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Effects of *Minthostachys mollis* essential oil and volatiles on seedlings of lettuce, tomato, cucumber and *Bidens pilosa*

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ABSTRACT

The extraction and chemical composition of essential oil of *Minthostachys mollis* (Kunth) Griseb (Lamiaceae) and its inhibitory effects on germination and shoot/root elongation of lettuce, tomato, cucumber and *Bidens pilosa* (L.) var *pilosa* are described. Hydrodistillation gave 2.9% (v/w) of essential oil (97.8% of terpenoids). Pulegone (83.6%), menthone (7.6%), limonene (2.0%) linaloo! (1.2%), and isomenthone (0.8%) were the major components of essential oil. This oil was applied at 1.0, 2.5 and 5.0 µl on filter paper disks for bioassay. The seed germination of all test spp was completely inhibited even with the lowest dose. Five days-old transplants of seedlings (7 days for *B. pilosa*) exposed to 5, 10 and 20 ppm of oil in the vapour phase inhibited the elongation of shoot more than root and its inhibition was concentration dependent. *B. pilosa* was most resistant and tomato the most sensitive plants. Shoot growth of *B. pilosa* was inhibited at 20 ppm with necrosis of hypocotyls.

Key words: Allelopathy, *Bidens pilosa*, cucumber, essential oil, growth inhibition, lettuce, menthone, *Minthostachys mollis*, pulegone, tomato, Venezuela.

INTRODUCTION

Minthostachys mollis (Kunth) Griseb. (Lamiaceae) is an indigenous shrub of the Andean range of South America and found from Venezuela to Argentina and Chile. Known in Perú and Bolivia as "Muña", "Khoa" and "Huaichcha", this plant is distributed between 1100 and 4200 m above sea level in moderately dry land but may be cultivated at lower altitudes, under temperate conditions. It is used as traditional medicine, spice (26) and essential oil (24). Peruvian farmers use its dry leaves to prevent potato sprout during storage. Inhibition of the tubers sprouting has been attributed to (-)-menthone, a component of *M. mollis* oil at dose of 4 µl/l of air (10). *Minthostachys* plants also have biological activities, viz., larvicidal against *Aedes aegypti* (*M. serosa*) (9), toxic to adult forms of *Rhodnius neglectus*, *R. infestans* (*M. andina*) (18), *Oncopelthus fasciatus* (*M. tomentosa*) (8), and to honeybee ectoparasite *Varroa destructor* (*M. mollis*) (30).

Besides, *M. mollis* is intensely aromatic and its volatiles may influence the trophic interactions. Either mechanical wounding (6) or by arthropods (33) in *M. mollis* changes the monoterpene composition of its essential oils. Some adaptive effect on plant-plant

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interactions may be anticipated but has not been studied. Previous work reported the phytotoxic effects of other essential oil bearing plants of the Florida scrub on sandhill grasses such as *Schizachyrium scoparium* Nash, *Andropogon gyrans* Ashe and *Leptochloa dubia* (HBK) (16). Also, purified essential oils from some aromatic species such as *Origanum syriacum* (L.), *Micromeria fruticosa* (L.) Druse, and *Cymbopogon cytratus* (DC.) Stapf inhibits the germination of several plant species between 20-80 ppm (13). Individual monoterpenes and their combinations in pairs also inhibits the germination and seedling growth in lettuce (*Latuca sativa*) (34). More information on the effect of plant essential oils on pantropical weeds is of importance.

MATERIALS AND METHODS

Aerial parts of *M. mollis* were collected near San Rafael de Mucuchíes, Mérida State at [3200 m above sea level (70° 52´W 8° 46´N) (688 mm rain/year)] on July, 2002, from highly acidic soil (pH 4.8). Fresh leaves of *M. mollis* (247 g) were blended with 2.0 l of distilled water and the volatile fraction was isolated by hydrodistillation for 3 h using a Clevenger-type trap. Test crops seeds of following cultivars were purchased from West Hill Seeds (Sutter, California): Lettuce (*Latuca sativa*) 'Great Lakes 659', Tomato (*Lycopersicum esculentum*) 'Rio Grande' and cucumber (*Cucumber sativus*) 'Pointset 76'. Seeds of *B. pilosa* were collected from the wild plants growing in meadows near the Mérida city, Venezuela, at 1500 m above sea level.

Bioassays

Germination: Twenty five seeds of lettuce, tomato, *Bidens pilosa,* or 10 seeds of cucumber, were placed in previously sterilized 9 cm dia petri dishes lined with moist filter paper (5 ml distilled water), at 24°C and 12/12 h light-dark cycle in growth chamber using 4x15 W fluorescent lamps and 1x25 W tungsten bulbs per 0.25 m^3 of chamber space. The air volume inside each dish was 63 ml. In center of each dish, 1 cm dia filter paper (Whatman N°1) discs were placed and 1, 2.5 and 5 µl of *M. mollis* essential oil was applied to them with mycrosyringe. The filter paper disc was placed on 2 cm aluminum foil disk to prevent direct contact of essential oil with the seed paper substrate. These dosages gave 16, 40 and 80 ppm of oil, respectively, in the air chamber. The oil was assumed to be in vapour phase, as no liquid remained in the disk after few hours. At time zero, petri dishes were sealed with parafilm. After 72 h the number of germinated seeds were recorded except for *B. pilosa* that were observed after 5-days, when controls showed 80 ± 5% germination. Treatments were replicates ten times.

Seedling growth: Seeds of lettuce, tomato, cucumber and *B. pilosa* were germinated on moist filter paper in capped 9 cm Petri dishes as above. Five-days old untreated seedlings of each species were transplanted at 3 cm spacing in hermetic growth plexiglass chambers (20x15x5 cm) containing 5 cm deep coarse sand (thoroughly washed, moist and maintained at 24°C). A 12/12 h photoperiod was used. *M. mollis* essential oil was applied on 1 cm filter paper discs, placed inside a 5 ml glass beaker on the sand surface, hence, without direct contact with the substrate. After few hours no oil was visible in the paper disc, showing that it has changed to the vapours inside the cage. The inner volume of the growth chamber with sand substrate was 1000 ml, therefore 1,2,5,10 and 20 µl doses

provided an atmosphere around the seedlings containing 1,2,5,10 and 20 ppm of volatiles. At time zero, each box was sealed and after five days seedlings were removed from the sand substrate. The root and shoot length were recorded after five days (lettuce, tomato, cucumber) and seven days (*B. pilosa*). The treatments were replicated ten times.

Isolation and identification of allelochemicals

Gas chromatography: A Perkin-Elmer AutoSystem gas chromatograph equipped with FID detector was used for Kováts indices determination. Two capillary columns of different polarities were used: a 5% phenylmethyl polysiloxane fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; HP-5, Hewlett-Packard, CA, USA) and a polyethylene glycol fused-silica column (60 m x 0.25 mm i.d., film thickness 0.25 μm; HP-WAX, Hewlett Packard, CA, USA). For the HP-5 column oven temperature was programmed from 60 to 200°C at 4°C/min. For the HP-WAX column the temperature was programmed from 50 to 230° C at 3°C/min. In both cases injector and detector temperatures were 200°C and 280°C, respectively. The carrier gas was helium at 0.8 ml/min. The samples (1.0 μl) were injected using a split ratio of 1:10. Retention indices were calculated relative to C_8–C_{18} n-alkanes. The percentage composition of the oils was calculated by the normalization method from the GC peak areas on the HP-5 column.

Gas Chromatography-Mass Spectrometry: GC-MS analyses were carried out on a Model 5973 Hewlett Packard GC-MS system fitted with a HP-5MS fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm, Hewlett Packard Corp.). Oven temperature program was the same used for the HP-5 column for GC analysis; the transfer line temperature was programmed from 150 to 280°C; source temperature, 230°C; quadruple temperature, 150°C; carrier gas, helium, adjusted to a linear velocity of 34 m/s; ionization energy, 70 eV; scan range, 40–500 amu; 3.9 scans/s. Samples (1.0 μl) were injected using a Hewlett Packard ALS injector with split ratio 100:1. The identity of the oil components was established from their GC retention indices (3,12,20) and by computer comparison of mass spectra with a Wiley MS Data Library (6^{th} ed.).

Statistical analysis

Calculations were performed with the Statistix V 7.0 package (Analytical Software, St. Paul, MN). The Kruskall-Wallis one-way ANOVA test of comparison of the means ($p < 0.05$ for statistical differentiation) was employed throughout.

RESULTS AND DISCUSSION

Essential oil

The essential oil content of *M. mollis* was 2.9% (v/w fresh plant). This is in agreement with previous results. Some wild plants from San Rafael were transplanted in Medicinal Plant Garden, Faculty of Pharmacy, Merida (1500 m asl, mean Temp 19°C), where they developed abundant foliage. A maximum of 3.15% (v/w) oil was obtained from wild plants harvested at San Rafael (March 1992) and 2.52% (v/w) from these specimens cultivated in Mérida (July 1993) (27). The identified oil components represented 97.8 % of the total oil (Table 1). The main components were pulegone (83.6%, July 2002, rainy season), menthone (7.6%), limonene (2.0%), linalool (1.2%) and

isomenthone (0.8%). It was reported later (28) that the pulegone content of oil from cultivated plants (75.2%) was lower than wild plants (79.3%). The same compounds were most abundant in our earlier studies, however, composition was different (28).

Table 1. Composition of the essential oil of *Minthostachys mollis* collected in San Rafael de Mucuchíes, Mérida, Venezuela

Component	RI_1	RI_2	Composition (%)	Identification method
Sabinene	972	1130	0.1	W-MS, RI_1, RI_2
β-pinene	978	1120	0.7	W-MS, RI_1, RI_2
3-octanol	990	-	0.2	W-MS, RI_1
Limonene	1028	1210	2.0	W-MS, RI_1, RI_2
Linalool	1095	1531	1.2	W-MS, RI_1, RI_2
1-octen-3-yl-acetate	1110	-	0.3	W-MS, RI_1
Menthone	1150	1475	7.6	W-MS, RI_1, RI_2
Iso-menthone	1164	1530	0.8	W-MS, RI_1, RI_2
Pulegone	1235	1660	83.6	W-MS, RI_1, RI_2
Piperitenone	1341	-	0.5	W-MS, RI_1
Eugenol	1360	2100	0.1	W-MS, RI_1, RI_2
β-caryophyllene	1419	1620	0.6	W-MS, RI_1, RI_2
α-humulene	1456	1710	0.1	W-MS, RI_1, RI_2

W-MS = compounds identified by computer comparison with Wiley MS Library (6^{th} Ed)
RI_1 = retention index on HP-5 capillary column, RI_2 = retention index on HP-Wax capillary column.

The content of pulegone and menthone was higher in the dry season (December – March) than during the rainy season, possibly because *M. mollis* leaves are covered with a dense layer of glandular trichomes, which produce oil. The exposure of this oil on the leaf surface may be washed/leached by rain and lost in evaporation due to heat and wind, as the exudate of trichomes were reduced during the rainy season. (personal observations.).

Effects on seeds and seedlings

Lettuce, cucumber and tomato: Germination of all these species was completely inhibited at 1 µl dose or 16 ppm concentration in the petri dish air chamber of *M. mollis* volatiles. By comparison with earlier work (13), *M. mollis* volatiles are much stronger than most known monoterpenes. Transplants of 5-days old seedlings of these cultivars exposed to 1 ppm of *M. mollis* essential oil for 7 days in the 1 litre growth chambers were also adversely affected. By contrast, lettuce roots remained unaffected at the highest concentration of *M. mollis* oil, whereas shoot growth was totally inhibited at 5 ppm dose. The air-exposed shoots of the three species showed greater growth inhibition than the roots (Table 2, Fig. 1) with lettuce being the least sensitive and tomato the most. Indeed, exposure to 5 ppm of *M. mollis* oil, lettuce shoots still developed roots 4.9 ± 0.9% in

Table 2. Effects of application of *M. mollis* essential oil on 5- days old seedlings growth of lettuce, tomato and cucumber

Oil concentration	Lettuce		Tomato		Cucumber	
(ppm)	Radicle	Shoot	Radicle	Shoot	Radicle	Shoot
0 (Control)	9.8[a]	28.3[a]	8.2[a]	29.0[a]	68.0[a]	34.6[a]
1	9.1[b]	20.0[b]	6.1[b]	7.6[b]	61.8[a]	13.8[b]
5	9.9[a]	1.4[c]	4.0[c]	3.0[c]	13.4[b]	6.1[c]
CD at 1%	NS	5.43	1.02	20.0	6.18	14.2

NS: no significant inhibition. Equal letters denote statistical undifferentiation in same column values (ANOVA $p<0.05$)

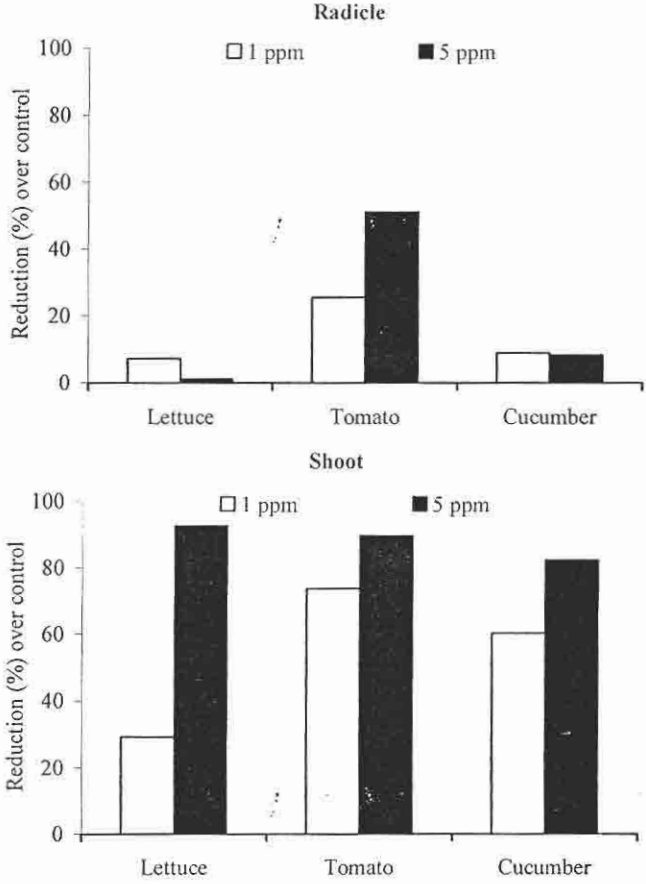

Figure 1. Inhibitory effects of applied *M. mollis* essential oil on radicle and shoot growth of test crops.

length relative to controls, whereas, the other species were almost completely inhibited showing necrosis of the emerging hypocotyls. When the seedlings were exposed to the low

concentration of volatiles, the inhibition was more in shoot than in root. This was possibly due to the more direct contact of the shoot with atmospheric components in the growth chamber that did not diffuse rapidly into the substrate sand. Indeed, the expansion of roots in seedlings allowed to grow with rootlets exposed to the air as on moist filter paper, were strongly inhibited (data not shown).

***Bidens pilosa*:** The germination of seeds of this plant was also inhibited by *M. mollis* volatiles in a concentration-dependent fashion (Table 3). Only radicles became barely visible, whereas, shoot development was completely stopped.

Table 3. Influence of applied *M. mollis* volatile monoterpenes on 5 days old seedlings growth of *B. pilosa*

Volatile monoterpene concentration (ppm)	Germination (%)	Radicle length (mm)
0 (Control)	90.0^{a}	3.3^{a}
1.0	88.0^{a}	2.7^{a}
2.5	63.0^{b}	1.5^{b}
5.0	0.0^{c}	0.0^{b}
CD at 1%	4.96	3.43

Equal letters denote statistical undifferentiation in same column values (ANOVA $p<0.05$)

The pre-germinated seedlings of *B. pilosa* were more resistant to the effects of *M. mollis* oil than lettuce. For this reason the seedlings were exposed to higher dosages of the volatiles (5, 10, and 20 ppm). The results in Figure 2 show that *B. pilosa* seedlings responded negatively even at 5 ppm of *M. mollis* volatiles and complete inhibition was recorded at 20 ppm. At this concentration necrosis of the emerging hypocotyls resulted. There was a differential response in the lengthening of root and shoot (Fig.e 2), the latter being much more affected. The steep growth reduction could also be recorded by the fresh and dry weights of radicles and aerial parts (Table 4).

Table 4. Influence of applied *M. mollis* essential oil on 10 days old seedlings growth of *B. pilosa*

Oil concentration (ppm)	Root			Shoot		
	Length (cm)	FW (mg)	DW (mg)	Length (cm)	FW (mg)	DW (mg)
0 (Control)	3.36^{a}	1.68^{a}	0.31^{a}	3.15^{a}	8.91^{a}	0.58^{a}
5	4.20^{b}	1.28^{b}	0.37^{a}	0.58^{b}	1.33^{b}	0.18^{b}
10	4.26^{b}	1.35^{b}	0.27^{a}	0.30^{c}	0.71^{c}	0.07^{c}
20	1.86^{c}	$<0.1^{c}$	$<0.1^{c}$	0.11^{d}	$<0.1^{d}$	$<0.05^{c}$
CD at 1%	2.391	2.177	1.984	1.461	1.285	2.022

FW: Fresh weight, DW: Dry weight. Equal letters denote statistical undifferentiation in same column values (ANOVA $p<0.05$)

Germination of *B. pilosa* seeds was also restrained by *M. mollis* volatiles, although it was less sensitive than test crops. However, the low concentrations (5 ppm) also decreased the length, fresh and dry weight of *B. pilosa*'s seedling (Table 3). Again the

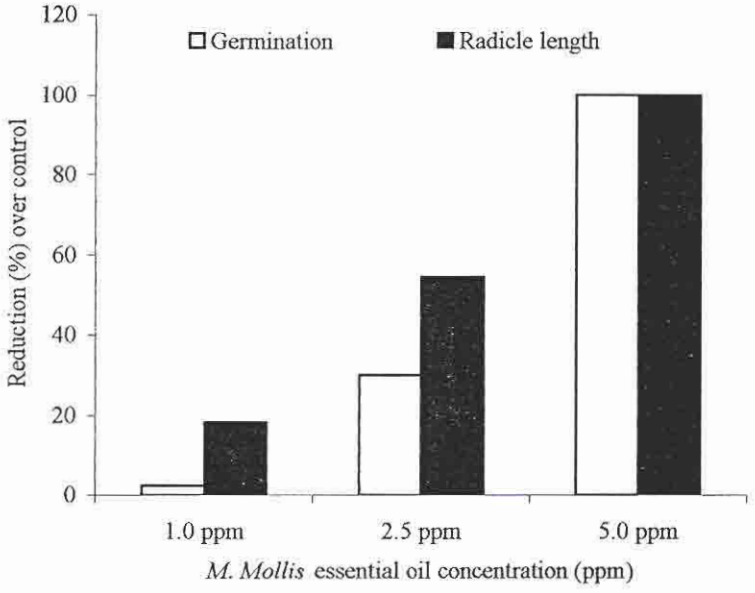

Figure 2. Influence of volatiles oil on germination and root growth (% of control) of *Bidens pilosa*.

reduction in shoot length relative to the control was more noticeable, probably due to the above reasons. *B. pilosa* apparently compensates the reduced growth of the shoot with increase in root length (Figure 1). Notably, while the decrease in shoot length was 70% its dry mass was reduced by 28% only. In fact, hypocotyls expanded laterally, although their surface was visibly reduced relative to the controls. At 10 ppm, the terpenoid fraction caused not only inhibition in size and mass but also deformed the hypocotyls. Besides, root mass was decreased much more dramatically than its length, indicating water retention in root cells. These result suggests that either menthone, pulegone or both cause severe osmotic alterations in the root system of *B. pilosa*.

Pulegone and menthone, the main components of the volatile fraction of *M. mollis*, moderately inhibited the germination and seedling growth in lettuce in the vapor phase (34) at 25 ppm. These compounds are also found in other species of the Lamiaceae (4,11,21,29,32). The pulegone depolarizes the root membrane potential in cucumber (23) and also changes the root and mitochondrial respiration at 0.080 and 0.12 mM concentration in growth medium (25). Other monoterpenes cause similar effects on maize (1,2). The seeds of wheat responds to this threat by degrading pulegone and other monoterpenes to less toxic products (14). Pulegone is transformed into menthone, isomenthone, pulegol and menthofuran. The toxicity of (+)-pulegone is decreased by (-)-menthone, (19), hence the biotransformation of pulegone to its antagonist by the wheat seed entails a compounded benefit for this plant.

The composition of essential oil of *M. mollis* native to other South American countries is significantly different. For instance, the pulegone content of *M. mollis* native to Ecuador was 0.8%, while, menthone was 24.0 % (5) and *M. mollis* from Argentina, had 8.9-58.3% pulegone and 24.1-73.3% menthone in the essential oil (7). Therefore, our

specimens appear to have high pulegone content. Thus higher contents of pulegone and menthone in *M. mollis* in the Venezuelan central Andes, opens new possibilities for its commercial cultivation to produce these compounds to control crop pests (weeds, insects, pathogens etc) (15,17,19,22,31,35).

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