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RESEARCH ARTICLE

ANTIFUNGAL POTENTIAL OF EXTRACTS FROM THREE PLANTS AGAINST TWO MAJOR PATHOGENS OF CELERY (*Apium graveolens* L.) IN CAMEROON

[1]Ndonkeu Mangoumou Ghislaine, [1]Nguefack Julienne, [1,2*]Galani Yamdeu Joseph Hubert, [1]Petchayo Tigang Sandrine and [2]Amvam Zollo Paul Henry

[1]Department of Biochemistry, Faculty of Science, University of Yaoundé 1, PO. Box 812, Yaoundé, Cameroon
[2]Department of Biochemistry, B.A. College of Agriculture, Anand Agricultural University, Anand-388110, Gujarat, India

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ABSTRACT

With the aim of contributing to natural control of plant pathogens, the antifungal activity of 11 extracts from 3 Cameroonian plants namely, *Drypetes gossweileri, Eucalyptus tereticornis* and *Sida acuta* was evaluated against *Acremonium apii* and *Colletotrichum dematium*, respectively causal agents of brown spot and anthracnose diseases of celery (*Apium graveolens* L.). The supplemented media technique was used to assess the inhibition of both fungi mycelial growth by essential oils, ethanol,hot water and cold water extracts. The essential oils exhibited the highest antifungal activity at 50 ppm with essential oil from *D. gossweileri*; and 6000 ppm and 7000 ppm, against *C. dematium and* A. *apii*, respectively, with essential oil from *E. tereticornis.* Ethanol and aqueous extracts displayed a moderate inhibitory activity with the best activity obtained from *D. gossweileri* ethanol extracts (90.31% and 67.53%, respectively, against *A. apii* and *C. dematium* at 10000 ppm). The fungitoxic potential of essential oils was comparative to the synthetic fungicide used as positive control. Phytochemical screening of solvent extracts revealed a diverse composition in secondary metabolites and stronger inhibitory effects were recorded with extracts rich in alkaloids, phenols, anthraquinones and saponines. These findings suggest a promising potential of essential oils and ethanol extracts for botanicals control of celery fungal pathogens.

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INTRODUCTION

Since it was introduced in Africa in the 19th century by the first Westerns, celery (*Apium graveolens* L.) has gain significant importance among gardening crops (Marquis, 2005). The leafy vegetable native to the Mediterranean region is classified as the 3rd most popular vegetables for salads and is very popular in is 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, the leafy vegetables including celery account for 11% of the value of final agricultural production of horticultural sector (Temple, 1999). In the country, celery is grown as market gardening crop and its culture represents a profitable employment for many families. It is also locally used as a condiment in seasoning, and also for decoration of various dishes. Celery plants are subjected to many pests and diseases. 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). In Cameroon, two predominant fungi have been identified: *Acremonium apii* and *Colletotrichum dematium*, respectively responsible of brown spots and anthracnose diseases, with incidences ranging from 12 to 60%. The pathogens are largely distributed in the centre area (Yaoundé) and are established to be responsible for significant losses in fields as well as the poor quality of marketable produce (Nguefack, University of Yaoundé 1, Cameroon, personal communication). Despite the effective results of synthetics fungicides for controlling the diseases, 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*, 2005).

The use of essential oils and solvent extracts against plant pathogenic fungi has been largely reported. However, few reports have shown the effects of plant extracts on celery pathogens. Petchayo *et al.,* (2013) demonstrated that essential oil from *O. gratissimum* inhibited *A. apii* and *C. dematium* at 400 ppm, *C. citratus* at 700 ppm and 800 ppm against *A. apii*

***Corresponding author: Galani Yamdeu Joseph Hubert**
Department of Biochemistry, B.A. College of Agriculture, Anand Agricultural University, Anand-388110, Gujarat, India.

and *C. dematium*, respectively, and *C. citrinus* at 6000 ppm against both pathogens. Ethanol extracts of *C. citrinus* at 10000 ppm reduced the radial growth of *A. apii* and *C. dematium* by 77.68% and 97.16% respectively. Besides, Sengo and Senthil (2006) have shown anti-insecticidal activity of *E. tereticornis. S. acuta* is shown to have antimalaria activity (Karou *et al*., 2003) and several alkaloids with pharmacological properties have been isolated from the plant (Karou *et al.,* 2007). Essential oil from *D. gossweileri* has exhibited strong antioxidant activity (Agnaniet *et al.,* 2003). Still, there is no report of the activity of these extracts against *A. apii* and *C. dematium*. Hence, in this study 11 plant extracts from these 3 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

The method of Petchayo *et al*. (2013) was used. Briefly, *A. apii and C. dematium* were isolated from celery (*Apium graveolens* L. cv. Grande) leaves showing brown spots and anthracnose dark necrosis symptoms respectively. Infected leaves 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

Three local plants (*Drypetes gossweileri, Eucalyptus tereticornis* and *Sida acuta* (Table 1) were selected based on the reported knowledge of their ethnobotanical uses and their previously demonstrated antimicrobial activities. Plant materials were collected at Yaoundé (Cameroon): leaves of *E. tereticornis* and the whole areal part of *S. acuta* were air-dried at room temperature (25-27°C) for 10 to 12 days; the cork of *D. gossweileri* was cut into small pieces and dried in oven at 50°C for 2 days.

Extraction of Essential Oils

Essential oils were extracted from dry plant material (*D. gossweileri* and *E. tereticornis*) by hydrodistillation method using a Clevenger-type apparatus. The collected oil was dehydrated through 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).

Preparation of solvent extracts

Shade-dried plant material of each species was coarsely powdered in a blender and then 100g of powder was first defatted by mixing with 300 mL of hexane for 90 min. After filtration the residue was spread for complete evaporation of the solvent. Lipid-free powder was then soaked and frequently stirred in 500 mL of cold distilled water, or 500 mL of hot water (100°C), or 500 mL of 70% ethanol for 90 min, respectively, followed by filtration first through a double folded cheese cloth, then through Whatman #1 filter paper. The filtrates were subsequently subjected to centrifugation at 7000 rpm for 10 min. Ethanol was totally evaporated from the extract using a rotary evaporator at 78°C. All supernatants were freeze dried using a lyophilisator and obtained powder of cold water extracts, hot water extracts and ethanol extracts preserved in refrigerator (4°C) into airtight brown bottles until further use. The yields of the solvent extracts were calculated as percent of dried plant material weight (% w/w).

Synthetic fungicides

Banko Plus® fungicide titrating 550g/L chlorotalonil and 100 g/L carbendanzime; and Penncozeb® containing 750 g/kg Mancozeb which are among the most used synthetic fungicides by Cameroonian farmers against celery fungal diseases were used in this study.

Antifungal activity tests

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 50 and 7000 ppm, the solvent extracts were tested at 1000, 5000 and 10000 ppm and the synthetic fungicide from 50 to 10000 ppm. Sterile double distilled water was used as negative control. Petri dishes sealed with parafilm were incubated in inverted position at 25±2°C in a 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 according to 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).

Phytochemical screening

Phytochemical tests for major secondary metabolites of the solvent extracts were performed. Plant extracts were screened for the presence of biologically active compounds namely alkaloids, anthocyanins and cardiac glycosides (Odebiyi and Sofowora, 1978); phenols and flavonoids (Harbone, 1976); triterpenes and sterols, (Schoppe, 1964), saponins (Wall *et al.,* 1952); anthraquinones, hydrolysable and condensed tannins (Trease and Evans, 1989) and coumarins (Kovac-Besović and Durić, 2003). Based on the intensity of coloration or the precipitate formed during the test, secondary metabolites

content was characterized as strongly present (+++), present (++), weakly present (+) and absent (-) when the test result was negative.

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

1. Plant extracts characteristics. Characteristics of essential oils and solvent extracts depend on the plant species, the solvent used and the extraction method. Cold water extracts gave highest yields, followed by hot water and ethanol extracts. The lowest were obtained from essential oils (Table 1).

2. Efficacy of essential oils

The 2 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 *D. gossweileri* was the most active, with 100% inhibition of both fungi at 50 ppm. With essential oil of *E. tereticornis* complete inhibition of both pathogens' growth was obtained at 6000 ppm on *C. dematium* and 7000 ppm against *A. apii* (Table 2).

3. Efficacy of ethanol extracts

All the ethanol extracts exerted a significant (p<0.05) antifungal activity against both pathogens at all the 3 concentrations tested. The highest inhibition was obtained from *D. gossweileri* at 10000 ppm against *A. apii* (90.31%) and against *C. dematium* (67.53%). In general, all the extracts were more active against *A. apii* and *S. acuta* extract has shown the lowest activity (Table 3).

Table 1. Characteristics of plants extracts

Plant species	Family	Organ used	Yield (%w/w)			
			EO	ETE	HWE	CWE
Drypetes gossweileri	*Euphorbiaceae*	Cork	0.29	10.03	10.93	12.43
Eucalyptus tereticornis	*Myrtaceae*	Leaves	0.53	7.05	8.56	9.60
Sida acuta	*Malvaceae*	Whole plant	-	2.10	5.30	6.05

EO: essential oil, ETE: ethanol extract, HWE: hot water extract, CWE: cold water extract

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

Essential oil concentration (ppm)	Percentage inhibition (%)			
	Acremonium apii		*Colletotrichum dematium*	
	Drypetes gossweileri	*Eucalyptus tereticornis*	*Drypetes gossweileri*	*Eucalyptus tereticornis*
50	100^{b}±0.00	0.00^{a}±0.00	100^{b}±0.00	0.00^{a}±0.00
100	100^{b}±0.00	0.00^{a}±0.00	100^{b}±0.00	0.00^{a}±0.00
300	100^{b}±0.00	0.00^{a}±0.00	100^{b}±0.00	0.00^{a}±0.00
500	100^{b}±0.00	0.00^{a}±0.00	100^{b}±0.00	0.00^{a}±0.00
1000	100^{b}±0.00	12.59^{a}±0.83	100^{b}±0.00	18.50^{a}±1.26
2000	100^{b}±0.00	28.97^{a}±1.32	100^{b}±0.00	19.09^{a}±3.00
4000	100^{b}±0.00	56.06^{a}±1.61	100^{b}±0.00	31.98^{a}±1.75
5000	100^{b}±0.00	75.75^{a}±8.12	100^{b}±0.00	66.54^{a}±8.64
6000	100^{b}±0.00	87.19^{a}±7.04	100^{a}±0.00	100^{a}±0.00
7000	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 concentration (ppm)	Percentage inhibition (%)					
	Acremonium apii			*Colletotrichum dematium*		
	Drypetes gossweileri	*Eucalyptus tereticornis*	*Sida acuta*	*Drypetes gossweileri*	*Eucalyptus tereticornis*	*Sida acuta*
1000	31.20^{b}±6.43	16.66^{a}±2.11	58.11^{c}±1.18	11.02^{a}±1.28	7.93^{a}±2.47	5.199^{a}±1.23
5000	78.94^{b}±10.63	55.05^{a}±3.35	62.6^{a}±2.29	25.63^{a}±16.67	24.02^{a}±8.41	8.98^{a}±1.36
10000	90.31^{b}±5.03	79.45^{a}±3.96	75.08^{a}±3.12	67.53^{c}±5.79	34.66^{b}±11.73	13.17^{a}±0.07

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

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

Cold water extracts concentration (ppm)	Percentage inhibition (%)					
	Acremonium apii			*Colletotrichum dematium*		
	Drypetes gossweileri	*Eucalyptus tereticornis*	*Sida acuta*	*Drypetes gossweileri*	*Eucalyptus tereticornis*	*Sida acuta*
1000	3.48^{a}±1.42	2.83^{a}±3.10	6.43^{a}±1.25	1.48^{a}±0.96	13.31^{b}±6.17	3.09^{a}±1.40
5000	40.80^{a}±2.13	19.14^{a}±12.12	10.51^{a}±1.36	40.73^{c}±0.73	23.54^{b}±1.46	8.43^{a}±2.30
10000	51.01^{b}±6.88	31.62^{a}±8.29	27.85^{a}±5.38	55.60^{c}±4.39	31.60^{b}±1.73	10.05^{a}±4.66

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

Table 5. Percentage of mycelial growth inhibition of *A. apii* and *C. dematium* by hot water extracts

Hot water extracts concentration (ppm)	Percentage inhibition (%)					
	Acremonium apii			*Colletotrichum dematium*		
	Drypetes gossweileri	*Eucalyptus tereticornis*	*Sida acuta*	*Drypetes gossweileri*	*Eucalyptus tereticornis*	*Sida acuta*
1000	$0.00^{a}\pm0.00$	$2.58^{a}\pm1.26$	$16.51^{b}\pm3.75$	$0.00^{a}\pm0.00$	$4.94^{a}\pm0.06$	$5.42^{a}\pm1.27$
5000	$27.64^{a}\pm1.54$	$36.62^{a}\pm11.78$	$27.36^{a}\pm14.88$	$16.47^{b}\pm5.88$	$12.35^{ab}\pm0.15$	$9.14^{a}\pm1.30$
10000	$58.25^{a}\pm8.41$	$44.47^{a}\pm12.64$	$47.95^{a}\pm3.92$	$28.79^{c}\pm1.76$	$25.30^{b}\pm0.30$	$20.19^{a}\pm0.43$

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

Table 6. Nature of inhibition of mycelial growth by essential oils (EO) and synthetic fungicide

EO/Fungicide	EO *E. tereticornis*	EO *D. gossweileri*	Banko Plus®	Penncozeb®
		Acremonium apii		
MIC (ppm)	7000	50	5000	50
MFC (ppm)	7000	50	10000	50
		Colletotrichum dematium		
MIC (ppm)	6000	50	5000	300
MFC (ppm)	6000	50	5000	300

4. Efficacy of cold water extracts

The mycelial growth of both pathogens was inhibited by all the cold water extracts. The most active extract was obtained from *D. gossweileri* at 10000 ppm (51.10% inhibition against *A. apii* and 55.60% inhibition against *C. dematium*). The extract from *S. acuta* was the less active (Table 4).

5. Efficacy of Hot water extracts

All the hot water extracts inhibited mycelial growth of both pathogens with the highest inhibition (58.25%) exhibited by *D. gossweileri* extract at 10000 ppm against *A. apii*. In general, the extracts were most active against *A. apii* (Table 5).

6. Efficacy of the synthetic fungicide

Banko Plus® completely inhibited the mycelial growth of both pathogens at 5000 ppm, while Penncozeb® was most active at 50 ppm against *A. apii* and 300 ppm against *C. dematium*.

7. Nature of the inhibition

The MIC and MFC of the essential oils and fungicide against the two pathogens remained the same, except for Banko Plus® against *A. apii*. (MIC =5000ppm and MFC=10000 ppm) (Table 6).

8. Preliminary phytochemical composition of solvent extracts

Phytochemical screening of solvent extracts showed that their secondary metabolites composition varies with botanical species, method and solvent used for extraction. Alkaloids, phenols, triterpenes, flavonoids, saponins and anthraquinones were largely distributed among the plant species and among the different extracts; sterols were present only in the ethanol extracts; no anthocyanins and coumarins were detected in all the extracts; cardiac glycosides were absent in all extracts from *D. gossweileri*; hydrolysable tannins were found in all extracts from *E. tereticornis* only; condensed tannins were present in all *D. gossweileri* extracts only.

Table 7. Preliminary phytochemical analysis of cold water, hot water and ethanol extracts

Secondary metabolites	Extract	*D. gossweileri*	*E. tereticornis*	*S. acuta*
Alkaloids	ETE	++	++	+
	CWE	+++	+++	+
	HWE	++	+	-
Phenols	ETE	+++	++	+
	CWE	+	+	-
	HWE	++	+	-
Triterpenes	ETE	+++	++	++
	CWE	+++	+	+
	HWE	+++	+	+
Sterols	ETE	+	+++	++
	CWE	-	-	-
	HWE	-	-	-
Flavonoids	ETE	+++	-	++
	CWE	+++	+	+++
	HWE	+++	+	++
Saponins	ETE	-	+++	+
	CWE	+++	+	++
	HWE	++	-	-
Anthocyanins	ETE	-	-	-
	CWE	-	-	-
	HWE	-	-	-
Anthraquinones	ETE	+++	+++	-
	CWE	+++	++	-
	HWE	++	++	+
Cardiac glycosides	ETE	-	+++	++
	CWE	-	+++	++
	HWE	-	+++	+
Coumarins	ETE	-	-	-
	CWE	-	-	-
	HWE	-	-	-
Hydrolysable tannins	ETE	-	+++	-
	CWE	-	+++	-
	HWE	-	+++	-
Condensed tannins	ETE	+	-	-
	CWE	++	-	-
	HWE	++	-	-

Strongly Present: +++; Present: ++; Weakly Present: +; Absent: -
ETE: ethanol extract, HWE: hot water extract, CWE: cold water extract

DISCUSSION

In this study the antifungal activity of essential oils and solvent extracts from 3 plants has been assessed against *A. apii* and *C. dematium* and and solvent extracts were screened for their secondary metabolites composition. Essential oils extraction yields varied with plant species: 053% yield were obtained from *E. tereticornis and* 0.29% from *D. gossweileri*. This yield

of *E. tereticornis* was different from the yield obtained by Abdellah *et al.* in 2002 (0.82%) from same species harvested in Morocco. Essential oil extraction yield and chemical composition can 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 (Zhira *et al.,* 1989; Bruneton, 1999). Further, yields obtained with solvent extracts were considerably higher than those of essential oils. The cold water extracts showed the highest yield, followed by hot water extracts and ethanol extracts. These differences can be explained by the extraction method used, the extraction solvent, and the relative solubility of the compounds in extraction solvents (Lapornik *et al.,* 2005). Both the essential oils used in this study shown antifungal activity against the two pathogens. Essential oils from *D. gossweileri* exhibited a strong activity (MFC=50 ppm) while moderate activity was observed with essential oil of *E. tereticornis*. It has been shown that the antimicrobial activity of an essential oil is related to its chemical composition and mainly its proportion in oxygenated monoterpenes (Hammer *et al.,* 2003; Nguefack *et al.,* 2012). The highest efficacy of *D. gossweileri* oil can be explained by its content in stigmasterol, and stearic acid. It was observed that solvent extracts were less active as compared to essential oils. This result is consistent with those obtained by previous authors. It was reported 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), *Phythophthora infestans* (Galani *et al*., 2013) and against *A. apii* and *C. dematium* (Petchayo *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 hot and cold water extracts; the ethanol extracts showed higher antifungal activity. According to Amvam *et al*., (1998) these differences can be explained by their different chemical compositions. Cold water extracts from *D. gossweileri* and *E. tereticornis* were more active against *C. dematium* than their respective hot water extracts. These cold water extracts contained more alkaloids, anthraquinones and saponines than the hot water extracts. These metabolites could be responsible of the antifungal activity of these extracts. Against *A. apii*, the hot water extracts from *D. gossweileri* richer in phenols were most active. Galani *et al*., (2013) have demonstrated that ethanol extracts with highest antifungal activity against *Phythophthora infestans* were rich in phenols, sterols, flavonoids, condensed tannins, coumarins and alkaloids. Phenolic compounds possess a very high antimicrobial activity (Lapornik *et al.,* 2005) and 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 inhibitory effect of *D. gossweileri* essential oil was comparable to Penncozeb® and was higher than Banko Plus® and the fungal strain *A. apii* appeared to be more sensitive to Penncozeb® than Banko Plus®. This difference in sensitivity can be explained by the difference in mechanism of action of extracts and/or the constitution of the two pathogens. The antimicrobial activity is strongly influenced by the physical, morphological and chemical characteristics of the components of the microbe (Hammer *et al*., 2003). So, to better understand the mode of action of these extracts, there is a need of more studies on the chemical and structural characterization of these pathogens.

Conclusion

Out of the 3 types of extracts used in this work, essential oils have shown the highest antifungal activity against *A. apii* and *C. dematium*, followed by ethanol extracts which were more active than cold water and hot water extracts. The degree of fungal growth inhibition recorded with essential oils was comparable or higher than the synthetic fungicides. These findings demonstrate a prospective source of compounds effective against these two serious pathogens of celery in Cameroon.

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