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Antifungal potential of extracts from four plants against *Acremonium apii* and *Colletotrichum dematium*, two major pathogens of celery (*Apium graveolens* L.) in Cameroon

Petchayo Tigang Sandrine[a], Nguefack Julienne[a], Galani Yamdeu Joseph Hubert[b]*, Fouelefack Francois Romain[a], Dakole Daboy Charles[a], Fotio Daniel[c] and Amvam Zollo Paul Henry[a]

[a]*Department of Biochemistry, Faculty of Science, University of Yaoundé 1, PO. Box 812, Yaoundé, Cameroon*

[b]*Department of Biochemistry, B.A. college of Agriculture, Anand Agricultural University, Anand-388110, Gujarat, India*

[c]*Institute of Agricultural Research for Development (IRAD), Box 2123, Yaoundé, Cameroon*

**Corresponding author: josephgalani@gmail.com; Phone: +912692225750*

Abstract

In order to contribute to a sustainable control of plant diseases through the use of natural compounds, the antifungal potential of 11 extracts from 4 Cameroonian plants (*Ageratum conyzoides*, *Callistemon citrinus*, *Cymbopogon citratus* and *Ocimum gratissimum*) was evaluated *in vitro* against *Acremonium apii* and *Colletotrichum dematium*, respectively the causal agents of brown spots and anthracnose diseases of celery (*Apium graveolens* L.). Inhibition of fungi mycelial growth by essential oils, ethanol and aqueous extracts was assessed by using the supplemented media technique. Essential oils exhibited comparable activities against both fungi with minimum inhibitory concentration between 400 and 6000 ppm. Essential oil from *O. gratissimum* showed the highest inhibitory activity against both pathogens (400 ppm) followed by *C. citratus* (700 ppm and 800 ppm against *A. apii* and *C. dematium*, respectively), and then *C. citrinus* (6000 ppm). Ethanol extracts exhibited after the essential oils, the higher inhibitory activity against the two pathogens. Extract of *C. citrinus* was the most active with reductions of radial growth of 77.68% and 97.16% respectively against *A. apii* and *C. dematium* at 10000 ppm. Aqueous extracts at the same concentrations of ethanol extracts had little or no activity against both fungi. The fungitoxic potential of essential oils was higher than the one of the synthetic fungicide used as positive control. Our results suggest a promising potential of essential oils and ethanol extracts for botanicals control of celery fungal pathogens.

Keywords: antifungal, plant extracts, celery, *Acremonium apii*, *Colletotrichum dematium*

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Introduction

Celery (*Apium graveolens* L.) is a leafy vegetable native to the Mediterranean region and was introduced in Africa in the 19th century by the first Westerns (Marquis, 2005). It is classified 3rd among the most popular vegetables for salads and is very popular in western countries where its productivity can reach up to 20 t/ha (Raid, 2004). In Europe, yields of 50 t/ha were achieved (Schippers, 2004). In Cameroon, celery is grown as market gardening crop and its culture represents a profitable employment for many families. The leafy vegetables account for 11% of the value of final agricultural production of horticultural sector (Temple, 1999). Celery is locally used as a condiment in seasoning, and also for decoration of various dishes. Growing celery is faces many limiting factors among which pests and diseases are

foremost. In Europe, the most damaging fungal disease is Septoria leaf spots caused by *Septoria apiicola*. The disease can cause more than 80% losses (Davis and Raid, 2002). An assessment of the phytosanitary status of celery cultures at Nkolondom, Yaoundé, Cameroon has revealed the predominance of two fungal strains, *Acremonium apii* and *Colletotrichum dematium*, respectively responsible of brown spots and anthracnose diseases, with incidences ranging from 12 to 60%. The pathogens were largely distributed in the area and were established to be responsible for significant losses in fields as well as the poor quality of marketable produce (Nguefack, 2006, personal communication).

Synthetics fungicides are usually used by farmers against these diseases, and have been proven effective. However, the limitations of this practice are numerous, including direct intoxication of users, environmental pollution (Deward et al., 1993) and the emergence of resistant strains. Moreover, analyzes of the local vegetables showed the presence of pesticide residues in consumable goods (Fotio and Monkiedje, 2005). Therefore, there is an urge to look for new control methods which are economically profitable, respectful to the environment and safe for the consumer. Botanicals, compared to synthetic pesticides have the advantage of low or no toxicity, easily biodegradable, eco friendly, and can therefore be a natural alternative control method against plant pathogenic fungi (Awuah, 1994; Mason and Mathew, 1996; Nguefack et al., 2008).

Several studies have reported the use of essential oils and solvent extracts against plant pathogenic fungi. Nguefack et al. (2012) demonstrated that at 1000 ppm, essential oil fraction of *O. gratissimum* was more active than *C. citrinus* fractions against *Penicillium expansum*. Bengyella et al. (2011) showed that *O. gratissimum* essential oil at 150 ppm inhibited by 86.17 and 100% the mycelial growth of *Bipolaris oryzae* and *Alternaria padwickii* respectively. The ethanol extract at 10000 ppm showed 80.92 and 61.54% growth inhibition of *B. oryzae* and *A. padwickii* respectively. Galani et al. (2013) reported that essential oils of *C. citratus* at 300 ppm, *O. gratissimum* at 400 ppm, and *C. citrinus* at 5000 ppm totally inhibited the growth of *Phythopthora infestans*. Additionally, the ethanol extracts of *A. conyzoides* and *C. citrinus* totally inhibited the pathogen at 5000 ppm, and that of *O. gratissimum* at 10000 ppm. However, there is no report of the effect of these extracts on celery pathogens *A. apii* and *C. dematium*. Therefore, in order to contribute to natural control of celery brown spots and anthracnose diseases, in this study 11 plant extracts from 4 Cameroonian plants were tested against these two pathogens under laboratory conditions to determine the effect of these extracts on their mycelial growth and find out the most effective extracts.

Materials and Methods

Pathogen's cultures

A. apii and *C. dematium* were isolated from celery (*Apium graveolens* L. cv. Grande) leaves showing brown spots and anthracnose dark necrosis symptoms respectively. The diseased material collected from field was sterilized and incubated on Potato Dextrose Agar (PDA) medium at 25ºC for 5 days. Cultures were purified by single hyphal tip method on Prune Lactose Yeast Agar (PLYA) and maintained at 25ºC. Cultures aged 7 days were used for antifungal tests.

Plant material

The 4 local plants used in this study (*Ageratum conyzoides, Callistemon citrinus, Cymbopogon citratus,* and *Ocimum gratissimum*) (Table 1) were selected based

Table 1. Characteristics of plants extracts

Plant species	Family	Organ used	EO colours	Yield (%w/w)		
				EO	ETE	CWE
Ageratum conyzoides	*Asteraceae*	Whole plant	Pale green	0.10	3.50	5.09
Callistemon citrinus	*Myrtaceae*	Leaves	Yellow	1.78	4.00	6.72
Cymbopogon citrates	*Poaceae*	Leaves	Yellowish	0.68	3.83	4.30
Ocimum gratissimum	*Lamiaceae*	Leaves	Yellowish	0.73	4.47	5.51

EO: essential oil - ETE: ethanol extract - CWE: cold water extract

on the reported knowledge of their ethnobotanical uses and their previously demonstrated antimicrobial activities. Plant materials were collected at Yaoundé, Cameroon in 2009 and air-dried at room temperature (25-27°C) for 10 to 12 days.

Extraction of essential oils

Essential oils were extracted from dry plant material by hydrodistillation method using a Clevenger-type apparatus. The collected oil was dried on a sodium sulphate column and preserved at 4°C into airtight amber bottles. The yields of oils were calculated as percent of plant material weight (% w/w) and essential oils from plants with higher yields (≥ 0.5% w/w) were used for antifungal tests.

Preparation of solvent extracts

Shade-dried plant material of each species was coarsely powdered in a blender and 100g of powder was first defatted in 300 mL of hexane for 90 min. The lipid-free powder was then extracted in 500 mL of distilled water or 500 mL of 70% ethanol for 90 min, respectively. After filtration, the filtrates were centrifuged at 7000 rpm for 10 min. Ethanol was evaporated from the ethanol extract using a rotary evaporator at 78°C. All supernatants were freeze-dried in a lyophilisator and the obtained powders of cold water extract and ethanol extract were kept at 4°C into airtight amber bottles. The yields of the solvent extracts were calculated as percent of dried plant material weight (% w/w).

Synthetic fungicide

Banko Plus® fungicide titrating 550 g/L chlorotalonil and 100 g/L carbendanzime, which is among the most used synthetic fungicides by Cameroonian farmers against celery fungal diseases was used in this study.

Antifungal activity test

The inhibitory effect of extracts and synthetic fungicide on mycelial growth of each pathogen grown on PLYA was evaluated using the supplemented media technique as described by Benjilali et al. (1986). Essential oils were added to media at concentrations ranging between 100 and 6000 ppm, the solvent extracts were tested at 1000, 5000 and 10000 ppm and the synthetic fungicide from 1000 to 5000 ppm. Sterile double distilled water was used as negative control. Petri dishes sealed with parafilm were incubated in inverted position at 25±2°C for 12 h alternating light-dark cycle during 13 days for *C. dematium* and 15 days for *A. apii*. The diameter of pathogen mycelial growth was recorded and results expressed as percentage of mycelial growth inhibition (% I) calculated as per the formula of Pandey et al. (1982): % I = (growth diameter in the control-growth diameter in the treatment sample) x 100 / growth diameter in the control.

Determination of the nature of inhibition

Fungal discs from plates in which no colony growth occurred after full incubation days were further checked to detect the fungicidal or fungistatic nature of the inhibition following the procedure of Mishra and Dubey (1994). The discs were re-inoculated onto the fresh PLYA medium and fungal growth was observed during 30 days of incubation. The inhibition was qualified as fungistatic if renewed mycelial growth was observed and the concentration was recorded as the minimum inhibitory concentration (MIC). If the contrary (no renewed mycelial growth) was observed, the inhibition was qualified as fungicidal and the concentration known as, minimum fungicidal concentrations (MFC).

Statistical analysis

Experiments were set in a Completely Randomized Design with three replications. Data were analysed using Statistical Package for Social science (SPSS) version 10.1 software by Analysis of Variance (ANOVA) paired to t-test of Student-Newman-Keuls (parametric) and differences among the means were determined for significance at $P<0.05$.

Results

Plant extracts characteristics

Characteristics of obtained essential oils and solvent extracts vary from one plant species to another and also depend on the solvent used as well as the extraction method. The highest yields were obtained from cold water extracts and the lowest from essential oils. The highest essential oil yield (1.78%) was obtained from *C. citrinus* and the lowest (0.10%) was recorded from *A. conyzoides* and the later was not enough to perform subsequent antifungal tests. Except essential oil of *A. conyzoides* which looked green, oil colours of other plants were yellow coloured (Table 1).

Efficacy of essential oils

All the 3 essential oils have shown significant antifungal activity against both fungi and inhibition of mycelial growth was dose- and plant species-dependant. The essential oil of *O. gratissimum* was the most active, with 100% inhibition at 400 ppm against both fungi. The essential oil of *C. citrinus* was the less active, complete inhibition of both pathogens' growth was obtained at 6000 ppm. Both *A. apii and C. dematium* have shown similar pattern of sensitivity to the 2 essential oils (Table 2).

Efficacy of ethanol extracts

All the ethanol extracts exerted antifungal activity against both pathogens at all the 3 concentrations tested. The extract of *C. citrinus* inhibited significantly ($P<0.05$) the growth of both pathogens with the highest activity (97.16%) recorded at 10000 ppm against *C. dematium*. Except the extract of *C. citratus* at 1000 and 5000 ppm; and *O. gratissimum* at 5000 ppm, all the extracts were in general most active against *C. dematium* (Table 3).

Efficacy of cold water extracts

The mycelial growth of both pathogens was not affected or was merely lightly inhibited by the majority of cold water extracts whereas some growth stimulation was observed with some extracts. The most active extract (12.44% inhibition) was obtained from *O. gratissimum* at 10000ppm against *C. dematium*. At all the tested concentrations, *C. citratus* extract stimulated the growth of *A. apii* and similar observation was obtained with *A. conyzoides* extract at 1000 ppm against *C. dematium*.

Efficacy of the synthetic fungicide

Synthetic fungicide Banko Plus® completely inhibited the

Table 2. Percentage of mycelial growth inhibition of *A. apii* and *C. dematium* obtained with essential oils

Essential oil (ppm)	Percentage inhibition (%)					
	Acremonium apii			*Colletotrichum dematium*		
	O. gratissimum	*C. citratus*	*C. citrinus*	*O. gratissimum*	*C. citratus*	*C. citrinus*
100	15.24^{b}±5.25	2.86^{a}±1.43	0.00^{a}±0.00	7.11^{b}±2.77	5.33^{b}±0.00	0.00^{a}±0.00
200	59.40^{c}±3.45	9.52^{b}±2.18	0.00^{a}±0.00	24.00^{c}±1.33	15.11^{b}±3.35	0.00^{a}±0.00
300	72.26^{c}±2.37	14.29^{b}±0.00	0.00^{a}±0.00	72.41^{c}±5.03	17.77^{b}±2.03	0.00^{a}±0.00
400	100^{c}±0. 00	16.19^{b}±2.18	0.00^{a}±0.00	100^{c}±0.00	21.78^{b}±2.77	0.00^{a}±0.00
500	100^{c}±0. 00	21.43^{b}±1.43	0.00^{a}±0.00	100^{c}±0.00	26.66^{b}±1.33	0.00^{a}±0.00
600	100^{c}±0. 00	37.14^{b}±1.43	0.00^{a}±0.00	100^{c}±0.00	31.55^{b}±2.03	0.00^{a}±0.00
700	100^{b}±0. 00	100^{b}±0.00	0.00^{a}±0.00	100^{c}±0.00	56.44^{b}±2.77	0.00^{a}±0.00
800	100^{b}±0. 00	100^{b}±0.00	0.00^{a}±0.00	100^{c}±0.00	100^{c}±0.00	0.00^{a}±0.00
900	100^{b}±0. 00	100^{b}±0.00	0.00^{a}±0.00	100^{c}±0.00	100^{c}±0.00	0.00^{a}±0.00
1000	100^{b}±0. 00	100^{b}±0. 00	0.95^{a}±0.82	100^{b}±0.00	100^{b}±0.00	6.64^{a}±0.04
3000	100^{b}±0. 00	100^{b}±0. 00	15.23^{a}±0.81	100^{b}±0. 00	100^{b}±0. 00	13.33^{a}±2.30
5000	100^{b}±0. 00	100^{b}±0. 00	34.76^{a}±1.64	100^{b}±0. 00	100^{b}±0. 00	55.33^{a}±0.00
6000	100^{a}±0. 00	100^{a}±0. 00	100^{a}±0. 00	100^{a}±0. 00	100^{a}±0. 00	100^{a}±0. 00

Values in same row followed by different letters are significantly different (P <0.05). Data are means ± SD of three experiments

Table 3. Percentage of mycelial growth inhibition of *A. apii* and *C. dematium* by ethanol extracts

Plant species	Ethanol extract concentration (ppm) Percentage inhibition (%)					
	Acremonium apii			*Colletotrichum dematium*		
	1000	5000	10000	1000	5000	10000
A. conyzoides	9.12^{a} ±0.05	36.96^{a} ±1.05	58.59^{b} ±0.23	14.22^{b}±0.11	42.69^{c}±0.28	64.52^{b}±0.40
C. citrinus	31.42^{c} ±3.34	67.38^{c} ±0.71	77.68^{d} ±0.16	40.05^{d}±0.08	80.66^{d}±1.15	97.16^{d}±0.28
C. citratus	14.07^{b} ±1.28	37.73^{a} ±0.07	46.58^{a} ±0.15	7.75^{a}±0.11	30.33^{a}±0.57	50.70^{a}±0.23
O. gratissimum	18.14^{b} ±0.08	45.40^{b} ±0.13	63.50^{c} ±0.23	20.11^{c}±0.22	39.95^{b}±0.08	73.49^{c}±0.28

Values in same column followed by different letters are significantly different (P <0.05). Data are means ± SD of three experiments

mycelial growth of *A apii* at 3000 ppm and *C. dematium* at 5000 ppm.

Nature of the inhibition

The MIC and MFC of *C. citratus* essential oil against the two pathogens remained the same, whereas for the other extracts and the fungicide, they were different (Table 4).

Discussion

Antifungal activity of essential oils and solvent extracts from 4 plants against *A. apii* and *C. dematium* has been assessed in this study. Essential oils showed the highest activity against both pathogens. Essential oils extraction yields varied with plant species.

Table 4. Percentage of mycelial growth inhibition of *A. apii* and *C. dematium* by cold water extracts

Plant species	Cold water extract concentration (ppm) Percentage inhibition (%)					
	Acremonium apii			*Colletotrichum dematium*		
	1000	5000	10000	1000	5000	10000
A. conyzoides	$0.00^{ab} \pm 0.00$	$1.43^{b} \pm 1.43$	$6.66^{c} \pm 2.17$	$-1.00^{a} \pm 1.31$	$1.73^{a} \pm 1.50$	$2.16^{a} \pm 1.98$
C. citrinus	$-2.73^{a} \pm 2.50$	$-7.03^{a} \pm 1.01$	$-7.58^{a} \pm 1.95$	$0.00^{a} \pm 0.00$	$2.67^{a} \pm 0.00$	$6.67^{b} \pm 0.00$
C. citratus	$0.00^{ab} \pm 0.00$	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.88^{a} \pm 0.76$	$1.77^{a} \pm 0.77$
O. gratissimum	$0.94^{b} \pm 0.81$	$0.94^{b} \pm 0.81$	$0.94^{b} \pm 0.81$	$0.88^{a} \pm 0.76$	$5.78^{b} \pm 1.54$	$12.44^{c} \pm 1.53$

Values in same column followed by different letters are significantly different ($P < 0.05$). Data are means ± SD of three experiments

Table 4. Nature of inhibition of mycelial growth by essential oils and synthetic fungicide

	O. gratissimum	*C. citratus*	*C. citrinus*	Banko Plus®
	Acremonium apii			
MIC (ppm)	400	700	6000	3000
MFC (ppm)	600	700	9000	5000
Colletotrichum dematium				
MIC (ppm)	400	800	6000	5000
MFC (ppm)	600	800	9000	7000

Higher yields of 0.73%, 0.68% and 1.78% were obtained from *O. gratissimum*, *C. citratus* and *C. citrinus* respectively whereas *A. conyzoides* yield (0.10%) was the lowest. Different yields were obtained by other authors from same plants: Nguefack et al. (2005) obtained 0.57% yield with *C. citratus* and Bengyella et al. (2011) have obtained 1.46% oil yield from *O. Gratissimum*. The plant materials used by these authors were harvested in different locations, at different period. However, our results are similar to the yields obtained by Galani et al. (2013) with plant material harvested from same location. According to Bruneton, (1999) essential oil extraction yield could be influenced by intrinsic factors such as botanical species and plant vegetative cycle; and extrinsic factors such as climatic conditions, soil type, place and time of harvest. Besides, yields obtained with solvent extracts were considerably higher than those of essential oils. The cold water extracts showed the highest yield ranging from 4.30 to 6.72% and the ethanol extracts yields varied from 3.50 to 4.47%. These differences are due to the extraction method used, solvent extraction, and the relative solubility of the compounds in extraction solvents (Lapornik et al., 2005).

All three essential oils used in this study exhibited antifungal activity against both pathogens. Essential oils from *O. gratissimum* and *C. citratus* showed strong activity while moderate activity was observed with *C. citrinus*. It has been shown that the antimicrobial activity of an essential oil is related to its chemical composition, mainly its proportion in oxygenated terpenes (Hammer et al., 2003; Nguefack et al., 2012). The highest efficacy observed with *O. gratissimum* oil as compared to other essential oils is due to its very high content of active phenolic compounds such as thymol (Nguefack et al.,

2007). The activity exhibited by *C. citratus* could be due to the action of its major components neral and geranial. The antifungal activity of 1,8-cineole, α-pinene and α-terpineol (94.90%) could be responsible for *C. citrinus* activity (Jazet et al., 2009; Dongmo et al., 2010) and was demonstrated by Laret and Barrandon (1998). Moreover, antifungal activity of essential oil could probably not only be due to the action of the major components, but also to the combined action of other compounds, these compounds may act synergistically (Nguefack et al., 2012)

It was noted a low activity of solvent extracts as compared to essential oils. This result is consistent with those obtained by previous authors. It was shown that essential oils compared to aqueous and ethanol extracts from same plants were more active against *Alternaria padwickii* and *Bipolaris oryzae* (Bengyella et al., 2011) and against *Phythophthora infestans* (Galani et al., 2013). The presence of active compounds in the extracts is influenced by the extraction method, the extraction solvent, the age of the plant and harvest time (Qasem and Abu-Blan, 1996; Lapornik et al., 2005). There was a significant difference in the activity of ethanol extracts as compared to cold water extracts against both pathogens; the ethanol extracts showed higher antifungal activity. The stronger activity was recorded with the extract of *C. citrinus*, with 97.16% inhibition against *C. dematium* and 77.78% inhibition against *A. apii* at 10000 ppm. According to Amvam et al., (1998) these differences can be explained by their different chemical compositions. Galani et al. (2013) have demonstrated that ethanol extracts with significant antifungal activity mainly contain phenols, sterols, flavonoids, condensed tannins, and to a lesser extent coumarins and alkaloids. Phenolic compounds possess a very high antimicrobial activity (Lapornik et al., 2005). Also, high activity of coumarins such as phytoalexins produced by plants in response to fungal attack has been reported by many authors (Cowan, 1999; Lapornik et al., 2005).

The stimulation of the growth of *A. apii* at all concentrations with *C. citrinus* cold water extract and *A. conyzoides* extract at 1000 ppm on *C. dematium* was observed in this study. Other reports mentioned stimulation of pathogens growth by plant extracts (Wang et al., 2001; Bengyella et al., 2011; Galani et al., 2013). This can be explained by their low content of phenolic compounds as revealed by Galani et al. (2013). According Mohapotra et al. (2000) small amounts of phenols (3-5 µg/ml) are required for normal metabolism of fungi, whereas the concentrations of 20 µg/ml or more become toxic. Moreover, the presence of glycosides shown by the analysis of the phytochemical composition of the extracts (Galani et al., 2013) can also explain the stimulation of growth of the pathogen. Glycosides can be considered as a potential source of glucose, needed by many pathogens. In fact, as the dose of the extract is increased, there is an increase of mycelial growth likely due to the increase in glycoside concentration in the medium.

The recovery of the mycelial growth of both pathogens was observed with essential oils from *C. citrinus* (MIC=6000 ppm; CMF=8000 ppm) and *O. gratissimum* (MIC=400 ppm; CMF=700 ppm). This reflects the fungistatic nature of inhibition from these two essential oils. There was no growth recovery of explants from the essential oil of *C. citratus* on both pathogens; this reflects its fungicidal character. The effectiveness of *O. gratissimum* and *C. citratus* essential oils on both pathogens was greater than synthetic fungicide Banko Plus® which was also more active on both pathogens than

the essential oil of *C. citrinus*. Additionally, Banko Plus® was more effective on *A. apii* (MIC=3000 ppm) as compared to *C. dematium* (MIC=5000 ppm). Moreover, the fungal strain of *C. dematium* appeared to be more sensitive to cold water and ethanol extracts as compared to *A. apii*. This difference in sensitivity between the two pathogens can be explained by the difference in mechanism of action of extracts and/or the constitution of the two pathogens. Hammer et al. (2003) stipulates that the antimicrobial activity is strongly influenced by the physical, morphological and chemical characteristics of the components of the microbe. Therefore, more studies on the chemical and structural characterization of these pathogens are required for a better understanding of the effect of plant extracts components on their metabolism.

In conclusion, among the 3 types of extracts used in this study, essential oils exhibited the strongest antifungal activity against *A. apii* and *C. dematium*, followed by ethanol extracts which were more active than cold water extracts. This is the first report of plant extracts from Cameroon as prospective source of compounds effective against these two serious pathogens of celery in the country. The degree of fungal growth inhibition recorded with essential oils was stronger than the synthetic fungicide. These findings pave the way towards isolation and characterization of antifungal compounds which can be used in crop protection strategies against fungal diseases of celery.

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