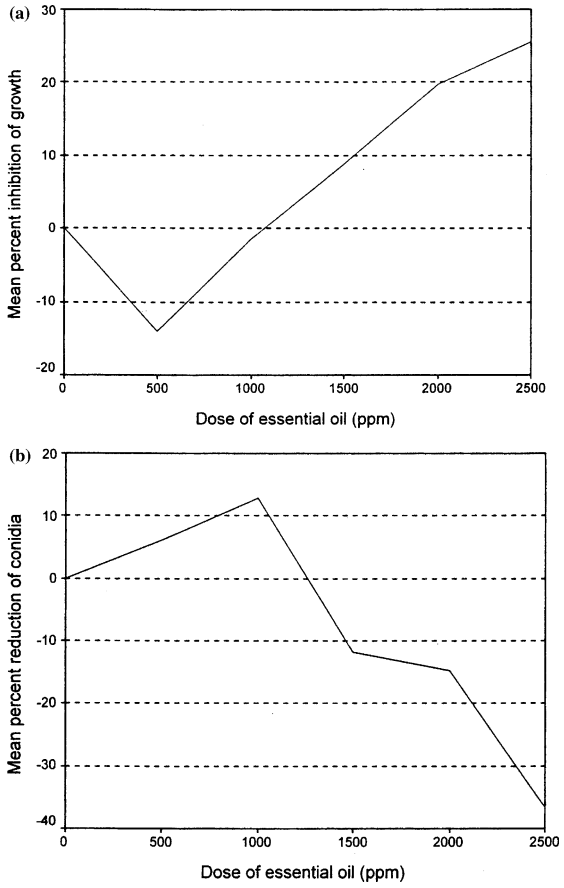

Figure 2. (a) Effect of changing dose of essential oils on percentage inhibition of *Phaeoramularia angolensis* 10 days after inoculation; (b) effect of changing dose of essential oils on the percentage reduction of conidia by *Phaeoramularia angolensis*.

counts on conidial production. There was a difference amongst the varieties (22 df, $F=76.23$, $P=0.0001$) and the dose levels (4 df, $F=13.2$, $P=0.0001$). No difference was observed as a result of plate number (1 df, $F=2.08$, $P=0.1495$) and counts (3 df, $F=0.43$, $P=0.513$). The optimum dose for decreasing conidial production was 1000 ppm (Figure 2b). The PRC due to essential oils was estimated and compared to the control plates and a ranking made (Table 3). At dose 1000 ppm, a linear and inverse relationship was observed between percentage reduction of conidia (PRC) and mean conidia produced per ml. In addition, using the PRC due to essential oils, five clusters were generated. The varieties were grouped into clusters (Table 3) which had a mean cluster PRC ranging from 87.87% to −37.50%.

Table 2. Percentage inhibition of radial growth of *Phaeoramularia angolensis* by essential oils of citrus varieties, their ranking and clustering

Variety code	Diameter on day 10 (mm)	Diameter inhibition on day 10 (mm)	Diameter on day 20 (mm)	Diameter inhibition on day 20 (mm)	Diameter on day 30 (mm)	Diameter inhibition on day 30 (mm)	Percentage inhibition of diameter of colony at dose = 2500 ppm after 30 days of incubation	Cluster
V4	3.00	0.61	3.00	0.80	3.00	0.87	76.62	1
V7	3.08	0.48	4.25	0.71	7.66	0.65	61.73	2
V3	3.00	0.61	5.50	0.63	10.83	0.56	60.70	2
V1	5.00	0.46	7.00	0.57	13.50	0.44	49.44	3
V9	3.00	0.65	8.83	0.42	15.50	0.31	46.71	3
V10	4.08	0.52	9.50	0.38	16.00	0.29	40.39	3
V16	5.58	0.45	11.16	0.26	15.41	0.37	36.90	3
V12	4.16	0.54	11.75	0.29	19.16	0.21	35.36	3
V20	5.75	0.42	12.91	0.16	20.50	0.16	25.25	3
V6	5.33	0.28	14.33	0.21	19.75	0.24	24.96	3
V21	6.58	0.33	13.33	0.13	21.50	0.12	19.97	4
V14	7.66	0.19	14.75	0.16	21.66	0.17	18.21	4
V19	6.58	0.34	13.41	0.12	22.66	0.07	18.18	4
V2	8.08	0.13	14.33	0.12	22.66	0.07	10.82	4
V15	9.33	0.08	13.50	0.12	23.08	0.06	9.13	4
V5	6.33	0.15	18.33	−0.00	24.25	0.07	7.23	4
V13	8.91	0.05	17.08	0.03	26.16	0.00	3.41	4
DMSO	8.41	0	15.92	0	24.04	0	0	
V11	9.83	−0.06	16.75	−0.00	24.25	0.01	−2.16	5
V17	7.50	−0.33	11.33	0.11	17.50	0.09	−4.07	5
V8	7.83	−0.31	14.33	0.03	22.16	0.01	−8.97	5
V18	7.16	−0.27	14.16	−0.10	22.66	−0.17	−18.53	5
	Cluster	Mean (percent Inhibition of radial growth)				Standard deviation		
	1	76.62						
	2	61.22				0.72		
	3	37.00				9.54		
	4	12.42				6.39		
	5	−8.43				7.31		

However, some of the varieties (e.g. V1) showed less than −100% reduction of conidia.

Discussion

Susceptibility groups and essential oil activity against P. angolensis

The varieties in the five clusters (Table 2) can be subdivided into their respective susceptibility groups to *P. angolensis* with respect to radial growth inhibition as follows: Cluster 1 has V4 which is a tolerant variety appearing to be the most effective in reducing radial growth (Table 1). Cluster 2 contains V7 and V3 both belonging to the susceptible group. Cluster 3 contains 4 varieties that are tolerant (V1, V9, V16 and V12), 1 susceptible (V20) and 2 highly susceptible (V10 and V6). Cluster 4 has 5 tolerant varieties (V21, V14, V19, V2 and V5) and 2 highly susceptible ones (V15 and V13). Finally cluster 5 contains 1 tolerant (V8) and 3 susceptible varieties (V11, V17 and V18) all with negative values of percentage inhibition of growth suggesting that oils from these varieties tend to promote fungal growth (Table 2). Furthermore, it was also noticed that changing from the optimal dose of oils had no marked effect on radial growth (data not shown).

Using the PIG at dose 2500 ppm, the Mann–Whitney test was used to compare susceptibility groups 10, 20 and 30 days after incubation. There was a difference observed in the mean PIG between the tolerant and susceptible groups ($P = 0.000$) and between the tolerant and highly susceptible groups ($P < 0.05$), throughout the period of incubation (Table 4a). In general, oils from the tolerant group appeared to be the most effective in reducing

Table 3. Effect of essential oils from different citrus varieties on the sporulation of *Phaeoramularia angolensis* and their ranking and clustering

Variety code	PRC (500 ppm)	Variety code	PRC (1000 ppm)	Variety code	PRC (1500 ppm)	Variety code	PRC (2000 ppm)	Variety code	PRC (2500 ppm)	Mean conidia ml^{-1} at 1000 ppm	Mean conidia ml^{-1} for controls	Coefficient of variation of conidia produced	Clusters based on PRC 1000 ppm
V7	86.62	V9	96.83	V1	100	V1	100	V1	100	18.62	582.88	28.69	1
V6	86.23	V7	78.91	V9	91.69	V12	100	V12	100	575.62	2758.25	17.92	1
V13	76.41	V13	77.91	V7	74.42	V9	100	V14	100	2.06.12	962.75	16.77	2
V8	51.78	V6	66.11	V6	54.49	V15	79.17	V9	100	924.62	2758.25	19.14	2
V3	49.46	V20	63.7	V13	38.47	V7	71.4	V15	73.64	290.50	810.00	7.14	2
V2	45.07	V8	41.58	V15	23.01	V6	57.2	V10	70.95	1604.50	2758.25	6.88	3
V15	39.02	V19	41.55	V8	14.38	V8	27.19	V7	66.76	870.75	1510.88	9.55	3
V11	30.21	V15	35.99	V17	11.19	V3	10.01	V11	37.83	797.37	1246.00	11.20	3
V19	26.62	V17	31.54	V3	11.12	V14	8.95	V19	33.57	2390.00	3533.38	7.74	3
V17	21.78	V18	26.79	V16	5.54	DMSO	0	V8	26.34	1091.50	1510.88	12.62	4
V12	19.25	V2	25.12	V19	2.66	V18	−6.32	V16	21.2	223.25	419.63	27.07	4
V18	18.07	V10	24.54	V21	1.43	V16	−12.31	V6	9.86	425.37	582.88	22.11	4
V9	11.77	V3	8.15	DMSO	0	V13	−13.85	V3	9.1	914.75	1052.00	10.39	4
DMSO	0	DMSO	0	V18	−3.63	V17	−14.08	V17	0.98	1388.14		12.40	
V21	−1.36	V21	−6.31	V12	−19.02	V19	−19.88	DMSO	0	842.75	810.00	7.17	5
V4	−8.43	V16	−7.62	V11	−20.4	V10	−36.24	V18	−19.62	3238.88	3533.38	18.71	5
V16	−16.27	V11	−12.04	V14	−24.2	V11	−36.32	V13	−24.57	636.75	582.88	18.51	5
V22	−19.35	V12	−15.76	V10	−28.19	V22	−73.71	V22	−58.11	1088.13	962.75	14.38	5
V20	−20.05	V22	−18.5	V20	−29.87	V5	−84.54	V20	−135.91	1102.50	931.63	10.39	5
V10	−35.8	V14	−24.18	V22	−80.68	V21	−111.31	V21	−171.49	1550.63	1246.00	18.96	5
V1	−49.49	V4	−27.39	V4	−107.45	V4	−123.6	V4	−185.02	1267.88	1052.00	9.81	5
V14	−111.88	V5	−86.58	V2	−156.17	V20	−125.64	V5	−261.02	1859.88	1052.00	49.26	5
V5	−163.52	V1	−139.21	V5	−220.01	V2	−219.35	V2	−701.71	661.00	419.63	24.50	5

Cluster	Mean (percent conidial reduction at 1000 ppm)	Standard deviation
1	87.87	12.67
2	69.23	7.5
3	37.66	4.85
4	21.15	8.71
5	−37.50	45.24

Table 4a. Susceptibility groups to *Phaeoramularia angolensis* and effect of oils on percentage inhibition of colony radial growth

	Susceptibility group	*N*	Mean percentage Inhibition at dose = 2500 ppm	Rank mean	Rank sum	Signification of Mann–Whitney test
Percentage inhibition after 10 days	Tolerant	300	34.45	236.87	79461.50	0.000
	Susceptible	180	14.10	199.89	35978.50	
	Tolerant	300	34.45	245.50	73650.00	0.000
	Highly susceptible	150	21.71	185.50	27825.00	
	Susceptible	180	14.10	156.91	28244.50	0.073
	Highly susceptible	150	21.71	175.80	26370.50	
Percentage inhibition after 20 days	Tolerant	300	28.44	255.45	76633.50	0.002
	Susceptible	180	25.39	215.59	38806.50	
	Tolerant	300	28.44	235.51	70654.00	0.021
	Highly susceptible	150	17.86	205.47	30821.00	
	Susceptible	180	25.39	161.34	29040.50	0.385
	Highly susceptible	150	17.86	170.50	25574.50	
Percentage inhibition after 30 days	Tolerant	300	27.01	259.64	77890.50	0.000
	Susceptible	180	21.96	208.61	37549.50	
	Tolerant	300	27.01	237.60	71279.50	0.005
	Highly susceptible	150	13.67	201.30	30195.50	
	Susceptible	180	21.96	159.51	28711.50	0.211
	Highly susceptible	150	13.67	172.69	25903.50	

growth with a PIG varying from 27% to 34.45%. However, there was no difference observed between the susceptible and highly susceptible groups ($P > 0.05$), throughout the period of incubation. These findings confirm what was suspected from previous results (Dongmo et al., 2002). There was a consistency in the behaviour of varieties irrespective of dose of oils as far as radial growth inhibition is concerned (Table 4a).

The varieties in the five clusters (Table 3) can be subdivided into their respective susceptibility groups to *P. angolensis* with respect to reduction of sporulation as follows: Cluster 1 has 1 tolerant variety (V9) and 1 susceptible variety (V7). Cluster 2 contains 2 highly susceptible varieties (V13 and V6) and 1 susceptible variety (V20). Cluster 3 contains 2 tolerant varieties (V8 and V19), 1 highly susceptible variety (V15) and 1 susceptible variety (V17). Cluster 4 contains 2 susceptible varieties (V3 and V18) and 2 highly susceptible varieties (V2 and V10). Finally cluster 5 contains 2 susceptible varieties (V11 and V22) and 7 tolerant varieties (V21, V16, V12, V14, V4, V5 and V1) all having negative values of percentage reduction of conidia (PRC). This suggests that oils from these varieties tend to promote sporulation (Table 3). However it was noticed that changing from the optimal dose (1000 ppm) induced a marked difference with respect to certain varieties notably V1, V12 and V14. It was possible for these varieties to induce up to 100% reduction in number of conidia produced at a dose of 2500 ppm as compared to the control DMSO (Table 3). This marked effect of dose on sporulation suggests that there may be different compounds acting when the dose changes. To our knowledge this is the first study of essential oils activity on *P. angolensis* involving conidial production with such a large number of varieties.

The PRC at dose 1000 ppm was compared amongst the susceptibility groups using the Mann–Whitney test. There was a difference observed in the mean PRC between the tolerant and highly susceptible groups ($P = 0.007$), and between the susceptible and highly susceptible groups ($P = 0.000$). However, there was no difference observed in the mean PRC between the tolerant and susceptible groups ($P = 0.831$) (Table 4b). Oils from the highly susceptible group appeared to be the most effective in reducing conidia with a mean PRC of 45.93%. Meanwhile, the tolerant group exhibited a negative mean PRC of −12.71 suggesting that there is a slight increase in sporulation as compared to the control DMSO. This is opposite to what was observed for radial growth inhibition where the tolerant group ranked first. But it should be noted that changing to higher doses resulted in a reverse situation with a positive 100% conidial reduction for oils from some of

Table 4b. Susceptibility groups of citrus and effect of oils on percentage reduction of *Phaeoramularia angolensis* sporulation

Susceptibility group	*N*	Mean percentage Inhibition at dose = 2500 ppm	Rank mean	Rank sum	Signification of Mann–Whitney test
Tolerant	400	−12.71	341.84	136737.50	0.831
Susceptible	280	25.50	338.58	94802.50	
Tolerant	400	−12.71	286.99	114794.49	0.007
Highly susceptible	200	45.93	327.53	65505.50	
Susceptible	280	25.50	219.48	61454.00	0.000
Highly susceptible	200	45.93	269.93	53986.00	

these varieties. As for the increase in sporulation observed with oils of some varieties notably V1, V5 and V4, this result is opposite to what was expected taking in account the antifungal properties of essential oils. A similar tendency was observed by Boccas and Laville (1978) who reported the case of a fungicide (Chlorineb) promoting the production of zoospores of *Phytophthora*. For many fungi, increase in sporulation may be induced by various sources of stress such as light, chemical compounds, crushing or grounding of mycelia (Boccas and Laville, 1978). In our study, oils (varieties) tending to promote sporulation at dose 1000 ppm do not show a marked activity in inhibiting radial growth at that same dose. So the increase in sporulation is not associated with a high inhibition of growth at that dose. However, increasing the dose to about 2500 ppm leads to a positive PRC value of 100%. The stress in this case may be neither light (same for all treatments) nor physical action on mycelium but may be due to chemical action. The chemical stress suspected is higher in the tolerant varieties reducing radial growth and inducing more conidial production than in the susceptible and highly susceptible varieties where there is increase in radial growth and less sporulation.

Botanical groups and essential oil activity against P. angolensis

The varieties in the five clusters (Table 2) can be subdivided into their respective citrus botanical groups with respect to radial growth inhibition as follows: Cluster 1 contains (V4) which is a pummelo (Table 2). Cluster 2 contains a tangerine (V7) and an orange (V3). Cluster 3 contains 2 limes (V1 and V12), a lemon (V9), a tangerine (V10), a hybrid of tangerine (V16), an orange (V20) and a grapefruit (V6). Cluster 4 contains 2 tangerines (V19 and 21), a lemon (V14), 2 grapefruits (V2 and V13), a hybrid of tangerine (V15) and a satsuma (V5). Cluster 5 contains an orange (V11), 2 tangerines (V17 and V8) and a tangerine hybrid (V18). From day 10 to day 20 after inoculation, oils from the pummelo, the limes and lemons remained the most effective in inhibiting radial growth of *P. angolensis* while the satsuma and the hybrids of tangerine appeared to be the least effective at dose 2500 ppm (Table 5a). But at day 30, oils from oranges showed more effectiveness as compared to those from lemons. The Kruskal–Wallis test shows a difference ($P = 0.000$) between these botanical groups with respect to inhibition of radial growth of colony. Except for the case of the satsuma group, the ranking observed is consistent with the behaviour of varieties in the field with respect to this disease (Rey et al., 1986, 1988; Bella-Manga et al., 1999).

The varieties in the five clusters (Table 3) can be subdivided into their respective citrus botanical groups with respect to reduction of sporulation as follows: Cluster 1 contains a lemon (V9) and a tangerine (V7). Cluster 2 contains 2 grapefruits (V13 and V6) and an orange (V20). Cluster 3 contains 3 tangerines (V8, V19 and V17) and a hybrid of tangerine (V15). Cluster 4 contains a hybrid of tangerine (V18), a grapefruit (V2), a tangerine (V10) and an orange (V3). Cluster 5 contains a tangerine (V21), a hybrid of tangerine (V16), an orange (V11), a lemon (V14), 2 satsumas (V5 and V22), a pummelo (V4), and 2 limes (V1 and V12). As regards reducing sporulation, the grapefruits and the tangerines are the most effective while the lemons and the satsumas are the least at dose 1000 ppm (Table 5b). The

Table 5a. Botanical groups of citrus and effect on percentage inhibition of radial growth of *P. angolensis* colonies

	Botanical group	*N*	Mean percentage inhibition at dose = 2500 μl l^{-1}	Rank mean	Kruskal–Wallis test
Percentage inhibition after 10 days	Pummelo	30	61.60	513.72	Chi square = 89.092, 7 df, $P = 0.000$
	Lime	60	50.56	424.56	
	Lemons	60	42.61	394.04	
	Oranges	90	32.56	349.72	
	Tangerine	180	17.32	274.71	
	Grape fruit	90	15.92	278.73	
	Satsuma	30	15.15	218.27	
	Hybrids	90	8.45	270.91	
Percentage inhibition after 20 days	Pummelo	30	80.37	441.22	Chi square = 65.217, 7 ,df, $P = 0.000$
	Lime	60	43.30	400.98	
	Lemons	60	29.92	386.51	
	Oranges	90	26.32	311.89	
	Tangerine	180	25.35	299.41	
	Grape fruit	90	12.56	313.29	
	Hybrids	90	10.33	251.39	
	Satsuma	30	-0.55	183.13	
Percentage inhibition after 30 days	Pummelo	30	87.89	454.53	Chi square = 45.432, 7 df, $P = 0.000$
	Lime	60	33.34	386.23	
	Oranges	90	24.91	325.76	
	Lemons	60	24.87	350.59	
	Tangerine	180	20.95	290.99	
	Grape fruit	90	10.73	317.01	
	Hybrids	90	8.72	252.34	
	Satsuma	30	7.10	266.02	

Table 5b. Botanical groups of citrus and effect of oils on percentage reduction of *P. angolensis* sporulation

Botanical group	*N*	Mean percentage reduction at dose = 1000 ppm	Rank mean	Kruskal–Wallis test
Grape fruit	120	64.24	485.67	Chi square = 210.219, 7 df, $P = 0.000$
Tangerine	240	24.94	476.56	
Oranges	120	19.87	380.52	
Hybrids of Tangerine	120	13.60	470.24	
Pummelo	40	−8.43	179.95	
Lime	80	−15.12	599.84	
Lemons	80	−50.05	565.55	
Satsuma	80	−91.44	155.00	

Kruskal–Wallis test shows a difference ($P = 0.000$) between these botanical groups in reducing sporulation. Contrary to what was observed for radial growth inhibition, the action of oils from different botanical groups in reducing sporulation at a dose of 1000 ppm does not match to what is observed for this disease on the field.

In conclusion, essential oils from the tolerant varieties of citrus appear to be generally the most effective in reducing radial growth of *P. angolensis* irrespective of the dose used, while those from the susceptible and highly susceptible groups are the least effective. However, there was apparently a marked dose effect as far as sporulation is concerned. In fact, the highly susceptible varieties are most effective in reducing sporulation at dose 1000 ppm while at higher doses of about 2000–2500 ppm the tolerant group can reduce

sporulation up to 100%. This suggests that there may be different chemical compounds acting in these essential oils. It would be interesting to identify and characterise such compounds. Furthermore, whether this action is quantitative, qualitative, antagonistic or synergistic is yet to be investigated. From the botanical perspective, the pummelo variety that was tested appeared to be the best in reducing radial growth at dose 2500 ppm (>87% inhibition) while the grapefruits are the most effective in reducing *P. angolensis* sporulation at dose 1000 ppm (>64% conidial reduction).

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